

Short Communication

Notch pathway signaling in the skin antagonizes Merkel cell development

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ABSTRACT

Merkel cells are mechanosensitive skin cells derived from the epidermal lineage whose development requires expression of the basic helix-loop-helix transcription factor *Atoh1*. The genes and pathways involved in regulating Merkel cell development during embryogenesis are poorly understood. Notch pathway signaling antagonizes *Atoh1* expression in many developing body regions, so we hypothesized that Notch signaling might inhibit Merkel cell development. We found that conditional, constitutive overexpression of the Notch intracellular domain (NICD) in mouse epidermis significantly decreased Merkel cell numbers in whisker follicles and touch domes of hairy skin. Conversely, conditional deletion of the obligate NICD binding partner RBPj in the epidermis significantly increased Merkel cell numbers in whisker follicles, led to the development of ectopic Merkel cells outside of touch domes in hairy skin epidermis, and altered the distribution of Merkel cells in touch domes. Deletion of the downstream Notch effector gene *Hes1* also significantly increased Merkel cell numbers in whisker follicles. Together, these data demonstrate that Notch signaling regulates Merkel cell production and patterning.

1. Introduction

Merkel cells are mechanosensitive skin cells derived from the epidermal lineage (Morrison et al., 2009) that require the basic helix-loop-helix transcription factor *Atoh1* for their development (Maricich et al., 2009; Ostrowski et al., 2015; Wright et al., 2015). Genes and signaling pathways that regulate Merkel cell specification are poorly understood.

Two pathways have recently been shown to regulate the development of Merkel cells around first-wave hair follicles. First, a cascade of Wnt, Eda, and SHH signaling drives development of first-wave hair follicles and initiates specification of surrounding Merkel cells (Xiao et al., 2016). Second, the polycomb repressor complex 2 (PRC2) inhibits development of Merkel cells around second-wave hair follicles (Bardot et al., 2013; Dauber et al., 2016; Perdigoto et al., 2016).

Notch signaling is vital for embryonic development and maintenance of many tissues including the skin (Massi and Panelos, 2012). In the epidermis, Notch signaling acts as a fate switch to induce differentiation of basal epidermal stem cells into mature keratinocytes (Blanpain et al., 2006). Canonical Notch signaling is initiated when a membrane-bound Notch ligand binds a Notch

receptor on an adjacent cell, triggering cleavage of the Notch intracellular domain (NICD). NICD translocates into the nucleus, where it pairs with Rbpj (Recombining binding protein suppressor of hairless) to promote transcription of downstream targets. In the epidermis, Notch promotes expression of *Hes1* (hairy and enhancer of split-1). Interestingly, both Notch signaling and *Hes1* expression directly inhibit *Atoh1* transcription in the inner ear, gut, and cerebellum (Kelley, 2006; Gerbe et al., 2011; Kim and Shivdasani, 2011; Zheng et al., 2011; Chonko et al., 2013).

We previously demonstrated that loss of Notch signaling in adult skin causes increased Merkel cell production (Ostrowski et al., 2015). Here, we investigated the role of Notch signaling on Merkel cell formation using transgenic mice that permit manipulation of Notch signaling components. We found that mice overexpressing NICD developed fewer Merkel cells in touch domes and whisker follicles. Mice that lack *RBPj* produced more whisker follicle Merkel cells and developed ectopic Merkel cells in the hairy skin. Further, we found that *Hes1*^{-/-} mice had more Merkel cells in whisker follicles. Together, these data demonstrate that Notch signaling antagonizes Merkel cell specification in the developing epidermis.

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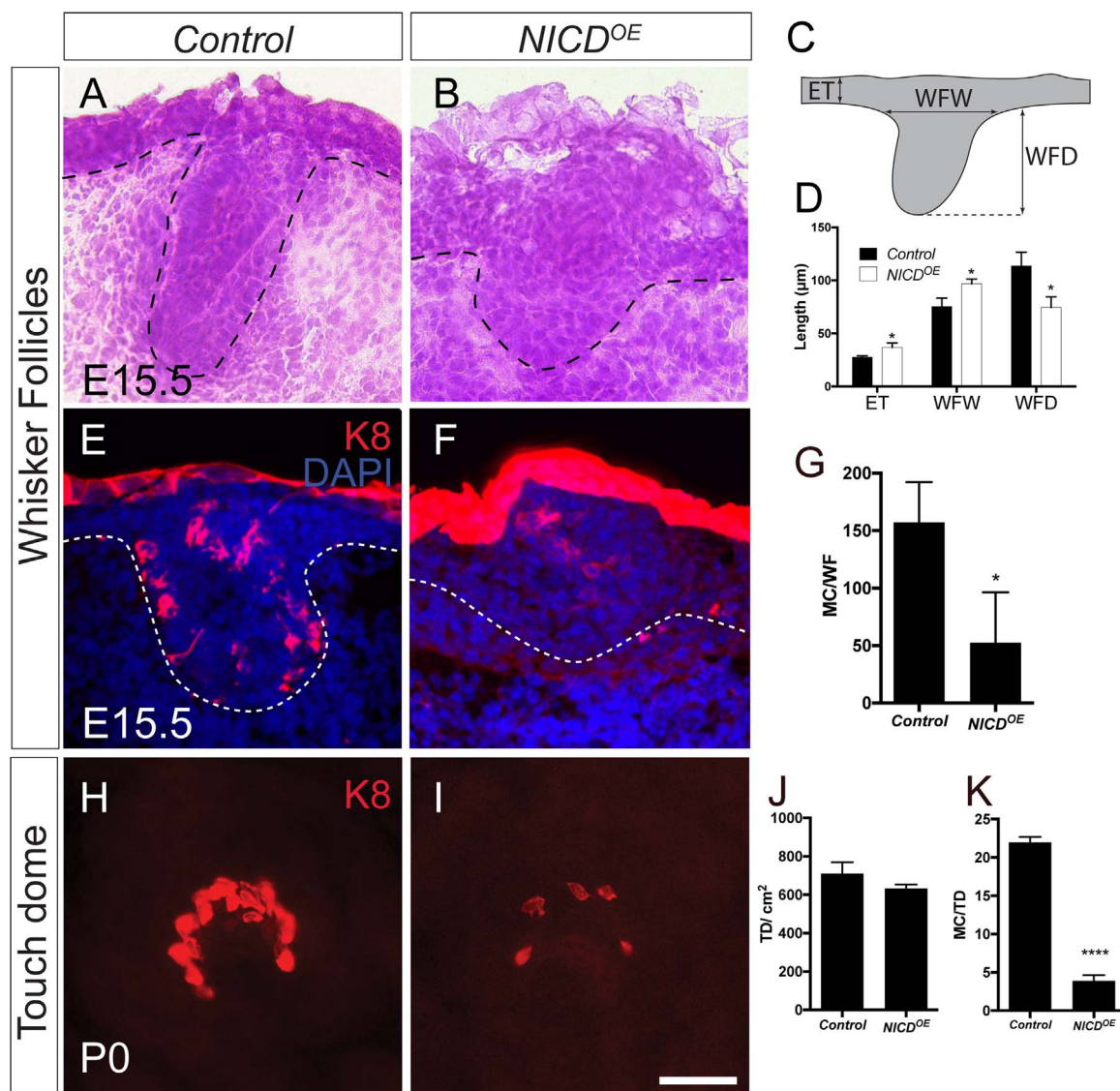


