RBPjk-Independent, NICD-Dependent Signals in Rbpj-/- Hair Follicle Keratinocytes Cause a Milder Phenotype in RBPjcKO Animals

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RBPjκ-Independent, NICD-Dependent Signals in Rbpjκ/κ Hair Follicle Keratinocytes

Cause a Milder Phenotype in RBPjκKO Animals

by

Mustafa Turkoz

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in St. Louis in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May 2014

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To my father and unborn child
Notch is a membrane-bound transcription factor that mediates signals between adjacent cells. It is the central member of a highly conserved pathway that is important for proliferation, cell fate decisions, and apoptosis not only during development, but also in adulthood acting either in stem cell maintenance or differentiation. In the skin, Notch is essential for epidermal differentiation, maintenance of hair follicle morphology and terminal differentiation of the inner and outer root sheath cells. Ligand-dependent activation, $\gamma$-secretase-dependent cleavage, and RBPjκ-mediated downstream transcriptional activities of Notch receptors constitute the “canonical” Notch signaling pathway. This implies that loss of $\gamma$-secretase (no Notch Intracellular Domain (NICD) release), Notch receptors (no NICD), or RBPjκ (no activation of downstream targets) should generate similar phenotypes based on the canonical model. Unexpectedly, keratinocyte-specific deletion of Rbpj gene (RBPjcKO) produced a significantly milder phenotype than the keratinocyte-specific deletion of both Notch1 and Notch2 genes (N1N2dcKO) or Presenilin1 and Presenilin2 genes (PSdcKO). Based on these observations, we
sought to determine the underlying mechanisms and investigated the potential role of non-canonical Notch signaling in generating these phenotypic differences.

First, we characterized N1N2dcKO, PSdcKO, and RBPjcKO embryonic skin. We found that all these mutants are similar at birth with no significant differences in the gross morphology of their epidermis and hair follicles as well as mRNA and protein expression patterns.

Secondly, we genetically dissected the Notch signaling pathway to define the elements ameliorating the phenotype. We showed that even a limiting amount of cleaved Notch1ICD is sufficient for the milder phenotype observed in RBPjcKO animals.

Lastly, we showed that the loss of Notch signaling in the hair follicles drives the severe phenotypes in PSdcKO and N1N2dcKO animals. Although the levels of epidermal TSLP expression are similar in all Notch mutants, RBPjcKO hair follicles are less distorted and produce less TSLP leading to lower serum TSLP levels. The reduced TSLP in serum results in less aggressive B cell expansion allowing RBPjcKO animals to live significantly longer than N1N2dcKO and PSdcKO animals. In conclusion, γ-secretase cleaved Notch1ICD likely stimulate RBPjκ-independent signals in the Rbpj−/− hair follicle keratinocytes causing a milder phenotype in RBPjcKO animals. Future experiments will interrogate whether RBPjκ-independent NICD activity directly generates a different transcriptional response or affects other signaling pathways.
CHAPTER I

INTRODUCTION

Author Contribution
The author wrote the text and prepared the figures in this chapter.
Multicellular organisms start their life as a single cell that is capable of giving rise to every cell type in the entire organism. During the transition from single cell to multicellular organism, cells proliferate, interact with their environment and each other, migrate, specialize, and even die in a precise manner to form organs. All of these cellular events require tightly regulated communication between cells. These highly complicated interactions and communications are mediated by signaling pathways. Notch, first described in 1914 by John S. Dexter, is the receptor for one of the major pathways. The Notch signaling pathway is essential for many processes during development of metazoans. This dissertation mainly focuses on a novel role for Notch signaling during mouse hair follicle development.

**Notch Signaling**

The Notch signaling pathway, conserved from metazoans to mammals, has been shown to be important for many different cellular functions including proliferation, cell fate decisions and apoptosis, not only during development, but also during adult stage for stem cell maintenance and differentiation (Artavanis-Tsakonas et al., 1995; Gridley, 1997; Weinmaster, 1997; Greenwald, 1998; Artavanis-Tsakonas et al., 1999; Miele and Osbourne, 1999; Radtke and Raj, 2003; Dang, 2012). At present, two receptors (Glp-1 and Lin-12) in *C.elegans*; one receptor (Notch) in *Drosophila melanogaster*, and four receptors (Notch 1-4) in mammals have been identified for this signaling pathway (Favier, 2000; Katoh 2007). Notch receptors are classified as type I transmembrane proteins (~300 kDa) that contain a large extracellular domain (ECD), one transmembrane domain (TMD), and intracellular domain (ICD) (Figure 1-1; Kopan and Ilagan, 2009). Receptor activation is mediated by the DSL (Delta and Serrate in Drosophila, Lag2 in *C.elegans*) family of ligands that are expressed on the surface of neighboring cells (Figure 1-1 & Figure 1-2). Mammals have 5 DSL ligands: Delta-like 1,3, and 4, and Jagged 1
Ligand binding triggers a conformational change in the Notch receptors exposing the negative regulatory region in the Lin12-Notch Repeats (LNR) and heterodimerization domain of the receptor (Figure 1-1). This allows the enzymatic cleavage by ADAM/TACE (a disintegrin and metalloprotease/TNF-\(\alpha\) converting enzyme) family proteins at the S2-cleavage site within the heterodimerization domain (Figure 1-1 & Figure 1-2). S2 cleavage generates the substrate for \(\gamma\)-secretase (multi-subunit protease complex consisting of PSEN1, PSEN2, nicastrin, APH-1, and PEN-2; Kaether et al., 2006), which further cleaves Notch at the S3 and S4 sites within the transmembrane domain of the receptor (Figure 1-1 & Figure 1-2). S3-cleavage is a critical step in the activation of the pathway, since it liberates the Notch intracellular domain (NICD) (Schroeter et al., 1998). NICD then translocates to the nucleus, where it interacts its DNA-binding partner, CSL (\(\text{CBF1/RBPj}\) in mammals, \(\text{Suppressor of Hairless}\) in \(Drosophila\) \textit{melanogaster}, \(\text{Lag1}\) in \(C.elegans\)) (Lecourtois et al., 1997). CSL normally functions as a repressor via interacting with and/or recruiting SKIP, SMRT, CoR, and HDAC proteins to form a transcriptional repressor complex (Kao et al., 1998; Zhou et al., 2000; Zhou and Hayward, 2001). NICD binding transforms CSL into a transcription activator by dissociating repressor complex and recruiting the coactivators such as Lag-3/mastermind, p300/CBP, and PCAF/GCN5 (Schuldt and Brand, 1999; Petcherski and Kimble, 2000; Oswald et al., 2001; Kurooka and Honjo, 2000; Wallberg et al., 2002) to initiate target gene transcription of basic helix-loop-helix (bHLH) transcription factors such as \(\text{Hairy Enhancer of Split}\) (HES) and HES-related proteins (Jennings et al., 1994; Jarriault et al., 1995; Nellesen et al., 1999; Bessho et al., 2001; Iso et al., 2003; Fischer et al., 2004). This linear pathway is called the canonical Notch signaling pathway (Figure 1-2).

To keep the terminology simple, I will use mammalian homolog names of Notch
signaling components (i.e. Delta 1,3,4 and Jagged 1,2; Notch 1-4, and RBPjk) for the rest of this dissertation.

**Non-canonical Notch Pathway**

Although canonical Notch signaling is well established and essential for many processes, several studies have suggested a possibility of non-canonical Notch signaling (Shawber et al., 1996; Wang et al., 1997; Giniger, 1998; Zecchini et al. 1999; Dumont et al., 2000; Ramain et al., 2001; Berechid et al., 2002; Crowner et al., 2003; Brennan and Gardner, 2002; Demehri et al., 2008; Le Gall et al., 2008; Sanders et al., 2009; Acosta et al., 2011; Kwon et al., 2011;). The simplest description of canonical Notch signaling entails DSL ligand-dependent activation of the Notch receptors, cleavage by γ-secretase and transcription mediated by RBPjk. Those pathways not conforming into the prototypical linear pathway scheme would be considered as non-canonical Notch signaling, which can be divided into four major different sub-categories:

a) **Delta/Jagged-independent activation of Notch receptor**

In this category of the non-canonical pathway, activation of Notch receptor is mediated by other ligands (Table 1-1 = D’Souza et al., 2008) such as F3/Contactin1 (Hu et al., 2003), NB-3 (Cui et al., 2004) or Delta/Notch-like EGF-related receptor (DNER) (Mukherjee et al., 2005), but not by canonical DSL family ligands. However, in this type of non-canonical signaling, the cleavage is still controlled by γ-secretase. After liberation of NICD from the cell membrane, NICD translocates to the nucleus and upregulates gene expression in an RBPjk-dependent or independent (Hu et al., 2003) manner (Table 1-1).

b) **Ligand Back Signaling**

Notch ligands, Delta and Jagged, also undergo proteolytic cleavages mediated by ADAM/TACE (Qi et al., 1999) and γ-secretase (Ikeuchi & Sisodia, 2003; LaVoie & Selkoe, ...
The cleavages of Delta/Jagged are thought to result in the production of a Ligand Intracellular Domain (LICD) that can translocate into the nucleus to mediate signal transduction in ligand-presenting cells (Ikeuchi & Sisodia, 2003; LaVoie & Selkoe, 2003; Duryagina et al., 2013; Bland et al., 2003; Six et al., 2003; Kolev et al., 2005).

c) Cleavage-independent receptor-dependent non-canonical Notch Signaling

In this model of non-canonical signaling, the activation of Notch receptors does not involve ligands and/or γ-secretase. A membrane-bound, uncleaved form of the receptor at the cell membrane/endosomal compartment mediates a non-canonical function of the receptor. Corbin’s group proposed a role for non-canonical Notch in muscle precursor fate determination (Rusconi and Corbin, 1998; Rusconi and Corbin, 1999); however the molecular nature of this function is still unknown. Martinez Arias’ group and Srivastava’s group suggested interaction between Notch and Wnt pathways (Brennan et al. 1999) involving a negative regulation of Armadillo/β-catenin. They claim that Axin and Apc involve in the trafficking of Notch receptor and compromising Notch trafficking elevates the activity of activated form of Armadillo (Hayward et al., 2005; Sanders et al., 2009; Muñoz-Descalzo et al., 2011; Kwon et al., 2011). In addition, Martinez Arias’ group also claimed another function of uncleaved-Notch inducing downregulation of JNK activity (Zecchini et al. 1999)

d) NICD-dependent, RBPjk-independent non-canonical Notch Signaling

In this type of non-canonical signaling, DSL ligand activation and γ-secretase dependent cleavage are required but interaction with DNA binding partner, RBPjk, is not necessary to accomplish the function of the NICD. Many different labs have showed evidence for this pathway. Although some those studies did not provide a specific mechanism (Shawber et al., 2003; Bland et al., 2003; Six et al., 2003; Zolkiewska, 2008) upon receptor-ligand engagement.
1996; Nofziger et al., 1999; Mizutani et al., 2007), others suggest involvement of Deltex (Ordentlich et al., 1998; Ramain et al., 2001; Yamamoto et al., 2001; Endo et al., 2002; Endo et al., 2003; Hu et al., 2003; Hori et al., 2004), Ras (Hodkinson et al., 2007), β-catenin (Acosta et al., 2011) or Rho/ROCK pathways (Yugawa et al. 2013).

**Skin Development in mammals**

Skin is the largest organ of the body in mammals covering the entire surface and functioning as a sensory organ and a protective barrier not only blocking harmful insults and dangerous threats from outside but also preventing water loss from inside out.

The major components of mammalian skin are the epidermis (four layers of epithelium), the epidermal appendages (e.g. hair follicles, sebaceous glands, sweat glands, mammary glands), and the dermis (Figure 1-3A). During early embryogenesis, surface ectoderm gives rise to the epidermis and epidermal appendages, whereas dermis is derived from mesoderm.

**Epidermal Development in mammals**

Mouse epidermal development begins around embryonic development day E8.5 - E9 from a single layer of ectodermal cells called surface ectoderm (Figure 1-3B). Surface ectodermal cells, expressing markers keratin 8 (K8) and K18, start to express K6 and K17 to give rise to periderm, an outer layer of epithelial cells (Figure 1-3B). Periderm is a transitory embryonic tissue that sheds off when epidermal differentiation is completed (McGowan and Coulombe, 1998; Sanes et al., 1986; M’Boneko and Merker, 1988). At the same time (E8.5-E9), some of the K8 and K18 positive ectodermal cells initiate the epidermal cell fate program by turning on the expression of K5 and K14 genes (Fuchs, 2007; Koster and Roop, 2007; Nagarajan et al., 2008). By E13, the progenitors, K5 and K14 positive epidermal basal cells cover the entire embryo in a single layer and are ready to initiate the epidermal differentiation program (Figure 1-
3B). Subsequently, at around E13.5 some of the basal keratinocytes lose their contact with the basement membrane and migrate towards the outer layer where they begin to express the spinous cell markers, K1 and K10 (Fuchs and Green, 1980; Byrne et al., 1994) (Figure 1-3B). Around E16.5, spinous cells continue to further differentiate and express loricrin, filaggrin, and transglutaminase, which are the markers for granulocytes (Figure 1-3B; Rothnagel et al., 1987). These specific markers are involved in the maturation of cornified layer, which serves as the protective barrier for the organism (Segre, 2006). By E18.5, epidermal differentiation is completed and all four layers of epidermis (basal layer, spinous layer, granular layer, and stratum corneum) are formed with a precise balance between proliferation and differentiation (Figure 1-3B; Blanpain and Fuchs, 2009).

**Function of Notch Signaling during Epidermal Development**

Activation of Notch receptors has been shown to be critical for cell cycle exit and keratinocyte differentiation (Lowell et al., 2000; Nickoloff et al., 2002; Rangarajan et al., 2001; Blanpain et al., 2006; Moriyama et al., 2008). Notch1 and Notch3 receptors are detectable in the basal layer at E13.5 and in suprabasal layers after E14.5, whereas expression of Notch2 receptor can be detected after E14.5 only in suprabasal layers (Figure 1-3D; Romano et al., 2012). Also, Jagged1 expression is restricted to the suprabasal layer; in contrast Jagged2 is detected in the basal cells (Powell et al., 1998).

During the early stage of epidermal differentiation, activated Notch1 inhibits growth in basal cells by activating p21\(^{WAF1/Cip1}\) expression in an RBPjk-dependent manner (Rangarajan et al., 2001). Notch1 activation in basal cells results in the downregulation of integrin α3/β1 and α6/β4 expression and loss of contact with the basement membrane, whereas Notch activation in spinous cells causes increased expression of early differentiation markers such as involucrin, K1
and K10 and suppression of late markers, loricrin and filaggrin (Rangarajan et al., 2001; Blanpain et al., 2006). The Notch-mediated induction of differentiation mechanisms in basal cells are controversial in terms of RBPjκ-dependence. Rangarajan et al. (2001) have proposed that activation of Notch1 or Notch2 can induce involucrin and K1 through an RBPjκ-independent mechanism, however Blanpain et al. (2006) have demonstrated that these inductions are RBPjκ and Hes1-dependent.

**Hair Follicle Development in mammals**

Hairs are one of the distinguishing characteristics of mammals. They function as a protective barrier from harmful external insults, conserve the body temperature from extreme temperature changes, serve as a sensory organ and provide a camouflaging coat. Mammalian hair follicles are formed during embryonic development and undergo cycles of growth (anagen), regression (catagen), and rest (telogen) throughout the lifespan of the animal. In this dissertation, I will focus more on the hair follicle morphogenesis since the phenotypes we are investigating manifest before the second hair cycle starts. Mouse hair follicles develop in three major waves during embryonic development:

- **Guard Hair** - Primary Hair Follicles: are formed starting from E13.5. (1-5% of hair coat)
- **Awl Hair** - Secondary Hair Follicles: are formed starting from E16-17 (~25%)
- **Zig-Zag Hair** - Third Wave: produces the tertiary placodes around E18.5 (~70%)

Morphogenesis of mouse hair follicles depends on reciprocal interactions between epithelial and mesenchymal cells (Sengel and Mauger, 1976; Hardy, 1992). Although earlier studies on skin development claimed that an unknown signal from the dermis is necessary to initiate hair follicle induction (Dhouailly, 1973; Hardy 1992), the signals first identified to be required for mouse hair follicle induction were Wnt signals (Wnt2, 7b, 10a, and 10b) emanating
from epidermal keratinocytes around E12.5-E13.5 (Noramly et al., 1999; Millar, 2002; Schmidt-Ullrich and Paus, 2005; Fuchs, 2007; Fu et al., 2013). These and additional Wnt ligands (Wnt3, 4, and 6) collectively activate Wnt/β-catenin signaling initially in the upper dermis (DasGupta et al., 1999; Fu et al., 2013), then within the future hair placode epithelium (Fuchs, 2007; Fu et al., 2013), and lastly in the dermal fibroblasts (Yang et al., 2012). Deletion of β-catenin in the basal (K5+/K14+) keratinocytes inhibits hair placode formation (Huelsken et al., 2001). The main function of the Wnts in the ectoderm is to inhibit Fibroblast Growth Factor (FGF) activity, thereby allowing increased levels of Bone Morphogenetic Protein (BMP) to induce the epidermal cell fate program (Stern, 2005; Fuchs, 2007).

Epithelial cells that receive Wnt signals turn on the expression of Ectodysplasin-A (EDA) and its receptor, EDAR (Durmowicz et al., 2002; Laurikkala et al., 2002). The combination of Wnt/β-catenin and EDA/EDAR activation in epithelial cells regulates expression of FGF20, which leads to reorganization of the underlying mesenchyme (Huh et al., 2013). All of these events trigger an epithelial thickening by downregulating E-cadherin expression (Jamora et al., 2003), condensation of dermal fibroblasts and induction of the hair follicle placode formation around E13.5 (Figure 1-3C; Hardy, 1992). The EDA/EDAR signaling pathway has been shown to be critical for primary hair placode induction and regulating hair follicle density (Mou et al., 2006; Pummila et al., 2007). It controls these processes in three major steps: (1) stabilization of β-catenin, thus controlling the expression pattern of Wnt/β-catenin pathway (Mou et al., 2006), (2) activation of NF-κB pathway at day E14.5 (Schmidt-Ullrich et al., 2006) and (3) activation of Sonic Hedgehog (Shh) through EDAR-associated death domain (EDARADD) and NF-κB (Tucker et al., 2000; Laurikkala et al., 2002; Pummila et al., 2007; Cui et al., 2011). Shh from epithelial cells activates Noggin expression in the dermal fibroblasts condensing beneath the
placode where Noggin carries out two important functions: (1) suppressing BMP signals in the epithelial cells (Mou et al., 2006; Pummila et al., 2007), and (2) activating Lef1 gene expression within placodes in combination with Wnt/β-catenin signaling (Botchkarev et al., 1999; Botchkarev et al., 2002; Jamora et al., 2003; Liu et al., 2004). Noggin and Lef1 have been shown to be critical for secondary hair follicle formation, since Noggin\(^{-/-}\) or Lef1\(^{-/-}\) animals can initiate primary hair follicles, but secondary hair follicle induction is completely blocked (van Genderen et al., 1994; Botchkarev et al., 2002).

After placode formation, epithelial cells start to invaginate towards the mesenchyme to form hair germ at around E15.5 (Figure 1-3C; Hardy, 1992). Shh and NF-κB signals are essential for epithelial proliferation and invagination, however they are not crucial for earlier induction events (Dahmane et al., 1997; St-Jacques et al., 1998; Xie et al., 1998; Grachtchouk et al., 2000; Mill et al., 2003; Huntzicker et al., 2006). Shh\(^{-/-}\) skin can form the hair placodes similar to wt skin; however due to the disrupted epithelial-mesenchymal crosstalk, hair placodes have impaired proliferation and invagination (Mill et al., 2003). In summary, Wnt/β-catenin, FGF, Shh, TGF-β/BMP and Eda/Edar/NF-κB signaling pathways together regulate early steps of hair morphogenesis and are essential for proper induction of hair follicles during embryonic development (St-Jacques et al., 1998; Botchkarev et al. 1999; Headon and Overbeek; 1999; Huelsken et al., 2001; Zhang et al., 2009; Woo et al., 2012;).

The reciprocal exchange of additional signals leads to hair placode growth downwards to form the hair germ around E15.5, the hair peg around E17.5 and the hair bulb around E18.5 for the primary hair follicles (Figure 1-3C; Hardy, 1992). The highly proliferative leading edge composed of the growing epithelial cells is called the matrix. These epithelial cells migrate downwards, surround the mesenchymal cells (dermal papilla) and form the hair bulb (Figure 1-
The dermal papilla functions as an inductive center for follicular growth and differentiation, not only during morphogenesis, but also for hair cycles during the adult stage (Hardy, 1992). Epithelial cells constantly interact with the dermal papilla and eventually give rise to seven terminally differentiated cell types. The outermost layer of cells constitutes the outer root sheath (ORS), which is continuous with the epidermis (Figure 1-3G). The next three layers of cells (Henle’s Layer, Huxley’s layer, and IRS cuticle) collectively form the inner root sheath (IRS) (Figure 1-3G). The innermost three layers of cells (cuticle, cortex and medulla) make up the hair shaft. (Figure 1-3G)

Hair follicle stem cells reside in the bulge (Figure 1-3C), an adjacent compartment of the hair follicles, contribute to reconstruction of the hair follicles during the anagen phase of the adult hair cycles (Cotsarelis et al., 1990)

**Notch Function during Hair Follicle Development**

During embryonic development, Notch1 mRNA (Kopan and Weintraub, 1993; Powell et al., 1998) and protein (Figure 1-3D) expression can be observed as early as at E13.5 in the single layer of epidermis, thereupon in the epidermal cells within the hair placode (E13.5-E14.5), and inner cells of the epidermal invaginations (E15.5-E16.5). However, mesenchymal cells do not express Notch1 (Powell et al., 1998) and Notch2 is not expressed in embryonic hair follicle cells (Favier et al., 2000). Delta1 protein expression is restricted to the mesenchymal cells within the bulb of dermal papilla (Powell et al., 1998). Jagged1 and Jagged2 mRNA expression overlaps with Notch1 transcripts in the embryonic hair follicle (Powell et al., 1998).

In neonatal hair follicles, Notch1 mRNA is found throughout the hair matrix cells and Notch2 mRNA in IRS cells, whereas Notch3 protein is detected in precursor cells, differentiating cortex, and cuticle cells (Figure 1-3F, G; Pan et al., 2004; Kopan and Weintraub, 1993; Powell et
al., 1998). Delta1 expression disappears from the dermal papilla in adult hair follicles (Estrach et al., 2008). Jagged1 and Notch1 expression are co-localized in the upper follicle bulb, whereas Jagged2 is detected in the inner bulb cells (Figure 1-3F,G; Powell et al., 1998) The Jagged1-Notch1 expression pattern is consistent with their role in regulating the differentiation of bulb matrix cells into hair shaft cortical and cuticle cells (Figure 1-3F,G; Powell et al. 1998).

Loss-of-function and gain-of-function studies suggest that Notch signaling is not essential for hair follicle induction, invagination, nor the formation of hair peg and hair bulb (Lin et al., 2000; Yamamoto et al., 2003; Pan et al., 2004; Uyttendaele et al., 2004; Blanpain et al., 2006; Estrach et al., 2006; Moriyama et al., 2006; Ambler and Watt, 2007; Lee et al., 2007; Estrach et al., 2008; Demehri et al., 2009). Interestingly, Notch pathway conditional-knock-out (cKO) animals are similar compared to the wild-type (WT) animals in their number and distribution of hair follicles (Blanpain et al., 2006; Demehri et al., 2009; Estrach et al., 2006; Lee et al., 2007; Pan et al., 2004; Yamamoto et al., 2003; Estrach et al., 2008; Vauclair et al., 2005). Moreover, the loss-of-Notch pathway components does not interfere with the expression patterns of signaling pathways (e.g. Wnt/β-catenin, FGF, Shh, TGF-β/BMP and EDA/EDAR/NF-κB) that participate in early placode formation and/or embryonic hair follicle development (Vauclair et al., 2005).

Even though Notch signaling is not required for embryonic development of hair follicles (i.e. initial induction, specification and differentiation), it does have a function in maintenance of follicular morphology (Pan et al., 2004) and hair follicle terminal differentiation (Blanpain et al., 2006). A complete loss-of-Notch signaling in hair follicle keratinocytes causes failure of IRS cellular identity as well as aberrant activation of the epidermal differentiation program in ORS cells, ultimately leading to the conversion of hair follicles into epidermal cysts (Pan et al., 2004).
Besides these functions in the mature hair follicles, Notch controls the expression of Wnt5a through canonical signaling in dermal papilla cells (Hu et al., 2010). Wnt5a from dermal papilla cells acts on hair keratinocytes and turns on FoxN1 (Hu et al., 2010), a key hair follicle differentiation gene (Mecklenburg et al., 2001).

**Consequences of loss-of-Notch Signaling in the epidermal keratinocytes**

To investigate the role of Notch signaling during mouse epidermal and hair follicle morphogenesis, we have utilized Msx2-Cre transgene-mediated deletion of Notch signaling components in skin keratinocytes (Pan et al., 2004; Lee et al., 2007; Demehri et al., 2008). Msx2 minimal promoter-controlled expression of Cre starts around E9.5 (Sun et al., 2000) within a cluster of cells in the primitive ectoderm, generating a chimeric pattern of deletion in the dorsal and ventral midline (Figure 1-4A; Demehri et al., 2008; Demehri et al., 2012).

Previous studies showed that keratinocyte-specific deletion of total Notch signaling causes differentiation defects in the epidermis (Figure 1-4B&C; Rangarajan et al., 2001; Pan et al., 2004; Blanpain et al., 2006; Demehri et al., 2008) and impaired hair follicle morphology manifested by loss of IRS cellular identity and activation of epidermal differentiation program in ORS cells (Pan et al., 2004; Demehri et al., 2008). These skin perturbations collectively induced the expression of thymic stromal lymphopoietin (TSLP) in suprabasal keratinocytes (Figure 1-4D; Demehri et al. 2008) and in defective ORS cells within the epidermal cysts (Figure 1-4E; Demehri et al., 2008). TSLP is a cytokine that has critical roles in immune cells during T-cell development and maturation (Omori et al., 2007; Rochman et al., 2008), B-cell development and differentiation (Scheeren et al., 2010), and activation of antigen presenting cells (Ziegler et al., 2013). Secretion of TSLP into the systemic circulation induces proliferation of pre-B cells originating from the fetal liver (Demehri et al., 2008). Eventually this hyperproliferation of
immature B cells results in B-cell Lymphoproliferative Disease (BLPD) in mutant animals, leading to their death around 30 days (Figure 1-4G&F; Demehri et al., 2008).

As canonical Notch signaling is thought to be a linear pathway, one would expect that the loss of γ-secretase (no NICD release), Notch receptors (no NICD) or RBPjk (no activation of downstream targets) should generate similar phenotypes. Unexpectedly, keratinocyte-specific deletion of Rbpj gene (RBPjcKO) produced a significantly milder phenotype than the keratinocyte-specific deletion of both Notch1 and Notch2 genes (N1N2dcKO) or both Presenilin1 and Presenilin2 genes (PSdcKO). Measurable differences in four phenotypes have been observed (Demehri et al, 2008).

1. **Lifespan:** N1N2dcKO and PSdcKO animals die around P25-P30, while RBPjcKO animals can live on average for 150 days. (Figure 1-4G)

2. **White Blood Cell (WBC) Counts:** N1N2dcKO and PSdcKO animals have 100k/ul WBC counts, whereas RBPjcKO animals have significantly lower (~25k/ul) WBC counts (Figure1-4G).

3. **Serum TSLP Levels:** Both N1N2dcKO and PSdcKO animals have very high serum TSLP levels (>5ng/mL) on average; however, RBPjcKO animals exhibit significantly lower (~0.65ng/mL) serum TSLP levels (Figure 1-4G).

4. **Total skin morphology** of RBPjcKO animals is significantly different from that of the N1N2dcKO or PSdcKO animals (Figure 1-4G). RBPjcKO skins contain less hair follicle cysts and immune-cell infiltration into the mutant skin is much less compared to the other mutants.

Based on these differential phenotypes, we hypothesized that RBPjk-independent signals may be responsible for these differences. We investigated the possibility of non-canonical Notch
signaling playing a role in these phenotypic differences. In Chapter II, we will characterize the N1N2dcKO, PSdcKO, and RBPjcKO mutants and showed that at birth, they are similar and have no significant differences in the gross morphology of their epidermis and hair follicles as well as mRNA and protein expression pattern. In Chapter III, we will dissect the Notch signaling pathway to find the necessary elements for the RBPjk-independent phenotype and showed that γ-secretase generated Notch1ICD molecule is necessary and sufficient to trigger a milder phenotype in RBPjcKO animals. In Chapter IV, we will provide evidence that levels of epidermal TSLP expression are similar in all Notch mutants. RBPjcKO hair follicles are less distorted and hence produce less TSLP. This, in turn, leads to less serum TSLP levels in RBPjcKO animals. A milder BLPD allows them to live significantly longer than N1N2dcKO and PSdcKO animals. In conclusion, NICD molecules likely stimulate RBPjk-independent signals in the Rbpj−/− hair follicles thereby causing a milder distortion hair follicle phenotype in RBPjcKO animals.
REFERENCES:


Kurooka H. and Honjo T. (2000) **Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5.** *J. Biol. Chem.* 275, 17211-17220


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Zhou S and Hayward SD. (2001) **Nuclear localization of CBF1 is regulated by interactions with the SMRT corepressor complex.** Mol. Cell. Biol. 21, 6222-6232

Mammalian DSL Ligands

Mammalian Notch Receptors

Figure 1-1
Both Notch receptors and DSL ligands are expressed on the cell surface. They contain EGF-like repeats in the extracellular domain (ECD). In addition, receptors have Lin12-Notch repeats (LNR) and heterodimerization domain (HD) in the ECD. S2-cleavage is mediated by ADAM-TACE family proteins, whereas S3 (and S4) cleavage(s) are mediated by γ-secretase complex. The intracellular domain (ICD) of the receptor consists of RBPjκ associated module (RAM), nuclear localization signals (NLS), ankyrin/cdc10 (ANK) repeats, transactivation domain (TAD), and Pro-Glu-Ser-Thr rich (PEST) domain. Note that Notch3 & Notch4 receptors do not carry TAD and Notch4 only has one NLS.

All ligands have a DSL domain in the ECD, whereas only Jagged1 and Jagged2 contain cysteine-rich domains proximal to the transmembrane domain. Jagged1, Dll1, and Dll4 ligands carry a PDZ* domain in the ICD of the ligands.

PDZ*: post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
Figure 1-2

Notch Target Genes

CoR

P300

GCN5

Cytoplasm

Nucleus

Notch

Delta/Jagged

Receptor Presenting Cell

Ligand Presenting Cell

γ-secretase

ADAM/TACE

S2 Cleavage

S3 Cleavage

S4 Cleavage

SKIP

SMRT

HDAc

RBPjk

PCAF

RBPjk

GCN5

MAML

P300

RBPjk
Figure 1-2. Overview of Canonical Notch Signaling Pathway.

Activation of Notch receptors triggered upon interaction with Notch ligands (Delta/Serrate family proteins) from the cell surface of the adjacent cells. Following ligand binding, receptors undergo conformational change which allows ADAM/TACE family proteins to access the S2 cleavage site within the HD. S2-cleavage generates the substrate for γ-secretase complex, which further cleaves the remaining receptor fragment at the S3 and S4 sites. This final cleavage liberates the NICD and allows translocation to the nucleus. In the nucleus, NICD finds its DNA binding partner, RBPjk, which normally functions as a repressor. NICD-RBPjk interaction displaces co-repressors and recruits coactivators leading to canonical target gene expression.
Surface Ectoderm (K8 & K18)
Basement Membrane
Periderm (K6 & K17)
Progenitor Basal Keratinocytes (K5 & K14)
Spinous layer cells (K1 & K10)
Granulocytes
(Loricrin, Filaggrin, & transglutaminase)
Cornified Layer
Granular Layer
(Loricrin, Filaggrin, & transglutaminase)
Spinous Layer (K1 & K10)
Basal Layer (K5 & K14)

E8.5-E9.5
E9.5-E13.5
E13.5-E16.5
E16.5-E18.5
E18.5

Figure 1-3
Figure 1-3. Expression pattern of Notch proteins during development of mammalian epidermis and hair follicles.

(A) Overview of mammalian skin and its appendages (adapted from Fuchs and Raghavan, 2002). (B) Schematic view of mouse embryonic epidermal development stages. (C) Schematic view of mouse primary hair follicle morphogenesis (modified from Paus et al., 1999). (D) Expression pattern of Notch1 (N1), Notch2 (N2), and Notch3 (N3) receptors in mouse epidermis at E13.5 (Courtesy of Dr. Zhenyi Liu from Kopan Lab). (E) Expression pattern of N1, N2, and N3 receptors in mouse epidermis at E15.5 (modified from Romano et al., 2012) (F) Expression pattern of Notch receptors and their ligands (Jagged1 (J1) and Jagged2 (J2)) during mouse hair follicle development. Co-expressions are designated with shaded areas (adapted from Watt, Estrach, & Ambler, 2008). (G) Expression patterns of Notch pathway members and some its targets during hair follicle growth in mouse (adapted from Aubin-Houzelstein, 2012). HS: hair shaft; IRS: inner root sheath; Me: medulla; Co: cortex; Ct: cuticle; Hu: Huxley layer; He: Henle layer; ORS: outer root sheath.
Figure 1-4

A. Msx2-Cre/+; Rosa26R

B. WT

C. K1/K14

D. DAPI / TSLP

E. TSLP

F. Notch1−/

G. Lifespan

WT  PSdcKO  RBPjcKO

WBC Counts

Serum TSLP Levels

WT  PSdcKO  RBPjcKO

WT  PSdcKO  RBPjcKO

N1N2dcKO  PSdcKO  RBPjcKO

WT  N1N2dcKO  PSdcKO  RBPjcKO
**Figure 1-4. Consequences of loss-of-Notch signaling in skin keratinocytes.**

(A) Activity and expression pattern of Msx2-Cre. Left: X-gal staining of an E15.5 Msx2-Cre +/-tg; Rosa26R embryo (adapted from Demehri et al. 2008). Right: live immunofluorescent imaging of newborn Msx2-Cre +/-tg; RosaEYFP pup. (B) H&E staining for newborn WT and PSdcKO skin samples. Due to the differentiation defects mutant skin has hyperproliferative epidermis. (C) Immunofluorescent staining for epidermal differentiation markers (K14, K1, Loricrin, Filaggrin) and integrins (β1 and β4) of WT and Notch1^-/- epidermis (21 days post OHT treatment). Notch1^-/- epidermis exhibits aberrant differentiation markers and integrin expression. Bar: 8 µm. (adapted from Rangarajan et al. 2001) (D) Suprabasal cells (adapted from Demehri et al., 2008) and (E) defective ORS cells that are converted into epidermal cysts (adapted Demehri et al., 2008) are positive for TSLP protein in the Notch signaling deficient epidermis and hair follicle, respectively. (F) Diagram depicting the consequences of events that are happening upon loss-of-total Notch signaling in the skin keratinocytes: (i) differentiation defects and impaired lipid biosynthesis. (ii) the integrity of the skin is abolished and lead to barrier defects (iii) Suprabasal cells and epidermal cells within the hair follicle cysts start to express TSLP which induces proliferation in fetal-liver originating pre-B cells. (iv) Expansion of immature B-cells leads to BLPD disease, which results in the death of animals by P30 (adapted from Demehri et al, 2008). (G) RBPjcKO animals are significantly different than other total-loss-of-Notch signaling mutants (N1N2dcKO & PSdcKO). These differences manifest in four different measures: (i) Lifespan (ii) WBC counts (iii) Serum TSLP levels and (iv) skin morphology.
<table>
<thead>
<tr>
<th>Lipid</th>
<th>Lipid structure</th>
<th>Notch-binding region of ligand</th>
<th>Ligand-binding region of Notch</th>
<th>Effect on Notch signalling</th>
<th>Proposed effector(s) of Notch signalling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dikk-1/Prex-1</td>
<td>EGF-like (6 oys)</td>
<td>EGF1-2 or EGF6-6</td>
<td>EGF10-41 or EGF12-13</td>
<td>cis-inhibition/ trans-activation?</td>
<td>CSL</td>
</tr>
<tr>
<td>DNER</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CSL or Delta</td>
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<tr>
<td>Jedl</td>
<td></td>
<td></td>
<td></td>
<td>Inhibitrin (no secreted protein)</td>
<td>CSL</td>
</tr>
<tr>
<td>F3/Contactin6</td>
<td>IgCAM</td>
<td>Full-length*</td>
<td>EGF1-13, EGF22-34</td>
<td>trans-activation</td>
<td>Detex</td>
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<tr>
<td>NB3/Contactin6</td>
<td></td>
<td>Full-length*</td>
<td>EGF22-34</td>
<td>trans-activation</td>
<td>Detex</td>
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</tbody>
</table>

**Table 1-1**
Table 1-1. Overview of non-canonical Notch ligands and their effects on Notch Signaling
(adapted from D'Souza et al. 2008)

Non-canonical ligands are structurally diverse and do not possess DSL domain (Delta/Serrate/LAG-2). Some of them include integral- and GPI-linked membrane proteins as well as secreted proteins.

EGF-like (6 cys), 6-cysteine epidermal growth factor-like repeats; cys, cysteine; EMI, emilin-like domain; Ig-CAM, immunoglobulin-containing cell adhesion molecule domain; FNIII, fibronectin type III domain; GPI, glycosylphosphatidylinositol; Q, glutamine-rich region; FReD, fibrinogen-related domain; DOS, Delta and OSM-11-like proteins; IGFBP, insulin-like growth factor-binding protein-like domain; VWF-C, von Willebrand factor type C-like domain; TSP-1, thrombospondin type 1-like domain; CTCK, C-terminal cysteine knot domain; MBD, matrix binding domain; RGD, integrin-binding motif; NT, N-terminal domain; CSD, cold shock domain, N1, Notch1; N2, Notch2; N3, Notch3; N4, Notch4.