Fig. 1. Epidermal *NICD* overexpression decreases Merkel cell numbers in developing whisker follicles and touch domes. (A,B) H & E stained whisker follicle sections from E15.5 control (A) and *K14^{Cre}; ROSA^{NICD}* (B, hereafter referred to as *NICD^{OE}*) mice. Dashed line indicates the epidermal-dermal border. (C) Diagram of three whisker follicle morphology parameters: 1) epidermal thickness (ET), 2) whisker follicle width (WFW), and 3) whisker follicle depth (WFD). (D) Quantification of E15.5 whisker follicle morphology. *NICD^{OE}* mice have an increased ET ($p = 0.044$, t -test) and WFW ($p = 0.039$, t -test), and a decreased WFD ($p = 0.038$, t -test). (E,F) K8 immunostaining of whisker follicle sections from E15.5 *NICD^{OE}* mice. (G) Average number of K8+ Merkel cells per whisker follicle ($p = 0.037$, paired t -test). (H,I) K8 wholemount immunostaining of touch domes from P0 control and *NICD^{OE}* mice. (J) Average touch domes density (touch domes per cm^2). (K) Average number of K8+ Merkel cells per touch dome. K8 expression in K8+ cells was qualitatively lower in whisker follicles and touch domes of *NICD^{OE}* mice compared to controls. Error bars are \pm SEM. Scale bar = 50 μm . * $p < 0.05$, **** $p < 0.0001$.

2. Results

2.1. Epidermal *NICD* overexpression decreases Merkel cell number

To test the effects of Notch signaling on Merkel cell development, we generated *K14^{Cre}; ROSA^{NICD}* (hereafter referred to as *NICD^{OE}*) mice that overexpress the Notch Intracellular Domain (NICD) in epidermal cells of the developing mouse (Blanpain et al., 2006). We first analyzed the whisker follicles of embryonic day (E)15.5 mice, when rapid Merkel cell production occurs (Morrison et al., 2009; Wright et al., 2015). Though epidermal *NICD* overexpression has been shown to alter epidermal morphology in body skin (Blanpain et al., 2006), its effect on whisker follicle morphology has not been described. To determine if *NICD* overexpression altered whisker follicle structure we measured three morphological parameters of H & E stained whisker follicle sections: 1) epidermal thickness (ET), 2) whisker follicle width (WFW), and 3) whisker follicle depth (WFD) (Fig. 1A–D). We found that ET in E15.5 *NICD^{OE}* mice was greater than that in control mice

($36.8 \pm 4.2 \mu\text{m}$ vs. $27.2 \pm 1.0 \mu\text{m}$, $p = 0.044$, t -test). WFW was larger ($96.8 \pm 4.5 \mu\text{m}$ vs. $75.5 \pm 7.9 \mu\text{m}$, $p = 0.039$, t -test) and WFD was decreased ($74.5 \pm 10.1 \mu\text{m}$ vs. $113.6 \pm 12.9 \mu\text{m}$, $p = 0.038$, t -test) in *NICD^{OE}* mice.

To determine whether *NICD* overexpression altered Merkel cell numbers, we immunostained E15.5 whisker follicle sections for the Merkel cell marker Keratin 8 (K8) (Fig. 1E–F), then reconstructed whisker follicles from serial sections and counted total numbers of Merkel cells per whisker follicle (Fig. 1G). Whisker follicles from *NICD^{OE}* mice had fewer K8+ cells per follicle than whisker follicles of control mice (52.4 ± 44 vs. 157.4 ± 35.2 , $p = 0.037$, paired t -test). To determine whether *NICD* overexpression similarly altered Merkel cell numbers in body skin, we wholemount immunostained the body skin of P0 *NICD^{OE}* mice and control siblings for K8. While touch dome density in *NICD^{OE}* mice was not significantly different from control mice ($310 \pm 38.7 \text{ TD}/\text{cm}^2$ vs. $391 \pm 15.9 \text{ TD}/\text{cm}^2$, $p = 0.062$, t -test, Fig. 1J), we found that *NICD^{OE}* mice had significantly fewer K8+ cells per touch dome than their control littermates (3.8 ± 0.7 vs. 22.0 ± 0.7 , $p < 0.0001$,

t-test, Fig. 1H–I,K). These results demonstrate that Notch signaling antagonizes Merkel cell development.