*Only full-length constructs were tested for binding

**Agonist of Jagged1 signaling

***Antagonist of Jagged1 signaling

**** Agonist of Dll4 (Delta-like 4) signaling
CHAPTER II

The phenotypes in the embryo and newborn animals
are due to the canonical Notch signaling

Publication & Copyright
Part of Figure 2-1 have been previously published in:

Author Contribution
The author performed all the experiments in this chapter except microarray and mass-spectroscopy experiments, which are carried by Genome Technology Access Center Core and Proteomics Core in the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis, MO.

The author wrote the text and prepared the figures in this chapter.
**SUMMARY**

Notch signaling plays a crucial role during epidermal differentiation and hair follicle maintenance. Demehri et al. (2008) showed that keratinocyte-specific deletion of the *Rbpj* (RBPjcKO) gene produced a significantly milder phenotype than keratinocyte-specific deletion of both Notch1 and Notch2 (N1N2dcKO) or both Preselin1 and Preselin2 (PSdcKO) genes, suggesting an RBPjκ-independent function of Notch signaling in the skin. To test the possibility whether RBPjκ-independent Notch function contributes to the differences between RBPjcKO and PSdcKO animals, I performed transcriptome (E18.5) and proteome (P0) analysis of the epidermis. Surprisingly, the analyses revealed no difference between *Rbpj*−/− and *PS*−/− epidermis. In addition, microarray analysis for newborn total skin samples from RBPjcKO and PSdcKO animals produced an indistinguishable signature at birth. Taken together, these results showed that the phenotypes in the embryo and newborn animals are due to the canonical Notch signaling suggesting that potential RBPjκ-independent Notch function occurs after birth.
INTRODUCTION

Epidermal development starts around E8.5 - E9 from a single layer of ectodermal cells in the mouse embryo. By E13, K5+ and K14+ progenitors cover the entire embryo and initiate the epidermal differentiation program. Notch signaling is essential for cell cycle exit and keratinocyte differentiation (Lowell et al., 2000; Rangarajan et al., 2001; Nickoloff et al., 2002; Blanpain et al., 2006; Moriyama et al., 2008). During the early stage of epidermal differentiation, activated Notch1 in basal cells inhibits growth by activating $p21^{WAF/Cip1}$ expression and downregulation of integrin $\alpha3/\beta1$ and $\alpha6/\beta4$ expression (Rangarajan et al., 2001). In spinous cells, Notch activation cause upregulation of early differentiation markers such as involucrin, K1 and K10 and suppression of late markers, loricrin and filaggrin (Rangarajan et al., 2001; Nickoloff et al., 2002; Blanpain et al., 2006).

Interestingly, Notch signaling is not essential for hair follicle induction, invagination and formation of hair peg and hair bulb. Keratinocyte-specific Notch pathway conditional-knock-out (cKO) animals do not display differences in the number or distribution of hair follicles compared to wild-type (WT) animals (Lin et al., 2000; Yamamoto et al., 2003; Pan et al., 2004; Uyttendaele et al., 2004; Vauclair et al., 2005; Blanpain et al., 2006; Estrach et al., 2006; Moriyama et al., 2006; Ambler and Watt, 2007; Lee et al., 2007; Estrach et al., 2008; Demehri et al., 2009). Furthermore, the loss-of-Notch pathway components does not interfere with the expression patterns of other signaling pathways (e.g. Wnt/β-catenin, FGF, Shh, TGF-β/BMP and Eda/Edar/NF-κB) that participate in early placode formation and/or embryonic hair follicle development (Vauclair et al., 2005). Although embryonic hair follicles can form without Notch signaling, Notch signaling functions in the maintenance of follicular morphology (Pan et al., 2004) and in hair follicle terminal differentiation (Blanpain et al., 2006). Complete loss-of-Notch
signaling induces conversion of hair follicles into epidermal cysts, due to the loss of cellular identity in IRS cells, and the activation of aberrant epidermal differentiation program in ORS cells (Pan et al., 2004).

The role of Notch signaling during skin development was analyzed using Msx2-Cre to create mosaic loss-of-function alleles with precise temporal and spatial resolution (Pan et al., 2004; Demehri et al., 2008). We have previously shown that Msx2-Cre-mediated skin keratinocyte-specific deletion of Notch1 and Notch2 genes (or Presenilin1 and Presenilin2 genes) leads to a significantly more severe phenotype compared to that of an Rbpj gene deletion (Demehri et. al., 2008). N1N2dcKO and PSdcKO animals exhibit higher serum TSLP levels (> 5 ng/mL) compared to RBPjcKO animals (Demehri et. al., 2008) translating into a higher white blood cell (WBC) counts. Their life expectancies are also dramatically different. N1N2dcKO and PSdcKO animals die around 30 days, but RBPjcKO animals live as long as 300 days (Figure 1-4G; Demehri et. al., 2008).

RBPjκ, the DNA-binding partner of all NICDs, mediates the transcriptional effects of Notch signaling. DSL ligand-dependent activation, γ-secretase cleavage, and RBPjκ-mediated transcriptional activity of Notch receptors define the “canonical” Notch signaling pathway (Hsieh et al., 1995; Furukawa et al., 1995; Jarriault et al., 1995). Therefore, deletion of Rbpj gene should result in a complete loss of canonical Notch signaling as in N1N2dcKO and PSdcKO animals. However, the milder skin phenotype of RBPjcKO animals suggests that there might be a function for RBPjκ-independent Notch signaling. Before investigating the RBPjκ-independent Notch function in skin keratinocytes, I tested the possibility that the milder RBPjcKO phenotype is not due to trivial explanations such as incomplete deletion of the Rbpj gene, incomplete elimination of the protein, or longer half-life of the RBPjκ protein, which leads to an “epigenetic
memory” causing a milder phenotype in RBPjeKO animals.
RESULTS AND DISCUSSION

**RBPjκ** protein is eliminated in *Rbpj<sup>−/−</sup>* keratinocytes.

To confirm that RBPjκ protein was depleted from skin keratinocytes, Demehri et al. (2008) performed RBPjκ immunohistochemistry on RBPjcKO skin at P0 and showed that RBPjκ protein was undetectable in epidermal keratinocytes (Figure 2-1A; Demehri et al., 2008). I confirmed these results in P9 animals (Figure 2-1A). Because these experiments relied on an antibody (T6709) specific for the C-terminus of the RBPjκ protein, we could not rule out the possibility that functional truncated proteins were generated from deleted *Rbpj<sup>floxed</sup>* (*Rbpj<sup>del</sup>*) allele. Unfortunately, this is the only available antibody known to detect mouse RBPjκ protein for immunohistochemistry. To examine if functional truncated protein(s) are made from the *Rbpj<sup>del</sup>* allele, I performed the following experiments.

**Rbpj<sup>del</sup>** gene is functionally null.

*Rbpj<sup>floxed</sup>* allele was constructed by inserting the floxed sites flanking exons 6 and 7 (Figure 2-1B; Han et al., 2002; Tanigaki et al. 2002) such that the regions encoding the DNA-binding and NICD-interacting domains of RBPjκ protein can be excised upon recombination. Thus, any potential RBPjκ<sup>del</sup> protein should not have any DNA binding and transcription activation ability. However, it has never been tested whether the *Rbpj<sup>del</sup>* gene is transcribed or whether a potential mutant protein still has some residual activity. To test whether *Rbpj<sup>del</sup>* gene is transcribed upon recombination, total mRNA from *Msx2-Cre*/tg; *Rbpj<sup>floxed/+</sup>* and wild-type (wt) epidermis were collected. I performed reverse transcription reaction to generate cDNA. Following cDNA production, I ran polymerase chain reactions (PCR) to assess whether *Rbpj<sup>del</sup>* cDNA is expressed. I was able to detect *Rbpj* cDNA lacking exons 6 and 7 (Figure 2-1C) establishing transcription of *Rbpj<sup>del</sup>* mRNA in skin keratinocytes. Sequencing the *Rbpj<sup>del</sup>* cDNA
confirmed that, in the absence of exons 6 and 7, exon 5 in $Rbpj^{del}$ mRNA was directly spliced to exon 8 and transcribed through to exon 11, which was the last exon in the $Rbpj^{wt}$ mRNA (Figure 2-1C). This resulted in a frameshift and a premature stop codon at beginning of exon8, leading to a truncated protein if no read-through occurs (Figure 2-1C).

I tested for potential residual activities in the putative products of $Rbpj^{del}$ transcripts by generating three expression vectors that mimic the expression of wild-type and mutant versions of $Rbpj$ mRNA (Figure 2-1D). In the first vector ($RBPj^{wt}$), the mouse $Rbpj203$ isoform, the predominant isoform, is expressed under CMV promoter and N-terminally tagged with 3xFLAG (Figure 2-1D). The second vector ($RBPj^{Δ67}$) is designed to express the transcript generated from $Rbpj^{del}$ alleles upon recombination. Although, there is a premature stop codon at exon8, this vector allowed us to test whether any protein produced from endogenous $Rbpj^{del}$ locus has any transcriptional activity (Figure 2-1D), particularly if read-through occurs. The third vector ($RBPj^{58S}$) is designed to express only the truncated mutant RBPjk protein that terminates at exon8 (Figure 2-1D).

To determine whether the potential truncated RBPjk proteins have any transcriptional activity, all the experiments were done in the OT11 ($RBPj^{−/−}$) cells (Kato et al., 1997). I co-expressed either $RBPj^{wt}$, $RBPj^{Δ67}$, or $RBPj^{58S}$ vectors with activated Notch1 receptor (Notch1$^{ΔE}$) and a Notch-responsive reporter (e.g. TP-1 luciferase reporter, 4xCSL luciferase reporter or HES-1 luciferase reporter). Co-expression of responsive $RBPj^{wt}$ and Notch1$^{ΔE}$ strongly induced activation of the TP-1 reporter by up to 100-fold; however, neither $RBPj^{Δ67}$ nor $RBPj^{58S}$ was able to activate the TP-1 reporter in the presence of Notch1$^{ΔE}$ (Figure 2-1E). Similar results were obtained with Hes1 (Figure 2-1E), 4xCSL, and Hes5 reporters (data not shown).

Based on the crystal structure, a portion of the DNA-binding domain of RBPjk resides
within the first five exons of the \textit{Rbpj} gene. To test whether the \textit{Rbpj}^{del} protein has any residual DNA binding ability, I designed a vector that expresses the mutant \textit{Rbpj}^{58} protein fused to a potent transactivation domain, VP16, to generate the \textit{Rbpj}^{58-VP16} fusion protein (Figure 2-1D). We know when VP16 domain is fused to \textit{Rbpj}^{wt} protein (Figure 2-1D); the fusion protein, \textit{Rbpj}^{wt-VP16}, can activate TP-1 reporter 200 folds even without the expression of Notch1^{AE} (Figure 2-1F). If \textit{Rbpj}^{58} has DNA binding ability, then \textit{Rbpj}^{58-VP16} vector should able to activate Notch-responsive reporters. Expression of Notch1^{AE} synergizes with \textit{Rbpj}^{wt-VP16} to activate the TP-1 reporter by up to 800-fold (Figure 2-1F). However, \textit{Rbpj}^{58-VP16} vector cannot activate TP-1 or 4xCSL reporter, alone or in the presence of Notch1^{AE} (Figure 2-1F).

To determine whether potential \textit{Rbpj}^{del} mutant protein can interfere with \textit{Rbpj}^{wt} or NICD^{wt} protein activity, I expressed \textit{Rbpj}^{58S} vector with \textit{Rbpj}^{wt-VP16} vector. Co-expression of \textit{Rbpj}^{58S} vector with \textit{Rbpj}^{wt-VP16} vector did not induce any significant reduction in the activation of TP-1 or 4xCSL reporter (Figure 2-1G). Similarly, overexpression of \textit{Rbpj}^{58S} together with Notch1^{AE} and \textit{Rbpj}^{wt} did not interfere with NICD activity to induce TP-1 or 4xCSL reporter (Figure 2-1G) suggesting that truncated \textit{Rbpj}^{del} protein cannot compete with \textit{Rbpj}^{wt} protein neither for DNA-binding nor NICD interaction.

These results indicate that the putative \textit{Rbpj}^{del} proteins produced from the \textit{Rbpj}^{del} alleles cannot activate Notch responsive reporter genes, either in the absence or presence of activated Notch receptor. Moreover, \textit{Rbpj}^{del} protein does not interfere with the function of \textit{Rbpj}^{wt} or NICD. Collectively, these results indicate that \textit{Rbpj}^{del} allele is a null allele and any potential protein that is made from this allele is not able to activate canonical Notch targets.
**RBPjk protein has 65-hour half-life.**

Another possible explanation for the differences among the total loss of Notch signaling mutants could be due to the “epigenetic memory” that is established through if the perdurance of RBPjk protein carries it through a critical developmental window. Conditional deletion of $Rbpj_{\text{floxed}}$ gene starts at E9.5 (Sun et al., 2000), 4 days before the induction of epidermal differentiation (Figure 2-2A). During the stratification (E13.5 - E18.5), any RBPjk protein that has not yet been degraded could result in activation of some canonical Notch pathway target genes. These targets can trigger an “epigenetic memory” in $Rbpj^{-/-}$ progenitors ultimately leading to the milder RBPjcKO phenotype.

To test this possibility, I measured the half-life of the RBPjk protein in mouse keratinocytes in vitro. I cultured epidermal keratinocytes isolated from $Rbpj_{\text{floxed/floxed}}$ animals. When keratinocytes reached to 80-90% confluency, I treated them with Adeno-Cre or Adeno-GFP. I started to observe loss of $Rbpj^{\text{wt}}$ mRNA within 12h post-infection and by 24h around 95% of the wt mRNA was eliminated from the keratinocytes (Figure 2-2B). By 60h after Adeno-Cre infection, 95% of the $Rbpj$ mRNA expressed corresponded to the $Rbpj^{\text{del}}$ (Figure 2-2B). I collected cell lysates for western blot analysis at multiple timepoints following infection. I did not observe a significant change in total RBPjk protein amount within 24h post-infection (data not shown). When I performed western blotting for lysates at later timepoints (40h-118h), I found that RBPjk protein had a very long perdurance (Figure 2-2C). The half-life of RBPjk protein was calculated to be around 65-hour in mouse keratinocytes (Figure 2-2D). In conclusion, there is a possibility that some of RBPjk protein still be present during stratification which can cause an “epigenetic memory” in $Rbpj^{-/-}$ keratinocytes and this memory can alter gene
expression in RBPjcKO epidermis. To test this possibility, I analyzed the epidermal transcriptomes of RBPjcKO, PSdcKO, and WT animals at E18.5, the results for which are described in the following section.

**No significant differences between Rbpj<sup>−/−</sup> and PS<sup>−/−</sup> epidermal transcriptomes at E18.5**

To address whether perdurance of RBPjκ protein caused an “epigenetic memory” in Rbpj<sup>−/−</sup> epidermal stem cells, we performed global gene expression profile analysis on total RNA samples extracted from E18.5 RBPjcKO, PSdcKO, WT<sup>RBPj</sup> (WT littermates of RBPjcKO animals) and WT<sup>PS</sup> (WT littermates of PSdcKO animals) epidermis. Three samples per genotype were examined. After labeling, samples were hybridized on Illumina MouseWG-6 v2.0 Expression BeadChip arrays. Samples from mutant animals (RBPjcKO and PSdcKO) were compared to each other and to their wild-type littermates.

I generated a heatmap and performed unsupervised hierarchical clustering to identify the similarities and/or dissimilarities between genes and between samples (Figure 2-2E). The dendrogram analyses revealed that samples from RBPjcKO epidermis cluster together with PSdcKO epidermal samples indicating that they were more similar to each other compared to the WT samples (Figure 2-2E). Then, I systematically characterized the amount of repression or induction for each gene for every sample represented in the microarray. A total number of 183 genes were significantly differentially expressed in Rbpj<sup>−/−</sup> and PS<sup>−/−</sup> epidermis (Table 2-1, Table 2-2, Table 2-3).

To further evaluate the potential relevance of differentially expressed genes in Rbpj<sup>−/−</sup> and PS<sup>−/−</sup> epidermis, I performed gene ontology (GO) and pathway enrichment analyses using GOminer (Zeeberg et al. 2003), GOstat (Beissbarth and Speed, 2004), GOtoolbox (Martin et al. 2004), DAVID (Huang et al. 2009) and TOPPFUN (Chen et al., 2009) analysis tools. All of
these analyses found no significant enrichment of GO terms relating to biological process, cellular component, or molecular function in the gene sets, suggesting that there were no major differences between gene expression patterns of \( \text{Rbpj}^{/-} \) and \( \text{PS}^{/-} \) epidermis.

**RBPjcKO and PSdcKO epidermal proteome are similar at birth**

Microarray analyses suggest that the gene expression patterns of \( \text{Rbpj}^{/-} \) and \( \text{PS}^{/-} \) epidermis are very similar at E18.5. This result raised the possibility that the phenotypic differences between RBPjcKO and PSdcKO animals could be due to differences at the protein level (e.g., post-transcriptional or post-translational modifications). To investigate this possibility, we have performed a proteomics analysis to identify the differentially expressed proteins in \( \text{Rbpj}^{/-}, \text{N1N2}^{/-}, \) and \( \text{PS}^{/-} \) epidermis from newborn animals.

I collected epidermal samples from newborn RBPjcKO, N1N2dcKO, PSdcKO, and WT animals. To avoid possible background effects, WT samples (WT\text{pool}) were generated by pooling one sample each of WT\text{RBPj}, WT\text{N1N2}, and WT\text{PS} epidermis. Three replicates per genotype were examined by mass spectrometry. Epidermis samples from RBPjcKO, N1N2dcKO and PSdcKO animals were compared to each other and to the wild-type epidermis pool.

We generated a heatmap and performed unsupervised hierarchical clustering to identify the similarities and/or dissimilarities between samples (Figure 2-3A). All replicates from the same genotypes clustered together tightly confirming the precision of the experimental procedure. Furthermore, samples from the \( \text{Rbpj}^{/-} \) epidermis clustered together with the \( \text{PS}^{/-} \) epidermal samples and were separated from N1N2dcKO and WT samples in the dendrogram analyses (Figure 2-3A) indicating that RBPjcKO epidermis was similar to PSdcKO epidermis in terms of protein expression profile. Surprisingly, N1N2dcKO epidermis clustered closer to the WT\text{pool} consistant with a functional role for Notch3 in the epidermal protein expression profile.
(N1N2dcKO animals still have Notch3-dependent canonical signaling).

Following dendrogram analysis, we further characterized the changes in abundance of peptides. Due to the technical limitations, we only analyzed proteins that were detected by mass spectrometry. A total number of 195 peptide sequences were significantly changed in abundance when \(Rbpj^{-/-}\) epidermis compared to \(N1N2^{-/-}\) or \(PS^{-/-}\) epidermis (Table 2-2). After careful evaluation of the data, we found no significant enrichment of proteins/pathways that could be responsible for phenotypic differences between RBPjcKO and PSdcKO (or N1N2dcKO) animals. We also examined differences in the post-translational modifications (e.g. phosphorylation, sumoylation, palmitoylation, and ubiquitination) on the peptides represented in the analysis, however we could not find a significant difference.

In summary, both E18.5 epidermal transcriptome and P0 proteome analyses suggest that \(Rbpj^{-/-}\) epidermis is very similar to \(PS^{-/-}\) (or \(N1N2^{-/-}\)) epidermis at birth.

There are no significant differences between RBPjcKO and PSdcKO total skin transcriptome in newborn pups

E18.5 epidermal transcriptome and P0 epidermal proteome analyses demonstrated that \(Rbpj^{-/-}\) epidermis was similar to \(PS^{-/-}\) (or \(N1N2^{-/-}\)) epidermis at birth. Both of these analyses focused on the epidermis, however Notch deletion was not limited to the epidermis. Msx2-Cre-mediated deletion also occurred within hair follicle keratinocytes. Therefore, there is a possibility that the differences in phenotypes between RBPjcKO and PSdcKO (or N1N2dcKO) animals could have resulted from differences in the hair follicle keratinocytes.

To address this, we performed microarray analysis on RNA samples isolated from total skin of RBPjcKO, PSdcKO, PSRBPjcKO and WT (WT littermates of RBPjcKO and PSdcKO animals) animals at P0. Demehri et al. (2008) previously showed that PSRBPjcKO animals
resembled PSdcKO animals in terms of lifespan, WBC counts, and serum TSLP levels. Four samples per genotype were examined. Samples were hybridized on Illumina MouseRef-8 v2.0 Expression BeadChip arrays following labeling. Mutant samples (RBPjcKO, PSdcKO, and PSRBpjcKO) were compared to each other and to their WT littermates.

To identify the similarities and/or dissimilarities between samples and between genes, we performed unsupervised hierarchical clustering and heatmap analysis (Figure 2-3B). Samples from WT epidermis clustered together to each other, however all other mutant samples interspersed each other (Figure 2-3B). We further evaluated the changes in gene expression in \( Rbpj^{-/-}, PS^{-/-}, PSR^{-/-}, \) and WT total skin samples. These analyses showed that 22 genes were found differentially expressed in \( Rbpj^{-/-} \) skin compared with those in \( PS^{-/-} \) and \( PSR^{-/-} \) skin (Table 2-2).

To determine the functional similarities among these gene products, we performed GO analysis using GOminer (Zeeberg et al. 2003), GOstat (Beissbarth and Speed, 2004), GOtoolbox (Martin et al. 2004), and DAVID (Huang et al. 2009). We were unable to identify a significant enrichment for the gene set indicating that RBPjcKO skin is similar to PSdcKO or PSRBpjcKO skin.
CONCLUSIONS

RBPjcKO animals have significantly different phenotypes compared with the N1N2dcKO and PSdcKO animals (Demehri et al., 2008). We demonstrated that canonical Notch signals are responsible for the Notch function during epidermal differentiation and hair follicle maintenance in the embryo. Both E18.5 epidermal transcriptome and P0 epidermal proteome analysis found no difference between RBPjcKO and PSdcKO epidermis. Moreover, global gene expression profile analysis for newborn total skin samples suggested that RBPjcKO and PSdcKO skin transcriptomes are indistinguishable at birth. All of these experiments suggested that there is no evidence of “epigenetic memory” induced in RBPjcKO animals and canonical Notch signals are responsible for the mutant phenotypes in the embryo.
EXPERIMENTAL PROCEDURES

Generation of mice

I have generated the following strains of mice using Msx2-Cre transgene-mediated deletion in skin keratinocytes (Pan et al., 2004). Animals were raised and kept in the Washington University Division of Comparative Medicine facility under Washington University Policy on Animal Care regulations.

Msx2-Cre \( ^{+/tg} \); Rbpj \( ^{floxed/floxed} \) (RBPjcKO)

Msx2-Cre \( ^{+/tg} \); Notch1 \( ^{floxed/floxed} \), Notch2 \( ^{floxed/floxed} \) (N1N2dcKO)

Msx2-Cre \( ^{+/tg} \); Presenilin1 \( ^{floxed/floxed} \), Presenilin2 \( ^{-/-} \) (PSdcKO)

Msx2-Cre \( ^{+/tg} \); Presenilin1 \( ^{floxed/floxed} \), Presenilin2 \( ^{-/-} \); Rbpj \( ^{floxed/floxed} \) (PSRBPjcKO)

Histology and H&E Staining

Skin samples were fixed in 4% paraformaldehyde (PFA) in PBS for overnight (o/n), washed 3x with PBS and washed for 15 min with 30% Ethanol (EtOH) and 50% EtOH, and then stored in 70% EtOH at 4°C. Skin samples were embedded in paraffin for sectioning at 8-12\( \mu \)m thickness. A standard H&E staining protocol was used for histology assessment.

Immunohistochemistry

Expression of the RBPjκ protein was detected on 4% PFA fixed paraffin sections with \( \alpha \)-RBPjκ rat monoclonal antibody T6709 (1:500, Cosmo Bio Co., Ltd) for o/n at 4°C. After o/n incubation with the primary antibody, several washes (3 x 5 min in 0.5% Triton X-100 in PBS) were done before a 2h-incubation with an anti-rat HRP-coupled secondary antibody. Horseradish-peroxidase-conjugated streptavidin and DAB substrate kit (Pierce) were used to visualize the signal. Sections were weakly counterstained with methyl green.
RNA isolation, cDNA preparation and quantitative RT-PCR

Skin samples were incubated in 5 mg/mL dispase for 3 hours at 4°C to separate the epidermis from the dermis. Total RNA was isolated from the E18.5 epidermis, P0 total skin, and keratinocytes cultured in vitro using TRIzol (Invitrogen) kit or RNeasy kit (Qiagen) according to the manufacturer's instructions. RNase-free DNase I (Invitrogen, USA) was used to remove any residual genomic DNA. RNA was dissolved in 30µl of RNase-free water and stored at -80°C after RNA quality control (gel electrophoresis) and concentration measurements. Reverse transcription was performed with 1µg of total RNA, random primers and SuperScript III RNase H-Reverse Transcriptase (Invitrogen) in a total volume of 20µl. Expression levels of transcripts were examined by real-time qPCR in a 7500 Real-Time PCR System using SYBR Green dye (Applied Biosystems), iQ SYBR Green Supermix (Bio-Rad) or TaqMan Probes. The results were analyzed by absolute quantification with a relative standard curve or relative quantification of expression according to the “ΔΔC methods” (Livak and Schmittgen, 2001) using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference for normalization. Primers were designed to amplify targets of 180–220 bp usually at the exon junctions to avoid amplification from genomic DNA contamination. The specificity of the PCR reactions was checked by recording a melting curve and by sequencing the amplicons.

Cloning of RBPj\textsuperscript{wt}, RBPj\textsuperscript{Δ67} and RBPj\textsuperscript{58S} cDNAs into p3xFLAG-CMV7 vector

Following total RNA isolation from Rbpj\textsuperscript{+/+} and Rbpj\textsuperscript{−/−} keratinocytes, cDNAs were prepared. The cDNA encoding the amino acid residues 1 through 465 of the mouse RBPj\textsuperscript{wt} gene was amplified by PCR using Klentaq LA DNA Polymerase (DNA Polymerase Technology, Inc.).
To construct RBPj\textsuperscript{wt} and RBPj\textsuperscript{Δ67} cDNA, the following primers were used:

\begin{align*}
\text{Rbpj-Fwd (5'}-'\text{-ATGCGAAATTATTTAAAAAGAAGGGGAT-3'}) \\
\text{Rbpj-Rev (5'}-	ext{-TTAGGACACCACGTTGTGATGGACGATGT-3'})
\end{align*}

To construct RBPj\textsuperscript{58S} cDNA, the following primers were used:

\begin{align*}
\text{Rbpj-Fwd (5'}-'\text{-ATGCGAAATTATTTAAAAAGAAGGGGAT-3'}) \\
\text{Rbpj58-Rev (5'}-	ext{-ACTTTCCTAATTATAGCATTCTT-3'})
\end{align*}

HindIII and BamHI sites were used to clone the cDNAs into the p3xFLAG-CMV7 (Sigma-Aldrich, St. Louis, MO) vector. DNA sequencing analysis was done to confirm the cloned vectors.

**OT11 (RBPjκ\textsuperscript{−/−}) cell culture and Luciferase Assay**

OT11 cells (a gift from Dr. Tasuku Honjo) were seeded at 5 x 10\textsuperscript{4} cells/mL density in 0.5mL/well (24-well plate) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, and 100 U/ml penicillin. The following day, cells at 60-70% confluency were transfected in Opti-MEM-I medium with 300ng reporter vector, 10ng Notch1\textsuperscript{AE} (if present), 100ng RBPj\textsuperscript{wt} (or RBPj\textsuperscript{58S}, RBPj\textsuperscript{Δ67}, RBPj\textsuperscript{VP16}, RBPj\textsuperscript{58-VP16}) and an appropriate amount of balance DNA (to 500ng total DNA/well) using Lipofectamine LTX and PLUS reagent according to the manufacturer’s instructions. Cells were harvested 48h after transfection and washed once with PBS. Cells were lysed in 100 µl of lysis buffer (100 mM KPO\textsubscript{4} buffer, pH 7.8; 0.2% Triton; 1 mM dithiothreitol (DTT); protease inhibitors) at room temperature for 10 min. 5 µl of lysate was used to determine β-galactosidase concentration (to normalize for transfection efficiency) according to the Tropix Galacton chemiluminescent substrate instructions. 50 µl of lysate was used to determine luciferase activity using assay buffer (30 mM Tricine, pH 7.8; 3 mM ATP; 15 mM MgSO\textsubscript{4}; 10 mM DTT; 0.2 mM CoA; 1 mM luciferin) and a Tropix TR717 luminometer.
For each reporter, fold stimulation was expressed relative to the activity of the empty vector control, which was normalized to a value of 1. All assays were performed in triplicate and error bars show standard deviation. The data reported in the figures were the representative of at least three independent experiments. Student’s t-test was used to determine the statistical significance.

**Primary Keratinocyte Culture**

Newborn (P0) pups were utilized to isolate keratinocytes from epidermis. Skin samples were incubated in 5 mg/mL dispase o/n at 4°C to separate the epidermis from the dermis. The next day, epidermis samples were collected and incubated with trypsin at 37°C for 10-15 min to obtain a single-cell suspension. Cells were passed through a 70µm filter to eliminate large tissue contamination. Finally, keratinocytes were transferred onto collagen-coated plates and incubated in Keratinocyte Serum-free Medium (KSFM) for 3-5 days until they reached 80-90% confluency.

**Adeno-Cre/Adeno-GFP infection of keratinocytes in vitro**

*Rbpj*\(^{floxed/floxed}\) animals were used to generate primary keratinocytes. When keratinocytes reached to 80-90% confluency, they were treated for 48 hours with Adeno-Cre, which mediates recombination of *Rbpj*\(^{floxed}\) gene or Adeno-GFP (control). Culture media was replaced with fresh KSFM. Samples for western blot analysis were collected at 0h, 6h, 12h, 24h, 40h, 48h, 60h, 80h, 90h and 118h post-infection.

**Western Blotting Analysis**

Cells were lysed in RIPA-Doc buffer (150mM NaCl, 50 mM Tris-HCl, 1% Triton-X, 0.1% SDS, 0.25% Sodium Deoxycholate) containing protease and phosphatase inhibitors (Roche Complete). Lysates were resolved through an 8% acrylamide gel for SDS-PAGE/Western Blotting analysis. Quantifications were done by using ImageJ software (NIH). Primary α-RBPjk
antibody, T 6709, (rat, 1:500, Cosmo Bio Co., Ltd) was resuspended in 5% milk PBS-T. α-rat secondary antibody conjugated with HRP was used at a 1/2000 dilution in 5% milk PBS-T and incubated for 1-2 hours at room temperature. Dura substrate (Thermo Scientific) was used to perform the enzymatic reaction before film development.

**Microarray, Partek Data Analysis, Hierarchical Clustering, and GO Analysis**

RNA samples were collected, handled independently and individually throughout the experiments, without pooling of samples. 28S and 18S ribosomal RNA bands were visualized for confirmation of the RNA integrity by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Genome Technology Access Center (WUSTL) performed the quality control for samples and labeling using Life Technologies Illumina-TotalPrep kits. Illumina MouseWG-6 v2.0 Expression BeadChip (45,281 probes) were used for E18.5 epidermis microarray and three samples per genotype are examined. Illumina MouseRef-8 v2.0 Expression BeadChip Kit (25,600 probes) were used for P0 total skin microarray and four samples per genotype are examined.

Partek Genomic Suite 6.4 software is used to analyze the data. I have performed one-way ANOVA across all samples and identified statistically significant genes by using mixed model analysis of variance with a false discovery rate (Benjamini and Hochberg, 1995) or p-value of $P<0.05$. Fold-change values $>|±1.5|$ were used for further analysis. To identify functional groups of genes, we have used unsupervised hierarchical clustering function of Partek software, which can reveal biologically meaningful patterns within the microarray data. Hierarchical clustering identifies the pair of genes that are most similar, connects them together, and then finds the next most similar pair of genes. This process continues until all of the genes are joined into one large cluster.
Enrichment analyses for significantly differentially expressed probes were done with GOminer (Zeeberg et al. 2003), GOstat (Beissbarth and Speed, 2004), GOtoolbox (Martin et al. 2004), and DAVID (Huang et al. 2009) gene enrichment analysis tools. The tools were used with the default option. The input gene set was consists of 184 genes for E18.5 analysis and 22 genes for P0 total skin analysis. Whole *Mus musculus* genome was used as reference background. Significance threshold was P value < 0.05 for a minimum of two genes from the input list in the enriched category.

**Mass Spectrometry Analysis (provided by MS Core-WUSTL)**

Skin samples were incubated in 5 mg/mL dispase for 3 hours at 4°C to separate the epidermis from the dermis. Epidermal samples were immediately stored at -80°C before submitting to the Mass Spectrometry Core (WUSTL). Details of the mass spectrometry methods (In-Solution Protein Digestion and Peptide Preparation, High Resolution MS1 and MS2 Mass Spectrometry, Data from in-solution digest) were adopted from Chen et al. (2012) based on the instructions from James P. Malone - Mass Spectrometry Core, Senior Scientist.
ACKNOWLEDGMENTS

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See more at: http://www.siteman.wustl.edu/contentpage.aspx?id=241#sthash.URFCbphp.dpuf
REFERENCES:


**Figure 2-1.** \(Rbpj^{del}\) gene is functionally null.

(A) Immunohistochemistry staining for mouse RBPjk protein in dorsal skin from wt and RBPjcKO mice confirming the absence of RBPjk protein in \(Rbpj^{−/−}\) keratinocytes at birth (left panel; Demehri et al. 2008) and at P9 (right panel). (B) Map of the mouse \(Rbpj\) gene and its three mRNA isoforms, LoxP sites are flanking Exon6 and Exon7. Upon recombination, \(Rbpj^{del}\) gene is generated. Isoforms 201, 202, and 203 (the latter being most abundant isoform) are expressed in skin. (C) \(Rbpj^{del}\) mRNA is not degraded by nonsense-mediated decay (NMD) and is therefore expressed in the skin. Sequencing of the mutant fragment confirms that exons 6 and 7 are deleted in \(Rbpj^{del}\) mRNA. Exon8 immediately follows Exon5 in \(Rbpj^{del}\) leading to a frameshift. A stop codon is generated after 2 aminoacids within Exon8 due to the frameshift. (D) Diagrams of \(Rbpj\) constructs used in the study. (E) Testing transcriptional activity of RBPjk^{del} proteins using Notch-responsive (TP-1 and Hes1) luciferase reporters. OT-11 cells (RBPjk^{−/−}) were transfected with TP1 or Hes1-luciferase reporters in addition to an activated form of Notch1 (Notch1^{ΔE}), RBPj^{wt}, and RBPj^{del} (RBPj^{58S} and RBPj^{Δ67}) expression vectors and +/- DAPT (γ-secretase inhibitor) as indicated in the table. (F) Testing the DNA binding ability of RBPjk^{del} protein by overexpression of RBPjk^{58S-VP16} fusion protein +/- Notch1^{ΔE}. (G) Testing dominant negative activity of RBPjk^{del} protein by overexpressing RBPj^{58S} together with RBPj^{VP16} or RBPj^{wt} in the presence of Notch1^{ΔE}.

**Abbreviations:** wt (wild type), 58S (Exon5 spliced to Exon8 followed by a premature STOP codon), Δ67 (deletion of Exon6 and 7), VP16 (a strong transactivation domain)
Figure 2-2

A. Msx2-Cre

B. RBPIk mRNA expression in RBPIk^flox^ keratinocytes post Adeno-Cre infection

C. RBPIk and beta-actin expression

D. Timelapse degradation of RBPIk protein

E. Hierarchical Clustering


data
Figure 2-2. RBPjk protein has a 65-hour half-life in skin keratinocytes.

(A) Timeline for mouse epidermal stratification. Msx2-Cre-mediated deletion of target floxed genes starts at E9.5 and a hypothetical time course RBPjk protein degradation occurring before stratification. (B) Recombination kinetics of $Rbpj^{flox/del}$ gene in keratinocytes. Graph represents average percentage of $Rbpj^{wt}$ and $Rbpj^{del}$ mRNA after infection with Adeno-Cre. (C) Western blotting for RBPjk protein. Protein samples were collected at the indicated time-points from $Rbpj^{flox/del}$ keratinocytes that were infected with Adeno-Cre. (D) Time course of the degradation of endogenous RBPjk protein in $Rbpj^{flox/del}$ keratinocytes following Adeno-Cre infection. (E) Heatmap and unsupervised hierarchical clustering for E18.5 epidermal microarray results. Red color indicates upregulation whereas green color represents downregulation. Distances within the dendrogram display the similarities/dissimilarities of samples (or genes).
Figure 2-3
Figure 2-3. Transcriptome and proteome analyses reveal that RBPjcKO is very similar to PSdcKO at birth.

(A) Heatmap and unsupervised hierarchical clustering for newborn epidermal mass-spectrometry results. Red color indicates relative increased abundance of corresponding peptide whereas green color represents decreased abundance. Distances within the dendrogram display the similarities/dissimilarities among samples. (B) Heatmap and unsupervised hierarchical clustering for newborn total skin global gene expression analysis results. Upregulation of genes is designated with red color whereas downregulation of genes are represented with green color. The degree of similarities/dissimilarities among samples are indicated by the distances within the dendrogram.
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<th>Change</th>
<th>RBPjcKO vs. WT</th>
<th>Change</th>
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<td>ILMN_1239055</td>
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<td>DOWN</td>
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<td>ILMN_2416620</td>
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<td>EG665S09</td>
<td>S100 calcium binding protein A11 (calcigizzarin) pseudogene</td>
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<td>DOWN</td>
<td>0.91</td>
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<td>0.97</td>
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Table 2-1. List of genes that are significantly altered in E18.5 PSdcKO epidermis but unchanged in RBPjcKO epidermis.