K8 is a relatively early marker of Merkel cells (Wright et al., 2015), but its expression is preceded by both *Atoh1* and *Sox2* (Maricich et al., 2009; Lesko et al., 2013). Therefore, failed Merkel cell differentiation following specification might explain the decreased Merkel cell numbers seen in *NICD^{OE}* mice. We therefore immunostained whisker follicles of *NICD^{OE}* and control mice for Sox2. In 5 whisker follicle sections from *n* = 2 E15.5 *NICD^{OE}* mice, we detected a total of 2 K8+, 3 Sox2+, and 0 K8+/Sox2+ Merkel cells. In 5 whisker follicle sections from *n* = 2 control mice, we detected a total of 13 K8+, 15 Sox2+, and 192 K8+/Sox2+ Merkel cells. We also immunostained body skin from P0 *NICD^{OE}* mice and found no Sox2+ touch dome cells, while control mice had 15.2 ± 0.32 Sox2+ cells per touch dome (*N* = 3 mice/genotype). The virtual absence of Sox2+ cells in the whisker follicles and hairy skin of *NICD^{OE}* mice suggests that NICD overexpression inhibits the earliest stages of Merkel cell development.

2.2. Conditional *RBPj* deletion in the epidermis increases Merkel cell numbers

Data from the *NICD^{OE}* mice suggested that Notch signaling directly inhibits Merkel cell development. However, disruptions in epidermal morphology (Fig. 1A,B) secondary to NICD overexpression could also secondarily disrupt Merkel cell development. To generate further support for our interpretation of the data, we next examined the effects of *RBPj* deletion on Merkel cell development.

We wondered whether disruption in Notch signaling would lead to opposite effects on Merkel cell numbers. We therefore conditionally deleted *RBPj*, an obligate binding partner of NICD, by generating *K14^{Cre}; RBPj^{flox/flox}* mice as previously described (Blanpain et al., 2006). *K14^{Cre}; RBPj^{flox/flox}* (hereafter referred to as *RBPj^{CKO}*) mice have decreased canonical Notch activity, shown by decreased expression of epidermal *Hes1*, a downstream target of canonical Notch signaling (Blanpain et al., 2006). We immunostained whisker follicle sections from E15.5 mice for K8 and reconstructed whisker follicles to determine total Merkel cell numbers per follicle (Fig. 2A–C). We found that whisker follicles of E15.5 *RBPj^{CKO}* mice had significantly more Merkel cells per whisker follicle than control mice (156.3 ± 24.75 vs. 127.3 ± 24.44 , *p* = 0.0026, *t*-test). In contrast to *NICD^{OE}* mice, E15.5 *RBPj^{CKO}* mice did not demonstrate changes in whisker follicle morphology (ET *p* = 0.288, WFW *p* = 0.673, WFD *p* = 0.725, Fig. 2C–D). These data demonstrate that disruption of canonical Notch signaling increases Merkel cell numbers in whisker follicles in the absence of disruptions in epidermal morphology.

In contrast, wholemount K8 immunostaining of back skin from P0 *RBPj^{CKO}; K14^{Cre}; RBPj^{flox/+}* mice (hereafter referred to as *RBPj^{HET}*) and *K14^{Cre}; RBPj^{+/+}* (hereafter referred to as control) mice demonstrated equivalent touch dome densities and Merkel cell numbers/touch dome in all three genotypes (Table 1, Fig. 2E–G, I, J). However, P0 *RBPj^{CKO}* body skin had significantly more Sox2+ cells in touch domes than littermate controls (17.9 ± 0.59 vs. 15.2 ± 0.32 , *p* = 0.0148, *t*-test, Fig. 2O–Q). This demonstrates that disruption of Notch pathway signaling increases the number of immature Merkel cells in touch domes, but does not change the number of mature Merkel cells.

The Notch pathway can also signal through non-canonical mechanisms, where cytosolic NICD antagonizes the Wnt/ β -catenin pathway by titrating active β -catenin (Andersen et al., 2012). Given that Wnt/ β -catenin promotes Merkel cell specification (Xiao et al., 2016), NICD overexpression decreases hairy skin Merkel cell numbers, and *RBPj* deletion does not affect K8+ Merkel cell numbers, we wondered whether non-canonical Notch signaling might explain the observed overexpression phenotype. We therefore generated *K14^{Cre}; RBPj^{flox/flox}; ROSA^{NICD} (RBPj^{CKO}; NICD^{OE})* mice to determine if the effects of overexpressing NICD depend on *Rbpj* (Fig. 2H). All values measured from wholemount immunostaining are summarized in Table 1. P0

RBPj^{CKO}; NICD^{OE}; RBPj^{CKO}; RBPj^{HET} and control mice had equivalent touch dome densities and numbers of Merkel cells/touch dome (Fig. 2H–J). These data suggest that Notch effects on Merkel cell production operate through canonical Notch signaling pathways.

Although numbers of K8 positive Merkel cells/touch dome were normal in P0 *RBPj^{CKO}; RBPj^{HET}* and *RBPj^{CKO}; NICD^{OE}* mice, we noticed two key differences between them and control mice. Increased numbers of ectopic, interfollicular K8+ cells were present outside of touch domes in hairy skin of all three genotypes (Table 1, Fig. 2E–H, K), similar to what was previously described in adult *K14^{CreER}; RBPj^{flox/flox}* mice (Ostrowski et al., 2015). The largest numbers of ectopic Merkel cells were seen in *RBPj^{CKO}* mice, with intermediate numbers present in *RBPj^{HET}* and *RBPj^{CKO}; NICD^{OE}* mice. The majority of ectopic K8+ cells ($67.1 \pm 2.4\%$) possessed dendritic projections similar to those seen in Merkel cells found in touch domes (Fig. 2E–H). Using DAPI to visualize hair follicles in K8-immunostained wholemount tissue, we found that $84.0 \pm 1.0\%$ of K8 positive ectopic cells were associated with hair follicles, where they were found in locations deep to the skin surface (Fig. 2M,M'). This finding recapitulates that seen following *RBPj* deletion in adult skin (Ostrowski et al., 2015). Also echoing that study, neither ectopic K8+ cells deep in hair follicles nor those found in interfollicular hairy skin were associated with cutaneous nerve branches at the resolution of wholemount NF200 immunostaining (Fig. 2N, N'). We also observed more ectopic Sox2 positive cells in the epidermis of *RBPj^{CKO}* mice (1695 ± 379.3 vs 231.8 ± 56.0 , *p* = 0.009, *t*-test, Fig. 2O,P,R). These results demonstrate that *RBPj*-dependent Notch signaling inhibits epidermal Merkel cell production outside of hairy skin touch domes.