Genome wide mRNA expression analysis was performed in E18.5 PSdcKO, RBPjcKO, WT<sup>PS</sup>, and WT<sup>RBPj</sup> epidermis. We identified 183 genes that had differential expression pattern in Rbpj<sup>−/−</sup> epidermis compared to PS<sup>−/−</sup> epidermis (<1.5 fold). 28 genes were upregulated in PSdcKO epidermis but were unchanged in RBPjcKO; whereas 18 genes were downregulated in PSdcKO, but were unchanged in RBPjcKO.
Table 2-2. Genes with upregulated expression in E18.5 RBPjkKO epidermis, but not changed in E18.5 PSdcKO epidermis

<table>
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<tr>
<th>PROBE_ID</th>
<th>TargetID</th>
<th>Gene Name</th>
<th>PSdcKO vs. WT Change</th>
<th>RBPjkKO vs. WT Change</th>
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<td>RIKEN cDNA 2210011C24 gene</td>
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<td>2610206P12RIK</td>
<td>RIKEN cDNA 2610206P12 gene</td>
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<td>4833444G19RIK</td>
<td>RIKEN cDNA 4833444G19 gene</td>
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<td>RIKEN cDNA 5730420E01 gene</td>
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<td>ribonuclease III, nuclear</td>
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<td>SCUBE2</td>
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<td>1.16</td>
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<td>ILMN_2725927</td>
<td>SERPINA3G</td>
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<td>1.04</td>
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<td>SET domain containing 5</td>
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<td>SORL1</td>
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<td>1.07</td>
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<td>SPEER1-PS1</td>
<td>predicted gene 6465; spermatogenesis associated glutamate (E)-rich protein 1</td>
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<td>TBN-PENDING</td>
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<td>1.12</td>
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<td>VEZT</td>
<td>vezatin, adherens junctions transmembrane protein</td>
<td>1.01</td>
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<td>WLS</td>
<td>wntless homolog (Drosophila)</td>
<td>1.18</td>
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<td>zinc finger protein 318</td>
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<td>ZFP709</td>
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Table 2-2. List of genes that are upregulated in E18.5 RBPjcKO epidermis but are unaltered in PSdcKO epidermis.

Genome wide mRNA expression analysis was performed in E18.5 PSdcKO, RBPjcKO, WT^{PS}, and WT^{RBPj} epidermis. We identified 183 genes that have differential expression pattern between \( Rbpj^{-/-} \) and \( PS^{-/-} \) epidermis (<1.5 fold). 70 genes were upregulated in RBPjcKO epidermis, but were unchanged in PSdcKO.
Table 2-3. Genes with downregulated expression in E18.5 RBPjKO epidermis, but not changed in E18.5 PSdcKO epidermis

<table>
<thead>
<tr>
<th>PROBE ID</th>
<th>TargetID</th>
<th>Gene Name</th>
<th>PSdcKO vs. WT</th>
<th>Change</th>
<th>RBPjKO vs. WT</th>
<th>Change</th>
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<tr>
<td>ILMN_1240567</td>
<td>1700060J05RIK</td>
<td>RIKEN cDNA 1700060J05 gene</td>
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<td>RIKEN cDNA 2310039H08 gene</td>
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<td>ILMN_2730311</td>
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<td>-</td>
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<td>RIKEN cDNA 4930448I06 gene</td>
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<td>-</td>
<td>0.56</td>
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<td>ILMN_2662113</td>
<td>4933414I15RIK</td>
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<td>ILMN_2658592</td>
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<td>0.66</td>
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<td>aldehyde dehydrogenase family 1, subfamily A1</td>
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<tr>
<td>ILMN_1218327</td>
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<td>asparagine-linked glycosylation 8 homolog (yeast, alpha-1,3-glucosyltransferase)</td>
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<td>-</td>
<td>0.62</td>
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<td>ILMN_2640082</td>
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<td>ATPase, H+ transporting, lysosomal V1 subunit G2</td>
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<td>-</td>
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<td>ILMN_1225813</td>
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<td>Cyclin-Dependent Kinase 17</td>
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<tr>
<td>ILMN_2659237</td>
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<td>camello-like 5</td>
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<td>PROBE_ID</td>
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<td>Gene Name</td>
<td>PSdcKO vs. WT</td>
<td>Change</td>
<td>RBPjcKO vs. WT</td>
<td>Change</td>
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<td>-</td>
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<td>-</td>
<td>0.63</td>
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<td>protein phosphatase 1, regulatory (inhibitor) subunit 3E</td>
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<td>-</td>
<td>0.59</td>
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<tr>
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<td>-</td>
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<td>ILMN_2682945</td>
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<td>-</td>
<td>0.62</td>
<td>DOWN</td>
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<td>RMND5B</td>
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<td>0.67</td>
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<td>-</td>
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<td>STARD7</td>
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<td>-</td>
<td>0.66</td>
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<td>ILMN_2942344</td>
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<td>stanniocalcin 1</td>
<td>1.03</td>
<td>-</td>
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<td>ILMN_3160610</td>
<td>SVAL2</td>
<td>seminal vesicle antigen-like 2</td>
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<td>TMEM33</td>
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<td>TTC32</td>
<td>tetratricopeptide repeat domain 32</td>
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<td>UBE2G2</td>
<td>ubiquitin-conjugating enzyme E2G 2</td>
<td>0.89</td>
<td>-</td>
<td>0.68</td>
<td>DOWN</td>
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<td>ILMN_1219688</td>
<td>VIIRD15</td>
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<td>YME1-like 1 (S. cerevisiae)</td>
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<td>zinc finger CCCH type containing 14</td>
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</tbody>
</table>
Table 2-3. List of genes that were downregulated in E18.5 RBPjcKO epidermis but were unchanged in PSdcKO epidermis.

Genome wide mRNA expression analysis was performed in E18.5 PSdcKO, RBPjcKO, WT<sup>PS</sup>, and WT<sup>RBPj</sup> epidermis. We identified 183 genes that have differential expression patterns between Rbpj<sup>−/−</sup> and P<sup>S−/−</sup> epidermis (<1.5 fold). 71 genes were downregulated in RBPjcKO epidermis but were not altered in PSdcKO.
### Table 2-4. Genes with altered expression in P0 RBPjKo skin, but not changed in P0 PSdcKo or PSR skin

<table>
<thead>
<tr>
<th>Probeset ID</th>
<th>SYMBOL</th>
<th>Gene Names</th>
<th>PSdcKO vs. WT</th>
<th>Change</th>
<th>PSR vs. WT</th>
<th>Change</th>
<th>RBPjcKO vs WT</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>160692</td>
<td>LOC434172</td>
<td>gamma-glutamyltransferase 5</td>
<td>1.05</td>
<td>-</td>
<td>1.02</td>
<td>-</td>
<td>1.68</td>
<td>UP</td>
</tr>
<tr>
<td>7040091</td>
<td>Ggt5</td>
<td>zinc finger protein 367</td>
<td>0.92</td>
<td>-</td>
<td>1.02</td>
<td>-</td>
<td>1.57</td>
<td>UP</td>
</tr>
<tr>
<td>5900605</td>
<td>Zfn37</td>
<td>sestrin 3</td>
<td>0.97</td>
<td>-</td>
<td>0.97</td>
<td>-</td>
<td>1.57</td>
<td>UP</td>
</tr>
<tr>
<td>610594</td>
<td>Abcb11</td>
<td>ATP-binding cassette, sub-family B (MDR/TAP), member 11</td>
<td>0.99</td>
<td>-</td>
<td>1.04</td>
<td>-</td>
<td>0.66</td>
<td>DOWN</td>
</tr>
<tr>
<td>7050678</td>
<td>V1rd20</td>
<td>1.01</td>
<td>-</td>
<td>1.01</td>
<td>-</td>
<td>0.65</td>
<td>DOWN</td>
<td></td>
</tr>
<tr>
<td>1190398</td>
<td>Ugt1a2</td>
<td>0.94</td>
<td>-</td>
<td>1.05</td>
<td>-</td>
<td>0.64</td>
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<td></td>
</tr>
<tr>
<td>3940255</td>
<td>Sos1</td>
<td>son of sevenless homolog 1 (Drosophila)</td>
<td>1.00</td>
<td>-</td>
<td>0.93</td>
<td>-</td>
<td>0.64</td>
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</tr>
<tr>
<td>2710386</td>
<td>Vwa1</td>
<td>von Willebrand factor A domain containing 1</td>
<td>0.96</td>
<td>-</td>
<td>0.92</td>
<td>-</td>
<td>0.63</td>
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<tr>
<td>4560634</td>
<td>Bmyc</td>
<td>brain expressed myelocytomatosis oncogene</td>
<td>1.04</td>
<td>-</td>
<td>0.91</td>
<td>-</td>
<td>0.60</td>
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<tr>
<td>60603</td>
<td>Havcr2</td>
<td>hepatitis A virus cellular receptor 2</td>
<td>0.95</td>
<td>-</td>
<td>0.98</td>
<td>-</td>
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### Table 2-5. Genes with altered expression in P0 PSdcKo or PSR skin, but not changed in P0 RBPjKo skin

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<thead>
<tr>
<th>Probeset ID</th>
<th>SYMBOL</th>
<th>Gene Names</th>
<th>PSdcKO vs. WT</th>
<th>Change</th>
<th>PSR vs. WT</th>
<th>Change</th>
<th>RBPjcKO vs WT</th>
<th>Change</th>
</tr>
</thead>
<tbody>
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<td>4180367</td>
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<td>UP</td>
<td>1.08</td>
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<td></td>
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<td>5390639</td>
<td>Cxcl2</td>
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<td>1.70</td>
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<td>0.97</td>
<td>-</td>
<td></td>
</tr>
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<td>4200368</td>
<td>Ctnbp2</td>
<td>1.61</td>
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<td>1.57</td>
<td>UP</td>
<td>1.01</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6420133</td>
<td>Zfn426</td>
<td>1.63</td>
<td>UP</td>
<td>1.52</td>
<td>UP</td>
<td>1.04</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1050092</td>
<td>Serpina3g</td>
<td>1.68</td>
<td>UP</td>
<td>1.49</td>
<td>UP</td>
<td>1.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4610010</td>
<td>Scta5g</td>
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<td>DOWN</td>
<td>0.69</td>
<td>DOWN</td>
<td>0.99</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2230465</td>
<td>Itih4</td>
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<td>DOWN</td>
<td>0.67</td>
<td>DOWN</td>
<td>1.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6450403</td>
<td>2410005O16Rik</td>
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<td>DOWN</td>
<td>0.66</td>
<td>DOWN</td>
<td>0.92</td>
<td>-</td>
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<tr>
<td>2810129</td>
<td>Paxip1</td>
<td>0.58</td>
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<td>0.66</td>
<td>DOWN</td>
<td>0.97</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>540463</td>
<td>Unc13c</td>
<td>0.66</td>
<td>DOWN</td>
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<td>DOWN</td>
<td>0.93</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4260152</td>
<td>Rorc</td>
<td>0.67</td>
<td>DOWN</td>
<td>0.58</td>
<td>DOWN</td>
<td>0.92</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Table 2-4 & 2-5. List of genes that are significantly altered in P0 PSdcKO and PSR vs. RBPjcKO total skin.

We have performed global gene expression profile analysis for P0 PSdcKO, PSR, RBPjcKO, WT total skin samples. 22 genes were identified to have significantly different expression patterns in RBPjcKO total skin vs PSdcKO (or PSR) total skin (<1.5 fold). 4 genes were upregulated in RBPjcKO skin but unchanged in PSdcKO and PSR skin whereas 7 genes were downregulated in RBPjcKO but unchanged in PSdcKO and PSR. In addition, 6 genes had increased expression and 5 genes had decreased expression in PSdcKO and PSR but were unchanged in RBPjcKO.
CHAPTER III

γ-secretase cleaved Notch1 ICD

is necessary and sufficient to drive

the milder phenotype in RBPjKO animals

Author Contribution
The author performed all the experiments, wrote the text, and prepared the figures in this chapter.
**SUMMARY**

In the epidermis, Notch signaling regulates epidermal integrity and barrier formation. In the hair follicle, it has a crucial function for the maintenance of hair follicle morphology. Loss of Notch signaling in embryonic skin keratinocytes causes differentiation defects in the epidermis, loss of cellular identity in the IRS and induction of epidermal differentiation program in hair follicle ORS cells. In response to these problems, suprabasal keratinocytes and epidermal cells within the hair follicle cysts collectively express TSLP, which is then released into the systemic circulation. The high level of TSLP induces expansion of immature B cells leading to BLPD and eventually death of the animal. Interestingly, RBPjcKO animals had a different phenotype compared to the N1N2dcKO and PSdcKO animals. We performed epistasis analysis to determine the factors that are necessary and sufficient to cause the milder phenotype in the RBPjcKO animals. We ruled out target derepression and Ligand Back Signaling (LBS) as the underlying mechanisms mediating the milder RBPjcKO phenotypes. Our genetic analyses suggest that γ-secretase-cleaved Notch1 ICD is necessary and sufficient to drive the milder phenotype in RBPjcKO animals.
INTRODUCTION

Notch1, Notch2 and Notch3 receptors play an important role in keratinocytes to maintain skin homeostasis. Loss-of-Notch signaling leads to differentiation defects in the epidermis (Lowell et al., 2000; Nickoloff et al., 2002; Rangarajan et al., 2001; Blanpain et al., 2006; Moriyama et al., 2008), and a failure in maintaining IRS cellular identity as well as inappropriate activation of the epidermal differentiation program in ORS cells (Pan et al., 2004, Demehri et al. 2008). Stepwise reduction of Notch dosage leads to progressive skin perturbations, ultimately leading to the conversion of hair follicles into epidermal cysts (Pan et al., 2004; Demehri et al., 2008). Differentiation defects in epidermal keratinocytes and activation of epidermal differentiation program in ORS cells collectively trigger expression of TSLP (Demehri et al. 2008). TSLP is an epithelial-derived cytokine originally discovered for its ability to promote activation of B lymphocytes (Friend et al., 1994; Levin et al., 1999), myeloid DCs (Reche et al., 2001; Soumelis et al., 2002) and Th2 cell differentiation of naive T cells (Watanabe et al., 2004). Secretion of TSLP into the systemic blood circulation induces expansion of pre-B cells resulting in B-cell Lymphoproliferative Disease (BLPD) in mutant animals (Demehri et al., 2008). Interestingly, serum TSLP levels in mutant animals inversely correlate with the dose of Notch in the skin (Demehri et al. 2008). Moreover, the severity of the BLPD during the first few weeks of life is directly correlated with the levels of TSLP in the sera (Demehri et al. 2008), such that extreme BLPD causes death in PSdcKO and N1N2dcKO animals (Demehri et al. 2008). As a result, TSLP levels in serum, WBC counts, and life expectancy of mutant animals showed a strong inverse correlation with the dose of Notch signaling (Demehri et al., 2008) allowing us to use these parameters to characterize the severity of the phenotype during the course of the
disease (Figure 1-4).

We have previously shown that keratinocyte-specific deletion of the Rbpj gene (RBPjcKO) produced a significantly milder phenotype than the keratinocyte-specific deletion of both Notch1 and Notch2 genes (N1N2dcKO) or Presenilin1 and Presenilin2 genes (PSdcKO) (Demehri et al., 2008). While dissecting the pathway to identify reasons for the milder phenotype, we proposed three possible explanations: (1) derepression of target genes, (2) ligand back signaling (LBS), and/or (3) cleavage-dependent RBPjk-independent Notch1 ICD activity. We provide evidence that γ-secretase cleaved Notch1 ICD is necessary and sufficient for the milder phenotype in the absence of RBPjk.
RESULTS AND DISCUSSION

Target derepression is not mediating ameliorated RBPjcKO phenotype

RBPjk can act as a repressor (Dou et al., 1994; Hsieh and Hayward, 1995). Therefore, loss of RBPjk protein might lead to the elimination of co-repressor complexes and derepression of some of the targets, thereby improving the phenotype in RBPjcKO animals compared to the N1N2dcKO or PSdcKO mutants. If target derepression is causing the milder phenotype, then removing Rbpj in the background of Notch1 and Notch2 loss (N1N2RBPjcKO) or Preselin1 and Presenilin2 loss (PSRBPjcKO) should resemble RBPjcKO mutant phenotype. To test the possibility of target derepression, Demehri et al generated triple mutants of N1N2RBPjcKO and PSRBPjcKO and showed that the N1N2RBPjcKO triple mutant animals were similar to N1N2dcKO mutants and the PSRBPjcKO animals were similar to PSdcKO mutants (Demehri et al., 2008). To confirm these findings, I compared the lifespans, WBC counts, serum TSLP levels, and overall skin morphology of these different mutants (Figure 3-2). I observed similar results suggesting that target derepression does not mediate milder RBPjcKO phenotype.

Ligand Back Signaling is not the reason behind the milder RBPjcKO phenotype

We confirmed that canonical Notch signaling is eliminated in RBPjcKO animals (Chapter 2) and target derepression was not the reason behind the milder phenotype (Figure 3-2; Demehri et al., 2008). Another possible explanation for the ameliorated RBPjcKO phenotype could be Ligand Back Signaling (LBS). In the absence of RBPjk, Notch ligands (Delta-like 1, 3, 4 and Jagged 1, 2) are still able to interact with Notch receptors. It has been previously shown that not only Notch receptors, but also DSL ligands undergo proteolytic cleavages upon receptor-ligand interaction (Qi et al., 1999; Ikeuchi & Sisodia, 2003; LaVoie & Selkoe, 2003; Bland et al.,
After cleavages of these ligands by ADAM metalloproteases and γ-secretase, Delta Intracellular Domain (Delta ICD), and/or Jagged Intracellular Domain (Jagged ICD) are produced and can translocate to the nucleus (Ikeuchi & Sisodia, 2003; LaVoie & Selkoe, 2003; Bland et al., 2003; Six et al., 2003; Zolkiewska, 2008; Duryagina et al. 2013). In the nucleus, Delta ICD can activate TGF-β/Activin signaling through Smads (Hiratochi et al., 2007; Bordonaro et al., 2011) and/or Jagged ICD can stimulate AP1-dependent gene expression (LaVoie & Selkoe, 2003).

To test whether LBS is mediating the milder phenotype in RBPjcKO animals, we decided to use a mouse model that expresses an allele of the Notch1 receptor carrying a fully functional extracellular domain but whose intracellular domain has been replaced with a heterologous protein, in this case the tamoxifen-inducible form of Cre-recombinase) CreERT (Figure 3-3A). Ligand binding can still trigger a conformational change in the Notch1-CreERT receptor (N1CreERT), and induce proteolytic cleavages, thereby releasing the CreERT molecule instead of Notch1ICD. The liberated CreERT molecule can translocate to the nucleus in the presence of tamoxifen and recombine floxed reporter alleles such as Rosa26R (Soriano, 1999). This can be visualized in the skin by X-gal assay (Figure 3-3B). Since ligand-receptor engagement is normal in the cells with N1CreERT, the ligand-presenting cells are expected to produce Delta ICD and/or Jagged ICD upon Notch-ligand interaction and able to activate Ligand ICD dependent gene expression.

To ask if the underlying cause for the milder phenotype in RBPjcKO animals is explained by LBS, we determined the impact of N1CreERT on the phenotype of mice lacking Notch1 and Notch2 receptors in keratinocytes and compare it to the RBPjcKO animals. The overall hair phenotypes in N1CreERTN2dcKO resembled N1N2dcKO animals, but not RBPjcKO animals.
(Figure 3-3C), supporting the idea that the milder phenotype is not due to LBS. Similarly, when we analyzed the histological sections from N1CreERTN2dcKO and RBPjcKO animals, we concluded that N1CreERTN2dcKO skin is significantly different than RBPjcKO skin (Figure 3-3D). N1CreERTN2dcKO skin had a higher number of cystic hair follicles with a greater severity in morphology and a lower number of adipocytes compared to RBPjcKO skin. Analysis of lifespan, WBC counts, and serum TSLP levels of N1CreERTN2dcKO animals were all similar to the N1N2dcKO animals (Figure 3-3E).

In summary, the LBS hypothesis was tested by utilizing a Notch receptor, Notch1CreERT (N1CreERT), that has functional Ligand-back-signaling, but lacking canonical Notch signaling. Animals that express N1CreERT receptor in N1\(^{-/-}\)N2\(^{-/-}\) skin keratinocytes were similar to N1N2dcKO animals in terms of their hair-loss phenotype, skin morphology, lifespan, WBC counts, and serum TSLP levels indicating that the ameliorated RBPjcKO phenotype is not due to LBS.

\textit{\textgamma-secretase cleaved unstable Notch1 ICD is sufficient to drive the milder phenotype in N1vgN2RBPjcKO animals}

We eliminated target derepression (Chapter 3.1) and LBS (Chapter 3.2) hypotheses by using triple mutants and N1CreERTN2dcKO animals, respectively. There is one possible explanation left to test: RBPj\(\kappa\)-independent \(\textgamma\)-secretase-cleaved Notch1 ICD is responsible for the amelioration of the phenotype in these animals.

We know that Notch ICD is necessary for the milder phenotype (N1N2dcKO and PSdcKO animals do not have NICD while RBPjCKO animals do). To determine whether Notch1ICD is sufficient to drive the RBPjcKO phenotype, we utilized another mouse line, Notch1 VG (Figure 3-4A). Notch1VG (N1vg) carries a point mutation in the \textit{Notch1} allele,
leading to a Valine to Glycine amino acid conversion at position 1744 of the receptor (Huppert et al., 2000) (Figure 3-4A). This allele has been characterized as a hypomorphic allele of Notch1 gene due to a shorter half-life of the Notch1ICD molecule upon proteolytic cleavage of the receptor by γ-secretase (Huppert et al., 2000; Blat et al., 2002; Tagami et al., 2008). Due to shorter half-life of the Notch1ICD, canonical Notch signaling in these animals is severely reduced such that N1vg/vg animals are embryonic lethal at E10.5 (Huppert et al., 2000). We decided to use the severely impaired N1vg allele to test whether unstable Notch1ICD is sufficient to drive the milder RBPjcKO phenotype.

If N1vgN2RBPjcKO animals are similar to RBPjcKO animals, then we would conclude that one allele of an unstable Notch1ICD is sufficient to ameliorate the N1N2RBPjcKO phenotype in an RBPjκ-independent manner.

We compared overall hair phenotypes, lifespans, WBC counts, and serum TSLP levels in N1vgN2RBPjcKO, N1N2dcKO and RBPjcKO animals. We found that N1vgN2RBPjcKO animals resembled RBPjcKO animals, not N1N2dcKO animals (Figure 3-4C). When we analyzed the histological sections from N1vgN2RBPjcKO, N1N2dcKO and RBPjcKO animals, we concluded that N1vgN2RBPjcKO skin was significantly different from the N1N2dcKO skin. N1vgN2RBPjcKO skin had less number of cystic hair follicles with a milder distorted morphology compared to N1N2dcKO skin (Figure 3-3D). Analysis of lifespan, WBC counts, and serum TSLP levels revealed that N1vgN2RBPjcKO animals were similar to the RBPjcKO animals (Figure 3-3E), suggesting that even unstable Notch1ICD is sufficient to rescue the phenotype.

In conclusion, I demonstrated that animals that express N1vg receptor in the background of N1N2-loss in skin keratinocytes were similar to RBPjcKO in terms of their hair loss.
phenotype, skin morphology, lifespan, WBC counts, and serum TSLP levels indicating that ameliorated RBPjcKO phenotype can be rescued by unstable Notch1 ICD molecule.
CONCLUSIONS

While dissecting the pathway to identify reasons for the milder phenotype, we proposed three possible explanations: (1) derepression of target genes, (2) ligand back signaling (LBS), and/or (3) γ-secretase-cleaved RBPjk-independent Notch1 ICD activity. We performed epistasis analysis to determine the factors that are necessary and sufficient for the RBPjcKO phenotype. We ruled out target derepression and LBS as the underlying mechanisms. We provide evidence that γ-secretase cleaved Notch1 ICD is necessary and sufficient to drive the milder phenotype in RBPjcKO animals.
EXPERIMENTAL PROCEDURES
Generation of mice

I have generated the following strains of mice using Msx2-Cre transgene-mediated deletion in skin keratinocytes (Pan et al., 2004). Animals were raised and kept in the Washington University Division of Comparative Medicine Animal Facility under Washington University Policy on Animal Care regulations.

- Msx2-Cre^{+/tg}; Rbpj^{floxed/floxed} (RBPjcKO)
- Msx2-Cre^{+/tg}; Notch1^{floxed/floxed}, Notch2^{floxed/floxed} (N1N2dcKO)
- Msx2-Cre^{+/tg}; Notch1^{floxed/floxed}, Notch2^{floxed/floxed}, Rbpj^{floxed/floxed} (N1N2RBPjcKO)
- Msx2-Cre^{+/tg}; Presenilin1^{floxed/floxed}, Presenilin2^{-/-} (PSdcKO)
- Msx2-Cre^{+/tg}; Presenilin1^{floxed/floxed}, Presenilin2^{-/-}; Rbpj^{floxed/floxed} (PSRBPjcKO)
- Msx2-Cre^{+/tg}; Notch1^{floxed/CreERT}, Notch2^{floxed/floxed} (N1CreERTN2dcKO)
- Msx2-Cre^{+/tg}; Notch1^{floxed/V-g}, Notch2^{floxed/floxed}, Rbpj^{floxed/floxed} (N1vgN2RBPjcKO)

Histology and H&E Staining

Skin samples were fixed in 4% paraformaldehyde (PFA) in PBS overnight (o/n), rinsed 3X in PBS and then washed with 30% Ethanol (EtOH) and 50% EtOH before storing them in 70% EtOH at 4°C. Skin samples were embedded in paraffin for sectioning at 8-12µm thickness. A standard H&E staining protocol was used for histology assessment.

Lifespan

For longevity analysis, mice were monitored on a daily basis for weakness, loss of appetite and morbidity. To provide a better nursing opportunity, unnecessary wt littermates were removed. Mutant animals were left with their mothers during the course experiment.
Measurement of WBC counts

Mandibular vein was used for blood sample collection. Complete blood counts including WBCs, platelets, red blood cells, white cell differential counts, and hemoglobin measurements were done with the Hemavet 950 analyzer (Drew Scientific).

Serum TSLP measurements

Total serum TSLP was determined using LEGEND MAX™ Mouse TSLP ELISA Kit (Biolegend) following the manufacturer’s instructions.
REFERENCES:


Figure 3-1
Figure 3-1. Epistasis analysis to determine the factors that are necessary and sufficient for the milder RBPjcKO phenotype.

While dissecting the pathway to identify reasons for the milder RBPjcKO phenotype, we proposed three possible explanations: (1) derepression of target genes, (2) ligand back signaling (LBS), and (3) cleavage-dependent RBPjk-independent Notch1 ICD activity. We have generated the indicated genotypes and provided evidence that γ-secretase cleaved Notch1 ICD is necessary and sufficient for the milder phenotype in the absence of RBPjk.
Figure 3-2. Derepression is not the mechanism behind the milder RBPjcKO phenotype.

Triple mutant of Notch1, Notch2, and Rbpj (N1N2RBPjcKO) or Presenilin1, Preselin2, and Rbpj (PSRPbPjcKO) are as severe as N1N2dcKO or PSdcKO, respectively. We have compared lifespans, WBC counts, serum TSLP levels (measured during second week of life) and overall skin morphology of indicated Notch mutants and wt animals.
Figure 3-3

A

Notch1

B

+ 4-OH Tamoxifen

C

WT N1N2dcKO N1CreERTN2dcKO RBPjcKO

D

WT N1N2dcKO N1CreERTN2dcKO RBPjcKO

E

Lifespan WBC Counts Serum TSLP Levels

Days K/uL ng/mL

WT N1N2dcKO N1CreERTN2dcKO RBPjcKO WT N1N2dcKO N1CreERTN2dcKO RBPjcKO WT N1N2dcKO N1CreERTN2dcKO RBPjcKO

n.s * n.s *

n.s : not significant
* : p< 0.0001
Figure 3-3. Ligand back signaling does not mediate the milder phenotype in RBPjcKO animals. (A) Diagram showing the structure of the Notch1CreERT (N1CreERT) receptor with respect to Notch1wt. (B) X-gal staining for 4-OH tamoxifen-injected N1CreERT Rosa26R/Rosa26R skin confirming N1CreERT receptor is functional and can be activated by ligands. (C) Overall dorsal hair phenotype and (D) histological analysis of the P9 skin samples of the WT, N1N2dcKO, N1CreERTN2dcKO, and RBPjcKO animals. (E) Analysis of lifespans, WBC counts, and serum TSLP levels (measured during second week of life) of WT, N1N2dcKO, N1CreERTN2dcKO, and RBPjcKO animals.
Figure 3-4

A

![Diagram showing Notch1 and Notch1vg domains]

B

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<th>WT</th>
<th>N1N2dcKO</th>
<th>N1N2RBPjcKO</th>
<th>RBPjcKO</th>
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C

![Histological images showing comparison of tissues](image6)

D

<table>
<thead>
<tr>
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<tr>
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</tr>
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</tr>
<tr>
<td>N1N2RBPjcKO</td>
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<td>10</td>
<td>n.s.</td>
</tr>
<tr>
<td>RBPjcKO</td>
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<td>29.48</td>
<td>23.26</td>
</tr>
<tr>
<td>N1vgN2RBPjcKO</td>
<td>100</td>
<td>6.15</td>
<td>6.90</td>
</tr>
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</table>

* p<0.0001
n.s: not significant
Figure 3-4. Cleavage-dependent unstable version of Notch1 ICD (N1vgICD) is sufficient to reconstitute RBPjcKO phenotype. (A) Diagram showing the structure of Notch1vg (N1vg) receptor with respect to Notch1<sup>wt</sup>. (B) Overall dorsal hair phenotype and (C) histological analysis of the P9 skin samples of WT, N1N2dcKO, RBPjcKO, and N1vgN2RBPjcKO animals. (E) Analysis of lifespans, WBC counts, and serum TSLP levels (measured during second week of life) of WT, N1N2dcKO, N1CreERTN2dcKO, and RBPjcKO animals.
CHAPTER IV

Hair follicles with Rbpj−/− keratinocytes express less TSLP thereby ameliorating the phenotype in RBPjcKO animals

Author Contribution
The author performed all the experiments, wrote the text, and prepared the figures in this chapter.
**SUMMARY**

High serum TSLP levels during the first two weeks of life induce uncontrolled pre-B cell proliferation, which ultimately results in premature death in N1N2dcKO and PSdcKO animals. These results suggest that TSLP is the key factor mediating these phenotypes. Therefore, I decided to determine the spatio-temporal expression pattern of TSLP mRNA in mutant animals. First, I showed that epidermal TSLP expression is similar in all Notch mutants (RBPjcKO, N1N2dcKO, and PSdcKO). Second, I established that RBPjcKO hair follicles express significantly less TSLP mRNA starting at P6. Finally, I demonstrated that RBPjcKO animals have a significant reduction in generation of keratinocytes cysts with the majority of Rbpj\(^{-/-}\) hair follicles being relatively mildly perturbed and containing significantly less TSLP (+) cells. In summary, TSLP mRNA expression and hair follicle phenotype analysis suggest that Rbpj\(^{-/-}\) hair follicles are less distorted and these follicle keratinocytes produce less TSLP ultimately leading to a milder phenotype in RBPjcKO animals.
INTRODUCTION

Keratinocyte-specific deletion of Rbpj gene (RBPjcKO) produced a significantly milder phenotype than the keratinocyte-specific deletion of both Notch1 and Notch2 genes (N1N2dcKO) or both Presenilin1 and Presenilin2 genes (PSdcKO) (Demehri et al., 2008). Genetic analyses of the Notch signaling pathway showed that even a limiting amount of γ-secretase-cleaved Notch1ICD molecules are sufficient for ameliorating the phenotype in RBPjcKO animals (Chapter 3) suggesting that Notch1ICD has other functions in the absence of RBPjκ. However, this function is not evident in the developing embryo, based on our analysis of embryonic skin from N1N2dcKO, PSdcKO, and RBPjcKO all share similar phenotypes at birth (Chapter 2). We showed that there are no significant alterations in the gross morphology of their epidermis and hair follicles between Notch pathway mutants. Our analyses examining mRNA and protein expression profiles from these mutants suggest that canonical Notch signaling is responsible for the phenotypes in the embryo and newborn animals (Chapter 2).

The differences between RBPjcKO animals and N1N2dcKO (or PSdcKO) animals are manifested in four phenotypic measures: lifespans, WBC counts, serum TSLP levels, and skin morphology (Demehri et al., 2008). Lifespan is directly correlated with the severity of the B-cell lymphoproliferative disease (BLPD), which is determined by the increased levels of pre-B cell expansion (Demehri et al., 2008). The pre-B cell expansion is a consequence of the high levels of TSLP in the sera (Demehri et al., 2008) suggesting that TSLP is a central factor underlying the phenotypic differences among all Notch signaling mutants. Therefore, I decided to examine the spatio-temporal expression pattern of TSLP mRNA in mutant animals.

First, I established that TSLP mRNA expression is similar in the epidermis of all Notch
mutants. Secondly, I provided evidence that expression of TSLP mRNA differs in the abnormal hair follicles starting postnatal day 6 (P6). RBPjcKO hair follicles express significantly less TSLP mRNA between P6 and P11 compared to N1N2dcKO or PSdcKO hair follicles. Finally, I characterized the distribution of the abnormal hair follicles in Notch mutant skin and showed that RBPjcKO animals have significantly less keratinocytes cysts. The majority of $Rbpj^{-/-}$ hair follicles was mildly perturbed and contains significantly less TSLP (+) cells. Taken together, TSLP mRNA expression analysis and hair follicle phenotype assessment suggest that $Rbpj^{-/-}$ hair follicles are less distorted and produce less TSLP ultimately leading to a milder phenotype in RBPjcKO animals.
RESULTS AND DISCUSSION

Similar levels of TSLP mRNA expressed in Rbpj−/−, N1N2−/− and PS−/− epidermis

The differences in lifespans, WBC counts and serum TSLP levels suggest that TSLP is the key factor driving these phenotypes in all Notch pathway mutants. Therefore, we hypothesized that levels of TSLP mRNA transcription might be different in RBPjcKO mutants compared to the other mutants. To test this hypothesis, I characterized the spatio-temporal expression levels of TSLP mRNA in mutant epidermis.

To determine the levels of TSLP mRNA expression in Notch mutant epidermis, I performed qRT-PCR analyses on extracted total RNA from WT, N1N2dcKO, PSdcKO, and RBPjcKO epidermal samples at ages E18.5, P0, P6, P9 and P11. Unexpectedly, RBPjcKO epidermis had higher levels of TSLP mRNA relative to N1N2−/− and PS−/− epidermis at E18.5 (Figure 4-2) contrary to the hypothesis that Rbpj−/− epidermis should express less TSLP mRNA. However, the postnatal (P0, P6, P9, and P11) expression of TSLP mRNA was similar in the epidermis of all Notch pathway mutants (Figure 4-2).

RBPjcKO hair follicles are less perturbed than those of N1N2dcKO and PSdcKO

To determine when the phenotype of hair follicle disruption is evident, I performed histological analysis on Notch mutant skin samples starting from E18.5 to P11. This analysis demonstrated that hair follicles started to have morphological problems at birth in all Notch pathway mutants (Figure 4-1A). To further evaluate the differences among RBPjcKO, N1N2dcKO and PSdcKO hair follicles, I have performed a quantitative assessment of hair follicle morphology in mutant animals. I performed K14 (epidermal basal cell marker), AE13 (hair cortex marker), IRS3.1 (Inner root sheath cell marker) (Figure 4-1A) and H&E staining of
Notch mutant skin samples starting from E18.5 to P13 (data not shown). Three independent blinded observers assigned a morphology ranking score for each hair follicle/keratin cyst within 10 different areas (3mm x 0.5mm) of dorsal skin. An unbiased scoring system (ranging from 1 => normal hair to 10 => keratin cyst; see the phenotype rankings in Figure 4-4) was used to define the severity of the abnormality for the hair follicle morphology (adapted from Demehri et al. 2009). Then I calculated the distribution of the phenotypic scores for each genotype. Detailed investigation of the hair follicle phenotypes revealed that the distribution of abnormal hair follicles in RBPjcKO skin was significantly different from that of N1N2dcKO and PSdcKO (Figure 4-4, Demehri et al., 2009). The majority of the hair follicles in N1N2dcKO and PSdcKO animals exhibited more of the cystic phenotype whereas the majority of the RBPjcKO hair follicles were only moderately distorted (Figure 4-4) supporting the notion that the hair follicles are the source of the difference.

**RBPjcKO hair follicles have significantly less TSLP expression compared to N1N2dcKO and PSdcKO hair follicles**

To determine whether differential expression of TSLP correlates with the degree of hair follicle disruption, I collected dorsal skin (includes both epidermis and hair follicles) samples from P0, P6, P9 and P11 animals and extracted total RNA. When I compared the TSLP mRNA levels at birth, I found that RBPjcKO total skin had similar levels of TSLP expression in comparison to N1N2dcKO or PSdcKO skin (Figure 4-3). However, N1N2dcKO (~950 fold) and PSdcKO (~850 fold) skin had significantly higher levels of TSLP transcripts compared to RBPjcKO (~300 fold) skin starting at P6 (Figure 4-3). These findings suggest that the differences between RBPjcKO and N1N2dcKO (or PSdcKO) animals originated in the mutant hair follicles and not the epidermis. The timing of increased TSLP expression also corresponds
to the onset of the phenotypes at P6.

**There are less TSLP (+) keratinocytes in RBPjcKO hair follicles**

To evaluate whether RBPjcKO skin has fewer number of TSLP-producing cells, I performed TSLP staining (Figure 4-1B) on dorsal skin samples from RBPjcKO, N1N2dcKO and PSdcKO animals. The number of TSLP (+) cells in the epidermis, abnormal hair follicles, and keratin cysts within 20 different areas (1mm x 0.5mm) of dorsal skin was determined. Analysis of these results indicated that RBPjcKO hair follicles possessed significantly fewer TSLP (+) cells (95 cells in RBPjcKO vs. 150-200 cells in N1N2dcKO and PSdcKO) (Figure 4-5) suggesting that the decreased abnormality in the hair follicles correlates with a lower number of TSLP (+) cells.
CONCLUSIONS

TSLP is the core factor mediating the phenotypes in all Notch pathway mutants phenotypes. Therefore, I decided to characterize the spatio-temporal expression pattern of TSLP mRNA in mutant animals. First, I demonstrated that epidermal TSLP expression is similar in all Notch pathway mutants. Second, I established that expression of TSLP mRNA differs in the abnormal hair follicles starting at P6. RBPjcKO hair follicles express significantly less TSLP mRNA between P6 and P11 compared to N1N2dcKO or PSdcKO hair follicles. Finally, I provided evidence that RBPjcKO animals have significantly less keratinocytes cysts and that the majority of Rbpj−/− hair follicles are mildly perturbed and contain significantly less TSLP (+) cells. In summary, Rbpj−/− hair follicles are less distorted and produce less TSLP ultimately leading to a milder phenotype in RBPjcKO animals.
EXPERIMENTAL PROCEDURES

Generation of mice

I have generated following strains of mice using Msx2-Cre transgene mediated deletion in skin keratinocytes (Pan et al., 2004). Animals were raised and kept in Washington University Division of Comparative Medicine facility under Washington University Policy on Animal Care regulations.

\[ Msx2-Cre^{+/tg}; Rbpj^{floxed/floxed} \] (RBPjcKO)
\[ Msx2-Cre^{+/tg}; Notch1^{floxed/floxed}, Notch2^{floxed/floxed} \] (N1N2dcKO)
\[ Msx2-Cre^{+/tg}; Presenilin1^{floxed/floxed}, Presenilin2^{−/−} \] (PSdcKO)

RNA isolation, cDNA preparation and quantitative RT-PCR

If epidermis was used for experiments, skin samples were incubated in 5 mg/mL dispase for 3 hours at 4°C to separate the epidermis from the dermis. Total RNA was isolated from E18.5, P0, P6, P9, and P11 epidermis and P0, P6, P9, and P11 total skin using TRIzol (Invitrogen) kit or RNeasy kit (Qiagen) according to the manufacturer's instructions. RNase-free DNase I (Invitrogen, USA) was used to remove any residual genomic DNA. RNA was dissolved in 30µl of RNase-free water and stored in -80°C after RNA quality control (gel electrophoresis) and concentration measurements. Reverse transcription was performed with 1µg of total RNA, random primers and SuperScript III RNase H-Reverse Transcriptase (Invitrogen) in a total volume of 20µl. Expression levels of transcripts were examined by real-time qPCR with a 7500 Real-Time PCR System using SYBR Green dye (Applied Biosystems), iQ SYBR Green Supermix (Bio-Rad) or TaqMan Probes. The results were analyzed by absolute quantification with a relative standard curve or relative quantification of expression according to the “ΔΔC
methods” (Livak and Schmittgen, 2001) using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference for normalization. Primers were designed to amplify targets of 180–220 bp usually at the exon junctions to avoid amplification from genomic DNA contamination. The specificity of the PCRs was checked by recording a melting curve and by sequencing the amplicons.

To perform SYBR Green-based qRT-PCR for TSLP mRNA, the following primers were used:

TSLP-Fwd (5’-CCAGGCTACCCTGAAACTGA-3’)
TSLP-Rev (5’-TCTGGAGATTGCATGAAGGA-3’)

To perform probe (FAM-TAMRA)-based qRT-PCR for TSLP mRNA, the following primers & probe were used:

TSLP-F1 (5’-ACTGCAACTTCACGTCAATTACG-3’)
TSLP-R1 (5’-AGTTCGAGCAAATCGAGGACT-3’)
TSLP-Probe (5’ FAM-GACCTGACTGGAGATTTGAAAGGGGCT-TAMRA 3’)

**Histology and H&E Staining**

Skin samples were fixed in 4% paraformaldehyde (PFA) in PBS o/n, washed 3X in PBS, washed with 30% EtOH and 50% EtOH for 15 min, before storing them in 70% EtOH at 4°C. Skin samples were embedded in paraffin for sectioning at 8-12µm thickness. A standard H&E staining protocol was used for histology assessment.

**Immunohistochemistry**

Expression of K14 (basal cell marker), IRS 3.1 (inner root sheath cell marker), AE13 (hair cortex marker) or TSLP proteins were detected on 4% PFA-fixed paraffin sections with α-K14 chicken polyclonal antibody (a gift from Julie Segre - NIH), α-IRS 3.1 rabbit polyclonal antibody (a gift from Dr. Rebecca Porter - University of Dundee, Scotland, UK), α-AE13 mouse
monoclonal antibody (a gift from Dr. Tung-Tien Sun - New York University), and α-TSLP goat polyclonal antibody (R&D Systems - Cat No: BAF555) by o/n incubation at 4C. The next day, several washes (3 x 5 min in 0.5% Triton X-100 in PBS) were done prior to a 2-hour incubation with fluorophore-coupled secondary antibody to visualize the signal. Sections were mounted with Vectashield for fluorescence with DAPI.
REFERENCES:


Figure 4-1

A

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**Figure 4-1. All Notch mutant hair follicles display morphological problems at birth.**

(A) Immunofluorescent staining for hair follicle markers at P0: K14 (purple, epidermal basal cell marker), AE13 (red, hair cortex marker), IRS3.1 (green, Inner root sheath cell marker), and DAPI (blue). All Notch mutants start to have morphological problems at Bar: 100 µm (B) Immunofluorescent staining for K14 (red), TSLP (green) and DAPI (blue) on P9 skin samples. TSLP is expressed in ORS cells (K14$^+$ cells) White Bars: 100 µm. Yellow Bar: 200 µm.
Figure 4-2

TSLP mRNA expression in epidermis at E18.5

WT  | N1N2dcKO | PSdcKO | RBPjcKO
---  | -------- | ------- | -------
1.04 | 67.18    | 105.09  | 189.79  

* : p<0.0001

TSLP mRNA expression in epidermis at P0

WT  | N1N2dcKO | PSdcKO | RBPjcKO
---  | -------- | ------- | -------
1.03 | 2965.54  | 3650.60 | 3199.96 

TSLP mRNA expression in epidermis at P6

WT  | N1N2dcKO | PSdcKO | RBPjcKO
---  | -------- | ------- | -------
1.12 | 1256.34  | 1582.84 | 1513.12 

TSLP mRNA expression in epidermis at P9

WT  | N1N2dcKO | PSdcKO | RBPjcKO
---  | -------- | ------- | -------
1.06 | 1862.77  | 1379.04 | 1492.69 

TSLP mRNA expression in epidermis at P11

WT  | N1N2dcKO | PSdcKO | RBPjcKO
---  | -------- | ------- | -------
1.01 | 1430.79  | 1225.14 | 1695.11 

Figure 4-2. *Rbpj*<sup>−/−</sup>, *NIN2*<sup>−/−</sup> and *PS*<sup>−/−</sup> epidermis expressed similar levels of TSLP mRNA. Real-time PCR analyses of TSLP mRNA in *Rbpj*<sup>−/−</sup>, *NIN2*<sup>−/−</sup>, *PS*<sup>−/−</sup> and WT epidermis from E18.5, P0, P6, P9, and P11 animals. Error bars indicate standard deviation.
Figure 4-3

TSLP mRNA expression in total skin at P0

WT  N1N2dcKO  PSdcKO  RBPjcKO

1.09  319.73  410.46  312.14

Fold Change

TSLP mRNA expression in total skin at P6

WT  N1N2dcKO  PSdcKO  RBPjcKO

1.05  958.97  865.59  302.85

* : p<0.001

TSLP mRNA expression in total skin at P9

WT  N1N2dcKO  PSdcKO  RBPjcKO

1.06  1805.75  1368.38  538.33

TSLP mRNA expression in total skin at P11

WT  N1N2dcKO  PSdcKO  RBPjcKO

1.01  2948.19  2996.20  1538.36

* : p<0.001
Figure 4-3. RBPjcKO skin has significantly less TSLP expression compared to N1N2dcKO or PSdcKO skin.

Real-time PCR analyses of TSLP mRNA in RBPjcKO, N1N2dcKO, PSdcKO and WT dorsal skin from P0, P6, P9, and P11 animals. Error bars indicate standard deviation.
### Distribution of Abnormal Hair Follicle Phenotypes in Notch mutants

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**Figure 4-4**

*Distribution of Abnormal Hair Follicle Phenotypes in Notch mutants*

- WT
- N1N2dcKO
- PSdcKO
- RBPjcKO
Figure 4-4. Distribution of abnormal hair follicle phenotypes in Notch pathway mutants.

A scoring system was used to compare the severities of the hair follicle phenotypes in a quantitative manner. Three independent blind observers scored each hair follicle within 10 random areas (3mm x 0.5mm) of dorsal skin at P9 to determine the average distribution of scores for each genotype. Error bars indicate standard deviation.
Figure 4-5

Distribution of TSLP (+) cells in the epidermis & abnormal hair follicles

- Epidermis
- Abnormal HFs
- Total Skin

* : p<10^-7
Figure 4-5. Distribution of TSLP (+) cells in the epidermis and aberrant hair follicles for Notch pathway mutants at P9.

Three independent observers counted TSLP (+) cells within the epidermis and hair follicles (avg. 10-13 hf per area) in 1mm x 0.5mm area of dorsal skin. 20 random areas were assessed to determine the average number of TSLP (+) cells per genotype. Error bars indicate standard deviation.
CHAPTER V

Conclusions

and

Future Directions

Author Contribution
The author wrote the text in this chapter.
The main focus of this dissertation was to investigate a novel role for RBPjk-independent Notch signaling during mouse skin development. The possibility of this novel role came from previous studies on different loss of Notch signaling mutants. In 2008, Demehri et al. showed that keratinocyte-specific deletion of Rbpj (RBPjcKO) gene produced a significantly milder phenotype than keratinocyte-specific deletion of both Notch1 and Notch2 (N1N2dcKO) or Preselin1 and Preselin2 (PSdcKO) genes. This observation suggested an RBPjk-independent function of Notch signaling in the skin.

The canonical Notch signaling pathway mediates Notch function in epidermal differentiation and hair follicle maintenance in the embryo

We tested several hypotheses in order to account for the milder phenotype in RBPjcKO animals. I performed E18.5 epidermal transcriptome, P0 epidermal proteome and P0 total skin transcriptome analyses that showed all of various Notch pathway mutants have an indistinguishable transcriptional signature at birth. These results suggested that canonical Notch signals are responsible for the Notch function during epidermal differentiation and hair follicle maintenance during embryonic development.

γ-secretase-cleaved Notch1 ICD is necessary and sufficient to drive the milder phenotype in RBPjcKO animals

While dissecting the Notch pathway to identify factors that may account for the milder RBPjcKO phenotype, we assessed three possible hypotheses: (1) Derepression of target genes, (2) Ligand back signaling (LBS), and/or (3) γ-secretase-cleaved RBPjk-independent Notch1 ICD activity. I generated triple mutants of N1N2RBPjcKO and PSRBPjcKO animals to test derepression of targets and utilized N1CreERTN2dcKO animals to test the LBS model. In each of these experiments the results clearly demonstrated that neither derepression nor LBS is the
mechanism behind the milder RBPjcKO phenotype. Finally, we have generated N1vgN2RBPjcKO animals to test whether a limiting amount of Notch1ICD was sufficient to drive the RBPjcKO phenotype. These analyses suggested that γ-secretase-cleaved Notch1ICD was necessary and sufficient to drive the milder phenotype in N1vgN2RBPjcKO animals.

**Hair follicles with Rbpj<sup>−/−</sup> keratinocytes express less TSLP ameliorating the phenotype in RBPjcKO animals**

In the first two weeks of Notch pathway mutant animals’ lives, high serum TSLP levels induce uncontrolled pre-B cell proliferation leading to a shorter lifespan for N1N2dcKO and PSdcKO animals suggesting that TSLP is the core factor mediating these phenotypes (Demehri et al., 2008). Therefore, I decided to determine the spatio-temporal expression pattern of TSLP mRNA in mutant animals. First, I demonstrated that epidermal TSLP expression is similar in all Notch pathway mutants. Second, I showed that RBPjcKO hair follicles express significantly less TSLP mRNA starting at P6. Finally, I provided evidence that RBPjcKO animals have significantly less keratinocytes cysts and that the majority of hair follicles with Rbpj<sup>−/−</sup> keratinocytes are mildly perturbed and contained significantly less TSLP (+) cells. In summary, TSLP mRNA expression and hair follicle phenotype analyses suggest that hair follicles with Rbpj<sup>−/−</sup> keratinocytes are less distorted and produce less TSLP ultimately leading to a milder phenotype in RBPjcKO animals.

**Discussion**

Experiments presented in this dissertation suggest that RBPjk-independent NICD-dependent signals ameliorate RBPjcKO hair follicle phenotype compared to the other Notch-loss-of-function hair follicles. Less distortion in overall hair follicle morphology leads to less TSLP production, consequently generating a milder BLPD and relatively longer lifespan for RBPjcKO
animals. To our knowledge, this is the first study that provides evidence for a non-canonical Notch function in the mouse hair follicles. It is still a mystery how these signals mediate a milder hair distortion phenotype in RBPjcKO animals. Previous publications suggest potential interactions between Notch ICD and other molecules such as Deltex (Ordentlich et al., 1998; Ramain et al., 2001; Yamamoto et al., 2001; Endo et al., 2002; Endo et al., 2003; Hu et al., 2003; Hori et al., 2004), Ras (Hodkinson et al., 2007), β-catenin (Acosta et al., 2011), or Rho/ROCK (Yugawa et al. 2013). However, we still do not have evidence regarding whether any of these molecules are playing a role during this process. We will further investigate the signaling pathways that are upregulated/downregulated in RBPjcKO hair follicles and compare them to N1N2dcKO or PSdcKO hair follicle transcriptome signature as suggested in Aim1 to identify Notch interacting pathways.

We will also try to identify molecules that are directly interacting with NotchICD in the absence of RBPjk (Aim 2). If these molecules are potential DNA binding partners, then we will also investigate the target genes that are responsible for the milder hair follicle phenotype (Aim3).

**A note on “Epigenetic Memory” - Caveat and Solutions**

Based on our transcriptome and proteome analysis at birth (Chapter 2), we claimed that there is no “epigenetic memory” in RBPjcKO animals. This statement still holds for epidermis, however there is still a possibility of “epigenetic memory” that is encrypted within Rbpjf/ hair follicle progenitor cells eventually leading to the milder distortion in RBPjcKO hair follicles. Notch signaling is necessary for maintaining IRS cellular identity in the hair follicle (Pan et al., 2004; Blanpain et al., 2006) and activation of Notch1 receptor within mouse hair follicles can be detected in the hair follicle cortex and cuticle precursors (Powell et al. 1998, Pan et al., 2004). Even though, ORS cells do not experience Notch activation, non-cell autonomous effects induce
an unexpected epidermal differentiation program within ORS cells in the absence of Notch signaling. Therefore, we think loss-of-Notch signaling within the IRS and hair cortex is critical for the phenotypic differences in the hair follicle morphology. To distinguish whether “epigenetic memory” accounts for milder phenotype in RBPjcKO hair follicles, we will generate a new animal that will have an inducible expression of Notch1 ICD within IRS cells in the background of triple mutant animals, N1N2RBpjKO or PSRBpjKO. We will transiently express Notch1 ICD between E18.5-P2 in which IRS cells expected to have active Notch signaling. If Notch1 ICD + PSRBpjKO animals resemble RBPjcKO or N1vgN2RBpjKO animals, then we will conclude that “epigenetic memory” is not the reason behind the phenotype. Otherwise, “epigenetic memory” could have a role in milder RBPjcKO hair follicle phenotype.
Future Directions

Aim 1: Determine whether TSLP expression is similar in \(Rbpj^{−/−}\), \(NIN2^{−/−}\), and \(PS^{−/−}\) hair follicle keratinocytes.

We want to know whether TSLP mRNA expression per cell is different in \(Rbpj^{−/−}\) hair follicle keratinocytes vs \(NIN2^{−/−}\) (or \(PS^{−/−}\)) hair follicle keratinocytes. To determine this, we will isolate total RNA from the same grade hair follicles (rank #8-9-10) of RBPjcKO, N1N2dcKO, and PSdcKO skin by using Laser Capture Microscopy (LCM) and perform qRT-PCR for TSLP expression.

1. If less TSLP mRNA is produced in \(Rbpj^{−/−}\) cells, then the question is limited to understanding TSLP gene regulation, which we will further investigate in ORS/cyst keratinocytes. We will perform global gene expression analysis/RNAseq to identify the genes that are correlated with less/more TSLP expression. We will also investigate the transcriptional regulation of TSLP gene by dissecting the core regulatory elements within the promoter and enhancer region of the TSLP gene.

2. If the levels of TSLP mRNA are unchanged between \(Rbpj^{−/−}\) and \(NIN2^{−/−}\) (or \(PS^{−/−}\)) hair keratinocytes, then they will phenotypically resemble \(Rbpj^{−/−}\), \(NIN2^{−/−}\), and \(PS^{−/−}\) epidermal keratinocytes. In this case, we will investigate the reasons for the differences in morphology between RBPjcKO and N1N2dcKO (or PSdcKO) hair follicles. The hair follicles from RBPjcKO are less distorted compared to other Notch mutant hair follicles. We will utilize LCM to determine which molecular pathways are altered in the RBPjcKO hair follicles that give rise to the milder phenotype. We will compare the transcriptional profiles of milder RBPjcKO hair follicles (rank #3-4-5-6) to the transcriptional profiles derived from severe keratin cysts (rank #8-9-10) of RBPjcKO skin, N1N2dcKO skin, or
PSdcKO skin. We hypothesize that the transcriptional differences are expected to be due
to the excess Notch1ICD molecules in RBPjκKO cells.

**Aim 2: Determine whether γ-secretase-cleaved Notch1ICD is interacting with any other molecule in the absence of RBPjκ.**

We showed that even limiting amounts of Notch1ICD is sufficient to give rise the milder phenotype in N1vgN2RBPjκKO animals. In the absence of RBPjκ, it is possible that Notch1ICD may interact with other molecules/proteins that are responsible for the milder phenotype observed in RBPjκKO animals. To identify potential Notch1ICD interacting molecules, we will utilize a screen using double-tagged Notch1-6myc6his (N16M6H) expressing animals and perform pull-down assays for N16M6H in the background of Rbpjκ−/− hair follicles. We will identify the proteins associated with Notch1ICD using mass spectrometry analysis. These animals were generated using a knock-in strategy and are viable as homozygous.

**Aim 3: Determine transcriptional target gene expression by Notch1ICD in the absence of its canonical DNA-binding partner, RBPjκ.**

In the absence of RBPjκ, we hypothesize that Notch1ICD could interact with other transcription factors and induce gene expression. In order to test this possibility, I will perform chromatin immunoprecipitation (ChIP) assays with the tagged N16M6H in Rbpjκ−/− hair follicles and identify DNA sequences that are bound by Notch1ICD in the absence of RBPjκ. We will also identify potential direct transcriptional targets of Notch1ICD in Rbpjκ−/− hair follicles by intersecting this candidate gene list with the genes from expression profile analysis proposed in Aim1.
CHAPTER VI

Elevated epidermal Thymic Stromal Lymphopoietin levels

establish an anti-tumor environment in the skin

Publication & Copyright
Results and text presented in this chapter have been previously published in:

Author Contribution
The author contributed only the following sections of this chapter:
• Generation of mice for Figure 6-1A.
• All the experiments in Figure 6-3, except H&E and α-RBPj antibody stainings.
• Part of the effort to perform the BM transplatation experiments in Figure 6-5 A & B, Figure 6-6 and Figure 6-7.

Dr. Shawn Demehri carried out majority of the other experiments described in this chapter with the assistance from other authors. Dr. Shawn Demehri and Kopan wrote the text of the published manuscript.
SUMMARY
Thymic Stromal Lymphopoietin (TSLP), a cytokine implicated in induction of T helper 2 (Th2) -mediated allergic inflammation, has recently been shown to stimulate solid tumor growth and metastasis. Conversely, studying mice with clonal loss of Notch signaling in their skin revealed that high levels of TSLP released by barrier-defective epidermis caused a severe inflammation resulting in gradual elimination of Notch-deficient epidermal clones and resistance to skin tumorigenesis. We found CD4+ T cells to be both required and sufficient to mediate these effects of TSLP. Importantly, TSLP overexpression in wild-type skin also caused resistance to tumorigenesis, confirming that TSLP functions as a tumor suppressor in the skin.

Significance
We demonstrate unequivocally that TSLP triggers a dominant anti-tumor response in a Th2-polarized inflammatory microenvironment in the skin. Importantly, the anti-tumor microenvironment created by TSLP-inducers like vitamin D agonists (e.g. Calcipotriol) can prevent and eliminate skin tumors in wild-type mice. Although our findings may reflect a skin-specific effect, it is intriguing to postulate that TSLP plays a common tumor suppressor role during the early stages of solid tumor development. Considering the emergence of TSLP as a potential new therapeutic target in treatment of solid cancers, this report points to an alternative utility for TSLP as an anti-tumor immune factor that can be utilized to optimally combat and ultimately prevent solid cancers.

Highlights
• Notch signaling-deficient skin with severe Th2 inflammation is resistant to tumorigenesis
• TSLP signaling is responsible for tumor resistance in Notch-signaling deficient skin
• TSLP acts through CD4+ T cells to establish tumor resistance in Notch-deficient skin
• TSLP upregulation in wild-type skin can produce in resistance to tumorigenesis
INTRODUCTION

The mammalian skin is a model organ with established propensity for solid tumor development. Used for decades in chemical carcinogenesis studies, it contributed to the recognition that carcinogenesis is a step-wise process (Hanahan and Weinberg, 2000). As in other models of cancer, skin tumors arise as a consequence of intrinsic changes in an initiated cell that are amplified through interactions with the tumor microenvironment (Campisi, 2005; Kessenbrock et al., 2010; Sneddon and Werb, 2007). Immune cells are one of the main components of tumor microenvironment. Although these cells can suppress tumor growth, their carcinogenesis-promoting role is becoming increasingly appreciated (Coussens and Werb, 2002; Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Moreover, because skin is the largest barrier organ in the body, it is under tight surveillance by the immune system, and even subtle changes in its cellular differentiation program can alter the overall susceptibility to cancer by eliciting a persistent inflammatory response (Quigley et al., 2009). This is partly due to a direct epidermal contribution to the inflammation via secretion of multiple cytokines, such as interleukin (IL) -1, IL-6 and TGF-b (Morasso and Tomic-Canic, 2005).

In the current report, the significance of inflammation in modulating tumorigenesis emerged from studying mice in which the Notch signaling pathway is inactivated in a subset of epidermal keratinocytes using the Msx2-Cre^{0w/+} line. This line expresses Cre recombinase early, transiently and only in the dorsal and ventral midline regions, generating a calico pattern of gene deletion (Figure 6-1A). This deletion pattern has allowed us to document the cell autonomous as well as the non-cell autonomous consequences of reducing Notch signal in the skin (Demehri et al., 2008; Demehri et al., 2009b). Notch is a transmembrane receptor that mediates short-range communication between adjacent cells (Kopan and Ilagan, 2009). Upon binding to the ligand
presented by a neighboring cell, Notch undergoes proteolysis by γ-secretase enzyme to release its intracellular domain (NICD). Subsequently, NICD translocates into the nucleus and binds to its DNA-binding partner, RBPjk, and regulates its downstream targets in a context-dependent manner (Kopan and Ilagan, 2009). Notch signaling plays multiple roles in skin development (Mascia et al., 2011), but in the context of carcinogenesis, the most relevant role is in promoting supra-basal differentiation (Blanpain et al., 2006; Demehri et al., 2009b; Nguyen et al., 2006; Nicolas et al., 2003b; Pan et al., 2004a; Rangarajan et al., 2001). Reduction in Notch signaling leads to aberrant epidermal differentiation and defective barrier formation, which creates a chronic wound-like environment prone to spontaneous skin tumors. For example, loss of Notch1 renders mice sensitive to 7,12-dimethylbenz[a]anthracene (DMBA)-induced cancer (Nicolas et al., 2003b) through induction of a non-cell autonomous feed-forward loop between the epidermis, dermal fibroblasts and inflammatory infiltrates (Demehri et al., 2009b). Consistent with these findings, mice and humans lacking the components of the γ-secretase complex (Pen2, PS1, Nestn) have elevated rates of spontaneous tumor development (Lapins et al., 2001; Li et al., 2007; Wang et al., 2010; Xia et al., 2001). Additionally, the γ-secretase inhibitor Semagacestat (LY450139) led to elevated incidence of skin cancer among the participants in a phase III clinical trial (Extance, 2010). Importantly, the latency for spontaneous tumor formation in the epidermis is determined by the degree of disruption in its differentiation program cased by Notch signaling loss (Demehri et al., 2009b). An epidermal-derived cytokine that rises as more of Notch signal is lost in the skin is Thymic Stromal Lymphopoietin (TSLP), which could contribute to the susceptibility of Notch-deficient skin to tumorigenesis (De Monte et al., 2011; Demehri et al., 2008; Demehri et al., 2009b; Olkhanud et al., 2011; Pedroza-Gonzalez et al., 2011).
TSLP is an interleukin (IL)-7-like cytokine studied mainly in the context of T helper 2 (Th2)-mediated allergic inflammation in the skin and lung (Leonard, 2002; Rochman et al., 2009; Ziegler, 2010); overexpression of TSLP is sufficient to promote the development of atopic dermatitis and asthma, respectively (Ziegler, 2010). Importantly, transient exposure to TSLP is sufficient to prime the skin and lung immune cells if an otherwise innocuous allergen is also present, creating long-lasting T cells that can trigger allergic inflammation at a later time (Zhang et al., 2009). In the gut, however, TSLP is continuously expressed and plays a physiological function by suppressing the Th1 response thus building tolerance to the gut microbiome (Zeuthen et al., 2008). Although TSLP is not expressed in skin under pathological conditions, skin keratinocytes are powerful secretors of TSLP in both humans (Lee et al., 2010) and mice, where chronic and severe barrier disruption can result in TSLP release into the serum up to 5000 folds over its baseline levels (Demehri et al., 2008; Dumortier et al., 2010; Zhang et al., 2009). Interestingly, this drives a leukemia-like B cell lymphoproliferative disease in newborn mice (Demehri et al., 2008) and constitutively active TSLP signaling causes acute B-lymphoblastic leukemia in children (Cario et al., 2010; Hertzberg et al., 2010; Shochat et al., 2011). In addition, TSLP has recently emerged as a pro-growth cytokine in breast and pancreatic cancers (De Monte et al., 2011; Olkhanud et al., 2011; Pedroza-Gonzalez et al., 2011). These findings suggest a therapeutic opportunity for TSLP-blocking agents, already in development for the treatment of allergic diseases (Schmitt, 2010), as cancer immunotherapeutic agents.

Unexpectedly, mice lacking RBPjκ in their skin released high levels of TSLP, but they did not develop spontaneous tumors and were resistant to chemical carcinogens. We demonstrated that at high concentrations, TSLP facilitates elevated immune surveillance by the adaptive immune system leading to rejection of the mutant cells and to tumor resistance. Global
deletion of the TSLP receptors (IL7rα or CRLF2 (TSLPR)) led to aggressive expansion of Notch-deleted epidermal clones and exposed the inherent predisposition of Notch signaling-deficient skin to tumor formation. Restricting TSLP reception exclusively to the CD4+ T cells was sufficient to maintain tumor resistance and elimination of Notch-deficient cells. In addition, elimination of only CD4+ T cells in an otherwise TSLP-responsive immune environment abolished this anti-tumor response. Importantly, induction of epidermal TSLP expression with a vitamin D analog, Calcipotriol (Dovonex™), endowed wild-type animals with marked resistance to chemical skin carcinogenesis. These effects can be directly attributed to TSLP since similar protection was seen in transgenic mice overexpressing this cytokine independent of Vitamin D receptor activation. Taken together, these findings demonstrate the ability of TSLP to create a T cell-based tumor-suppressing microenvironment in the skin. Considering the current view of TSLP antagonists as potential new therapeutic agents in treatment of solid cancers, this report highlights the potential use of TSLP or its agonists towards establishing an anti-tumor immune response.
RESULTS

Mice lacking all canonical Notch signaling in the epidermis are resistant to skin carcinogenesis

Step-wise removal of Notch alleles in epidermal keratinocytes with Msx2-Cre is associated with a corresponding decline in skin-barrier function, creation of a wound-like environment and increased susceptibility to carcinogenesis ((Demehri et al., 2009b) and Figure 6-1A&B). Initially, susceptibility to carcinogenesis was not tested in mice with complete loss of Notch signaling in their skin (e.g. Msx2-Cre\textsuperscript{tg/+}; PS1\textsuperscript{flox/flox}; PS2\textsuperscript{-/-} (PSDCKO; loss of \(\gamma\)-secretase enzyme function)) because they die shortly after birth, not allowing enough time for skin tumor development (Figure 6-1B). Rbpj-deficient animals (Msx2-Cre\textsuperscript{tg/+}; Rbpj\textsuperscript{flax/flax} or RBPjCKO), however, live for ~100 days on average, which is comparable to animals lacking all but one Notch2 allele in their skin (Msx2-Cre\textsuperscript{tg/+}; Notch1\textsuperscript{flox/flox}; Notch2\textsuperscript{flox/+}; Notch3\textsuperscript{-/-} or N1N2hN3CKO). Surprisingly, while 20% of N1N2hN3CKO mice developed spontaneous skin tumors, none (0/40) of the RBPjCKO mice that have been examined developed spontaneous tumors (Figure 6-1B).

To further rule out the possibility that the short life span of RBPjCKO mice masked their cancer susceptibility, we subjected young RBPjCKO and their wild type littermates to a multistage chemical skin carcinogenesis model (Yuspa et al., 1994). The RBPjCKO cohort was maintained in a mixed genetic background (FVB, CD1, and C57BL/6) to facilitate tumor development within a short treatment period. Three-week-old RBPjCKO and wild type littermates (defined as all mice not inheriting the Cre transgene) were treated with a single initiating dose of DMBA, followed by a twice-weekly dose of 12-O-tetradecanoylphorbol-13-acetate (TPA) for 14 weeks. Surprisingly, whereas the majority of wild type littermates...
developed more than 20 papillomas/mouse, no tumors were detected in the DMBA/TPA-treated RBPjCKO animals after 15 weeks of follow-up (n=7 in each group, p <0.0001, Figure 1C&D). These results contrast starkly to the enhanced susceptibility to tumor formation seen in other Notch-deficient animals (Figure 6-1B; (Demehri et al., 2009b)). The resistance of RBPjCKO mice to skin carcinogenesis might reflect a reduced growth potential of RBPj-deficient keratinocytes (Blanpain et al., 2006). However, even if this were the case, we would expect initiated wild-type cells adjacent to RBPjCKO epidermis to form tumors (Demehri et al., 2009b). Alternatively, the global resistance to tumorigenesis in RBPj-deficient skin may be due to a switch from a tumor-promoting environment in N1CKO (Demehri et al., 2009b) to a tumor-suppressing environment in RBPjCKO skin.

**Wild-type keratinocytes replace their Notch signaling-deficient neighbors over time**

The calico pattern of gene deletion in Msx2-Cre-positive animals allowed us to notice a second, potentially related phenotype in RBPjCKO mice. As these mice aged, the mutant epidermal clones on their dorsal and ventral surfaces shrank, and Rbpj-deficient epidermal clones eventually disappeared in the oldest individuals (Figure 6-2A and S1). Although Msex2-Cre<sup>Wt/+; Notch1<sup>flox/flox</sup>, Notch2<sup>flox/flox</sup> (N1N2CKO) and PSDCKO mice die at weaning due to a lethal B-lymphoproliferative disorder (Demehri et al., 2008), they can survive longer if we controlled their B-LPD with a sublethal dose of irradiation (Figure 6-2B, (Demehri et al., 2008)). When lethality was rescued in this manner we observed a similar regression of Notch1/2- or PS1/2-deleted epidermal territories as N1N2CKO and PSDCKO animals aged. This could reflect the passive proliferative disadvantage of Notch signaling-deficient keratinocytes or the active process of rejection.
H-Ras infected Notch-deficient keratinocytes are highly tumorigenic in immune-compromised mice

To ask if the resistance of RBPjCKO mice to carcinogenesis and the loss of mutant keratinocytes are due to a low proliferative capacity of Rbpj-deficient keratinocytes, we evaluated their tumor-forming potential in the nude mouse environment in response to the activated H-Ras oncogene (Nicolas et al., 2003b). First, we isolated keratinocytes from newborn Rbpj^fl/fl and Rbpj^fl/+ littermates. Cells were then infected with activated H-Ras-expressing retrovirus, allowed to recover for 24 hours and then infected with Cre-expressing adenovirus. H-ras-infected, Cre expressing, Rbpj^-/- or Rbpj^+/+ cells (1.5 x 10^6) were injected subcutaneously into nude mice and tumor development was monitored over time. Importantly, RBPjcko (Rbpj^-/-) keratinocytes formed large tumors in 30 days while wild-type (Rbpj^+/+) keratinocytes did not form a significant tumor mass (Figure 6-3). Similar results were obtained using γ-secretase-deficient (PSDCKO) keratinocytes (Figure 6-3). The formation of massive tumors by Notch signaling-deficient keratinocytes demonstrates that these cells are intrinsically competent to form tumors in a T cell-deficient environment. Therefore, we hypothesized that the apparent resistance of Notch signaling-deficient animals to skin tumorigenesis is most likely due to the activation of a tumor-suppressing environment in their skin.

Notch-deficient animals develop severe allergic skin inflammation caused specifically by epidermal TSLP overexpression

Inflammation can either promote or suppress tumorigenesis depending on its magnitude and the immune cells involved (Coussens and Werb, 2002; Schreiber et al., 2011). As previously shown (Demehri et al., 2009a; Dumortier et al., 2010), the severe skin-barrier defect in
Notch/RBPj-deficient mice leads to the development of a full-blown atopic dermatitis (AD)-like allergic inflammation. This level of skin-barrier defect and Th2 inflammation is not detected in mice with at least one allele of Notch2 remaining (N1N2hN3CKO; Figure 6-4A, (Demehari et al., 2009b)). In sharp contrast to the mild inflammation, dermal fibroplasia and angiogenesis, which generated a tumor-promoting environment in N1CKO skin (Demehari et al., 2009b), RBPjCKO skin exhibited a significant accumulation of leukocytes (CD45+ cells) beneath the Rbpj-deficient epidermal clones (Figure 6-4A). This high level of dermal inflammation could have a suppressing effect on tumor growth (Coussens and Werb, 2002) perhaps by inducing anti-tumor immune responses and thus explain the loss of Notch/RBPj-deficient cells over time. Therefore, we reasoned that reducing the inflammation could restore an environment permissive to and/or promoting carcinogenesis. To address the role of extreme inflammation as the key factor preventing carcinogenesis in Rbpj-deficient skin, we first examined the effect of immunosuppressant drugs on DMBA-treated RBPjCKO mice. Treatment with the maximum tolerable doses of Dexamethasone or Methotrexate did not significantly reduce inflammation in RBPjCKO mice nor did it affect the rejection of mutant skin cells or allow tumor formation (Figure 6-S2 online).

RBPjCKO keratinocytes produced significantly higher levels of TSLP than those seen in the tumor-prone N1CKO and N1N2hN3CKO animals (Figure 6-4B). High TSLP levels are known to drive the severe Th2 inflammation seen in RBPjCKO skin (Demehari et al., 2009a; Dumortier et al., 2010; He et al., 2008). Therefore, we next examined the effect of loss of TSLP signaling on inflammation by generating RBPjCKO mice that lack the IL7rα arm of the TSLP receptor (Msx2-Cre+; Rbpj–/–; IL7rα+/– or RBPjCKO;IL7rα–/– (Demehari et al., 2009a)). IL7rα mice were used in place of TSLPR deficient mice because the Rbpj and CRLF2 (TSLPR) genes
are linked on the same arm of chromosome 5, and thus we were unable to generate RBPjCKO;TSLPR<sup>−/−</sup> animals. Deleting *IL7rα* in RBPjCKO or N1N2CKO (*Msx2-<sup>Cre<sub>tg</sub>/+</sup>; Notch<sup>1<sub>flax/flax</sub></sup>, Notch<sup>2<sub>flax/flax</sub></sup>, *IL7rα*<sup>−/−</sup> or N1N2CKO; *IL7rα*<sup>−/−</sup>) mice led to a marked reduction in skin inflammation (Figure 6-4C and Figure 6-S3 online). Flow cytometry on dermal cells showed that CD4+ T cell population were significantly reduced (Figure 6-S7 online). To confirm that this effect was specific to TSLP and not an indirect consequence of reduced lymphocyte number caused by *IL7rα* deletion (Peschon et al., 1994), we deleted the TSLPR arm of the TSLP receptor in PSDCKO animals (and *Msx2-<sup>Cre<sub>tg</sub>/+</sup>; *PS1<sub>flax/flax</sub>, *PS2*<sup>−/−</sup>; TSLPR<sup>−/−</sup> or PSDCKO; TSLPR<sup>−/−</sup>), which significantly prolonged their lifespan (Dumortier et al., 2010). As with N1N2CKO;*IL7rα*<sup>−/−</sup> and RBPjCKO; *IL7rα*<sup>−/−</sup>, inflammation was greatly reduced in PSDCKO; TSLPR<sup>−/−</sup> animals (Figure 6-S3 online). Taken together, these results demonstrate a central role for TSLP in regulating the level of inflammation in Notch-deficient skin.

**Blocking TSLP signaling in Notch-deficient animals results in the expansion of the mutant skin and tumorigenesis**

RBPjCKO; *IL7rα*<sup>−/−</sup> animals showed a clear reversal of the two phenotypes unique to RBPjCKO mice. First, RBPj-deficient epidermal clones expanded dramatically in RBPjCKO; *IL7rα*<sup>−/−</sup> mice and formed numerous hypertrophic cysts (Figure 6-5A). Second, RBPjCKO; *IL7rα*<sup>−/−</sup> mice developed spontaneous invasive dermal and exophytic tumors over time (Figure 6-5 and S4 online). N1N2CKO; *IL7rα*<sup>−/−</sup> and PSDCKO; TSLPR<sup>−/−</sup> animals also showed expansion of their mutant skin territories (Figure 6-5A, (Dumortier et al., 2010) and the accompanying paper by Di Piazza et al). All RBPjCKO; *IL7rα*<sup>−/−</sup> and PSDCKO; TSLPR<sup>−/−</sup> animals eventually developed cancerous lesions that invaded through the subcutaneous muscle layer at 10 to 15 weeks of age (Figure 6-5B and S4 online). Treating the skin of these animals
with a single dose of DMBA further revealed their susceptibility to tumorigenesis (Figure 6-5B). Therefore, reducing the inflammation by eliminating TSLP reception restored a tumor-promoting environment reminiscent of the N1CKO mice and uncovered the underlying cancer-prone phenotype in mice lacking canonical Notch signaling in their skin.