The second key difference that we observed was that the patterning of Merkel cells in touch domes was disrupted in P0 *RBPj^{CKO}* and *RBPj^{CKO}; NICD^{OE}* mice. Specifically, we found that touch dome Merkel cells in control and *RBPj^{HET}* mice were restricted to the characteristic crescent occupying $281 \pm 4.3^\circ$ and $273 \pm 6.1^\circ$, respectively, around the guard hair follicles (Fig. 2E, F, L). In contrast, Merkel cells of *RBPj^{CKO}* and *RBPj^{CKO}; NICD^{OE}* mice occupied $331 \pm 6.2^\circ$ and $328 \pm 5.7^\circ$, respectively, around guard hairs (Table 1, Fig. 2G, H, L). These data demonstrate that Notch signaling plays a role in restricting Merkel cell position within the touch dome structure.

2.3. The Notch downstream target *Hes1* inhibits Merkel cell specification

In the epidermis, Notch signaling promotes expression of *Hes1*, a transcription factor that inhibits *Atoh1* expression in the inner ear, cerebellum, and secretory cells of the gut (Kelley, 2006; Gerbe et al., 2011; Kim and Shivasani, 2011; Zheng et al., 2011; Chonko et al., 2013). Since *Atoh1* is required for Merkel cell production (Maricich et al., 2009), we hypothesized that Notch-induced *Hes1* expression might antagonize *Atoh1* expression in the epidermis and subsequently inhibit Merkel cell production.

Hes1 expression is increased in the epidermis of hairy skin of *NICD^{OE}* mice and decreased in the hairy skin of *RBPj^{CKO}* mice (Blanpain et al., 2006); however *Hes1* expression has not been described in whisker follicles. Whisker follicles of control *RBPj^{CKO}*, and *NICD^{OE}* littermates were immunostained for *Hes1* (Fig. 3A–C'). We observed qualitatively stronger nuclear *Hes1* staining throughout the whisker follicles of *NICD^{OE}* mice and qualitatively weaker nuclear *Hes1* staining in whisker follicles of *RBPj^{CKO}* mice, both relative to control littermates. These data demonstrate that *Hes1* expression is driven by Notch signaling in a similar fashion in whisker follicles and hairy skin.

To determine whether epidermal *Hes1* expression regulated Merkel cell production, we counted numbers of K8+ cells in whisker follicles of E15.5 *Hes1^{-/-}* mice and control littermates (Fig. 3D–F). *Hes1^{-/-}* mice had significantly more Merkel cells per whisker follicle than control mice (238.5 ± 18.46 vs. 175.5 ± 17.47 , *p* = 0.0234, *t*-test). We also observed the presence of ectopic Merkel cells residing outside of