To test whether another prominent proinflammatory cytokine shown to be overexpressed in Notch signaling-deficient skin (Dumontier et al., 2010) contribute to skin rejection and the tumor resistant phenotype, we blocked TNFα signaling in RBPjCKO animals. RBPjCKO animals lacking TNFα signaling (Msx2-Cre<sup>tg/+; Rbpj<sup>fl<sup>ox</sup>/</sup>; TNFRI<sup>−/−</sup>; TNFRII<sup>−/−</sup>) were indistinguishable from their RBPjCKO littermates (Figure 6-S5 online). Therefore, we conclude that high levels of inflammation triggered by TSLP are required to initiate the process that leads to elimination of the Notch-, Rbpj- or Presenilin-deficient keratinocytes, which are otherwise proliferative, and prevents tumorigenesis in this background. This supports the hypothesis that skin tumor resistance and mutant cell rejection phenotypes are specific to TSLP-responsive effectors.

**The adaptive immune system mediates the effects of TSLP on skin rejection and tumor resistance**

In order to determine if bone marrow (BM)-derived immune cells mediated the effects of TSLP on skin rejection and resistance to tumorigenesis, we reconstituted the immune system of lethally irradiated RBPjCKO; IL7<sup>α−/−</sup> and PSDCKO; TSLPR<sup>−/−</sup> mice with BM from their wild-type littermates and monitored their response to DMBA treatment. Interestingly, wild-type BM restored resistance to DMBA-induced tumors in RBPjCKO; IL7<sup>α−/−</sup> and PSDCKO; TSLPR<sup>−/−</sup> mice; concomitantly, the mice gained the ability to eliminate their mutant skin cells (Figure 6-6). In a complementary set of experiments, we reconstituted the immune system of lethally
irradiated RBPjCKO and PSDCKO mice with BM from their \( \text{IL}7r^\alpha^- \) and \( \text{TSLPR}^- \) littermates, respectively, to determine if a pro-tumor environment would be re-established. This immune reconstitution, however, failed to establish tolerance, and no DMBA-induced tumors formed (Figure 6-S6 online). This could be explained by the presence of irradiation-resistant activated and memory T cells in the mutant animals at the time of transplantation (Figure 6-S6 online). Together, these findings demonstrate that BM-derived immune cells act downstream of TSLP, and suggest that the effector cell type(s) formed in RBPjCKO and PSDCKO are resistant to lethal dose of irradiation (Figure 6-S7 online).

Based on the findings above, we focused on the immune cell repertoire in RBPjCKO (Figure 6-S8 online; (Demehri et al., 2009b)) and targeted cell types that are known to be resistant to irradiation ((Murphy et al., 1987) and personal communications from Dr. Lisa Coussens), are known to express TSLP receptors, and can mediate tumor resistance and skin rejection (Vesely et al., 2011). Based on these criteria, we chose to delete CD8\(^+\) Cytotoxic T lymphocytes (CTLs) and mast cells in RBPjCKO animals. Genetic depletion of these cells in RBPjCKO; CD8\(^a^-\) and RBPjCKO; Kit\(^{wsh/wsh}\) did not cause any alteration in the RBPjCKO skin phenotypes, suggesting that neither cell type is necessary to mediate the effects of TSLP (Figure 6-S9 online). In a separate set of experiments, we used previously described depleting antibodies (Rogers and Unanue, 1993; Shankaran et al., 2001) to deplete CD4\(^+\) T cells, CD8\(^+\) T cells, natural killer (NK) cells, or granulocytes in RBPjCKO animals. Although we achieved the complete depletion of these cell types, we failed to alter rejection or tumor resistance phenotypes in RBPjCKO mice (Figure 6-S10 online). From these sets of experiments, we concluded that the irradiation-resistant immune cell type(s) mediating the effects of TSLP were either: (a) resistant to depletion by irradiation and antibodies, which is characteristic of activated/memory T cells.
(Jamali et al., 1992; Murphy et al., 1987), or (b) not present among the cell types depleted with the antibodies we used.

To distinguish these possibilities systematically, we focused on RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− mice, which never establish TSLP-dependent tumor resistance and thus lack activated effector cells. Having established that Wild-type BM can reconstitute the skin rejection and tumor resistance in RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− animals (Figure 6-6), we examined whether the adaptive or innate immune system mediated these effects downstream of TSLP. To accomplish this, we reconstituted the immune cells of lethally irradiated RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− mice with BM from Rag2−/− donors that lack adaptive immunity. DMBA-treated RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− animals transplanted with Rag2−/− BM failed to reject mutant skin, which formed hypertrophic cysts and developed tumors (Figure 6-6), in contrast to RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− reconstituted with wild-type bone marrow that repress tumor development. This clearly demonstrates that adaptive immune cells respond to epidermal-derived TSLP are required for the tumor resistance and mutant skin rejection in Notch signaling-deficient animals.

**CD4+ T cells are both required and sufficient to mediate the effects of TSLP on skin rejection and tumor resistance**

Among adaptive immune cell types, activated T cells are known to be resistant to irradiation and persist after antibody depletion (Jamali et al., 1992; Murphy et al., 1987). Considering that mice lacking CD8+ CTLs retained their tumor resistance and skin rejection phenotypes (RBPjCKO; CD8a−/−; Figure 6-S9 online), CD4+ T cells emerged as the prime candidate mediating the effects of TSLP in Notch signaling-deficient skin. To test this hypothesis, 2 x 10⁶ CD4+ or CD8+ T cells from wild-type littermates were transferred to
sublethally irradiated RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− newborns. Adoptive transfer of wild-type CD4+ T cells to RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− mice re-established the tumor resistance and mutant skin rejection phenotypes in these animals, but adoptive transfer of wild-type CD8+ CTLs did not (Figure 6-7A). This finding demonstrates that CD4+ T cells are sufficient to receive TSLP signal and initiate the effects of TSLP in Notch signaling-deficient skin.

To ask if CD4+ T cells were required to initiate the effects of TSLP in Notch signaling-deficient skin, lethally irradiated RBPjCKO; IL7rα−/− mice were transplanted with c-Kit+ BM cells from their wild-type littermates and treated continually with anti-CD4, anti-CD8 or IgG control antibodies (Figure 6-7B). Injecting the RBPjCKO; IL7rα−/− animals with depleting antibodies weekly resulted in effective and sustained depletion of targeted cell types (Figure 6-7C). Importantly, only the RBPjCKO; IL7rα−/− mice that received wild-type BM progenitors plus anti-CD4 antibody retained their mutant cells, formed hypertrophic cysts, and developed skin tumors in response to DMBA (Figure 6-7D). Taken together, these data demonstrate that CD4+ T cells constitute the cell type receiving the TSLP signal and coordinating the immune response necessary to reject Notch signaling-deficient skin and prevent tumorigenesis in this context.

**Epidermal TSLP overexpression in wild-type skin prevents skin tumorigenesis**

A critical question that emerged from our findings is whether or not the tumor suppressor function of TSLP is specific to Notch signaling-deficient skin. To test if TSLP overexpression can mobilize the anti-tumor immune response of an otherwise wild-type animal, we used chemical and genetic approaches to upregulate epidermal TSLP expression in wild-type animals and investigate their response to the multistage chemical skin carcinogenesis model. First, we used the topical application of calcipotriol (low-calcemic Vitamin D3 analog; known also as
MC903 or Dovonex) to induce epidermal TSLP expression in CD1 female mice (Li et al., 2006). CD1 genetic background was chosen for these studies because the skin inflammation caused by TSLP overexpression downstream of calcipotriol treatment did not result in a full-blown AD-like disease (Demehri et al., 2009a). Mice were treated with a single initiating dose of DMBA (125mg), followed by a twice-weekly dose of TPA (4mg) for 14 weeks. The test group was also treated with topical calcipotriol (32nmol) and controls with carrier only (EtOH) five times a week during the 14 weeks of TPA application. Strikingly, the majority of carrier-only treated animals developed papillomas, whereas only two of the calcipotriol-treated animals transiently developed papillomas during 15 weeks of follow-up (n = 10 for each group, p <0.0001, Figure 6-8A&B).

To investigate the effect of high TSLP expression on existing skin tumors, we randomly divided the tumor-bearing CD1 animals from the carrier-only treatment group of the calcipotriol study (Figure 6-8A&B) into two groups at the end of the 15-week TPA treatment. One of the subgroups received calcipotriol five times weekly whereas the other received carrier only. We monitored their tumors for an additional 7-weeks. While the carrier-treated cohort maintained their tumor counts, the calcipotriol-treated animals lost their papillomas over time (p <0.05 at week 6 and 7, Figure 6-8C). In addition, the tumors from the carrier-treated cohort grew bigger over time, unlike the remaining calcipotriol-treated tumors that shrank (Figure 6-8D).

Considering that calcipotriol is a low-calcemic Vitamin D3 analog, it is possible that the effects observed above are related to vitamin D signaling independent of its upregulation of TSLP expression in the skin. Therefore to confirm that the anti-tumor effects observed are mediated through TSLP, we used animals that genetically overexpress TSLP in their basal keratinocytes. Importantly, K14-TSLPtg animals treated with DMBA-TPA also showed
significant resistance to tumorigenesis compared to their wild-type littermates (n=22 for K14-TSLP\textsuperscript{tg} group and 21 for wild-type group, p <0.0001, Figure 6-8E&F). The serum TSLP measurements confirmed the overexpression of TSLP in Calcipotriol-treated wild-type and K14-TSLP\textsuperscript{tg} animals (Figure 6-8G). These results clearly demonstrate that the tumor-suppressor effect of TSLP in the skin is not specific to Notch mutants and that TSLP overexpression not only prevents tumorigenesis, but it can also inhibit growth of existing tumors present in an otherwise wild-type background.
DISCUSSION

The main finding of this report is that upregulation of epidermal TSLP generates a dominant anti-tumor T cell response in a Th2 inflammatory microenvironment (Demehri et al., 2009a) protecting animals from spontaneous and chemically-induced skin tumors. The anti-tumor CD4+ T cells that receive the TSLP signal orchestrate the recognition and elimination of proliferating pre-cancerous cells. This is in stark contrast to the pro-tumor function commonly attributed to the Th2-polarized inflammation (Johansson et al., 2008) and to recent observations that TSLP upregulation promotes the growth and metastasis of breast and pancreatic cancers through a Th2 response (De Monte et al., 2011; Olkhanud et al., 2011; Pedroza-Gonzalez et al., 2011). Importantly, we show that chemical induction of TSLP expression in wild-type animals with DMBA-induced papillomas results in tumor regression. This latter finding suggests that TSLP upregulation may provide therapeutic benefits in treating skin tumors and perhaps for other solid tumors.

TSLP is a pleiotropic cytokine involved in several immune processes (Ziegler and Artis, 2010). Most notably, in the skin and the lung TSLP is expressed in response to barrier disruption (Demehri et al., 2008), where it can skew CD4+ T cell differentiation towards Th2 subtype (Al-Shami et al., 2004; Tanaka et al., 2009; Ziegler and Artis, 2010) and promote Th2-mediated allergic inflammation (Leonard, 2002). We previously identified TSLP as a sensitive readout for the degree of disruption in epidermal differentiation and skin barrier integrity (Demehri et al., 2008). Animals with epidermal Notch1 deletion and mild disruption in epidermal differentiation develop a tumor-promoting environment dominated by Gr-1+ CD11b+ myeloid suppressor cells and soluble factors including TGF-β (Demehri et al., 2009b). The contribution of TSLP to this tumor-promoting skin environment remains to be determined. Conversely, once all canonical
Notch-signaling is removed, these animals develop a potent tumor-suppressing environment mediated by TSLP. While others have shown that TSLP is responsible for promoting tumor growth and metastasis in pancreatic and breast cancer (De Monte et al., 2011; Olkhanud et al., 2011; Pedroza-Gonzalez et al., 2011), we are the first to show an anti-cancer role for TSLP. There are several possible explanations for these conflicting findings. First, the protective effect of TSLP may be skin-specific. Alternatively, TSLP may have a protective effect on the early stages of tumorigenesis but a promoting effect on later stages of tumor growth and metastasis. However, the observation that calcipotriol treatment is capable of shrinking pre-existing tumors suggests that this may not be the case. Finally, following the trend of our allelic series of Notch-deficient mice, TSLP levels may determine whether it promotes or inhibits tumor development and growth, and the tipping point may vary according to the strength of the pro-tumor environment in a specific context. Support for this concept comes for the observations that higher TSLP levels are needed to establish a tumor-resistant phenotype in the otherwise tumor-prone Notch mutant animals than the otherwise wild-type K14-TSLPtg animals. Below this context-specific threshold level, TSLP may have a neutral or tumor-promoting effect, but above the threshold, it acts as a potent tumor-suppressor. These concepts need to be formally tested in future studies. Nonetheless, here we report that in both a neutral and a pro-tumor microenvironment, TSLP concentrations capable of creating a CD4+ T cell-mediated anti-tumor response can be reached. Once achieved, activated CD4+ T cells not only identify and prevent growth of transformed cells but also mediate the rejection of Notch signaling-deficient cells.

In our mutant animals, large territories of mutant tissue are embedded in wild-type skin. Therefore, the global resistance of calico RBPj-deficient skin to chemical carcinogens suggests that the cancer-initiated wild-type keratinocytes are being suppressed alongside the initiated
mutant cells. Uninitiated mutant cells with three different Notch-deficient genotypes (i.e. RBPjCKO, N1N2CKO or PSDCKO) are also rejected; but importantly, TSLP-activated CD4⁺ T cells do not target the normal skin cells in the same animal. Based on these observations, we conclude that CD4⁺ T cells that are trained in high local TSLP concentrations must recognize immunogenic epitope(s) arising independent of Notch signaling in cells with abnormal differentiation and/or proliferation profiles. This protective activity does not arise in CD4⁺ T cells that lack the TSLP receptor or in animals from which CD4⁺ T cells are cleared before the wild-type immune cells encounter the Notch-deficient skin. It is important to note that CD4⁺ T cell activation by TSLP can occur even if all other tissues and hematopoietic lineages, including dendritic and Langerhans cells, are rendered blind to TSLP by germline deletion of either TSLP receptor arms (IL7rα or CRLF2/TSLPR). These data establish that TSLP-responsive CD4⁺ T cells are both sufficient and required to create the tumor suppressing microenvironment, likely by recruiting several cytotoxic immune effector cells including CTLs, NK cells and macrophages to the skin. Considering that TSLP promotes Th2 differentiation (Al-Shami et al., 2004; Tanaka et al., 2009; Ziegler and Artis, 2010) and Th2 cells predominate in Notch signaling-deficient animals (Demehri et al., 2009a), we propose that the CD4⁺ Th2 cells are initiating the effects of TSLP. The exact nature of the TSLP-responsive CD4⁺ T cells and the tumor antigens they are reacting to remain to be indentified in future studies. Once activated, these CD4⁺ T cells form a lasting pool of “memory” cells that could not be eradicated with anti-CD4 antibody or irradiation. This observation is very exciting as it suggests that lasting anti-tumor immunity can be achieved by targeting antigens that are specific to tumor cells. We show that this anti-tumor immunity can be achieved even after the tumor is formed; application of a TSLP-inducer, Calcipotriol, halted growth of fully developed skin tumors in wild-type animals. It remains to be
determined if TSLP can also stimulate the regression of other solid tumors besides the ones formed in the skin.

In an accompanying paper, a similar set of observations is reported. Both studies report an anti-tumor function for TSLP in Notch-deficient skin and both demonstrate that this function of TSLP can also be elicited in animals with intact Notch signaling. However, our colleagues report that CD8\(^{+}\) CTLs together with CD4\(^{+}\) T cells mediate the tumor resistance phenotype. In their study, Notch proteins are deleted globally in the skin after birth using an inducible basal keratinocyte Cre, resulting in an intact hair follicle bulge and an immune system that matured in the absence of TSLP or tumor antigens. This deletion paradigm precludes monitoring the rejection of mutant skin clones. In our mice, the immune system matures in the presence of TSLP, which begins to accumulate as soon as incomplete differentiation becomes evident (at E16.5; (Demehri et al., 2008)), and in the presence of antigens presented by mutant cells that may be shared by the cancer-initiated cells later on. Moreover, in our mice the hair follicle is destroyed by P10 and bulge never forms because the outer root sheath of hair follicles adopts an epidermal fate right after birth (Demehri et al., 2008). It is conceivable that some of the difference between the two studies reflects methodological differences, but it is hard to explain why CD8\(^{+}\) CTLs do not contribute in our system yet provide the bulk of protection in their Notch-deficient mice. The explanation may lie in the difference between the tumor cells of origin, the timing of the Notch signaling deletion, or reflect the involvement of multiple cytotoxic cell types in our model. The differential role of CTLs in these two experimental paradigms remains to be addressed in future studies. The accompanying study demonstrates that the cystic tumors arise via a Wnt/b-catenin-dependent mechanism from bulge-derived hair follicles whereas our tumors originate from epidermal cells that do not appear to accumulate
nuclear β-catenin prior to tumor formation (Demehri et al., 2009b). Taken together, however, the differences between the studies support the conclusion that TSLP can provide immunological protection against skin cancer and support the speculation that it may do the same in other types of solid tumors.

In summary, this study and the accompanying paper by Di Piazza et al identify a previously unrecognized role for TSLP in inducing a robust anti-tumor response in several experimental paradigms (Notch loss of function, Wnt gain of function (Di Piazza et al) and DMBA-induced tumors). Moreover, these studies demonstrate that TSLP exerts its anti-tumor effects through CD4+ T cells in a Th2-dominant inflammatory environment. Therefore, our findings may explain why individuals who suffer from Th2-dominant allergic disorders display reduced risk of developing certain types of cancers including non-melanoma skin cancers (Gandini et al., 2005; Hwang et al., 2012; Prizment et al., 2007; Vajdic et al., 2009; Wang and Diepgen, 2005). Importantly, therapeutic exploitation of this mechanism seems within reach given the efficacy of calcipotriol, an FDA-approved drug and a potent inducer of TSLP, in blocking DMBA-induced carcinogenesis.
EXPERIMENTAL PROCEDURES

Mice

The following mutant animals were generated according to the methods outlined in our previous report (Pan et al., 2004b):

Msx2-Cre<sup>tg/+</sup>; Rosa-LSL-EYFP (Srinivas et al., 2001)

Msx2-Cre<sup>tg/+</sup>; Notch1<sup>flax/flax</sup> (N1CKO)

Msx2-Cre<sup>tg/+</sup>; Notch1<sup>flax/flax</sup>, Notch2<sup>flax/+</sup> (N1N2hCKO)

Msx2-Cre<sup>tg/+</sup>; Notch1<sup>flax/flax</sup>, Notch2<sup>flax/+</sup>, Notch3<sup>−/−</sup> (N1N2hN3CKO)

Msx2-Cre<sup>tg/+</sup>; Notch1<sup>flax/flax</sup>, Notch2<sup>flax/flax</sup>, IL7ra<sup>−/−</sup> (N1N2CKO)

Msx2-Cre<sup>tg/+</sup>; PS1<sup>flax/flax</sup>, PS2<sup>−/−</sup> (PSDCKO)

Msx2-Cre<sup>tg/+</sup>; PS1<sup>flax/flax</sup>, PS2<sup>−/−</sup>, TSLPR<sup>−/−</sup> (PSDCKO;TSLPR<sup>−/−</sup>)

Msx2-Cre<sup>tg/+</sup>; Rbpj<sup>flax/flax</sup> (RBPjCKO)

Msx2-Cre<sup>tg/+</sup>; Rbpj<sup>flax/flax</sup>, IL7ra<sup>−/−</sup> (RBPjCKO;IL7ra<sup>−/−</sup>)

Msx2-Cre<sup>tg/+</sup>; Rbpj<sup>flax/flax</sup>, CD8a<sup>−/−</sup> (RBPjCKO;CD8a<sup>−/−</sup>)

Msx2-Cre<sup>tg/+</sup>; Rbpj<sup>flax/flax</sup>, Kit<sup>wsh/wsh</sup> (RBPjCKO;Kit<sup>wsh/wsh</sup>)

Msx2-Cre<sup>tg/+</sup>; Rbpj<sup>flax/flax</sup>, TNFRI<sup>−/−</sup> (RBPjCKO;TNFRI<sup>−/−</sup>)

Msx2-Cre<sup>tg/+</sup>; Rbpj<sup>flax/flax</sup>, TNFRI<sup>−/−</sup>, TNFRII<sup>−/−</sup> (RBPjCKO;TNFRI<sup>−/−</sup>;TNFRII<sup>−/−</sup>)

K14-TSLP<sup>tg</sup>

The pedegreed RBPjCKO cohort was maintained in mixed FVB, C57BL/6 and CD1 genetic backgrounds, which were overall more susceptible to DMBA/TPA skin carcinogenesis. All other animals were kept in mixed C57BL/6 and CD1 genetic backgrounds and therefore were
relatively more resistant to chemical skin carcinogens. In all cancer experiments, age-matched littermates were compared.

All the mice were housed in Washington University animal facility in accordance with animal care regulations. *Msx2-Cre<sup>lox/lox</sup>; Rosa-LSL-EYFP* mouse was imaged at P0 using Leica stereoscopic fluorescence microscope with regular light (rendered magenta in Figure 6-1a) or YFP filter. In studies related to mutant skin rejection, spontaneous/DMBA-induced tumorigenesis and life span, mice were photographed with a digital camera weekly and monitored for onset, number and size of tumors and any sign of failure to thrive. Moribund mice are euthanized and skin, tumors, blood, spleen and lymph nodes were harvested.

**Chemical Skin Carcinogenesis Studies**

For RBPjCKO DMBA-TPA experiments, 3-week-old mutant mice and Cre-negative wild-type littermates were treated with standard protocol for skin chemical carcinogenesis model as previously described (Nicolas et al., 2003a). RBPjCKO mice received one dose of 25mg DMBA (Sigma, St. Louis, MO) followed in a week by biweekly treatment with 4mg TPA (Sigma, St. Louis, MO) for 14 weeks. In *K14-TSLP*<sup>tg</sup> and CD1 carcinogenesis experiments, mice were treated with 125mg DMBA (CD1, Calcipotriol treatment) or 100mg DMBA (*K14-TSLP*<sup>tg</sup>). In all the experiments, adult mice were shaved under anesthesia 2 days prior to treatment with DMBA to ensure the hair follicles are in the second telogen.

In studies where tumorigenesis was induced with one dose of DMBA, the mutant animals received a single dose of 125mg DMBA in 100ml of acetone during the second week of life, after the mice were subjected to any other experimental procedures including irradiation, BMT or adoptive T cell transfer.
Bone marrow transplantation (BMT)

The recipient mice were lethally irradiated with 950-cGy in second week of life and transplanted with unfractionated BM cells from their littermates, Rag2<sup>−/−;</sup>gc<sup>−/−</sup> or Rag2<sup>−/−</sup> animals as previously described (Zhang and Ren, 1998). All transplanted animals were maintained on antibiotics containing water to prevent infection.

Adoptive T cell transfer

For T cell isolation, splenocytes were positively selected for CD4 or CD8 surface expression using CD4 or CD8 MicroBeads, respectively, followed by negative selection to remove any CD11c<sup>+</sup> dendritic cells using CD11c MicroBeads (Miltenyi Biotec Inc., Auburn, CA). RBPjCKO;IL7r<sup>α−/−</sup> and PSDCKO;TSLPR<sup>−/−</sup> mice were irradiated with 450-cGy during the second week of life and injected intravenously with ~2 x 10<sup>6</sup> CD4<sup>+</sup> or CD8<sup>+</sup> T cells isolated from the spleen of their littermates.

BMT and Antibody Depletion

RBPjCKO;IL7r<sup>α−/−</sup> newborn mice received intraperitoneal injection of 750mg anti-CD4 (GK1.5; Bio X Cell, West Lebanon, NH), anti-CD8 (YTS; Bio X Cell, West Lebanon, NH) or Control IgG antibody (Sigma, St. Louis, MO) in second week of life. 48hr later, the mutant animals received BMT using c-Kit<sup>+</sup> bone marrow progenitors (lacking T cells) from their wild-type littermates isolated using CD117 MicroBeads and magnetic columns according to manufacturer’s protocol (Miltenyi Biotec Inc., Auburn, CA). Each recipient was injected intravenously with 2x10<sup>6</sup> c-Kit<sup>+</sup> BM cells in 100µl PBS+2%FBS. The mutant animals were then treated topically with one dose of DMBA and continued to receive 250mg of the depleting antibody weekly. Mice were monitored for skin rejection and tumor formation weekly and harvested at P90.
**Histology and Immunohistochemistry**

Hematoxylin and eosin (H&E), toluidine blue and immunostainings were performed on paraffin-embedded 5µm skin sections. Skin samples were fixed in 4% paraformaldehyde in PBS, dehydrated with ethanol and embedded in paraffin as previously described (Pan et al., 2004b). For Immunohistochemistry, anti-panleukocytic marker (CD45; BD PharMingen, San Diego, CA) and anti-RBPj antibody (clone T6709, Institute of Immunology Co., Tokyo, Japan) were used. After treatment with biotinylated secondary antibody, HRP-conjugated streptavidin (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) and DAB substrate kit (Pierce, Rockford, IL) were applied to visualize the signal.

**Flow cytometry (FC)**

Single cell suspensions from dermis or peripheral blood were prepared as described (Demehri et al., 2009b). The following antibodies were used for FC analysis: anti-CD45 conjugated to peridinin chlorophyll-a protein-cyanin 5.5 (PerCP-Cy5.5), anti-CD4, anti-CD8b, anti-B220, anti-Ly6G, anti-NK1.1, and anti-Gr-1 conjugated to phycoerythrin (PE) and anti-CD11b conjugated to fluorescein (FITC; all from BD PharMingen); anti-CD4 conjugated to Allophycocyanin (APC), anti-CD44 conjugated to PE and anti-CD62L conjugated to phycoerythrin-Cy7 (PE-Cy7; from eBioscience, San Diego, CA). BD FACScan Flowcytometer (Cytek Development) was used for FC, and FC analysis was performed using FlowJo software (Ashland, OR).

**ELISA**

Quantikine mouse TSLP kit (R&D Systems, Minneapolis, MN) was used to measure serum TSLP concentrations.
Statistical analysis

Except for Calcipotriol studies performed on wild type CD1 animals, all the animals used in this report were kept on outbred genetic backgrounds in a pedigreed colony (i.e. all animals are logged into a database). To minimize the confounding effects of strain or family background on tumor outcomes, we only compared gender-matched littermates in each cancer study. Using the power analysis described previously (Demehri et al., 2009b), we determined the number of animals needed in each chemical skin carcinogenesis study.

As the test of significance between the study groups, we used long rank test for “Time to tumor onset” and Student’s t-test for tumor counts, serum TSLP levels and other quantitative measurements.
ACKNOWLEDGMENTS

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REFERENCES:


Associated Lethal Myeloproliferative Disorder Arise from Loss of Notch Signaling in the Murine Skin. PLoS ONE 5, e9258.


Figure 6-1. Mice lacking Rbpj gene in portion of their epidermis are resistant to skin tumorigenesis. (A) The calico pattern EYFP expression (green) induced by Msx2-Cre-mediated gene deletion in Msx2-Cre, Rosa (LoxP_Stop_LoxP)-EYFP newborn. Bright light image is shown in magenta. After birth, mutant clones become evident due to hair phenotypes. (B) Reduction in Notch signaling dosage in the skin leads to the corresponding shortening in life span and in spontaneous tumor latency. This trend, however, does not extend to mice lacking RBPj (green arrow), which live ~100 days but do not develop any skin tumor (n>20 in each group; % indicates the percentage of mice that developed skin tumors; **: p <0.01, student’s t-test; modified from (Demehri et al., 2009b)). (C,D) RBPjCKO mice are resistant to chemical carcinogens. Treating RBPjCKO and wild-type littermates with standard DMBA-TPA protocol from 3 to 18 weeks of age results in (C) tumor formation in majority (6/7) of wild-type mice and (D) increase in the number of their tumors over this time. In contrast, no persistent tumor is formed in the RBPj-deficient mice (n=7 for each group; error bars represent SEM; p <0.0001, log-rank test). Genotypes: Msx2-Cre<sup>tg/+</sup>; Notch<sup>1</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>1</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>2</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>1</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>3</sup>fl<sup>x</sup>fl<sup>x</sup>; (N1N2h3CKO), Msx2-Cre<sup>tg/+</sup>; Notch<sup>1</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>2</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>1</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>3</sup>fl<sup>x</sup>fl<sup>x</sup>; (N1N2hCKO), Msx2-Cre<sup>tg/+</sup>; Notch<sup>1</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>2</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>1</sup>fl<sup>x</sup>fl<sup>x</sup>; Notch<sup>2</sup>fl<sup>x</sup>fl<sup>x</sup> (N1N2CKO), Msx2-Cre<sup>tg/+</sup>; PS1fl<sup>x</sup>fl<sup>x</sup>; PS2<sup>/−</sup> (PSDCKO), Msx2-Cre<sup>tg/+</sup>; Rbpjfl<sup>x</sup>fl<sup>x</sup> (RBPjCKO).
A 3wks

RBPj CKO

RBPj-deleted Skin

Wild-type Skin

1cm

50 µm

B 3wks

N1N2CKO

PSDCKO

10wks

*
**Figure 6-2. Notch signaling-deficient epidermal clones regress with age.** (A) The red dotted line and arrowheads delineate the boundaries of the $Rbpj$-deficient dorsal epidermis as determined by hair/epidermal phenotype. α-RBPj antibody staining confirms that RBP-j depleted keratinocytes, still present in the midline, were replaced by wild-type cells in the periphery. (B) An identical phenomenon is observed in Notch1&2 or PS1&2-deficient animals that are rescued from their lethal blood disease (Demehri et al., 2008) by a sublethal dose of irradiation (*). Scale bars: 1cm (mice pictures), 50µm (histology).
Figure 6-3. H-Ras-infected RBPjKO or PSDKO keratinocytes are highly tumorigenic in nude mice. (A) Schema showing the experimental procedure. Cells are infected with retrovirus containing oncogenic H-Ras and then with Adeno-Cre to delete the floxed alleles. (B) 1.5x10^6 H-Ras-infected RBPjKO or PSDKO cells injected subcutaneously into nude mice formed large tumors after 30 days (red circle) whereas 1.5x10^6 H-Ras-infected cells with one remaining allele of RBPj or PS1 formed small nodules. Histological analysis confirmed that poorly differentiated squamous cell carcinomas are devoid of RBPj (scale bar: 50µm).
Figure 6-4. Elevated TSLP expression in RBPjCKO epidermis causes a severe skin inflammation. (A) RBPjCKO animals have much higher level of skin inflammation compared to the tumor-prone N1CKO and N1N2hN3CKO mice. (B) Reduction in Notch alleles in the skin correlates with a corresponding increase in circulating TSLP during second week of life (error bars represent standard deviation around the mean; *: p <0.05, student’s t-test; modified from (Demehri et al., 2008)). (C) Loss of TSLP signaling in RBPjCKO; IL7rα−/− animals significantly reduced CD45-positive dermal leukocytes (scale bar: 50µm).
Figure 6-5. Loss of TSLP receptors results in mutant skin expansion and tumor formation in Notch signaling-deficient animals. Deleting the IL7rα arm of the TSLP receptor in RBPjCKO and N1N2CKO animals, or the TSLPR arm in PSDCKO animals, leads to (A) expansion of mutant skin, and (B) the development of skin tumors. Treatment of RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− animals with one dose of 125mg DMBA during the 2nd week of life leads to earlier onset of tumorigenesis and formation of multiple exophytic tumors. Note that although N1N2CKO; IL7rα−/− and PSDCKO; TSLPR−/− can survive to adulthood (Dumortier et al., 2010), they are sublethally irradiated alongside their N1N2CKO and PSDCKO littermates to control for the irradiation effects (*:recipients of a sublethal dose of irradiation in the second week of life; Insets in (A): invasive cancers penetrating the muscle layer; scale bars: 1cm (mice pictures), 200µm (histology)).
A

Donor: Rag2-/-, γc-/-

Recipient: Rag2-/-, γc-/-

C

Donor: Rag2-/-, γc-/-

Recipient: Rag2-/-

B

Donor: IL7ra-/-

Recipient: IL7ra-/-

D

Donor: TSLPR-/-

Recipient: TSLPR-/-

E

Recipient(+DMBA)

Donor

Exophytic Tumor #

RBPjCKO;IL7ra-/-

Wt

PSDCKO;TSLPR-/-

Wt

RBPjCKO;IL7ra-/-

IL7ra-/-

PSDCKO;TSLPR-/-

IL7ra-/-

RBPjCKO;IL7ra-/-

γc-/-

PSDCKO;TSLPR-/-

γc-/-

RBPjCKO;IL7ra-/-

Rag2-/-

PSDCKO;TSLPR-/-

Rag2-/-

F

Recipient(+DMBA)

Donor

Exophytic Tumor #

RBPjCKO;IL7ra-/-

Wt

PSDCKO;TSLPR-/-

Wt

RBPjCKO;IL7ra-/-

IL7ra-/-

PSDCKO;TSLPR-/-

IL7ra-/-

RBPjCKO;IL7ra-/-

γc-/-

PSDCKO;TSLPR-/-

γc-/-

RBPjCKO;IL7ra-/-

Rag2-/-

PSDCKO;TSLPR-/-

Rag2-/-
Figure 6-6. Adaptive immune cells mediate the effects of TSLP on Notch-deficient skin rejection and tumor resistance. (A) A schematic diagram depicting the bone marrow transplantation (BMT) procedure used to determine if bone marrow (BM)-derived cells mediate the effects of TSLP in Notch and TSLP receptor-deficient mice. (B) BMT from wild-type donors is sufficient to reconstitute the mutant skin rejection and tumor resistance phenotypes in host RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− littermates. TSLP receptor-deficient BM is used in the control experiments. (C&D) BM from (C) Rag2−/−,gc−/− and (D) Rag2−/− donors fails to reject mutant keratinocytes and prevent tumorigenesis in RBPjCKO; IL7rα−/− and PSDCKO; TSLPR−/− animals. (E&F) DMBA treatment following BMT in the 2nd week of life confirms that (E) RBPjCKO; IL7rα−/− and (F) PSDCKO; TSLPR−/− animals receiving TSLP-responsive BM are resistant to tumorigenesis. In contrast, mice transplanted with TSLP-responsive, T and B cell-deficient BM develop exophytic DMBA-dependent tumors. Tumors were scored at 10wks. The success of BMT in each experiment is confirmed by endpoint genotyping of the hematopoietic cells in the recipients (Figure 6-S11 online). Representative mice and H&E-stained skin pictures are shown (p <0.05 compared to wild-type donor group; n>4 for each group, error bars represent standard deviation; scale bars: 1cm (mice), 200µm (histology)).
Exophytic Tumor # P60

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Adoptive T Cell Transfer

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84 11 10 2 10 3 10 4 10 5

17.2% 11.2% 0.309%

10 1 10 2 10 3 10 4 10 5

16.6% 15.8%

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Figure 6-7. CD4+ T cells mediate the effects of TSLP on mutant skin rejection and tumor resistance in Notch-deficient animals. (A) 2-week-old RBPjCKO; IL7ra−/− and PSDCKO; TSLPR−/− pups are irradiated with 450-cGy and injected intravenously with 2x10^6 wild-type CD4+, wild-type CD8+ or TSLP receptor-deficient CD4+ T cells isolated from the spleens of their littermates. Following adoptive T cell transfer, mutant mice are treated with DMBA. Only the mutant animals that received wild-type CD4+ T cells gain the ability to reject their mutant skin and suppress DMBA-induced tumorigenesis (Representative P60 mice pictures are shown; exophytic tumor count were done at P90). (B) Schematic diagram outlining the experimental method used to test if T cells are required to mediate the effects of TSLP in Notch signaling-deficient skin. 2-week-old RBPjCKO;IL7ra−/− mice are treated with depleting antibody (750mg anti CD4 or 750mg anti CD8α; black arrows), lethally irradiated 2 days later and transplanted with T cell-depleted c-Kit+ BM progenitor cells from their wild-type littermates. 2 days later the animals are treated with one dose DMBA while continuing to receive a weekly intraperitoneal injection of the indicated depleting antibody for 10 weeks. (C) Histograms confirming that CD4+ or CD8+ T cells are completely absence from RBPjCKO; IL7ra−/− mice transplanted with c-Kit+ wild-type BM and treated with anti-CD4 or anti-CD8a antibodies, respectively. Note that PE-conjugated anti-CD8b antibody is used to detect CD8+ cells, and the samples were collected one week after the last antibody treatment. Blood and spleen CD45+ leukocytes are used to generate the histograms. (D) Only RBPjCKO; IL7ra−/− mice transplanted with CD4+ cell depleted c-Kit+ BM cells retain their mutant keratinocytes, develop hypertrophic cysts and form skin tumors. RBPjCKO;IL7ra−/− mice transplanted with CD8+ cell depleted c-Kit+ BM cells reject mutant keratinocytes and do not develop any tumors in response to DMBA, as seen with transplantation with wild-type BM (Figure 6-6). The success of adoptive T cell transfer and BMT in each
experiment is confirmed by endpoint genotyping of the hematopoietic cells in the recipients (Figure 6-S11 online). Mice are followed up to 90 days of age for exophytic tumor count (Representative pictures are shown; at least five mice are analyzed in each group; scale bars: 1 cm).
Figure 6-8. TSLP creates a tumor-suppressing environment in wild-type skin. (A&B) Topical treatment of CD1 wild-type animals with calcipotriol, a known inducer of TSLP (Li et al., 2006), resulted in an acquired resistance to chemical skin carcinogenesis compared to littermates receiving only the carrier (EtOH). This tumor resistance is demonstrated by (A) longer time to tumor onset (p <0.0001, log-rank test) and (B) much smaller average number of tumors among tumor-bearing calcipotriol-treated animals (n=10 for each group; error bars represent SEM; *: p <0.01, student’s t-test). Mice are treated with 125ug DMBA once followed by 4ug TPA twice weekly for a total of 15 weeks treatment period. Animals in the test group received 32nmol calcipotriol five times a week while controls received only EtOH during the 15-week DMBA-TPA treatment period. (C&D) Calcipotriol-treatment halts tumor growth and induces tumor regression in tumor-breeding wild-type skin. The 8 tumor-bearing carrier-treated mice in (A&B) were randomly divided into two groups at the end of the 15-week DMBA-TPA treatment course. The “test” group of 4 animals was treated with 32nmol calcipotriol and the “control” group continued to receive carrier only 5 times per week. After additional 7-week follow-up period, the calcipotriol-treated mice (C) show significant reduction in skin tumor numbers and (D) have smaller tumors compared to the carrier-treated mice (n=4 in each group; tumors are highlighted with blue circles; error bars represent standard deviation; *: p <0.05, student’s t-test; representative pictures are shown). (E&F) K14-TSLP\textsuperscript{tg} animals are resistant to chemical skin carcinogenesis. K14-TSLP\textsuperscript{tg} female mice treated once with 100ug DMBA followed by 4ug TPA twice weekly show (E) markedly longer time to tumor onset (p <0.0001, log-rank test) and (F) significantly fewer tumors among tumor-bearing animals compared to their wild-type female littermates (n=22 for K14-TSLP\textsuperscript{tg} group and n=21 for wild-type group; error bars represent SEM; *: p <0.01, student’s t-test). (G) TSLP levels are elevated in calcipotriol-
treated and K14-TSLP\textsuperscript{tg} animals compared to their controls, reflected in the elevation of serum TSLP levels (error bars represent standard deviation; *: p <0.0001, student’s t-test).
CHAPTER VII

Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens.

Publication & Copyright
Results and text presented in this chapter have been previously published in:

Author Contribution
The author contributed only the following sections of this chapter:
• Generation and maintenance of Rbpj\textsuperscript{vav} and Psen1\textsuperscript{vav}Psen2\textsuperscript{vav} mice.
• Flow cytometry experiments for splenocytes from Rbpj\textsuperscript{vav} and Psen1\textsuperscript{vav}Psen2\textsuperscript{vav} in Figure 7-3.

Dr. Ansu T. Satpathy carried out majority of the other experiments described in this chapter with the assistance from other authors. Dr. Ansu T. Satpathy and Kenneth M. Murphy wrote the text of the published manuscript.
ABSTRACT

Defense against attaching-and-effacing bacteria requires the sequential generation of interleukin 23 (IL-23) and IL-22 to induce protective mucosal responses. Although CD4+ and NKp46+ innate lymphoid cells (ILCs) are the critical source of IL-22 during infection, the precise source of IL-23 is unclear. We used genetic techniques to deplete mice of specific subsets of classical dendritic cells (cDCs) and analyzed immunity to the attaching-and-effacing pathogen *Citrobacter rodentium*. We found that the signaling receptor Notch2 controlled the terminal stage of cDC differentiation. Notch2-dependent intestinal CD11b+ cDCs were an obligate source of IL-23 required for survival after infection with *C. rodentium*, but CD103+ cDCs dependent on the transcription factor Batf3 were not. Our results demonstrate a nonredundant function for CD11b+ cDCs in the response to pathogens *in vivo*.
INTRODUCTION

The cytokines interleukin 23 (IL-23) and IL-22 are critical for immune responses that maintain mucosal integrity against infections by attaching-and-effacing bacterial pathogens\textsuperscript{1,2}. Isolated lymphoid follicles (ILFs) in the small and large intestine contain dendritic cells (DCs), B cells and innate lymphoid cells (ILCs) that orchestrate protection against those pathogens\textsuperscript{2,3,4}. During such infections, ILCs produce IL-22, which promotes barrier integrity by inducing the production of antimicrobial peptides, including RegIII\textbeta and RegIII\textgamma, by epithelial cells\textsuperscript{2,5,6}. The importance of IL-22 is indicated by the susceptibility of \textit{Il22}\textsuperscript{−/−} mice to the attaching-and-effacing bacterium \textit{Citrobacter rodentium}, a model for the infection of humans with enteropathogenic and enterohemorrhagic \textit{Escherichia coli}\textsuperscript{2,6,7}.

IL-22-producing ILCs are heterogeneous and include a CD4\textsuperscript{+} subset\textsuperscript{5} and a CD4\textsuperscript{−}NKp46\textsuperscript{+} subset\textsuperscript{8}. Both subsets express the transcription factor ROR\textgamma\textsuperscript{t}, which is required for their development\textsuperscript{9,10}. An important unresolved question is the identity of the cells that stimulate ILCs to produce IL-22. ILCs do not directly detect infection by attaching-and-effacing pathogens but instead seem to depend on IL-23 produced by other innate cells for their activation\textsuperscript{2,8}. It has been suggested that in response to \textit{C. rodentium}, either macrophages or DCs may be the main source of IL-23 (refs.11, 12). A role for macrophages was proposed on the basis of the greater pathogen burden of mice deficient in the chemokine receptor CX3CR1 (\textit{Cx3cr1}\textsuperscript{−/−} mice), as well as the greater susceptibility of CD11c-DTR mice (which, after treatment with diphtheria toxin, are selectively depleted of cells that express diphtheria toxin receptor (DTR) under the control of the promoter of the gene encoding the integrin CD11c) to such infection\textsuperscript{12}. Alternatively, a role for classical DCs (cDCs) was proposed on the basis of the greater pathogen burden and lower IL-23 production in mice that lack the lymphotoxin-\textbeta receptor (LT\textbeta R) in CD11c-expressing
cells\textsuperscript{11} and by the separate observation that cDCs are the main source of IL-23 after stimulation of Toll-like receptor 5 (ref. 13).

The opposing conclusions of those studies emphasize the difficulty in distinguishing the roles of macrophages and cDCs \textit{in vivo}, particularly for studies that rely on depletion methods based on CD11c\textsuperscript{14}. Zbtb46 has been identified as a transcription factor expressed in cDCs but not macrophages, monocytes or plasmacytoid DCs (pDCs), and expression of DTR under the control of the \textit{Zbtb46} promoter (\textit{Zbtb46}DTR) allows selective depletion of cDCs\textsuperscript{15,16}. Other systems have been developed that also allow selective depletion of individual cDC subsets \textit{in vivo}\textsuperscript{17,18,19,20}. Mice deficient in the transcription factor Batf3 lack the CD8\textsuperscript{α+} cDC subset that is identified by its expression of CD103 in the periphery; as a result, these mice have defective CD8\textsuperscript{+} T cell responses to several viral pathogens\textsuperscript{17,21} and are susceptible to infection with \textit{Toxoplasma gondii}\textsuperscript{22}. Similarly, mice with selective depletion of pDCs have defects in the production of type I interferon and are susceptible to chronic infection by viruses such as lymphocytic choriomeningitis virus\textsuperscript{19,23}. Thus, studies using systems of selective depletion have identified nonredundant roles for CD8\textsuperscript{α+} cDCs and pDCs but not for the third major subset of DCs, the CD11b\textsuperscript{+} cDCs.

A subset of CD11b\textsuperscript{+} cDCs \textit{in vivo} expressing the adhesion molecule ESAM undergoes depletion after conditional deletion of the gene encoding the signaling receptor Notch2 (ref. 18). That study proposed that Notch2 signaling is selectively required for the development of splenic CD11b\textsuperscript{+}ESAM\textsuperscript{+} cDCs and intestinal CD103\textsuperscript{+}CD11b\textsuperscript{+} cDCs derived from the precursor of the cDC (pre-cDC), analogous to the unique dependence of CD8\textsuperscript{α+} cDCs on Batf3 (ref. 17). However, CD11b\textsuperscript{+}ESAM\textsuperscript{−} cDCs persist in Notch2-deficient mice, which prompts the question of how those cells are related to CD11b\textsuperscript{+}ESAM\textsuperscript{+} cDCs and whether they provide compensatory
functions. Furthermore, although that study suggested that Notch2 specifically regulates the CD11b<sup>+</sup> branch of cDCs, some evidence indicated a developmental defect in the CD8α<sup>+</sup> branch as well, although that was not further analyzed<sup>18</sup>. Unexpectedly, mice that lack CD103<sup>+</sup>CD11b<sup>+</sup> cDCs have only 50% less production of IL-17 by T cells stimulated ex vivo, and responses during infection have not been examined to show a specific function for such cells in immunological defense.

Here we demonstrate a selective function for CD11b<sup>+</sup> cDCs in immunological defense against pathogens. Through the use of several genetic models with selective depletion of various subsets of cDCs in vivo, we found that Notch2-dependent intestinal CD103<sup>+</sup>CD11b<sup>+</sup> cDCs provided nonredundant protection against infection with attaching-and-effacing pathogens, but macrophages or Batf3-dependent CD103<sup>+</sup>CD11b<sup>-</sup> cDCs did not. Notch2-dependent cDCs were required for host survival mediated by the local generation of IL-23-dependent antimicrobial responses early after infection. Developmentally, Notch2 regulated the terminal differentiation of both the CD8α<sup>+</sup> and CD11b<sup>+</sup> branches of cDCs, which allowed their subsequent homeostatic population expansion via signaling through LTβR. Our results demonstrate that intestinal CD103<sup>+</sup>CD11b<sup>+</sup> cDCs detect infection with attaching-and-effacing pathogens and secrete IL-23, which is critical for IL-22 production by ILCs and the maintenance of mucosal integrity.

**RESULTS**

**cDCs mediate defense against attaching-and-effacing pathogens**

The generation of Zbtb46<sup>GFP</sup> mice, which express green fluorescent protein (GFP) driven by the Zbtb46 promoter (Zbtb46-GFP), as well as Zbtb46<sup>DTR</sup> mice, has allowed the selective visualization and depletion of cDCs, which can help in distinguishing the requirements for cDCs and macrophages in immune responses in vivo<sup>15,16</sup>. We sought to determine whether expression
of Zbtb46-GFP could be used to distinguish cDCs from macrophages in the intestinal lamina propria and mesenteric lymph nodes (MLNs) (Figure 7-1a,b). Among MHCII⁺CD11c⁺ lamina propria cells, CD103⁺CD11b⁻ cells and CD103⁺CD11b⁺ cells were uniformly GFP⁺, consistent with their identity as cDCs. Both CD103⁻CD11b⁺ cells and CD103⁻CD11b⁻ cells also included a substantial GFP⁺ fraction (Figure 7-1a), in agreement with published studies suggesting that cDCs may also reside in those gates. In contrast, F4/80⁺ macrophages were present mainly in the MHCII⁻CD11c⁺ gate and did not express GFP (Figure 7-1a). In the MLNs, migratory cDCs were uniformly GFP⁺ and were mainly in the CD103⁻CD11b⁻ and CD103⁻CD11b⁺ gates (Figure 7-1b), consistent with the ability of cDCs, but not macrophages, to migrate to the MLNs. Resident cDCs were also uniformly GFP⁺, but were predominantly in the CD103⁻CD11b⁻ or CD103⁻CD11b⁺ gate. Thus, expression of Zbtb46 seemed to distinguish cDCs from macrophages in the intestine independently of their expression of the integrins CD103 (αEβ7) or CD11b (αM). Histological assessment of the small intestine of wild-type mice reconstituted with Zbtb46ᵍᵉᵖ bone marrow (Zbtb46ᵍᵉᵖ chimeras) showed that GFP⁺ cDCs were present in organized lymphoid structures, including Peyer's patches and ILFs, as well as at points of antigen encounter in intestinal villi (Figure 7-1c). In the large intestine, GFP⁺ cDCs were also present in colonic patches, ILFs and surrounding villi (Figure 7-1c).

The results reported above suggested that the administration of diphtheria toxin to Zbtb46ᵈᵗʳ mice should selectively eliminate cDCs but spare macrophages. Indeed, treatment of the Zbtb46ᵈᵗʳ chimeras with diphtheria toxin resulted in a significantly lower frequency of both CD103⁺CD11b⁻ cDCs and CD103⁺CD11b⁺ cDCs in the lamina propria and the F4/80⁻ populations of CD103⁻CD11b⁻ cDCs and CD103⁻CD11b⁺ cDCs, but did not affect F4/80⁺ macrophages (Figure 7-2a,b). In addition, lymphocytes and ILCs in the lamina propria
did not undergo depletion after treatment of $\text{Zbtb46}_{\text{DTR}}$ chimeras with diphtheria toxin and did not express $\text{Zbtb46-GFP}$ (Supplementary Figure 7-1a–c). Thus, we used this system to determine whether cDCs were required for protection against $\text{C. rodentium}$. We found that $\text{Zbtb46}_{\text{DTR}}$ chimeras treated with diphtheria toxin were unable to recover after challenge with $\text{C. rodentium}$ and died within 8–12 d of infection (Figure 7-2c). In contrast, wild-type mice reconstituted with wild-type bone marrow (wild-type chimeras) and $\text{Zbtb46}_{\text{DTR}}$ chimeras not treated with diphtheria toxin survived beyond 15 d; they underwent some weight loss but recovered normal weight by 30 d after infection (Figure 7-2c,d).

To confirm a specific role for cDCs and to investigate a possible role for monocyte-derived cells in protection from $\text{C. rodentium}$, we next analyzed mice deficient in the cytokine Flt3L ($\text{Flt3l}^{-/-}$ mice) and mice deficient in the chemokine receptor CCR2 ($\text{Ccr2}^{-/-}$ mice). $\text{Flt3l}^{-/-}$ mice have fewer lamina propria cDCs but maintain normal numbers of macrophages and monocytes. Similar to diphtheria toxin–treated $\text{Zbtb46}_{\text{DTR}}$ chimeras, $\text{Flt3l}^{-/-}$ mice succumbed to $\text{C. rodentium}$ infection after 10–16 d (Figure 7-2e,f). In contrast, $\text{Ccr2}^{-/-}$ mice have a specific defect in the recruitment of monocytes to the intestinal lamina propria but maintain normal populations of cDCs. Although $\text{Ccr2}^{-/-}$ mice underwent more weight loss than did their wild-type ($\text{Ccr2}^{+/+}$) counterparts, only 25% succumbed to infection (Figure 7-2e,f). These results demonstrated that cDCs, rather than macrophages or monocyte-derived cells, were required for early innate defense against $\text{C. rodentium}$, but did not indicate whether a particular cDC subset was required.

**Notch2-deficient mice lack CD11b$^+$ cDCs in vivo**

Notch2 is required for the development of the CD11b$^+$ESAM$^+$ cDC fraction. However, the specific function of those cells has not been determined in vivo. To characterize how Notch2
signaling influences cDC development, we examined the requirement for Notch2 and its transcriptional partner RBPjκ, as well as the γ-secretase complex composed of presenilin 1 (PSEN1) and PSEN2, in the development of splenic CD8α+ or CD11b+ cDCs (Figure 7-3a). Hematopoietic loss of either Rbpj or Notch2 mediated by Cre recombinase expressed from the hematopoietic compartment–specific promoter Vav1 (Vav1-Cre) resulted in a lower frequency of CD11c+MHCII+ cDCs and CD8α+ cDCs (identified by expression of the surface marker CD24) and elimination of CD11b+ESAM+CD4+cDCs (also identifiable by expression of the signal-transduction molecule CD172a rather than CD11b; Figure 7-3a). Vav1-Cre–mediated deletion of Psen1 and Psen2 also resulted in a similarly lower frequency of CD11c+MHCII+ cDCs and CD8α+ cDCs and elimination of ESAM+CD4+cDCs (Figure 7-3a). These results indicated that deletion of RBPJ affected cDC development through loss of canonical Notch signaling rather than through derepression of genes that are not targets of Notch. Histologically, deletion of Notch2 eliminated the characteristic clusters of CD11b+cDCs in the marginal zone and bridging channels of the spleen32, which left a distribution of cDCs loosely scattered throughout the T cell zones and red pulp (Figure 7-3b).

As loss of Notch2 also affects the development of additional hematopoietic lineages33, we used Cre expressed from the promoter of the gene encoding CD11c (CD11c-Cre) instead of the Vav1-Cre system to restrict the effect of Notch2 deletion to myeloid lineages for analysis of cDC function during infection in vivo. Deletion of Notch2 by CD11c-Cre (Notch2cKO) generated defects in cDC development identical to those that resulted from Vav1-Cre–induced deletion of Notch2 (Figure 7-3c,d) but prevented effects on other hematopoietic lineages (Supplementary Figure 7-2 online). Notch2cKO mice had fewer splenic cDCs than did their Notch2-sufficient counterparts and had no ESAM+CD4+cDCs (Figure 7-3c,d). A fraction of CD24+cDCs also
expressed ESAM and was dependent on Notch2 signaling (Figure 7-3c,d), which suggested that Notch2 acted in a similar way, although to a different extent, in the development of the CD11b+ and CD8α+ subsets of cDCs. Notch2-dependent ESAM+cDCs were abundant not only in the spleen but also in the resident cDC fraction in the MLNs (Supplementary Figure 7-2a online). Similarly, a small fraction of resident ESAM+, Notch2-dependent cDCs was present in skin-draining lymph nodes (Supplementary Figure 7-2a online). In contrast, migratory dermis-derived cDCs in skin-draining lymph nodes and peripheral tissue–resident cDCs in the lungs and kidneys were not affected by Notch2 deletion18 (Supplementary Figure 7-2a,b online). In the intestinal lamina propria, deletion of Notch2 resulted in a much lower frequency of CD103+CD11b+ cDCs (Figure 7-3e). In contrast, Batf3−/− mice selectively lacked the complementary lamina propria CD103−CD11b− cDC subset (Figure 7-3e) but retained normal numbers of CD103+CD11b+ cDCs34. Similarly, Notch2cKO mice had considerably fewer migratory CD103+CD11b+ cDCs in the MLNs, and Batf3−/− mice had no CD103+CD11b− cDCs (Figure 7-3e). The development of cryptopatches and ILFs containing CD11c+ cDCs and ILCs was unaffected in Notch2cKO mice (Figure 7-3f and Supplementary Figure 7-2d online), consistent with a published report showing that CD11b+ DCs are present mainly in the lamina propria and not in ILFs35. Thus, Notch2cKO mice provided an in vivo system for the analysis of CD11b+ cDC function in the presence of intact lymphoid structures.

**Notch2 controls the terminal differentiation of cDC subsets**

Although the role of Notch2 in cDC development is thought to be limited to the CD11b+ subset of cDCs18, we observed that Notch2cKO mice also had defects in the CD8α+ cDC subset (Figure 7-3c,d). As CD8α expression can be altered by manipulation of the Notch pathway36, we used expression of CD24 and the DC marker DEC-205 (CD205) to identify this
subset of cDCs (Supplementary Figure 7-3a online). To assess the transcriptional effects of Notch2, we analyzed gene expression in both CD11b⁺cDCs and DEC-205⁺cDCs from mice with \textit{loxP}-flanked \textit{Notch2} alleles (\textit{Notch2}^\textit{fl} mice) and \textit{Notch2}^\textit{cKO} mice (Figure 7.4a and Supplementary Figure 7-3a online). As expected, we noted substantial differences between \textit{Notch2}^\textit{fl} CD11b⁺cDCs and \textit{Notch2}^\textit{cKO} CD11b⁺cDCs. In particular, the expression of known Notch targets such as \textit{Hes1} and \textit{Dtx1} was lower in \textit{Notch2}^\textit{cKO} CD11b⁺cDCs, as was the expression of cDC-specific genes such as \textit{Lphn3}, \textit{Spint1} and \textit{Dnase1l3} (Figure 7-4a). Most of the genes regulated by Notch2 in CD11b⁺cDCs were also regulated by Notch2 in DEC-205⁺cDCs (Figure 7-4a). In CD11b⁺cDCs, Notch2 influenced gene expression in both the ESAM⁺ and ESAM⁻ fractions (Supplementary Figure 7-3b online), which suggested that Notch2 acted early after differentiation of the CD11b⁺cDC subset from pre-cDCs, before the induction of ESAM expression, and that its actions were not simply restricted to the development of an ESAM⁺ subset. Likewise, in DEC-205⁺cDCs, Notch2 regulated the same set of genes in both the ESAM⁺ and ESAM⁻ fractions, which largely overlapped the genes regulated in CD11b⁺cDCs (Supplementary Figure 7-3b online).

Principal-component analysis showed that similar genes were influenced by Notch2 deficiency in CD11b⁺cDCs and DEC-205⁺cDCs. Principal component 1 (PC1) segregated cDCs by lineage subset, which distinguished CD11b⁺cDCs from DEC-205⁺cDCs (Figure 7-4b). In contrast, PC2 segregated both cDC lineages by the presence or absence of Notch2 signaling, which distinguished Notch2-sufficient cDCs from Notch2-deficient cDCs of each lineage (Figure 7-4b). Next we assessed the expression of genes most heavily weighted in PC2 along the developmental pathway from common myeloid progenitor to splenic cDC. Genes with the highest 'loadings' in PC2, which were thus induced by Notch2, had high expression in terminally
differentiated cDCs but low expression in DC progenitors (Figure 7-4b–d). Conversely, genes with the most negative 'loadings' in PC2 had high expression in progenitor cells. Through the use of reporter mice with sequence encoding GFP knocked into two such genes, Ccr2 and Cx3cr1, we found that CD11b⁺ESAM⁺cDCs, which underwent depletion in Notch2KO mice, were distinguished from CD11b⁺ESAM⁻cDCs on the basis of GFP expression (Figure 7-4e, top). Similarly, two populations were also distinguished on the basis of their expression of GFP and CD4 (Figure 7-4e, bottom). These subsets probably reflected true differences in the maturity of CD11b⁺cDCs, as Zbtb46, which has intermediate expression in cDC progenitors and is upregulated in mature cDCs, had higher expression in the ESAM⁺ fraction of CD11b⁺ cDCs than in the ESAM⁻ fraction (Figure 7-4d,e). Accordingly, both ESAM⁺ cDCs and ESAM⁻ cDCs developed from wild-type common DC progenitors and pre-DCs in vivo (Supplementary Figure 7-4a,b online), which supported a model in which Notch2 influenced the terminal cDC differentiation of pre-DC–derived cells.

To evaluate the stage at which Notch2 first affected cDC development, we analyzed competitive mixed chimeras generated by reconstitution of wild-type mice with bone marrow from wild-type donors and donors with Vav1-Cre–mediated deletion of Notch2 (Notch2vav), in which Notch2 is deleted at an early stage of hematopoietic development (Figure 7-4f). We observed equal competition between wild-type and Notch2vav progenitors for cells in the lineage-negative (Lin⁻) Sca-1⁺c-kit⁺fraction and up to the pre-DC stage of DC development (Figure 7-4f and Supplementary Figure 7-5a online). However, in the mature splenic cDC compartment, wild-type cells outcompeted Notch2vav cells (Figure 7-4f and Supplementary Figure 7-5b online), which indicated that Notch2 first affected development after the pre-DC. We noted this effect in both the CD11b⁺ and CD8α⁺ subset of mature cDCs and, furthermore, in both the ESAM⁺ and
ESAM fraction of each cDC subset. In summary, our analyses of gene expression and competitive bone marrow chimeras indicated that Notch2 acted in the terminal differentiation of CD11b+ cDCs and CD8α+ cDCs.

**LTβR mediates the population expansion of Notch2-dependent cDCs**

As Notch2 signaling controls the terminal differentiation of cDCs in the spleen and intestine, its effects should be subsequent to those of Flt3L signaling, which can be observed in the population expansion of DC progenitors in the bone marrow. To test that hypothesis, we compared the development of Notch2-dependent cDC subsets in wild-type and Flt3l−/− mice (Supplementary Figure 7-6a,b). As expected, Flt3l−/− mice had a much lower abundance of all subsets of cDCs, including CD11b−ESAM+ cDCs. However, treatment of wild-type mice with Flt3L resulted in 25-fold more CD11b−ESAM− cDCs but only 2.5-fold more CD11b−ESAM+ cDCs (Supplementary Figure 7-6c,d online). Thus, Flt3L was necessary but not sufficient for development of CD11b−ESAM+ cDCs, which suggested that Notch2 regulated a step in cDC development subsequent to the actions of Flt3L.

Because the lymphotoxin LTα1β2 is required for the proliferation of splenic CD8− cDCs located in the marginal zone and bridging channels, we investigated whether signaling via both Notch2 and LTβR selectively influenced the same cDC subset. We compared the development of splenic cDC subsets in wild-type, Notch2cKO and LTβR-deficient (Ltbr−/−) mice. Ltbr−/− mice, similar to Notch2cKO mice, had fewer CD11c+MHCII+ cDCs and CD8α+ cDCs and considerably fewer ESAM+cDC4+ cDCs (Figure 7-5a). Similarly, Nik−/− mice, which are deficient in activation of the noncanonical pathway of the transcription factor NF-κB downstream of LTβR signaling, and Nfkb1−/− mice, which lack the p105 subunit of NF-κB, showed selective loss of ESAM+ cDCs but retained ESAM− cDCs (Supplementary Figure 7-7a,b online). We did not
observe those defects in mice lacking the costimulatory receptor CD40 (Supplementary Figure 7-7a online), a member of the tumor-necrosis factor receptor family that can also activate NF-κB39. As Ltbr−/− mice lack lymph nodes and Peyer's patches40, we sought to determine whether the loss of ESAM+ cDCs in these mice was cell intrinsic by analyzing mixed chimeras generated by reconstitution of wild-type mice with wild-type and Ltbr−/− bone marrow. We observed substantial outcompetition of Ltbr−/− bone marrow by wild-type bone marrow in the generation of splenic and MLN-resident CD11b+ cDCs (Figure 7-5b,c), as well as outcompetition of Ltbr−/− CD8α+ cDCs by wild-type CD8α+ cDCs in this setting (Figure 7-5b,c). Thus, Ltbr−/− bone marrow and Notch2cKO bone marrow had a similar competitive disadvantage relative to that of wild-type bone marrow in the generation of both branches of cDCs.

To evaluate the epistatic relationship between Notch2 and LTβR signaling, we examined cDC development in mixed chimeras generated by the reconstitution of wild-type mice with Notch2cKO and Ltbr−/− bone marrow. In these mixed chimeras, CD11b+ cDCs in the spleen and MLNs developed equally from Notch2cKO bone marrow and Ltbr−/− bone marrow (Figure 7-5b,c). Similarly, CD8α+ cDCs from either donor also developed equally. These results suggested that Notch2 and LTβR acted along a similar pathway in cDC development. Furthermore, in these mixed chimeras, Notch2cKO bone marrow did not generate any ESAM+cDCs, whereas Ltbr−/− bone marrow generated a small population of ESAM+cDCs (Figure 7-5b), which suggested that the requirement for Notch2 preceded the requirement for LTβR. In this model, Ltbr−/− cDCs were able to activate Notch2 signaling and progress to an ESAM+ subset but were unable to undergo homeostatic expansion, which resulted in diminished fitness relative to that of wild-type cDCs; in contrast, Notch2cKO cDCs were unable to progress to ESAM+ cells or undergo LTα1β2-mediated population expansion (Supplementary Figure 7-7c online). Given
those results, we sought to determine if LTβR also had a similar role in development of Notch2-dependent CD103⁺CD11b⁺ cDCs in the lamina propria. Indeed, in mixed chimeras generated by the reconstitution of wild-type mice with wild-type and Ltbr⁻/⁻ bone marrow, we noted outcompetition of Ltbr⁻/⁻ bone marrow by wild-type bone marrow in the production of CD103⁺CD11b⁺ cDCs, unlike mice deficient in the receptor for interferon-αβ (Ifnar1⁻/⁻ mice), which did not show defects in cDC development (Figure 7-5d,e). Thus, LTβR signaling was required for the population expansion of Notch2-dependent cDC subsets.

A nonredundant role for CD11b⁺ cDCs in C. rodentium infection

As Notch2cko and Batf3⁻/⁻ mice lacked CD103⁺CD11b⁺ cDCs and CD103⁺CD11b⁻ cDCs, respectively, we sought to determine whether either subset was specifically required for host defense against an attaching-and-effacing bacterial pathogen. We compared the survival of Notch2ef, Batf3⁻/⁻ and Notch2cko mice after oral infection with C. rodentium. Although Notch2ef and Batf3⁻/⁻ mice were resistant, Notch2cko mice were highly susceptible to infection, which caused death within 7–10 d (Figure 7-6a). Unlike Notch2ef or Batf3⁻/⁻ mice, Notch2cko mice underwent rapid weight loss after infection, and when killed at day 9, they had significantly greater pathogen burden and shorter colons (Figure 7-6b–d and Supplementary Figure 7-8a,b online). Colons of Notch2cko mice had considerable infiltration of inflammatory cells and crypt elongation, scattered loss of mucosal architecture, and ulceration and coagulation necrosis, unlike those of Notch2ef mice (Figure 7-6e,f). The development of Batf3-independent CD8α⁺ cDCs during infection with intracellular pathogens such as Listeria monocytogenes has been shown to occur as a result of compensation by the AP-1 factor Batf2 (ref. 41). However, CD103⁺CD11b⁻ cDCs were not restored in Batf3⁻/⁻ mice during infection with C. rodentium (data not shown), and Batf2⁻/⁻ mice survived infection without substantial weight loss.
(Figure 7-6g,h), which suggested that resistance to this attaching-and-effacing pathogen did not require compensatory development of CD8α⁺ cDCs.

To determine whether CD103⁺CD11b⁺ cDCs had to migrate to draining lymph nodes to provide protection against C. rodentium, we studied mice deficient in the transcription factor IRF4 (Irf4⁻/⁻ mice) or the chemokine receptor CCR7 (Ccr7⁻/⁻ mice)²⁶,⁴². Irf4⁻/⁻ mice have a selective defect in the migration of CD11b⁺ cDCs from tissues to draining lymph nodes⁴². We confirmed that migratory CD103⁺CD11b⁺ cDCs were absent from the MLNs of Irf4⁻/⁻ mice, whereas lamina propria CD103⁺CD11b⁺ cDCs were present but less abundant (Supplementary Figure 7-8c online). Irf4⁻/⁻ mice survived infection with C. rodentium until at least day 28 (Figure 7-6g,h), which excluded the possibility that early resistance required cDC migration. However, those mice did eventually succumb to C. rodentium by day 42 after infection (Figure 7-6g,h), perhaps because of defects in antibody responses²,⁴³. Next we analyzed Ccr7⁻/⁻ mice, which have a general defect in cDC migration²⁶ but intact antibody responses. These mice were completely resistant to infection with C. rodentium (Figure 7-6g,h), which suggested that protection against such infection was provided by the local action of CD103⁺CD11b⁺ cDCs.

To determine whether the defects in mucosal immunity in Notch2cko mice were specific to infection with C. rodentium, we infected these mice with T. gondii. In contrast to Batf3⁻/⁻ mice, which are highly susceptible and uniformly succumb by 9d after infection²², Notch2cko mice survived infection and were indistinguishable from their wild-type counterparts (Supplementary Figure 7-8d online). These results demonstrated that CD103⁺CD11b⁺ cDCs were not required for resistance to T. gondii and that the function of Batf3-dependent CD103⁺CD11b⁻ cDCs seemed to be unaffected in Notch2cko mice.
CD11b+ cDCs are not essential for healing mucosal wounds

We sought to determine whether the enhanced susceptibility of Notch2cKO mice to C. rodentium reflected local defects in colonic wound repair rather than defects in pathogen-specific immunological defense. Colonic wound repair involves local prostaglandin production by the cyclooxygenase COX-2 (encoded by Ptgs2), which supports epithelial proliferation required for the resolution of inflammation. We noted that CD103+CD11b+ cDCs and macrophages in the intestine expressed Ptgs2 (Figure 7-7a), which suggested that either cell type might be involved in supporting wound repair. We used endoscopy-guided mucosal excision to induce colonic injury and found that Notch2cKO mice had normal wound repair, as did Notch2f/f and Batf3−/− mice, in contrast to Notch2f/f mice treated with the COX-2 inhibitor NS-398 (Figure 7-7b,c). Inhibition of COX-2 after mucosal excision resulted in failed regeneration of the β-catenin-positive epithelial layer and impaired maintenance of the underlying α-smooth muscle actin-positive muscularis propria layer (Figure 7-7d), as reported before. In contrast, Notch2f/f, Notch2cKO and Batf3−/− mice had normal epithelial regeneration and maintenance of the muscularis propria at day 6 after mucosal excision (Figure 7-7e). Thus, cDCs were not required for the prostaglandin production involved in mucosal healing, which suggested that the susceptibility of Notch2cKO mice to infection with C. rodentium was not due to any defects in wound repair.

CD11b+ cDCs regulate IL-23-dependent mucosal immunity

To determine the basis of the susceptibility of Notch2cKO mice to infection with C. rodentium, we analyzed differences in gene expression in colonic cells isolated from Notch2f/f, Batf3−/− and Notch2cKO mice 9 d after infection (Figure 7-8a). Overall, for colonic gene expression, the difference between Notch2cKO mice and Notch2f/f mice was greater than the
difference between Batf3−/− mice and Notch2ff mice (Figure 7-8a and Supplementary Table 1 online). Ptgs2 expression was not lower in Notch2cko mice 9 d after C. rodentium infection (Figure 7-8a,b), consistent with the intact wound healing noted above (Figure 7-7e). However, Notch2cko mice had upregulation of various genes encoding inflammatory molecules, including Il1a, Il1b, Il33 and Ccl3, and substantial downregulation of the genes Reg3b and Reg3g, which encode antimicrobial peptides (Figure 7-8a–c). Notably, among the set of genes shown to be induced by the stimulation of colonic cells with IL-22ex vivo, only Reg3b, Reg3g and Mup1 had lower expression in the colons of infected Notch2cko mice than in their Notch2ff counterparts (Figure 7-8c), which indicated the importance of the molecules encoded by those genes in the defense against attaching-and-effacing pathogens. Genes encoding the antimicrobial peptides S100A8 and S100A9 had higher expression in Notch2cko mice than in Notch2ff mice (Figure 7-8c), consistent with a published report indicating the insufficiency of S100A8 and S100A9 in the defense against C. rodentium. Consistent with the findings reported above, IL-22 production was much lower in Notch2cko mice, but not in Batf3−/− mice, 9 d after infection with C. rodentium (Figure 7-8d).

To eliminate the effect of inflammation on gene expression, we also assessed changes in gene expression at day 4 after infection, a time at which no change in histology, pathogen dissemination, colonic length or death had occurred (Figure 7-8e and Supplementary Figure 7-8e,f online). Again, Notch2cko mice had lower expression of Il22 and of the IL-22-responsive genes Reg3b and Reg3g (which, as noted above, encode antimicrobial peptides) (Figure 7-8e and Supplementary Table 1 online). Accordingly, ILCs isolated from Notch2cko mice had lower intracellular expression of IL-22 protein at day 4; however, that effect was 'rescued' by the addition of IL-23 ex vivo (Figure 7-8f), which indicated that the lower IL-22 production was
extrinsic of ILCs in Notch2<sup>cKO</sup> mice and instead probably resulted from a defect in the stimulation of ILCs.

Mice deficient in the p19 subunit of IL-23 (Il23a<sup>−/−</sup> mice) are susceptible to infection with <i>C. rodentium</i><sup>1,2</sup>, and intestinal CD103<sup>+</sup>CD11b<sup>+</sup> cDCs have been reported to produce IL-23 <i>in vivo</i> after stimulation of Toll-like receptor 5 (ref. 13). However, the requirement for those cells as an obligate source of IL-23 during infection with attaching-and-effacing bacteria remains unclear. Functional IL-23 is a heterodimeric protein composed of p19 (encoded by <i>Il23a</i>) and p40 (encoded by <i>Il12b</i>). In the lamina propria, we found that <i>Il23a</i> was expressed specifically in CD11b<sup>+</sup>CD103<sup>+</sup>cDCs in the steady state and 2 d after infection by <i>C. rodentium</i>, but this transcript was undetectable in macrophages in either setting (Figure 7-8g). In addition, we found that intracellular expression of p40 was undetectable in CD11b<sup>+</sup>cDCs at steady state but increased substantially after activation (Supplementary Figure 7-8g online). The inducible expression of <i>Il12b</i> (encoding p40) was not dependent on the presence of CD8α<sup>+</sup>cDCs or on the transcription factors Batf, Batf2 or Batf3 (Supplementary Figure 7-8g,h online). Thus, expression of IL-23 was both inducible after activation and restricted to the CD11b<sup>+</sup>cDC subset.