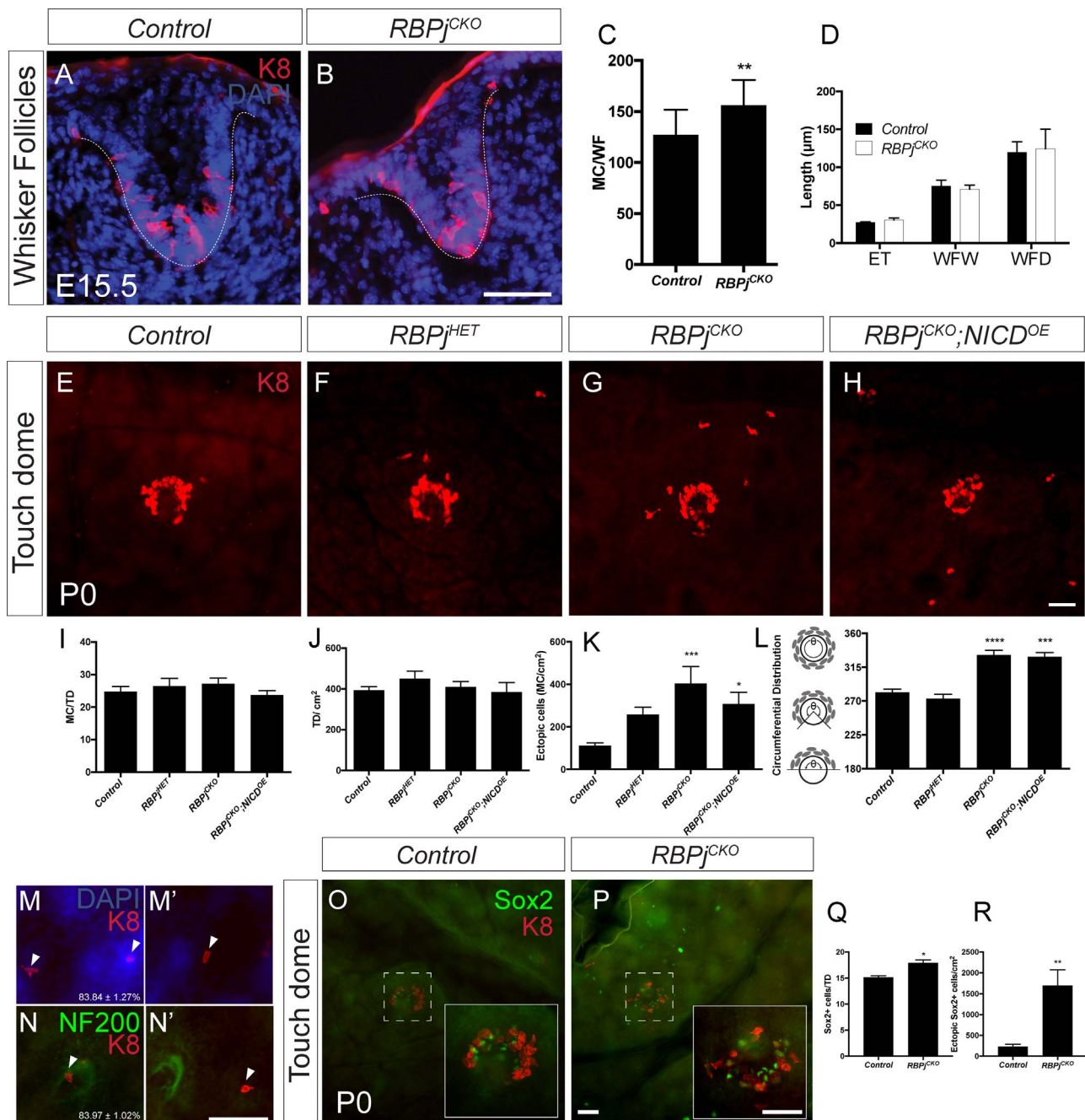


Fig. 2. Disruption of Notch signaling increases Merkel cell numbers in whisker follicles and body skin. (A,B) K8 immunostaining in whisker follicle sections of control and $K14^{cre}; RBPj^{flax/flax}$ mice (hereafter referred to as $RBPj^{CKO}$ mice). Dashed line labels epidermal-dermal border. (C) Average numbers of K8+ Merkel cells in reconstructed whisker follicles. $RBPj^{CKO}$ mice have more Merkel cells per whisker follicle than control mice ($p = 0.0026$, paired t -test). (D) No differences in quantitative measures of whisker follicle morphology were seen between $RBPj^{CKO}$ and control mice (ET $p = 0.288$, WFW $p = 0.673$, WFD $p = 0.725$). (E–H) K8 immunostaining of wholemount back skin of control, $K14^{cre}; RBPj^{flax/+}$ (hereafter referred to as $RBPj^{HET}$), $RBPj^{CKO}$, and $RBPj^{CKO};NICD^{OE}$ mice. (I) Average numbers of Merkel cells per touch dome ($p = 0.589$, one-way ANOVA). (J) Touch dome density ($p = 0.659$, one-way ANOVA). (K) Density of ectopic Merkel cells. $RBPj^{CKO}$ and $RBPj^{CKO};NICD^{OE}$ mice have significantly more ectopic Merkel cells per cm² than control mice (ANOVA $p = 0.0005$, control vs. $RBPj^{CKO}$ $p = 0.0002$, control vs. $RBPj^{CKO};NICD^{OE}$ $p = 0.013$, Dunnett's post-hoc comparison). (L) Average circumferential distribution of Merkel cells in touch domes. Touch domes from control and $RBPj^{HET}$ mice surround $281.3^{\circ} \pm 4.3$ of the hair follicle. Touch domes from $RBPj^{CKO}$ and $RBPj^{CKO};NICD^{OE}$ mice have a significantly larger central angle than control mice (ANOVA $p < 0.0001$, $RBPj^{CKO}$ vs. control $p < 0.0001$, $RBPj^{CKO};NICD^{OE}$ vs. control $p = 0.0001$). (M,M') High magnification images of K8+ ectopic Merkel cells (white arrows) associated with hair follicles (M) or interfollicular skin (M'). (N,N') High magnification images of K8+ ectopic Merkel cells (white arrows) associated with hair follicles (N) or interfollicular skin (N') are not innervated by NF200+ cutaneous afferents. (O,P) Wholemount Sox2 immunostaining of P0 control and $RBPj^{CKO}$ mice with high magnification image of touch dome in inset. (Q) Average number of Sox2 positive cells per touch dome. (R) Ectopic Sox2+ cell density in hairy skin. Error bars are \pm SEM. Scale bars = 50 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

whisker follicles in $Hes1^{-/-}$ mice. These results suggest that *Hes1* is the downstream Notch effector in the epidermis that regulates Merkel cell production during embryogenesis. Unfortunately, very few $Hes1^{-/-}$ mice survive past E15.5 (Ishibashi et al., 1995), so we were unable to measure Merkel cell numbers in body skin of these mice.

3. Discussion

Our results identify Notch signaling as an important pathway that regulates Merkel cell production during embryogenesis. Fewer Merkel cells are produced in mice that overexpress NICD, while more Merkel

Table 1
Summary of data from Fig. 2I–L.

| | MC/TD | | TD/cm ² | | Ectopic cells/cm ² | | Circumferential Distribution | |
|---|---------------|----------------------|--------------------|----------------------|-------------------------------|----------------------|------------------------------|----------------------|
| | Average ± SEM | p-value ^a | Average ± SEM | p-value ^a | Average ± SEM | p-value ^a | Average ± SEM | p-value ^a |
| Control | 24.8 ± 1.5 | | 394 ± 17 | | 112 ± 12 | | 281 ± 4.3° | |
| <i>RBPj^{HET}</i> | 26.5 ± 2.4 | 0.8404 | 450 ± 37 | 0.3313 | 258 ± 35 | 0.0503 | 273 ± 6.1° | 0.5674 |
| <i>RBPj^{CKO}</i> | 27.2 ± 1.8 | 0.6202 | 410 ± 26 | 0.9488 | 404 ± 80 | 0.0002*** | 331 ± 6.2° | < 0.0001**** |
| <i>RBPj^{CKO};NICD^{OE}</i> | 23.7 ± 1.4 | 0.9561 | 384 ± 48 | 0.9911 | 308 ± 55 | 0.0133* | 328 ± 5.7° | < 0.0001**** |
| ANOVA | | 0.5888 | | 0.6593 | | 0.0005*** | | < 0.0001**** |

n = 4 mice/genotype.
* p < 0.05.
*** p < 0.001.
**** p < 0.0001.
^a Compared to control.