To determine whether Notch2-dependent cDCs were the obligate source of IL-23 during infection with <i>C. rodentium</i>, we generated mixed chimeras by reconstituting wild-type mice with Notch2<sup>eff</sup> bone marrow and Il23a<sup>−/−</sup> bone marrow or with Notch2<sup>cKO</sup> bone marrow and Il23a<sup>−/−</sup> bone marrow and analyzed resistance to <i>C. rodentium</i>. In Notch2<sup>cKO–Il23a<sup>−/−</sup></sup> mixed chimeras, intestinal CD103<sup>+</sup>CD11b<sup>+</sup>cDCs developed only from the Il23a<sup>−/−</sup> bone marrow, but all other hematopoietic populations were unaffected in their ability to generate IL-23. The survival of Notch2<sup>eff–Il23a<sup>−/−</sup></sup> chimeras after infection by <i>C. rodentium</i> was similar to that of chimeras generated by transplantation of wild-type bone marrow alone; however, the survival
of Notch2<sup>cKo</sup>−Il23a<sup>−/−</sup> chimeras was similar to that of chimeras generated with Il23a<sup>−/−</sup> bone marrow alone, which succumbed to C. rodentium at 7–11 d after infection (Figure 7-8h). The observation that Notch2<sup>cKo</sup>−Il23a<sup>−/−</sup> mixed chimeras were as susceptible to C. rodentium infection as were chimeras generated with Il23a<sup>−/−</sup> bone marrow alone suggested that Notch2-dependent CD11b<sup>+</sup>CD103<sup>+</sup> cDCs were the critical source of IL-23 required for protection.
DISCUSSION

Efforts to distinguish the functions of macrophages and DCs have been limited by the availability of systems for the selective depletion of each lineage in vivo. Genetic models have been generated that have led to the characterization of nonredundant roles for CD8α+ cDCs and for pDCs, but a unique role for CD11b+ cDCs has not been studied by a selective depletion model in vivo so far, to our knowledge. Here we used the Notch2 dependence of CD11b+ cDCs to analyze their role in host defense. We observed that Notch2-dependent CD103+CD11b+ cDCs were required for IL-23 production to protect the host from early susceptibility to infection with C. rodentium, but Batf3-dependent CD103+CD11b− cDCs were not. That role in innate defense was pathogen specific, as CD103+CD11b+ cDCs were not necessary for resistance to T. gondii or for the healing of intestinal mucosa after injury. These findings settle an unresolved question about the critical source of IL-23 required for driving IL-22-dependent antimicrobial responses to infection with attaching-and-effacing pathogens. As IL-22 also acts in other host-defense processes, such as in chronic inflammatory skin disease, future studies should examine the requirement for CD11b+ cDCs in those settings as well.

Given our observation that Notch2 also regulated gene expression in CD8α+ cDCs, we investigated whether defects in these cDCs could account for early susceptibility to C. rodentium. However, Batf3−/− and Batf2−/− mice showed normal resistance to C. rodentium. We also excluded the possibility of a role for other Notch2-independent cDCs. In the absence of Notch2, some CD11b+ cDCs developed in the spleen; likewise, Zbtb46-expressing CD103−CD11b+ cDCs may have also developed in the intestine. However, any 'Notch2-inexperienced' CD11b+ cDCs remaining in Notch2−/− mice were insufficient for IL-23
production and protection against infection with *C. rodentium*. Notch signaling also regulates development of IL-22-producing ILCs, but we found that ILC function was not affected by CD11c-Cre–mediated deletion of *Notch2*. Nonetheless, this dual role of Notch signaling in defense against infection with attaching-and-effacing pathogens might represent a coordinated immunological strategy involving the intentional expression of Notch ligands in pathogen-responsive cellular niches.

Antibody blockade of LTβR *in vivo* has also been found to diminish IL-23 production during infection with *C. rodentium*, and conditional deletion of *Ltbr* by CD11c-Cre increases susceptibility to this pathogen. In addition, a published study has reported a requirement for LTβR in the homeostasis of CD4+ cDCs in the spleen. Such results would suggest a possible relationship between signaling via Notch2 and signaling via LTβR in cDC differentiation. Indeed, we found that LTβR selectively influenced the development of splenic ESAM+ cDCs and intestinal CD103+CD11b+ cDCs, but Flt3L did not. It has been shown that loss of LTβR signaling directly impairs *Il23a* expression by cDCs. However, our results suggest that LTβR signaling may also control the development of CD103+CD11b+ cDCs, which are a source of IL-23, independently of any effects on *Il23a* expression.

The tissue-resident CD11b+ cDCs critical for early defense against *C. rodentium* were affected in Zbtb46<sup>DTR</sup>, *Notch2<sup>SKO</sup>* and *Flt3<sup>−/−</sup>* mice and thus seemed to derive from pre-cDCs, rather than from monocytes. However, our demonstration that Notch2-dependent CD11b+ cDCs were required for IL-23-dependent protection against *C. rodentium* does not exclude the possibility of contributions from macrophages and monocyte-derived cells. Indeed, monocytes serve a protective role in the eradication of *C. rodentium* after their recruitment to the lamina propria during later stages of infection. So, whereas cDC-derived IL-23 may be critical early,
monocyte-derived cells\textsuperscript{31} can produce IL-6 (ref. 52), which could support CD4\textsuperscript{+} T cell–derived IL-22 production later during infection\textsuperscript{53}. Our observation that Ccr2\textsuperscript{-/-} mice had diminished mortality relative to that of Notch2\textsuperscript{cKO} mice, yet still lost weight, is also in agreement with a model in which monocytes are important in immune responses to \textit{C. rodentium}. Thus, future studies should characterize the nonredundant functions of pre-cDC–derived and monocyte-derived cells in resistance to infection with attaching-and-effacing bacteria.

The role of cDCs in immunological defense has been thought to reside mainly in their ability to prime the responses of CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells\textsuperscript{54}. Our results presented here, along with the similar requirement for CD8\textalpha\textsuperscript{+} cDCs in IL-12 production for early innate protection against \textit{T. gondii}, demonstrate that both branches of cDCs are also critical for the initiation of innate immunity. These findings may indicate that the DC lineage separated from monocytes and macrophages before the emergence of adaptive immunity and that it has a dedicated role in orchestrating the reactions of the expanding family of ILCs\textsuperscript{3}. 
**EXPERIMENTAL PROCEDURES**

**Mice**

All animals were bred and maintained in a specific pathogen-free animal facility according to institutional guidelines and with protocols approved by the Animal Studies Committee at Washington University in St. Louis. The generation of Zbtb46gfp and Zbtb46DTR mice has been described\(^1\)\(^5\),\(^6\). For depletion of cDCs from Zbtb46DTR mice, diphtheria toxin (40 ng/g; Sigma) was administered on day −3 and day −1 before analysis on day 0. For depletion of cDCs during infection with *C. rodentium*, mice were injected with 20ng/g diphtheria toxin 1d before infection, and doses of 5ng/g were given every 2–3d thereafter to maintain depletion.

Mice of the following genotypes were from Jackson Laboratories:

- CD11c-cre (B6.Cg-Tg(Itgax-cre)1-1Reiz/J),
- Vav1-cre (B6.Cg-Tg(Vav1-cre)A2Kio/J),
- *Notch2*\(^{ef}\) (B6.129S-Notch2<sup>tm3Grid</sup>/J),
- *Ccr2*\(^{-/-}\) (B6.129S4-Ccr2<sup>tm1Ifc</sup>/J),
- *Cd40*\(^{-/-}\) (B6.129P2-Cd40<sup>tm1Kik</sup>/J),
- *Nfkb1*\(^{-/-}\) (B6;129P-Nfkb1<sup>tm1Bal</sup>/J),
- *Ccr7*\(^{-/-}\) (B6.129P2(C)-Ccr7<sup>tm1Rfor</sup>/J) and
- *Cx3cr1*<sup>gfp</sup> (B6.129P-Cx3cr1<sup>tm1Lit</sup>/J).

The generation of Rbpj<sup>ef</sup>, Psen1<sup>ef</sup> and Psen2<sup>−/-</sup> mice has been described\(^5\)\(^6\),\(^5\)\(^7\). Flt3L<sup>−/-</sup> (C57BL/6-flt3L<sup>tm1Imx</sup>) mice were obtained from Taconic. The generation of Il23<sup>−/-</sup> mice\(^2\), Ltbr<sup>−/-</sup> mice\(^4\)\(^0\) and Batf<sup>−/-</sup>, Batf2<sup>−/-</sup> and Batf3<sup>−/-</sup> mice\(^1\)\(^7\),\(^4\)\(^1\) has been described. The generation of Nik<sup>−/-</sup> mice has been described\(^5\)\(^8\); these mice were a gift from B. Sleckman. Ifnar1<sup>−/-</sup> mice were a gift from T. Watts.
For the generation of \textit{Ccr2}\textsuperscript{eGFP} 'knock-in' mice, a targeting construct was designed to insert a DNA fragment encoding enhanced GFP (eGFP), followed by a polyadenylation signal and a \textit{loxP}-flanked neomycin-resistance cassette at the translation start site of \textit{Ccr2}. The construct was transfected by electroporation into LK-1 (C57BL/6J) embryonic stem cells\textsuperscript{59}, and one correctly targeted clone identified by Southern blot analysis was used for the generation of chimeras. Chimeras were bred to mice with transgenic expression of Cre recombinase from the cytomegalovirus (CMV) promoter (B6.C-Tg(CMV-Cre)1Cgn/J, Jackson Laboratories)\textsuperscript{60} for removal of the neomycin-resistance cassette. For the generation of \textit{Irf4}\textsuperscript{−/−} mice, \textit{Irf4}\textsuperscript{f/f} mice (B6.129S1-\textit{Irf4}\textsuperscript{tm1Rdf}/J; Jackson Laboratories) were crossed with CMV-Cre mice. For bone marrow–chimera experiments, the CD45.1\textsuperscript{+} B6.SJL (B6.SJL-\textit{Ptprc}\textsuperscript{a}Pepc\textsuperscript{b}/BoyJ) mice were from Jackson Laboratories. Unless otherwise indicated, experiments used sex-matched littermates at 8–12 weeks of age. Unirradiated mice used for \textit{C. rodentium} experiments weighed less than 20 g when infected, unless specified otherwise.

\textbf{DC preparation}

DCs were collected from lymphoid organs and nonlymphoid organs and were prepared as described\textsuperscript{16}. Spleens, MLNs, skin-draining (inguinal) lymph nodes and kidneys were minced and digested for 30 min at 37 °C, with stirring, in 5 ml Iscove's modified Dulbecco's medium plus 10\% FCS (cIMDM) with 250 \(\mu\text{g/ml}\) collagenase B (Roche) and 30 U/ml DNase I (Sigma-Aldrich). Cells were passed through a 70-\(\mu\text{m}\) strainer before red blood cells were lysed with ammonium chloride–potassium bicarbonate lysis buffer. Cells were counted on a Vi-CELL analyzer (Beckman Coulter), and \(5\times10^6\) to \(10\times10^6\) cells were used for each antibody-staining reaction. Lung-cell suspensions were prepared after perfusion by injection of 10 ml Dulbecco's PBS (DPBS) into the right ventricle after transection of the lower aorta. Dissected and minced
lungs were digested for 1 h at 37 °C, with stirring, in 5 ml cIMDM with 4 mg/ml collagenase D (Roche). Suspensions of cells from the small intestine were prepared after removal of Peyer's patches and fat. Intestines were opened longitudinally, washed of fecal contents and cut into pieces 1 cm in length and were incubated for 40 min at 37 °C, with rotation at 1g, in Hank's balanced-salt solution (Life Technologies) plus 5 mM EDTA. Tissue pieces were washed in DPBS and minced and were incubated for 90 min at 37 °C, with stirring, in 25 ml RPMI medium plus 2% FBS with collagenase VIII (100 U/ml; C2139; Sigma). Cell suspensions were pelleted, suspended in 40% Percoll (P4937; Sigma), overlaid on 70% Percoll and centrifuged for 20 min at 850g. Cells in the interphase were recovered, washed in DPBS and stained. For DC population expansion in vivo, 10 µg Flt3L (250-31L; PeproTech) was injected intraperitoneally for two consecutive days and organs were collected after 7 d. For induction of p40, splenocyte samples were enriched for CD11c+ cells through the use of MACS beads (Miltenyi). After enrichment, cells were stimulated for 24 h ex vivo with 1 µg/ml LPS (L2630; Sigma) and 50 ng/ml interferon-γ (315-05; PeproTech). Brefeldin A (1 µg/ml) was added for the final 4 h. Cells were then washed, stained for the expression of surface markers (antibodies identified below), permeabilized with 0.25% saponin and stained for intracellular p40.

**Antibodies and flow cytometry**

Samples were stained at 4 °C in the presence of Fc Block (2.4G2; BD Biosciences) in flow cytometry buffer (DPBS plus 0.5% BSA plus 2 mm EDTA). The following antibodies were from BD Biosciences: allophycocyanin-conjugated antibody to CD4 (anti-CD4 (RM4-5)), V450–anti-GR1 (RB6-8C5), fluorescein isothiocyanate–anti-CD3ε (145-2C11), fluorescein isothiocyanate–anti-CD45.1 (A20), fluorescein isothiocyanate–anti-CD21/CD35 (7G6), phycoerythrin-indotricarbocyanine–anti-CD24 (M1/69), phycoerythrin-indotricarbocyanine–
anti-CD8α (53-6.7) and allophycocyanin–anti-CD172a (anti-SIRPα; P84). The following antibodies were from eBioscience: peridinin chlorophyll protein–cyanine 5.5–anti-CD11b (M1/70), allophycocyanin–anti-CD90.2 (53-2.1), allophycocyanin–eFluor 780–anti-CD11c (N418), phycoerythrin–anti-CD23 (B3B4), phycoerythrin–anti-RORγt (AFKJS-9), phycoerythrin–anti-IL22 (1H8PWSR), phycoerythrin–anti-IL12/23 p40 (C17.8), phycoerythrin–anti-ESAM (1G8), eFluor 450–anti-MHCII (I–A–I–E; M5/114.15.2), phycoerythrin–anti-CD103 (2E7), peridinin chlorophyll protein–cyanine 5.5–anti-CD16/32 (93), allophycocyanin–anti-CD45.2 (104), allophycocyanin–eFluor 780–anti-CD117 (2B8), phycoerythrin–anti-CD135 (A2F10), V500–anti-B220 (RA3-6B2), allophycocyanin–anti-F4/80 (B-M8), Alexa Fluor 700–anti-Sca1 (D7) and peridinin chlorophyll protein–eFluor710–anti-Siglec-H (eBio440C). Phycoerythrin– and allophycocyanin–conjugated anti-CD205 (anti-DEC-205; NLDC-145) were from Miltenyi. Biotin anti-CD127 (A7R34) was from BioXCell. In general, all antibodies were used at a dilution of 1:200; anti-DEC-205 was used at a dilution of 1:20. Cells were analyzed on a FACSCanto II or FACSARia II (BD) and data were analyzed with FlowJo software (TreeStar). For immunofluorescence histology experiments, the following reagents were used: rabbit polyclonal antibody to GFP (A11122), Alexa Fluor 488–conjugated antibody to rabbit IgG (A11034) and streptavidin–Alexa Fluor 555 (S32355; all from Invitrogen); Alexa Fluor 647–anti-CD11c (N418; BioLegend); biotin–anti-F4/80 (BM8; Caltag); Alexa Fluor 488– and biotin-conjugated anti-B220 (RA3-6B2), biotin–anti-IgD (11-26) and biotin–anti-MADCAM-1 (MECA-367; all from eBioscience); and rabbit polyclonal antibody to β-catenin (C2206) and indocarbocyanine–conjugated antibody toα-smooth muscle actin (1A4; both from Sigma).
**C. rodentium**

Mice were infected by intraoral inoculation of $2 \times 10^9$ colony-forming units of *C. rodentium*, strain DBS100 (American Type Culture Collection) as described\(^2\). Survival and weight loss were monitored for 30–45 d. Survival studies were done in accordance with institutional guidelines and with protocols approved by the Animal Studies Committee at Washington University in St. Louis. Colon lengths were measured for mice infected for 4 or 9 d. For histology, distal colons were collected and fixed overnight at 25 °C in 10% buffered formalin phosphate (4% (wt/wt) formaldehyde, 0.4% (wt/vol) sodium phosphate (monobasic monohydrate), 0.65% (wt/vol) sodium phosphate (dibasic anhydrous) and 1.5% (wt/vol) stabilizer methanol; SF100-4; Fisher Scientific), embedded in paraffin, sectioned and stained with hematoxylin and eosin. For analysis of colony-forming units, spleens and colons from mice infected for 4 or 9 d were weighed and homogenized and serial dilutions were plated for 24 h at 37 °C in duplicate onto MacConkey agar plates (M7408; Sigma). The severity of colitis (by histology of samples assessed by a researcher 'single-blinded' to sample identity) was assigned a score on a scale from 1 to 7, which corresponded to the following: 1, no evidence of inflammation; 2, little cellular infiltration in <10% of the field; 3, moderate cellular infiltration in 10–25% of the field, crypt elongation, bowel-wall thickening and no evidence of ulceration; 4, substantial cellular infiltration in 25–50% of field, thickening of the bowel wall beyond the muscular layer and high vascular density; 5, substantial infiltration in >50% of field, crypt elongation and distortion and transmural bowel thickening with ulceration; 6, complete loss of mucosal architecture with ulceration and loss of mucosal vasculature; and 7, coagulation necrosis. For analysis of IL-22 expression in ILCs, MLN cells isolated from mice infected for 4 d were stimulated for 4 h with 50 ng/ml PMA (phorbol 12-myristate 13-acetate; P1585; Sigma)
and 1 µM ionomycin (I0634; Sigma), with or without 10 ng/ml recombinant IL-23 (1887-ML-010; R&D Systems), in the presence of 1 µg/ml brefeldin A (B6542; Sigma). Cells were washed with DPBS and stained for surface expression of specified markers (antibodies identified above). Cells were then fixed in 2% methanol-free paraformaldehyde (PFA), permeabilized with 0.25% saponin and stained for intracellular IL-22.

*T. gondii*

The type II Prugniaud strain of *T. gondii* expressing a transgene encoding firefly luciferase and GFP (PRU-FLuc-GFP) used was provided by J. Boothroyd. The parasites were grown in human foreskin fibroblasts cultures as described. For infection, freshly egressed parasites were filtered, counted, and 100 tachyzoites were injected intraperitoneally into mice. Survival was monitored for 30 d.

**Endoscopy-guided mucosal excision**

Mice were anesthetized, and colon lumens were visualized with a high-resolution miniaturized colonoscope system. After inflation of the colon with DPBS, 3F flexible biopsy forceps were inserted into the sheath adjacent to the camera. Three to five full-thickness areas of the entire mucosa and submucosa were removed from along the dorsal side of the colon. For this study, wounds that averaged approximately 1 mm² (equivalent to 250–300 crypts) were evaluated. Wounded mice were killed 2 or 6 d after injury, and each wound was frozen individually in optimal cutting temperature compound. Sections were prepared and fixed in 4% PFA, boiled in 10 mmol/l citrate buffer and rinsed in DPBS, then nonspecific binding was blocked by incubation for 20 min with 3% bovine serum albumin and 0.1% Triton X-100, followed by incubation for 1 h with primary antibodies (identified above). Slides were then rinsed with DPBS, incubated with secondary antibodies (identified above), stained with bis-
benzimide and then mounted with Mowiol 4-88 (EMD Chemicals). Sections were viewed with a Zeiss (Oberkochen, Germany) Axiovert 200 microscope equipped with an Axiocam MRM camera. The selective COX-2 inhibitor NS-398 (Cayman Chem) was dissolved in dimethyl sulfoxide for preparation of the stock solution. Stocks were further diluted in 5% NaHCO₃, and doses of 5 mg per kg body weight were administered intraperitoneally daily after wounding.

**Isolation and transfer of bone marrow progenitor cells**

Bone marrow was collected from the femur, tibia, humerus and pelvis. Bones were fragmented by mortar and pestle, and debris were removed by gradient centrifugation with Histopaque 1119 (Sigma). Cells were passed through a 70-µm strainer and red blood cells were lysed with ammonium chloride–potassium bicarbonate lysis buffer. Cells were counted on a Vi-CELL analyzer, and 5 × 10⁶ to 10 × 10⁶ cells were stained for analysis or the entire bone marrow was stained for sorting. The gates used to define common myeloid progenitors, common DC progenitors and pre-DCs were based on published studies: common myeloid progenitors were identified as Lin⁻c-KitʰSca-1⁻CD11c⁻CD135⁺CD16/32⁻CD127⁻ cells; common DC progenitors were identified as Lin⁻c-KitⁱⁿᵗSca-1⁻CD135⁺CD16/32⁻CD127⁻ cells; pre-DCs were identified as Lin⁻c-KitʰSca-1⁻CD11c⁺CD135⁺CD16/32⁻CD127⁻ cells; and cDC-restricted pre-cDCs were identified as Zbtb46-GFP⁺ cells in the pre-DC gate. FACSria II (BD Biosciences) was used for cell purification. Cells were sorted into DPBS supplemented with 0.5% BSA and 2 mM EDTA. Cell purities of at least 95% were confirmed by analysis after sorting. For transfer experiments, purified cell populations were transferred retroorbitally into congenically marked mice (CD45.1⁺) after sublethal irradiation with 600 rads of whole-body irradiation.
Expression microarray analysis

Total RNA was isolated from cDCs with an RNAqueous-Micro kit (Ambion) or from colonic cells with an RNeasy Mini kit (Qiagen). For Mouse Gene 1.0 ST Arrays, RNA was amplified with a WT Expression kit (Ambion) and labeled, fragmented and hybridized with a WT Terminal Labeling and Hybridization kit (Affymetrix). Data were processed by robust multiarray average summarization and quantile normalization, and expression values were modeled with ArrayStar software (DNASTAR version 4). For principal-component analysis, microarray data sets were pre-processed by ArrayStar with robust multiarray average summarization and quantile normalization, and then replicates were grouped by sample. Either log-transformed expression values from each replicate or mean log-transformed expression values from each replicate group were exported in table format, imported into software of the R project for statistical computing (version 2.13.2), mean-centered by gene, root-mean-square-scaled by sample, transposed and subjected to principal-component analysis computed by singular value decomposition without additional centering or scaling. Scores were plotted in R software.

Quantitative RT-PCR

Gene expression was analyzed in colonic cells isolated from mice infected for 2 or 9 d with *C. rodentium*. Bone marrow–derived DCs cultured for 9 d with Flt3L (200 ng/ml) were stimulated with LPS and interferon-γ as described above. RNA and cDNA were prepared with an RNeasy Mini kit (Qiagen), Superscript III reverse transcriptase (Invitrogen) and Oligo(dT)20 Primer (Invitrogen). For analysis of *Il23a* expression in cDCs and macrophages, CD11b+ cDCs (Aqua−CD45−B220−CD11c+MHCII+CD103+CD11b+) and macrophages (Aqua−CD45−B220−CD11c+MHCII−CD11b+F4/80+) from mice infected for 2 d were sorted to a
purity of >95%. Total RNA was isolated from sorted cells with an RNAqueous-Micro kit (Ambion). The StepOnePlus Real-Time PCR system was used according to manufacturer's instructions (Applied Biosystems) with the relative quantitation standard-curve method with HotStart-IT SYBR Green qPCR Master Mix (Affymetrix/USB). PCR conditions were as follows: 10 min at 95 °C, followed by 40 two-step cycles consisting of 15 s at 95 °C and 1 min at 60 °C. Primers used for measurement of Il22, Reg3g, Il23a, Il12a, and Il12b expression were as follows:

IL22-F, 5′-TGACGACCAGAACATCCAGA-3′;
IL22-R, 5′-CGCCTTGATCTCTCCACTCT-3′;
HPRT-F, 5′-TCAGTCAACGGGGGACATAA-3′;
HPRT-R, 5′-GGGGCTGTACTGCTTAACCAG-3′;
IL23a-F, 5′-AATAATGTGCCCCGTATCCA-3′;
IL23a-R, 5′-GGATCCTTTGCAAGCAGAAC-3′;
IL12a-F, 5′-GTGAAGACGGCCAGAGAAA-3′;
IL12a-R, 5′-GGTCCCGTGTGATGTCTTC-3′;
IL12b-F, 5′-AGCAGTAGCAGTCCCCTGGA-3′;
IL12b-R, 5′-AGTCCCTTTGGTCCAGTAGT-3′;
Reg3g-F, 5′-ATCATGT CCTGGATGCTGCT-3′; and
Reg3g-R, 5′-AGATGGGGCATCTTTCTTGG-3′.

Immunofluorescence microscopy

Spleens and intestines were fixed for 8 h in 2% PFA, incubated overnight in 30% sucrose in H2O and embedded in optimal cutting temperature compound, and then cryosections 8 µm in thickness produced. After two washes in DPBS, nonspecific binding in sections was blocked by
incubation for 10 min at 21 °C in CAS Block (00-8120; Invitrogen) containing 0.2% Triton X-100. Sections were stained with primary and secondary reagents (identified above) diluted in CAS Block containing 0.2% Triton X-100 and were mounted with ProLong Gold Antifade reagent containing DAPI (4,6-diamidino-2-phenylindole; P36935; Invitrogen). For staining of CD11c, spleens were frozen without fixation and then were fixed in acetone for 15 min at 4 °C before being washed in DPBS and stained. A Zeiss AxioCam MRn microscope equipped with an AX10 camera was used for four-color epifluorescence microscopy. Monochrome images were acquired with AxioVision software with either a 10× or a 20× objective and then were exported into ImageJ software for subsequent color balancing and overlaying.

**Bone marrow chimeras**

Bone marrow cells from donor mice were collected as described above, and 5×10⁶ to 10×10⁶ total bone marrow cells were transplanted by retroorbital injection into wild-type B6.SJL recipients. For developmental competition experiments, recipients received a single dose of 1,200 rads of whole-body irradiation on the day of transplantation, and mice were analyzed 8–10 weeks later. For infection experiments, recipients received a total of two doses of 525 rads of whole-body irradiation, with an interval 4 h between the doses, on the day of transplantation, and mice were infected 8–10 weeks later. For mixed–bone marrow competition, bone marrow was obtained from mice of two genotypes, then cells were counted and mixed at a ratio of 1:1, and Lin⁻Sca-1⁺c-Kit⁺ cells were analyzed by flow cytometry to confirm 1:1 chimerism before transplantation. *Il23a⁻/⁻* bone marrow was distinguished from *Notch2⁷ff* or *Notch2⁷KO* bone marrow by GFP expression. *Ltbr⁻/⁻* bone marrow was distinguished by expression of CD45.1.
Statistical analysis

In general, differences between groups were analyzed by unpaired, two-tailed Student's $t$-tests. Results with a $P$ value of 0.05 or less were considered significant (Prism; GraphPad Software). Survival studies were analyzed by the log-rank Mantel-Cox test. For multiple comparisons, data were analyzed by the one-way ANOVA followed by Tukey's multiple-comparisons test. The appropriate nonparametric test was used when data failed to meet assumptions for parametric statistics.

Accession codes

GEO: microarray data, GSE45698.
ACKNOWLEDGMENTS

We thank B. Sleckman (Washington University in St. Louis) for Nik−/− mice; T. Watts (University of Toronto) for Ifnar1−/− mice; J. Boothroyd (Stanford University) for the plasmid PRU-FLuc-GFP; the Immunological Genome Project consortium for use of their database54; and the Alvin J. Siteman Cancer Center at Washington University School of Medicine for use of the Center for Biomedical Informatics and Multiplex Gene Analysis Genechip Core Facility. Supported by the Howard Hughes Medical Institute, the US National Institutes of Health (AI076427-02 to K.M.M., R01 GM55479 to R.K., R01 DE021255-01 and U01 AI095542-01 to M.C., R01 DK071619 to T.S.S. and R01 DK064798 to R.D.N.), the US Department of Defense (W81XWH-09-1-0185 to K.M.M.), the American Heart Association (12PRE8610005 to A.T.S. and 12PRE12050419 to W.K.), the Canadian Institutes of Health Research (MOP 67157 to J.L.G. and FRN 11530 to C.J.G.) and the National Cancer Institute (P30 CA91842 for the Alvin J. Siteman Cancer Center).

Contributions

REFERENCES


11. Tumanov, A. V. et al., Lymphotoxin controls the IL-22 protection pathway in gut innate


21. Torti, N. et al., Batf3 transcription factor-dependent DC subsets in murine CMV infection:


52. Rivollier, A. et al., Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *Journal of*


Figure 1. \(ζb\theta 40\text{arp}\) identifies intestinal cDC populations.

a Lamina propria

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b MLN

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C

Small intestine

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Figure 7-1: Zbtb46-GFP identifies intestinal cDC populations.

(a,b) Flow cytometry of live cells from the lamina propria (a) and MLNs (b) of Zbtb46<sup>gfp</sup>/+ mice (n = 9); pregating, above plots. Numbers adjacent to outlined areas indicate percent cells in each throughout. MHCII, major histocompatibility complex class II. (c) Fluorescence microscopy of Zbtb46-GFP, CD4, B220 and β-catenin, and of staining with the DNA-intercalating dye DAPI, in sections of small intestine (top) and colon (bottom) from chimeras (n = 5) generated by the reconstitution of wild-type mice with Zbtb46<sup>gfp</sup> bone marrow. Scale bars, 200 µm (Peyer's patch and colonic patch) or 100 µm (isolated lymphoid follicles (ILF) and villi). Data are representative of three independent experiments (a,b) or two independent experiments (c).
Figure 2. Zbtb46+ cDCs are essential for the control of C. rodentium infection.

a) Flow cytometry plots showing changes in MHCII and CD11c expression in the spleen and lamina propria of DT-treated and non-treated mice.

b) Bar graph showing the number of cells per 10^6 CD45.2+ cells in various cell populations.

c) Survival curve showing the percentage of survival over days post infection for WT and Zbtb46^/- mice.

d) Body weight change curve showing the change in body weight over days post infection for WT, Zbtb46^/-, and WT + DT-treated mice.

e) Survival curve showing the percentage of survival over days post infection for Cor2^+/-, Cor2^-/-, FLt3^+/-, and FLt3^-/- mice.

f) Body weight change curve showing the change in body weight over days post infection for Cor2^+/-, Cor2^-/-, FLt3^+/-, and FLt3^-/- mice.
Figure 7-2: Zbtb46+ cDCs are essential for survival after infection with C. rodentium.

(a) Expression of CD103 and F4/80 (right) in lamina propria cells from chimeras generated by the reconstitution of wild-type (WT) mice with Zbtb46DTR bone marrow, left untreated (− DT) or treated with diphtheria toxin (40 ng per gram body weight (ng/g)) on day −3 and day −1 (+ DT) and assessed on day 0 (CD45+B220+ cells pregated at left). (b) Quantification of cDCs and macrophages (MΦ) in the lamina propria of the chimeras in a (Zbtb46DTR => WT (donor=>recipient); n = 4), presented per 1 × 10^6 lamina propria cells. (c,d) Survival (c) and weight loss (d) of chimeras (key: donor=>recipient; n = 8 per group, except WT => WT + DT, n = 5) given oral inoculation of C. rodentium (2 × 10^9 colony-forming units) and otherwise left untreated (− DT) or treated with diphtheria toxin (20 ng/g) 1 d before infection and every third day (5 ng/g) for the remainder of the experiment (+ DT). (e,f) Survival (e) and weight loss (f) of C. rodentium–infected mice (n = 5 (Ccr2+/+), 4 (Ccr2−/−) or 10 (Flt3l+/+ and Flt3l−/−)). NS, not significant; *P < 0.05 and **P < 0.001 (Student's t-test (b,d,f) or log-rank Mantel-Cox test (c,e)). Data are from two independent experiments (error bars (b,d,f), s.e.m.).
Figure 3. Canonical Notch2 signaling is required for splenic and intestinal CD11b⁺ cDC development.

(a) Flow cytometry dot plots showing the expression of various markers in WT, Rbpᴍ³, Psen1⁻/⁻ Psen2⁻/⁻, and Notch2⁻/⁻ MHCI⁺ cells. The plots compare CD11c, CD24, and ESAM expression.

(b) Immunofluorescence images of CD11c⁺ and MHCI⁺ cells in Notch2⁺⁺ and Notch2⁻/⁻ conditions, showing differences in the expression of CD11c and MHCI in the bone marrow and WACAM-1 in the intestine.

(c) Flow cytometry dot plots depicting the expression of markers in Notch2⁺⁺, Notch2⁻/⁺ CD11c-cre, and Notch2⁻/⁻ MHCI⁺ cells. The plots show CD11c, CD24, and ESAM expression.

(d) Bar graph comparing the number of cells per 1X g splenocytes in Notch2⁺⁺ and Notch2⁻/⁻ conditions. The graph shows a significant increase in the number of cells in Notch2⁻/⁻ samples for CD11c⁺, CD24⁺, and MHCI⁺ markers.

(e) Flow cytometry dot plots illustrating the expression of markers in Notch2⁺⁺, Notch2⁻/⁻, and Batf³⁻⁻. The plots compare CD11c, MHCI, and CD45⁺CD11c⁺ in the lamina propria and MLN.

(f) Immunofluorescence images showing the distribution of CD11c⁺ and CD24⁺ cells in Notch2⁺⁺ and Notch2⁻/⁻ conditions, with DAPI staining for nuclei.
Figure 7-3: Canonical Notch2 signaling is required for the development of splenic and intestinal CD11b+ cDCs.

(a) Flow cytometry of live splenocytes from wild-type mice and mice with Vav1-Cre–mediated deletion of Rbpj (Rbpj\textsuperscript{vav}), Psen1 and Psen2 (Psen1\textsuperscript{vav}Psen2\textsuperscript{vav}) or Notch2 (Notch2\textsuperscript{vav}), pregated as MHCII\textsuperscript{+}CD11c\textsuperscript{+} (left) and CD172a\textsuperscript{+}CD24\textsuperscript{−} (middle) cells. (b) Fluorescence microscopy of CD11c, IgD and B220 (top) or CD11c and MAdCAM-1 (bottom) in sections of spleen from Notch2\textsuperscript{f/f} and Notch2\textsuperscript{vav} mice. Scale bars, 200 µm. (c) Flow cytometry of splenocytes from Notch2\textsuperscript{f/f} mice (top), mice with CD11c-Cre–mediated deletion of a single loxP-flanked Notch2 allele (Notch2\textsuperscript{f/+}; middle) and Notch2\textsuperscript{cKO} mice (bottom), stained for various markers (plot margins). (d) Quantification of cDC development in the Notch2\textsuperscript{f/f} and Notch2\textsuperscript{cKO} mice in c, presented per 1 × 10\textsuperscript{6} splenocytes. (e) Flow cytometry of cells from the lamina propria of Notch2\textsuperscript{f/f}, Notch2\textsuperscript{cKO} and Batf3\textsuperscript{−/−} mice, stained for various markers (plot margins). (f) Immunofluorescence microscopy of cryptopatches (CP) and ILFs from sections of small intestine from Notch2\textsuperscript{f/f} and Notch2\textsuperscript{cKO} mice, stained for CD11c and CD90 and with DAPI. Scale bars, 50 µm. *P < 0.01 and **P < 0.001 (Student's t-test). Data are representative of three independent experiments (a,c,e; n = 6–8 (a), 7 (c) or 3–5 (e) mice per group) or two independent experiments (b,f; n = 4 (b) or 5 (f) mice) or are from three independent experiments (d; n = 7 mice per group; error bars, s.e.m.).
Figure 4. Notch2 controls the terminal differentiation of CD11b+ and DEC205+ cDCs.
Figure 7-4: Notch2 controls the terminal differentiation of CD11b⁺ and DEC-205⁺ cDCs.

(a) Microarray analysis of cDC subsets from Notch2⁺ff (f/f) and Notch2⁺cKO (KO) mice, sorted as MHCII⁺CD11c⁺CD24⁻CD11b⁺ cells (CD11b⁺ cDCs; left) or MHCII⁺CD11c⁺CD24⁺CD11b⁻DEC-205⁺ cells (DEC-205⁺ cDCs; right); P values (vertical axes), Welch's t-test. Colors indicate expression more than twofold higher (red) or lower (blue) in Notch2⁺ff cells than in Notch2⁺cKO cells. (b) Principal-component analysis (left) of Notch2⁺ff and Notch2⁺cKO CD11b⁺ cDCs (CD11b) and DEC-205⁺ cDCs (DEC-205), analyzed by individual replicate (f/f1–f/f3 and KO1–KO3; variance: PC1, 54.0%; PC2, 21.0%), and gene expression (right) in progenitors (common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP) and common DC progenitors (CDP)) and in CD8⁺ and CD4⁺ cDC subsets, derived from the Immunological Genome Project database for probe sets corresponding to the 20 most positive and negative 'loadings' in PC2 (log-transformed values). (c) Gene expression in DC progenitors and subsets of probe sets corresponding to the 50 most positive and negative 'loadings' in PC2 (presented as mean-centered, log-transformed values). Each symbol represents an individual gene; small horizontal lines indicate the mean. (d) Gene expression of selected probe sets, analyzed as in c. (e) Flow cytometry of splenic CD11b⁺ cDCs from Ccr2⁺gfp mice (left), Cx3cr1⁺gfp mice (middle) and Zbtb46⁺gfp mice (right). (f) Contribution of donor cells to bone marrow progenitors and splenocytes in mixed chimeras generated with CD45.2⁺ Notch2⁺ff and CD45.1⁺ wild-type bone marrow (Notch2⁺ff + WT) or CD45.2⁺ Notch2⁺vav bone marrow and CD45.1⁺ wild-type bone marrow (Notch2⁺vav + WT), analyzed 8–10 weeks after lethal irradiation and transplantation; results are presented as the ratio of Lin⁻Sca-1⁺c-Kit⁺ chimerism in the same mouse (percent contribution of CD45.2⁺ cells / percent CD45.2⁺ cells among Lin⁻Sca-1⁺c-Kit⁺ cells), with chimerism of monocytes, neutrophils and cDCs analyzed among splenocytes.
Each symbol represents an individual mouse; small horizontal lines indicate the mean. *$P < 0.01$ and **$P < 0.001$ (Kruskal-Wallis test with Dunn's multiple comparison test (c) or Student's $t$-test (f)). Data are from two independent experiments (a,b, left; $n = 3$ biological replicates per cell type), three independent experiments (b, right, c,d; $n = 3–5$ biological replicates per cell type) or two independent experiments (f; $n = 3–5$ mice per group) or are representative of three independent experiments (e; $n = 5–6$ mice per group).
Figure 7-5: LTβR signaling mediates the homeostatic population expansion of Notch2-dependent cDCs.