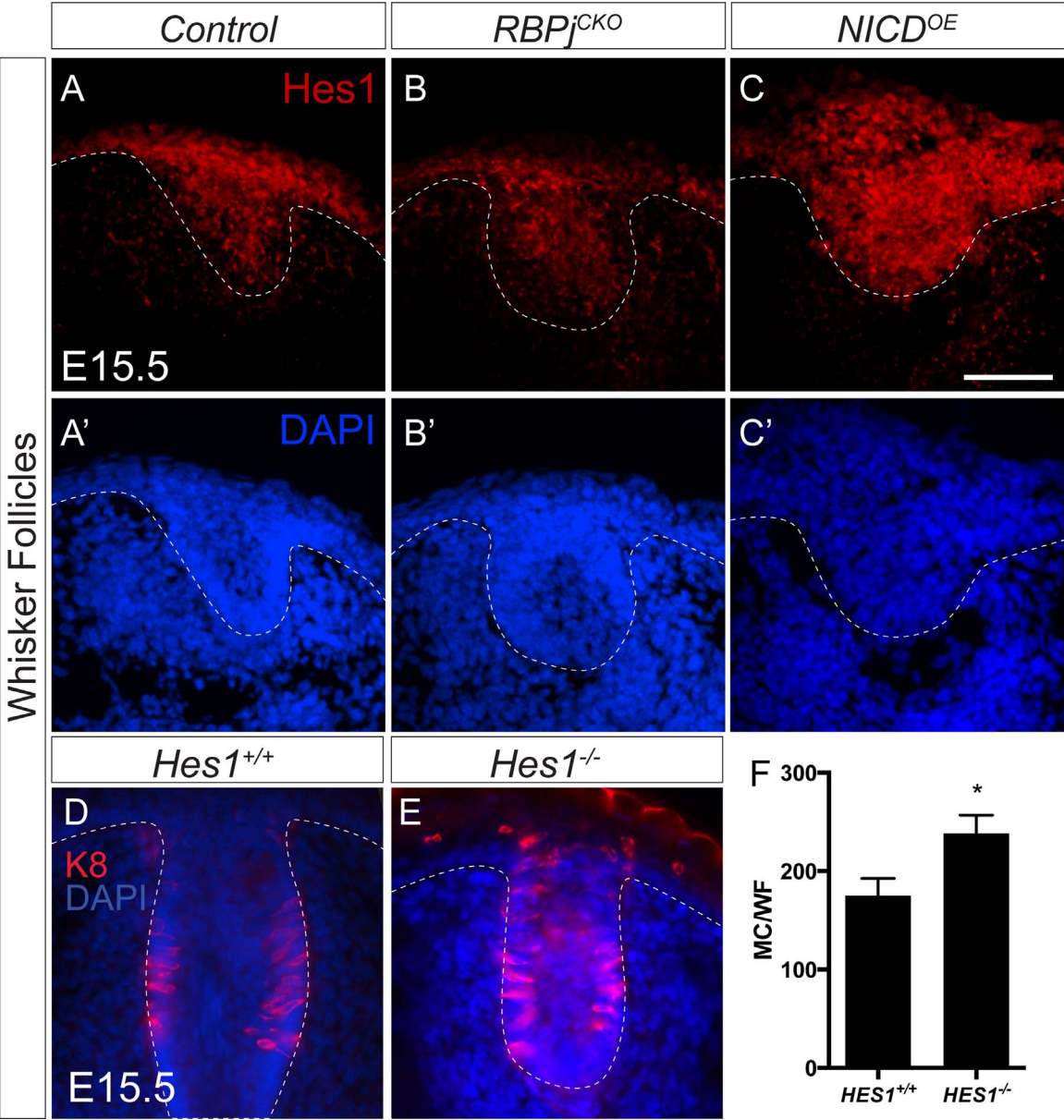


Fig. 3. Hes1-null mice have more whisker follicle associated-Merkel cells. (A,B) (A–C) Hes1 immunostaining of whisker follicles from E15.5 control (A), *RBPj^{CKO}* (B), and *NICD^{OE}* (C) mice. (A'–C') Corresponding DAPI staining. Images are representative of n = 3 mice/genotype. (D,E) K8 immunostaining of whisker follicle sections from control and *Hes1^{-/-}* mice. Dashed line labels the epidermal-dermal border. (F) Average numbers ± SEM of K8 positive Merkel cells from reconstructed whisker follicles (p = 0.023, *p < 0.05) Scale bar = 50 μm.

cells are produced in the whisker follicle of embryonic *RBPj^{CKO}* mice, and ectopic Merkel cells are produced in the body skin of *RBPj^{CKO}* mice. Epidermal Notch signaling promotes *Hes1* expression, and *Hes1*^{-/-} mice produce more Merkel cells in embryonic whisker follicles. Together, these data demonstrate that Notch-induced *Hes1* expression regulates Merkel cell production during embryogenesis.

We can conclude that Notch signaling affects Merkel cell development; however, it is unclear whether Notch signaling inhibits Merkel cell specification, proliferation, survival, or maturation. We detected few Merkel cells in *NICD^{OE}* mice at E15.5, when Merkel cell specification occurs, so it is likely that Notch inhibits Merkel cell specification. We also observed an increase in immature Sox2+ Merkel cells in *RBPj^{CKO}* mice, which suggests that Notch affects Merkel cell maturation. Measuring Merkel cell death and proliferation will be necessary to understand how Notch signaling affects Merkel cell development.

Since Notch signaling and *Hes1* expression directly inhibit *Atoh1* transcription in the inner ear, gut, and cerebellum (Kelley, 2006; Gerbe et al., 2011; Kim and Shivdasani, 2011; Zheng et al., 2011; Chonko et al., 2013), we hypothesize that Notch signaling antagonizes Merkel cell development by inhibiting expression of *Atoh1*. However, it is important to note that we do not directly show that Notch signaling affects *Atoh1* expression, so alternative hypotheses are possible. We observe that *RBPj^{CKO}* mice produce immature Sox2+ K8- cells, but ectopic expression of *Atoh1* results in production of mature K8+, Sox2+ Merkel cells (Ostrowski et al., 2015). This suggests that *Atoh1* is not the only downstream target of Notch signaling. An alternative hypothesis is that Notch overexpression, through its promotion of premature differentiation of embryonic epithelial precursors (Blanpain et al., 2006) decreases the population of progenitor cells competent to become Merkel cells. As reliable markers of Merkel cell progenitors remain to be identified, this latter possibility is difficult to exclude at this time. However, the increases in K8+ and/or Sox2+ cell numbers seen in the whisker follicles and hairy skin of *RBPj^{CKO}* mice in the absence of effects on skin morphology suggest, in our view, that Notch signaling directly affects Merkel cell production.

Notch signals through a juxtacrine mechanism, whereby a cell with a Notch ligand binds to an adjacent cell with a Notch receptor to initiate the signaling cascade (Guruharsha et al., 2012). Mammals have four isoforms of the Notch receptor (Notch1-4) and 5 isoforms of the Notch ligand (Jagged1-2 and Delta-like1,3, and 4), each with different potential to activate the Notch signaling cascade (Gordon et al., 2009; Andersson et al., 2011). Notch signaling can promote lateral inhibition, in which the fate of two adjacent cells become defined when one cell expresses high levels of a Notch receptor and the other expresses high levels of a Notch ligand. Through this mechanism Notch can regulate embryonic patterning (Perrimon et al., 2012). In *RBPj^{CKO}* mice, the presence of ectopic Merkel cells and loss of the typical crescent-shaped distribution within touch domes suggests that Notch plays a role in Merkel cell patterning (Fig. 2). Interestingly, a circular distribution of Merkel cells around the hair follicles is also observed in *Frizzled6* knockout (*Fz6*^{-/-}) mice, where disruption of the Wnt signaling pathway leads to loss of hair follicle polarity (Chang et al., 2016). Hair follicle polarity was not altered in *RBPj^{CKO}* mice (data not shown), suggesting that this mechanism does not explain touch dome disruption in these mice. Further experiments are necessary to determine if the Notch pathway interacts with the Wnt signaling pathway in other ways that might affect Merkel cell patterning.

Several notch receptors and ligands such as Notch 1–3, Jagged 1–2, and Delta-like 1 are expressed in the epidermis and are involved in regulating differentiation of basal epidermal stem cells, but their expression in touch domes and whisker follicles has not been described (Watt et al., 2008). The differentiation of other *Atoh1*-positive progenitors into secretory cells of the gut or hair cells of the inner ear requires the expression of Notch ligands. Gut secretory cell differentia-

tion is promoted by Jagged-1 (Kim and Shivdasani, 2011; Gomi et al., 2016), and inner ear hair cell differentiation is promoted by Delta-like1 and Jagged-2 (Kelley, 2006; Kiernan, 2013). We predict that Merkel cell differentiation requires expression of Notch ligand(s); further experiments are necessary to understand which ligands regulate this process.

A previous study by our lab showed that the inhibitory role of Notch on Merkel cell production persists into adulthood (Ostrowski et al., 2015). In that study, epidermal *RBPj* deletion in adult mice led to the appearance of a modest number of ectopic, interfollicular Merkel cells. Here, we observed around 10-fold greater density of ectopic Merkel cells following epidermal *RBPj* deletion (404 ± 80 vs. 36 ± 16 Merkel cells/cm²). One possibility for this discrepancy could be an increased competence of epidermal cells in younger animals to become Merkel cells. A second possibility is that additional genes and/or signaling pathways that promote Merkel cell specification during embryogenesis are downregulated in postnatal animals.

Recently, a cascade of Wnt, Eda, and Shh signaling was shown to be essential for touch dome formation (Xiao et al., 2016). Wnt initiates the cascade by promoting hair follicle development and inducing expression of Eda and SHH. Shh subsequently promotes formation of touch dome Merkel cells. Hair follicles of *NICD^{OE}* mice appear to develop normally (Blanpain et al., 2006), indicating that Notch must be downstream of Wnt signaling. Notch and Shh interact cooperatively to promote differentiation of neural progenitors (Dave et al., 2011; Kong et al., 2015), but it is unlikely that they work synergistically to regulate Merkel cell development since the two pathways have opposing effects on Merkel cell specification. Further experiments are needed to clarify how Notch signaling interacts with the Wnt/Eda/SHH cascade.

The polycomb repressor complex 2 (PRC2) has been suggested to restrict Merkel cell generation to first-wave primary hair follicles by preventing Merkel cell creation in secondary hair follicles that develop later in embryogenesis (Bardot et al., 2013; Dauber et al., 2016; Perdigoto et al., 2016). Our data raise the possibility that Notch signaling and PRC2 may act cooperatively to restrict Merkel cell formation and patterning during skin development, similar to the way that Notch and Polycomb proteins synergize to inhibit *Rb* expression in *Drosophila* (Ferres-Marco et al., 2006). Notch/PRC2 synergy may also play a role in the modest increases seen when *RBPj* is deleted in adult mice (Ostrowski et al., 2015). Further exploration of the relationship between Notch and PRC signaling is warranted.