(a) Flow cytometry of live splenocytes from wild-type, Notch2cKO and Ltbr−/− mice, stained for various markers (plot margins; pregating, left and middle). (b) Flow cytometry of live splenocytes from mixed chimeras generated with CD45.1+ Ltbr−/− and CD45.2+ wild-type bone marrow (Ltbr−/− + WT) or with CD45.1+ Ltbr−/− and CD45.2+ Notch2cKO bone marrow (Ltbr−/− + Notch2cKO), analyzed 8–10 weeks after lethal irradiation and transplantation (pregating, above plots; neutrophils, CD11c−CD11b+CD24+ cells). Bottom row, red numbers in top right corners indicate chimerism ratio (percent contribution of CD45.1+Ltbr−/− cells/percent CD45.1+Ltbr−/− cells among splenic neutrophils). (c) Contribution of Ltbr−/− bone marrow to each cell type in the mixed chimeras in b, presented as a ratio of neutrophil chimerism in the same mouse. Each symbol represents an individual mouse; small horizontal lines indicate the mean. (d) Contribution of wild-type or Ltbr−/− bone marrow to CD103+CD11b+cDCs in the lamina propria from chimeras generated by the reconstitution of CD45.1+CD45.2+ wild-type mice with CD45.1+CD45.2+ wild-type bone marrow (WT => WT), CD45.1+ Ltbr−/− bone marrow (Ltbr−/− => WT) or a mixture of Ltbr−/− and wild-type bone marrow (Ltbr−/−+WT => WT). (e) Quantification of chimerism in mixed chimeras generated with a mixture of Ltbr−/− and wild-type bone marrow (as in d) or Ifnar1−/− and wild-type bone marrow, calculated as follows: (percent contribution of mutant cells to intestinal cDCs / percent contribution of wild-type cells to intestinal cDCs) / (percent contribution mutant cells to splenic T cells / percent contribution of wild-type cells to splenic T cells). Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.e.m.). *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test). Data are representative of three independent experiments.
(a; \(n = 6\) mice per group) or five independent experiments (d; \(n = 10\)–11 mice per group) or are from two independent experiments (b,c; \(n = 2\)–3 mice per group) or five independent experiments (e; \(n = 10\) mice (\(Ltb^{/-}\)) or 5 mice (\(Ifnar1^{/-}\))).
Figure 6. Notch2-dependent CD11b+ cDCs are required for host defense against C. rodentium infection.

a) Survival (%) over Days post infection for different strains.

b) Body weight change (%) over Days post infection for different strains.

c) Spleen CFU/g (Log_{10}) for Notch2^-^, Notch2^2^-^, and Batf3^+^.

d) Colon CFU/g (Log_{10}) for Notch2^-^, Notch2^2^-^, and Batf3^+^.

e) Histology scores for Notch2^-^ and Notch2^2^-^.

f) Histology score for Notch2^-^, Notch2^2^-^, and Batf3^+^.

g) Survival (%) over Days post infection for different strains.

h) Body weight change (%) over Days post infection for different strains.
Figure 7-6: Notch2-dependent CD11b+ cDCs are essential for host defense against infection with *C. rodentium*.

(a,b) Survival (a) and weight loss (b) of *Batf3*−/−, *Notch2*eff and *Notch2*cko mice (n = 9–10 per group) orally inoculated with *C. rodentium* (2×10⁹ colony-forming units). (c) *C. rodentium* titers in the spleen (left) and colon (right) of mice (n = 6–7 per group) 9 d after inoculation as in a, presented as colony-forming units (CFU). (d) Colon lengths in mice (n = 3 per group) 9 d after infection as in a. (e) Hematoxylin-and-eosin staining of colon sections from *Notch2*eff and *Notch2*cko mice (n = 6–7 per group) 9 d after infection as in a. Scale bars, 200 µm. (f) Histological scores of the sections in e (and similar sections from *Batf3*−/− mice). (g,h) Survival (g) and weight loss (h) of mice (n = 8 (wild-type), 5 (*Ccr7*−/−, *Batf2*−/− and *Batf2*+/+) or 6 (*Irf4*−/−)) infected with *C. rodentium* as in a. *P < 0.05, **P < 0.01, ***P < 0.001 (log-rank Mantel-Cox test (a,g), Student's *t*-test (b,h), one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test (c,d) or Kruskal-Wallis test with Dunn's multiple-comparison test (f)). Data are from three independent experiments (a,b) or two independent experiments (c,d,g,h) or are representative of two independent experiments (e,f; error bars (b–d,f,h), s.e.m.).
Figure 7. Notch2-dependent cDCs are dispensable for colonic wound repair.
Figure 7-7: Notch2-dependent cDCs are dispensable for colonic wound repair.

(a) Ptgs2 expression in various intestinal cell types, derived from the Immunological Genome Project database\textsuperscript{55}, assessed by microarray. (b) Whole-mount images of Notch2\textsuperscript{f/f} and Notch2\textsuperscript{cKO} colonic wounds 6 d after excision (left), and quantification of epithelial coverage in individual wound beds at day 6 after excision, assessed in whole-mount images (right). Outlined areas (left) indicate the initial injury site. Scale bars (left), 1 mm. Each symbol (right) represents an individual mouse (n = 6 per group); small horizontal lines indicate the mean (± s.e.m.). (c) Loss of α-smooth muscle actin (SMA) underlying wound beds at day 6 after excision from Notch2\textsuperscript{f/f}, Notch2\textsuperscript{cKO} and Batf3\textsuperscript{−/−} mice, and from Notch2\textsuperscript{f/f} mice treated with NS-398 (far right), measured in histological sections (gap length / wound bed length). Each symbol represents an individual mouse (n = 2–6 per group); small horizontal lines indicate the mean (± s.e.m.). (d) Colonic sections from an untreated Notch2\textsuperscript{f/f} mouse (left) or Notch2\textsuperscript{f/f} mice treated with NS-398 (5 µg/g; right), stained for β-catenin and α-smooth muscle actin (αSMA) and with DAPI on day 2 (left) or day 6 (right) after excision (n = 2–3 mice per group). Scale bars, 200 µm. (e) Colonic sections from Notch2\textsuperscript{f/f}, Notch2\textsuperscript{cKO} and Batf3\textsuperscript{−/−} mice (n = 3–6 per group) stained for β-catenin, α-smooth muscle actin and F4/80 and with DAPI at 6 d after excision. Scale bars, 200 µm. Statistical results (b, right), Student’s t-test. Data are from one experiment with two to four replicate arrays (a; mean and s.e.m.) or two independent experiments (b,e) or are representative of two independent experiments (d,e).
Figure 8. CC11b⁺ cDCs regulate IL-23-dependent antimicrobial responses to C. rodentium.

(a) Graphs showing the relationship between Notch2⁺ cell counts and Nnitch2⁺ cell counts with varying conditions.

(b) Bar graph showing the fold change of Notch2⁺ cell counts in different conditions.

(c) Bar graph showing the fold change of Notch2⁺ cell counts in different conditions.

(d) Graphs showing the expression levels of IL-22 and Reg3g in Notch2⁺ cDCs.

(e) Graphs showing the effect of IL-23 on Notch2⁺ cell counts.

(f) Graphs showing the effect of IL-23 on IL-22 expression in uninfected and infected conditions.

(g) Graphs showing the gene expression levels of IL-23a in different cell types.

(h) Graph showing the survival rates of different infection conditions over time.
**Figure 7-8: Notch2-dependent CD11b⁺ cDCs regulate IL-23-dependent antimicrobial responses to *C. rodentium.*

(a) Microarray analysis of gene expression in colonic cells from mice 9 d after infection with *C. rodentium*, presented as M-plots. Colors indicate higher (red) or lower (blue) expression in *Notch2*KO cells (left) or *Batf3*⁻/⁻ cells (right) than in *Notch2*ff cells. (b,c) Expression of selected inflammatory molecule–encoding genes from a with higher expression in *Notch2*KO (KO) mice than in *Notch2*ff (ff) mice (b), and expression of IL-22-stimulated genes (c). (d) Quantitative RT-PCR analysis of *Il22* and *Reg3g* mRNA in colons from *Notch2*ff, *Notch2*KO and *Batf3*⁻/⁻ mice (n = 6–7 per group) 9 d after infection with *C. rodentium*, presented in arbitrary units (AU) relative to expression of the housekeeping gene *Hprt*. (e) Microarray analysis of gene expression in colonic cells from *Notch2*ff and *Notch2*KO mice 4d after infection with *C. rodentium*, presented as an M-plot (left), and hematoxylin-and-eosin staining of colons from those mice (n = 4 per group; right). Scale bars (right), 100 µm. (f) Intracellular IL-22 expression in ILCs obtained from the MLNs of uninfected *Notch2*ff mice (UI) or *Notch2*ff and *Notch2*KO mice (n = 4 per group) 4 d after infection with *C. rodentium* (day 4) and then stimulated *ex vivo* with the phorbol ester PMA and ionomycin in the presence (+ IL-23) or absence (− IL-23) of IL-23 (pregating, above plots). (g) Quantitative RT-PCR analysis of *Il23a* mRNA in CD11b⁺ cDCs (CD11c⁺MHCII⁺CD103⁺CD11b⁺) and macrophages (CD11c⁺MHCII⁻CD11b⁺F4/80⁺) sorted from the lamina propria of wild-type uninfected mice (UI) or wild-type mice 2 d after infection with *C. rodentium* (day 2), presented as in d (n = 3 mice per group). (h) Survival of mixed chimeras (n = 5–6 per group) generated by the reconstitution of wild-type mice with *Il23a*⁻/⁻ bone marrow (*Il23a*⁻/⁻ => WT), a mixture of *Il23a*⁻/⁻ and *Notch2*KO bone marrow (*Il23a*⁻/⁻+*Notch2*KO => WT) or *Il23a*⁻/⁻ and *Notch2*ff bone marrow (*Il23a*⁻/⁻+*Notch2*ff => WT),
or wild-type bone marrow (WT => WT), orally inoculated with $2 \times 10^9$ colony-forming units C. 
rodentium 8–10 weeks after lethal irradiation and transplantation. ND, not detected. 
* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA with Tukey's multiple-comparison test 
(d), Student's t-test (f) or log-rank Mantel-Cox test (h)). Data are from one experiment (a–c; 
average of two to three biological replicates per sample), three independent experiments 
(d; bars, s.e.m.), one experiment with two biological replicates per sample (e, left) or two independent 
experiments (g,h; error bars (g), s.e.m.) or are representative of two independent experiments (e, 
right, f).
CHAPTER VIII

The absence of a microbiota enhances TSLP expression in mice with defective skin barrier but does not affect the severity of their allergic inflammation.

Publication & Copyright
Results and text presented in this chapter have been previously published in:

Author Contribution
The author contributed only the following sections of this chapter:
• Generation and the maintenance of the some of the germ-free RBPjcKO and WT mice.
• Serum TSLP measurements in Figure 8-3B.
• WBC-count measurements in Figure 8-4A.

Ms. Laura J. Yockey and Dr. Shadmehr Demehri carried out majority of the other experiments described in this chapter with the assistance from other authors. Ms. Laura J. Yockey, Dr. Shadmehr Demehri, and Dr. Raphael Kopan wrote the text of the published manuscript.
ABSTRACT

Evidence is accumulating to suggest that our indigenous microbial communities (microbiota) may have a role in modulating allergic and immune disorders of the skin. To examine the link between the microbiota and atopic dermatitis (AD), we examined a mouse model of defective cutaneous barrier function with an AD-like disease due to loss of Notch signaling. Comparisons of conventionally raised and germ-free (GF) mice revealed a similar degree of allergic skin inflammation, systemic atopy, and airway hypersensitivity. GF mutant animals expressed significantly higher levels of thymic stromal lymphopoietin, a major proinflammatory cytokine released by skin with defective barrier function, resulting in a more severe B-lymphoproliferative disorder that persisted into adulthood. These findings suggest a role for the microbiota in ameliorating stress signals released by keratinocytes in response to perturbation in cutaneous barrier function.
INTRODUCTION

Atopic dermatitis (AD) is a common allergic disease that affects about 10% of children in the United States and generally resolves by puberty (Spergel, 2010). However, up to 70% of individuals with a history of AD go on to develop asthma and other atopic disorders later in life, a phenomenon called the “atopic march” (Spergel and Paller, 2003; Spergel, 2010). AD flare-ups are known to be associated with overgrowth of bacterial species, including *Staphylococcus aureus*, in barrier-defective lesional skin (Boguniewicz and Leung, 2011; Kong et al., 2012). Paradoxically, increased AD prevalence correlates with improvements in human hygiene in the last century. One explanation for this correlation is that improved hygiene and increased use of antibiotics have led to decreases in host exposure to microorganisms (Kalliomaki et al., 2001; Umetsu et al., 2002; Yazdanbakhsh et al., 2002; Romagnani, 2004; Macia et al., 2012), which may in turn alter immune responses leading to an increase in atopy.

In this study, we used a mouse model of AD generated by removing the floxed alleles of *RBPj*, the DNA-binding partner of Notch, in the skin via expression of *Msx2-Cre* transgene (Demehri et al., 2008; Dumortier et al., 2010). RBPj and Notch signaling are essential for coordinating terminal differentiation of epidermal keratinocytes and for formation of a competent barrier (Blanpain et al., 2006). The *Msx2-Cre* transgene (Demehri et al., 2008; Dumortier et al., 2010) deletes *RBPj* flox in a mosaic (calico) pattern along the back and stomach of the mouse, allowing juxtaposed patches of RBPj-deficient and wild-type skin to be compared and contrasted (Demehri et al., 2008). These mice exhibit many of the hallmarks of AD including T helper (Th) 2–mediated skin inflammation, high serum IgE levels, and greater susceptibility to asthma (Demehri et al., 2009; Dumortier et al., 2010). In addition, these mice
develop a marked, transient B-cell expansion early in life and a myeloproliferative disorder in adulthood (Demehri et al., 2008, 2009; Dumortier et al., 2010).

Epidermal-derived thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine, is responsible for driving inflammation, B-cell expansion, and allergen sensitization in these animals (Demehri et al., 2009; Zhang et al., 2009; Han et al., 2012). Moreover, TSLP is both necessary and sufficient to drive asthma in another mouse model, and it is upregulated in several models displaying cutaneous barrier defects (Demehri et al., 2008; Comeau and Ziegler, 2010; Ziegler and Artis, 2010).

Germ-free (GF) mice are widely accepted as being an invaluable tool to characterize the impact of indigenous microbial communities on host physiology. For example, comparisons of GF mice and mice colonized with gut microbiota harvested from conventionally raised (CONV-R) mouse donors that have been subjected to deliberately manipulated host and environmental factors, or from human donors representing a variety of physiological or disease states, have provided important insights about the causal relationship between the microbiota and obesity (Turnbaugh et al., 2006; Turnbaugh et al., 2009; Liou et al., 2013), immune development (Ivanov et al., 2009), metabolic syndrome (Vijay-Kumar et al., 2010), cardiovascular disease (Wang et al., 2011), and severe acute malnutrition (Smith et al., 2013). To investigate the role that indigenous microbes have in modulating local as well as systemic inflammation, we generated GF Msx2-Cre/+; RBP-j^flx/flx and wild-type littermate controls. GF status did not affect allergic skin inflammation and asthma susceptibility in mutant or wild type mice contrary to the prediction of the hygiene hypothesis. However, GF mutant mice produced significantly higher levels of epidermal TSLP than their microbe-laden, CONV-R counterparts, leading to a persistent expansion of immature B cells. These results suggest a role for indigenous microbial
communities in controlling TSLP levels and helping mitigate some deleterious effects such as B-lymphoproliferative disorder (B-LPD). They also highlight the role of the microbiota in modulating cytokine production by keratinocytes.
RESULTS

Morphogenesis and maintenance of the epidermis and hair follicle in GF mice is unaltered

To assess the contribution of indigenous microbial communities to the phenotypes documented in our animals, we re-derived Msx2-Cre/+; RBPj\(^{\text{flox/flox}}\) and wild-type littermate control embryos into pseudo pregnant sterile dams using an embryo-transfer technique described in an earlier publication (Faith et al., 2010). The GF status of the resulting mice was confirmed by aerobic and anaerobic culture as well as by PCR assays targeting conserved regions of the bacterial 16S rDNA gene. Skin morphology and histology in wild-type GF and CONV-R animals were indistinguishable (Figure 8-1a and c). At postnatal day 9 (P9), Msx2-Cre/+; RBPj\(^{\text{flox/flox}}\) (RBPjCKO) mutant skin had a thickened epidermis, a highly cellular dermis and hair-follicle-derived epidermal cysts (Figure 8-1a). These changes became more pronounced at P30 (Figure 8-1c) and occurred independently of the presence or absence of the microbiota. Immunocytochemical staining for filaggrin, involucrin, and keratin-14 revealed that GF and CONV-R wild-type mice expressed these differentiation markers in the granular, spinous, and basal epidermis, respectively (Figure 8-1b and d). Conversely, RBPjCKO mice showed expansion of keratin-14 into suprabasal cells at P9 (Figure 8-1b). At P30, filaggrin and involucrin expression overlapped, an indication of abnormal differentiation (Figure 8-1d). Trans-epidermal water loss measurements detected a barrier defect of similar magnitude in P9 GF and CONV-R RBPjCKO mice (Supplementary Figure 8-S1 online). Collectively, these observations indicate that morphological and functional characteristics of the skin and the dysfunction caused by the absence of Notch signaling are not modulated by the microbiota. This allowed us to ask whether the microbiota has a role in skin inflammation and atopy in the RBPjCKO model.
**Skin inflammation and AD-like disease persist in GF RBPjCKO mice**

To better understand the role that indigenous microbial communities have in skin inflammation and the systemic diseases associated with cytokine production by the skin with a chronic barrier defect, we analyzed RBPjCKO mice at 10–13 weeks, the age at which the AD-like phenotype develops (Demehri *et al.*, 2009; Dumortier *et al.*, 2010). GF RBPjCKO mice were morphologically and histologically indistinguishable from their CONV-R RBPjCKO counterparts (Figure 8-2a), with all mice displaying the same macroscopic signature of AD, including excoriation and scaling. Histological analysis of hematoxylin and eosin–stained sections revealed similar levels of hyperkeratosis, parakeratosis, and acanthosis. Staining for CD45 (a pan hematopoietic cell marker) and toluidine blue staining revealed similar levels of skin inflammation and mast cell infiltration, respectively, in both GF and CONV-R animals (Figure 8-2a and b). Tumor resistance and loss of mutant keratinocytes are TSLP and T cell–dependent consequences of the severe inflammatory response that develops in RBPjCKO mice over a period of 75–200 days (Demehri *et al.*, 2012; Di Piazza *et al.*, 2012). The absence of a microbiota did not affect this inflammatory, Th2-mediated response.

Finally, systemic atopy, as measured by serum IgE levels, was also not significantly different between the GF and CONV-R RBPjCKO mice (Figure 8-2c); effector/memory CD4+ T cells (CD44hiCD62Llo) were elevated to similar levels in RBPjCKO mice in the spleen, skin-draining lymph nodes, and mesenteric lymph nodes (Figure 8-2d and Supplementary Figure 8-S2 online). RBPjCKO animals had higher percentages of FoxP3+ Treg cells among CD4+ T cells compared with their wild-type littermates in the mesenteric and skin-draining lymph nodes, as well as in the spleen (Figure 8-2e and Supplementary Figure 8-S2 online).
GF RBPjCKO mice have elevated TSLP levels but similar asthma susceptibility

TSLP produced by the perturbed epidermis is a significant driver of skin inflammation and many of the systemic effects in this mouse model (Demehri et al., 2008, 2009, 2012). Given the similar degree of disruption of epidermal differentiation and cutaneous barrier function (Figures 8-1 and 8-2, and Supplementary Figure 8-S1 online), we were surprised to find 2-fold higher epidermal Tslp mRNA levels in the GF RBPjCKO mice compared with CONV-R controls at P9 (P<0.05; n=6–10 mice/group; Figure 8-3a). Elevated Tslp mRNA levels in the skin were associated with elevated serum TSLP levels at P9 and P30 (Figure 8-3b).

To determine whether the microbiota ameliorated the severity of asthma in CONV-R mice, we subjected CONV-R and GF mice to ovalbumin (OVA) sensitization and intranasal challenge. Wild-type GF and CONV-R littermates displayed similar mild responses to OVA challenge (Figure 8-3c). Similarly, no significant differences in the severity of the asthmatic response were detected between GF and CONV-R-RBPjCKO animals, as judged by lung histology and total counts of leukocytes in lung washings obtained by bronchoalveolar lavage (Figure 8-3d, e). These data suggest that although the microbiota might well regulate skin and serum levels of TSLP in the absence of Notch signaling in the skin, the microbiota does not modulate AD and asthma in this model.

GF RBPjCKO mice have elevated white blood cell counts and persistent B-cell expansion

Epidermal-derived TSLP directly drives B-LPD in newborns. B-LPD severity mirrors TSLP levels early in life but is resolved after P30 despite persistently high TSLP levels (Demehri et al., 2008). Myeloproliferative disorder (MPD) replaces B-LPD, persisting into adulthood (Demehri et al., 2009; Dumortier et al., 2010). Surprisingly, white blood cell (WBC) counts did not normalize in young (P30-P45) GF RBPjCKO animals (Figure 8-4a). At P80,
WBC counts in GF RBPjCKO mice remained significantly higher than in CONV-R animals, splenomegaly was more severe (Figure 8-4a–c), B-cell numbers were elevated (CD45⁺B220⁺; Figure 8-4d), and T-cell numbers were unchanged (CD45⁺CD3⁺; Figure 8-4d) in the blood. As previously reported, MPD in RBPjCKO animals coincided with an increase in granulocyte colony-stimulating factor (G-CSF). Although G-CSF levels were significantly lower in GF RBPjCKO mice (Figure 8-4f), MPD (CD45⁺Ly6G⁺) persisted to the same extent as it did in CONV-R RBPjCKO animals (Figure 8-4d).

The hematocrit in both the GF and CONV-R RBPjCKO mice was significantly lower than in their wild-type littermates (Figure 8-4h). We hypothesized that epidermal-derived TSLP may drive a B-cell-mediated autoimmune hemolytic anemia (AIHA), which could cause low hematocrit (Iseki et al., 2012). To test whether AIHA occurs in our mice, we measured the levels of lactate dehydrogenase (LDH) and haptoglobin. In AIHA, release of LDH from lysed red blood cells elevates its level in serum, and because haptoglobin binds to hemoglobin released from lysed red blood cells, its level plummets (Tabbara, 1992). We detected no significant difference in LDH levels between wild-type and RBPjCKO (Figure 8-4i, Supplementary Figure 8-S3A online), and serum haptoglobin levels were significantly higher in both GF and CONV-R RBPjCKO compared with wild-type mice (Figure 8-4j, Supplementary Figure 8-S3B online). We concluded that it is unlikely that the anemia is due to AIHA. An alternative explanation for the low hematocrit is that an expanded B-cell and granulocytic compartment may cause a reactive contraction in other cell types without autoimmunity.

Analysis of P80 GF RBPjCKO mice identified both mature (B220⁺CD23⁺CD93⁻; Supplementary Figure 8-S2 online) and immature (IgD⁻IgM⁺) B cells (Figure 8-4e). High levels of TSLP in newborn GF RBPjCKO animals could affect neonatal B1 cell production dynamics.
or the activities of skin B1 cells in adults (Vosshenrich et al., 2004; Geherin et al., 2012). Accordingly, we detected a significantly elevated level of resting anti-phosphorylcholine IgM, most of which is made by B1 cells, in the sera of GF RBPjCKO mice (Figure 8-4g). This is not solely a result of losing microbial antigens, as GF wild-type mice have significantly lower levels of anti-phosphorylcholine IgM than their CONV-R counterparts (Figure 8-4g). Collectively, these data establish that the microbiota has effects on B cells, but not T cells, in mice with chronic cutaneous barrier defects.
DISCUSSION

The role of indigenous microbial communities in skin development and function is only beginning to be explored (Lai et al., 2009; Gallo and Nakatsuji, 2011; Grice and Segre, 2011; Naik et al., 2012). In this study, mice deficient in Notch signaling in their skin that develop severe, chronic skin inflammation and systemic atopy were re-derived as germ free. Morphologically and histologically, skin pathology was unaffected by the presence of a microbiota, and lack of a microbiota did not modulate skin inflammation in these animals. Surprisingly, despite these similarities, we observed an increase in the production of TSLP by the skin and the amount of TSLP in the serum of GF animals. These results indicate that the microbiota has a role in suppressing TSLP production in the barrier-defective skin.

TSLP is emerging as an essential cytokine in the development of allergic responses (Yoo et al., 2005; Zhou et al., 2005; Demehri et al., 2009; Zhang et al., 2009; Han et al., 2012; Jiang et al., 2012). Local TSLP expression in the skin is sufficient for the progression from AD to asthma (Han et al., 2012). Consistent with the finding that TSLP is upregulated in utero (Demehri et al., 2008), microbial products are not necessary to induce TSLP expression in barrier-defective skin but may instead limit its expression level or duration.

In contrast to the transient B-LPD that is apparent in neonatal CONV-R RBPjCKO mice and transitions to MPD in adulthood, both B-LPD and MPD persist to adulthood in GF animals (Demehri et al., 2008). Previously, we proposed that fetal B cells are responsive to TSLP but marrow-derived B cells are not (Demehri et al., 2008). The present study demonstrates that marrow-derived B cells continue to be responsive to TSLP in GF mice. It is possible that in GF mice, B-cell maturation is delayed or altered in the presence of TSLP, establishing a persistent
B-LPD and expansion of B1 cells. One mechanistic explanation may relate to the lower G-CSF levels that we document in GF RBPjCKO animals; G-CSF has been shown to suppress B cells in the bone marrow (Dumortier et al., 2010; Winkler et al., 2012) and could thus contribute to resolving B-LPD. These observations also indicate that the link between TSLP and G-CSF (Dumortier et al., 2010) does not require the presence of a microbiota.

Despite an increase in TSLP and WBC counts, we see no evidence for a more severe allergic disease in GF RBPjCKO animals. These findings do not support the hypothesis that complete lack of a microbiota will create a runaway Th2-skewed immune response in this model, but understanding the generality of this observation will depend on the results of similar experiments with other AD models. While our findings clearly demonstrate that the microbiota modulates expression of at least one proinflammatory cytokine in barrier-defective skin, a task ahead is to identify which microbial species or species consortia from which body habitat-associated communities mediate these observed effects and through which signaling pathways. Colonization of the GF mice we describe in this report with components of the skin and/or gut microbiota should provide a path ahead for addressing these questions.
EXPERIMENTAL PROCEDURES

Mice

All experiments involving mice were conducted using protocols approved by the Animal Studies Committee of Washington University. Msx2-Cre/+; RBPj^flox/flox (RBPjCKO) mice were generated as previously described (Pan et al., 2004). GF mice were derived by embryo extraction, sterilization, and transfer into a pseudo pregnant sterile dam (Faith et al., 2010) and maintained in flexible film gnotobiotic isolators under a strict 12-h light cycle on a standard plant polysaccharide–rich low-fat chow diet fed ad libitum (B&K Universal; England, UK). Maintenance of GF status in the colony was confirmed using culture-independent methods (i.e., negative PCR assays of fecal DNA using universal primers directed against bacterial 16S rRNA genes), or culture-based approaches (culturing feces in brain heart infusion broth, nutrient broth, or Sabouraud dextrose broth under anaerobic conditions). We analyzed age- and sex- matched GF wild-type littermates and their CONV-R siblings (i.e., mice derived from the same founders but reared in a mouse barrier facility in a specified pathogen-free state, and acquiring microbes from their environment beginning at birth).

OVA treatment

Airway challenge was performed on GF and CONV-R mice as outlined previously (Zhou et al., 2005). Six- to 8-week-old female mice were sensitized by intraperitoneal injection of 250 µl of a solution containing 50 µg OVA (Sigma A5503; St Louis, MO) dissolved in phosphate-buffered saline and 1.3 mg aluminum hydroxide gel (Sigma) on experimental days 1 (d1) and d14. Mice were challenged intranasally with 150 µg of OVA dissolved in 40 µl of
phosphate-buffered saline on d21, d22, and d23. Controls were given the same regimen without the addition of OVA antigen. Mice were killed on d24.

**Histology, immunohistochemistry, and immunofluorescence**

After killing, lungs were inflated with 4% paraformaldehyde at 25 cm water pressure. Dorsal skin and lung tissues were fixed in 4% paraformaldehyde overnight, dehydrated in ethanol and embedded in paraffin. Five-µm thick sections were used for all analyses. To assess inflammation and skin morphology, sections were stained with hematoxylin and eosin or toluidine blue. Mast cell infiltration in the skin was quantified by counting the average number of toluidine blue positive cells in three randomly selected microscopic fields at 200x magnification. Paraffin embedded sections were rehydrated and stained with biotinylated anti-keratin 14 (clone LL002, Neomarkers), chick anti-filaggrin, and rabbit anti-involucrin, and antigen-antibody complexes were visualized with Cy5-conjugated streptavidin, Cy3-conjugated anti-rabbit Ig, and Alexa488-conjugated anti-chicken Ig (all from Jackson ImmunoResearch Laboratory). CD45 staining was performed as previously described (Demehri *et al.*, 2012).

**Bronchoalveolar and WBC analyses**

Bronchoalveolar fluid was collected by infusing 1 ml of phosphate-buffered saline into the lung at killing using a Surflo catheter (Terumo Medical; Somerset, NJ) (Demehri *et al.*, 2009). Bronchoalveolar fluid was spun down, the cell pellet was stained with Giemsa, and at least 100 cells were counted to determine the differential leukocyte cell counts. Blood was diluted 1:1 with 10 mM EDTA in phosphate-buffered saline, and total white blood cell count and hematocrit were measured using a Hemavet 950 analyzer (Drew Scientific; Waterbury, CT).
Serology

Serum IgE was determined using Mouse IgE ELISA (Immunology Consultants Laboratory, Portland, OR). Serum TSLP and G-CSF levels were measured using Mouse TSLP ELISA (Biolegend; San Diego, CA) and Mouse G-CSF ELISA (R&D Systems; Minneapolis, MN), respectively. Serum anti-phosphorylcholine IgM was measured by coating 96-well flat-bottom EIA/RIA plates (Costar; Tewksbury, MA) overnight at 4 °C with 2 µg ml⁻¹ of PC-BSA and performing ELISA assays (Foote and Kearney, 2009). To test for hemolytic anemia, serum LDH was determined using LDH cytotoxicity assay (Cayman Chemical Company; Ann Arbor, MI), and serum haptoglobin was measured using Mouse Haptoglobin Elisa Test Kit (Life Diagnostics; West Chester, PA).

Flow cytometry

This method was applied to single-cell suspensions prepared from spleen, skin-draining lymph nodes, or mesenteric lymph nodes. Cells were surface stained with the reagents listed in Supplementary Materials and Methods, fixed overnight at 4 °C using FoxP3 fixation/permabilization buffer (eBioscience; San Diego, CA), and stained intracellularly using FoxP3 Permeabilization buffer as per the manufacturer’s instructions. Samples were acquired on a BD FACS can or BD LSR II flow cytometer. Data were analyzed using FlowJo software (TreeStar; Ashland, OR).

PCR and quantitative real-time reverse-transcriptase–PCR

To genotype mice, PCR was performed on DNA extracted from toes (Demehri et al., 2009). For gene expression analysis, mice were euthanized on P9, the skin was removed and placed on dry ice. The epidermis was scraped off using a scalpel, homogenized, and RNA was
isolated using RNeasy Mini Kit (Qiagen; Germantown, MD). cDNA was generated using Superscript RT-II kit (Invitrogen; Grand Island, NY). Quantitative real-time reverse-transcriptase–PCR was performed for mouse Tslp and Hprt mRNA (control) as previously described (Lee et al., 2007; Demehri et al., 2008).

**Statistical analysis**

An unpaired Student’s $t$-test was used to test statistical significance; $P<0.05$ was considered significant. Mean values±SEM are presented in all graphs.
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REFERENCES


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Jiang H, Hener P, Li J et al. (2012) Skin thymic stromal lymphopoietin promotes airway sensitization to inhalant house dust mites leading to allergic asthma in mice. Allergy 67:1078–82


Lee J, Basak JM, Demehri S et al. (2007) Bi-compartmental communication contributes to the opposite proliferative behavior of Notch1-deficient hair follicle and epidermal keratinocytes. Development 134:2795–806


Macia L, Thorburn A, Binge L et al. (2012) Microbial influences on epithelial integrity and immune function as a basis for inflammatory diseases. Immunological Reviews 245:164–240


Pan Y, Lin M, Tian X et al. (2004) Gamma-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. Dev Cell 7:731–43


Vijay-Kumar M, Aitken JD, Carvalho FA et al. (2010) **Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5.** Science 328:228–31

Vosshenrich CA, Cumano A, Muller W et al. (2004) **Pre-B cell receptor expression is necessary for thymic stromal lymphopoietin responsiveness in the bone marrow but not in the liver environment.** Proc Natl Acad Sci USA 101:11070–5


Winkler IG, Bendall LJ, Forristal CE et al. (2012) **B-lymphopoiesis is stopped by mobilizing doses of G-CSF and is rescued by overexpression of the antiapoptotic protein Bcl2.** Haematologica 98:325–33


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**A**

- **P9**
  - H&E
  - K14 INV FLG

**B**

**C**

**D**

**P30**
Figure 8-1: Epidermal and hair follicle structure are unaffected in germ-free (GF) mice compared with their conventionally raised (CONV-R) counterparts.

Hematoxylin and eosin (H&E) staining at (a) P9 and (c) P30 shows skin morphology and inflammation levels in GF and CONV-R RBPjCKO and wild-type mice. Bar, 50 µm. Immunocytochemical staining for keratin-14 (K14), involucrin (INV), and filaggrin (FLG) at (b) P9 and (d) P30 shows skin architecture in GF and CONV-R wild-type and RBPjCKO animals. Both GF and CONV-R RBPjCKO animals exhibit similar barrier defects. Bar, 10 µm.
Figure 8-2: Skin inflammation and atopic dermatitis (AD)–like disease persists in germ-free (GF) RBPjCKO animals. (a) Conventionally raised (CONV-R) and GF P80 mice. Scale bar, 1 cm. Hematoxylin and eosin (H&E), CD45 immunohistochemistry, and toluidine blue staining reveal skin inflammation and mast cell infiltration in both CONV-R and GF wild-type (Wt) and RBPjCKO (Mut) mice. Bar, 50 µm. (b) Quantification of mast cells viewed in three randomly selected microscopic fields at × 200 (CONV-R Wt, n=4; CONV-R Mut, n=15; GF Wt, n=4; GF Mut, n=15). (c) Serum IgE levels in P80 Wt mice (CONV-R, n=10; GF, n=6) and RBPjCKO animals (CONV-R, n=23; GF, n=23). (d) Flow cytometry gated on CD4+ T cells in the spleen to detect effector (CD44hi CD62Llo) and naive (CD44lo CD62Lhi) cells in RBPjCKO (CONV-R, n=9; GF, n=9) and Wt mice (CONV-R, n=4; GF, n=4). A compilation of results from four independent experiments is shown. (e) Flow cytometry gated on CD4+ T cells detects regulatory T cells (Treg, Foxp3+) in RPBjCKO (GF and CONV-R, n=6) and Wt mice (CONV-R and GF, n=3). A compilation of three independent experiments is shown. Asterisks mark statistical significance; *P<0.05, **P<0.01, ***P<0.001 (unpaired Student’s t-test).
Figure 8-3: Asthma in germ-free (GF) RBPjCKO mice is not more severe but thymic stromal lymphopoietin (TSLP) levels are higher than in their conventionally raised (CONV-R) counterparts. (a) Tslp mRNA levels in P9 CONV-R wild-type (Wt; n=6), CONV-R RBPjCKO (n=7), GF Wt (n=10), and GF RBPjCKO (n=10) mice. (b) Serum TSLP levels in P9 RBPjCKO (CONV-R, n=7; GF, n=10), P30 (CONV-R, n=7; GF, n=14), and P80 (CONV-R, n=19; GF, n=24). (c) Hematoxylin and eosin staining of the lungs after ovalbumin (OVA) challenge at 6–8 weeks shows increased inflammatory infiltrate (arrows) in the RBPjCKO mice. Bar, 50 µm. (d) White blood cell count in bronchial alveolar lavage fluid (BAL) of Wt (CONV-R, n=6; GF, n=10) and RBPjCKO (CONV-R, n=8; GF, n=8) mice. (e) The percentage of eosinophils, macrophages, lymphocytes, or neutrophils in BAL from RBPjCKO (CONV-R, n=11; GF, n=9) or Wt (CONV-R, n=3; GF, n=4) mice. The differences between RBPjCKO and wild-type animals remain evident in a GF state. *P<0.05, **P<0.01, ***P<0.001 (unpaired Student’s t-test). PBS, phosphate-buffered saline.
Figure 8-4: B-cell expansion persists in germ-free (GF) RBPjCKO animals. (a) White blood cell (WBC) counts in P30 RBPjCKO mice (conventionally raised (CONV-R), n=7; GF, n=14) and P80 (CONV-R, n=33; GF, n=43). (b–c) Spleen size mirrored WBC levels, reported as a percentage of body weight (wild-type (Wt), CONV-R n=13; GF, n=19; Mut CONV-R, n=34; GF, n=43). Bar, 1 cm. (d) B-cell (B220+ CD45+), myeloid (Ly6G+), and T cell (CD3+) expansion in 10- to 12-week-old mice, quantified by flow cytometry (Wt CONV-R, n=5; GF, n=4; RBPjCKO CONV-R, n=13, GF, n=12). (e) The percentage of immature B220+ cells (IgM+ IgDlo) in GF RBPjCKO animals. Data compiled from five independent experiments (Wt CONV-R, n=5, GF, n=4; RBPjCKO CONV-R and GF, n=11). (f) Granulocyte colony-stimulating factor (G-CSF) levels in serum from GF and CONV-R mice (Wt CONV-R, n=5, GF, n=10; RBPjCKO CONV-R, n=11; GF n=14). (g) Anti-phosphorylcholine (anti-PC) IgM levels (Wt CONV-R, n=6; GF, n=10; RBPjCKO CONV-R, n=9; GF n=12). (h) RBPjCKO mice show lower hematocrit than Wt counterparts (Wt CONV-R, n=13; GF, n=17; RBPjCKO CONV-R, n=34; GF n=43). (i) Serum lactate dehydrogenase (LDH) levels (Wt CONV-R and GF, n=6; RBPjCKO CONV-R and GF, n=12). (j) Serum haptoglobin levels (Wt CONV-R and GF, n=6; RBPjCKO CONV-R and GF, n=12). *P<0.05, **P<0.01, ***P<0.001 (unpaired Student’s t-test).