Understanding the intricacies of the multiple signaling pathways regulating Merkel cell development could provide insight into the biology of Merkel cell carcinoma (MCC), a deadly skin cancer (Tang and Toker, 1978; Leonard et al., 2002; Eng et al., 2007; Tilling and Moll, 2012). We postulate that disrupting Notch inhibition of Merkel cell formation could contribute to MCC initiation and/or progression. In support of this hypothesis, Notch1 is expressed by most MCC tumors (Panelos et al., 2009), and miR-375, the most highly expressed micro RNA in MCC tumors, post-transcriptionally represses *Rbpj* and Notch2 (Abraham et al., 2016). More research is needed to understand what role Notch signaling plays in MCC progression.

4. Conclusion

Canonical Notch signaling inhibits Merkel cell maturation in whisker follicles and touch domes during embryogenesis. Epidermal deletion of *RBPj* induces ectopic intrafollicular Merkel cell production. The Notch downstream target *Hes1* is detected at higher levels in whisker follicles of Notch overexpressing mice, and lower levels in *RBPj^{CKO}* mice. *Hes1*-null mice have more Merkel cells in whisker follicles. Taken together we conclude that Notch signaling induces *Hes1* expression, which inhibits Merkel cells maturation.

5. Methods

5.1. Mice

Mice were housed per University of Pittsburgh Institutional Animal Care and Use Committee guidelines. *K14^{Cre/+}* mice (Dassule et al., 2000, Jax#004782) were bred to *ROSA^{NICD/+}* mice (Murtaugh et al., 2003, Jax#004782) to produce *NICD^{OE}* mice and littermate controls. *RBPJ^{flox/+}* mice (Han et al., 2002) were bred with *K14^{Cre/+}*; *RBPJ^{flox/+}* to generate *RBPJ^{CKO}* mice. *K14^{Cre/+}*; *RBPJ^{flox/+}* mice were bred to *RBPJ^{flox/flox}*; *ROSA^{NICD/+}* mice to generate *RBPJ^{CKO}*; *NICD^{OE}* mice. *Hes1^{-/-}* mouse embryos were generously provided by Dr. Nadean Brown (UC Davis, Davis, CA).

5.2. Tissue processing

Adult mice were sacrificed by cervical dislocation under anesthesia with isoflurane. For embryonic ages, the day of plug detection was designated E0.5. E15.5 mice were dissected out of pregnant dams. Embryos were decapitated before processing. Fresh frozen tissue was collected for all E15.5 tissue by embedding in OCT compound (Tissue-Tek) and kept at -80°C . To obtain sections of whisker follicles, E15.5 heads were sectioned at $25\ \mu\text{m}$ through the horizontal plane. Tissue sections were fixed with acetone for 10 min before immunostaining. PO embryos were drop-fixed in 4% PFA overnight.

5.3. Histology

Sectioned tissues were stained on glass slides using shandon coverplates (Thermo Scientific). Tissues were rehydrated in 1xPBS for 2 min and then incubated in PBS/0.3% H_2O_2 for 15 min at room temperature. Slides were then blocked in PBS with 0.3% Triton-X and 5% normal donkey serum (Millipore) for 30 min. Primary antibodies were diluted in blocking buffer and tissue sections were incubated for one hour at room temperature with the following antibodies: rat anti-keratin 8 (1:20; TROMA-1; Developmental Studies Hybridization Bank), rabbit anti-Sox2 (1:400, ab97959), NF200 (1:500; Sigma-aldrich, N4142) and rabbit anti-Hes1 (gift of Ben Stanger, Zong et al., 2009). After primary incubation, slides were washed 3x5 min at room temperature and incubated for 30 min in secondary antibodies: Cy3 conjugated anti-rat and cy3 conjugated anti-rabbit (1:250). Nuclei were stained with DAPI (1:1000; Thermo Fisher Scientific). PO tissue was processed and stained by wholemount with a four day primary incubation and a two day secondary incubation, as described previously (Wright et al., 2017). All tissues were mounted in Prolong Gold (Invitrogen) on glass coverslips.

5.4. Whisker follicle morphology parameters

To assess whisker follicle morphology, we measured three parameters from H & E stained tissue of whisker follicle sections. Each of the following parameters were measured from 5 whisker follicles per mouse (3 mice/genotype). 1) Epidermal thickness (ET) was measured as distance from the basal lamina to the most superficial side of the epidermis. 2) Whisker follicle width (WFW) was measured at the base of the interfollicular epidermis. 3) Whisker follicle depth (WFD) was measured from the base of the interfollicular epidermis to the deepest point of the whisker follicle. One way *t*-tests were used to compare transgenic mice to controls for each of the parameters.

5.5. Cell counts

To quantify number of Merkel cells per whisker follicle, intact embryonic heads were serial sectioned and 7–10 whisker follicles per mouse ($n = 3$ mice/genotype, $n = 4$ mice/genotype for *Hes1^{-/-}* and control mice, $n = 2$ mice/genotype for *NICD^{OE}* mice) reconstructed

based on location and morphology. We counted the total number of Sox2+, K8+, and Sox2+/K8+ cells from 5 sections of whisker follicles in two E15.5 *NICD^{OE}* and two control mice; total cell numbers are reported in each case. To determine the number of Merkel cells per touch dome in wholemount tissue, K8+ or Sox2+ cells were counted in 25 touch domes per mouse ($n = 4$ mice/genotype for K8 counts, $n = 3$ mice/genotype for Sox2 counts). Touch dome and ectopic cell density was measured by total number of touch domes and ectopic cells in a $5\times 5\ \text{mm}$ area of skin ($n = 4$ mice/genotype). To determine the central angle of touch domes, 10 images of touch domes were captured from each mouse ($n = 4$ mice/genotype), and ImageJ used to measure the angle formed from the crescent-shaped touch dome. One-way ANOVAs were performed for all measurements in wholemount tissue. All parameters were measured blinded to the genotypes of the mice. Images were adjusted for contrast and brightness for publication.

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Author contributions

GJL and SMM designed the study; GJL, MCW, and ACK performed the experiments; GJL and SMM analyzed the data; and GJL and SMM wrote the manuscript with input from the other authors. Financial support

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