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Article

Reciprocal Requirements for EDA/EDAR/NF-κB and Wnt/β-Catenin Signaling Pathways in Hair Follicle Induction

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SUMMARY

Wnt/β-catenin and NF-κB signaling mechanisms provide central controls in development and disease, but how these pathways intersect is unclear. Using hair follicle induction as a model system, we show that patterning of dermal Wnt/β-catenin signaling requires epithelial β-catenin activity. We find that Wnt/β-catenin signaling is absolutely required for NF-κB activation, and that *Edar* is a direct Wnt target gene. Wnt/β-catenin signaling is initially activated independently of EDA/EDAR/NF-κB activity in primary hair follicle primordia. However, Eda/Edar/NF-κB signaling is required to refine the pattern of Wnt/β-catenin activity, and to maintain this activity at later stages of placode development. We show that maintenance of localized expression of *Wnt10b* and *Wnt10a* requires NF-κB signaling, providing a molecular explanation for the latter observation, and identify *Wnt10b* as a direct NF-κB target. These data reveal a complex interplay and interdependence of Wnt/β-catenin and EDA/EDAR/NF-κB signaling pathways in initiation and maintenance of primary hair follicle placodes.

INTRODUCTION

Hair follicle development requires reciprocal communication between surface epithelial cells and the underlying mesenchyme that is mediated by sequential signaling pathways (Schmidt-Ullrich and Paus, 2005). The first morphological sign of hair follicle development is the formation of epithelial placodes, known as hair follicle placodes (Hardy, 1992). Whether this initiating dermal signal is broadly expressed or patterned is unknown. Signaling from the placodes promotes clustering of underlying dermal fibroblasts, forming dermal condensates that are the precursors of hair follicle dermal papillae (Schmidt-Ullrich and Paus, 2005). Further signaling interactions between the hair placode and the nascent dermal papilla lead to placode down-growth and hair follicle morphogenesis. Mouse hair follicle development occurs in several waves, with primary (guard) hair follicle placodes appearing at approximately E14.5, and secondary (awl and zigzag hair) placodes forming between E16.5 and birth (Schmidt-Ullrich and Paus, 2005).

Among known signaling mechanisms involved in hair follicle development, the Wnt/β-catenin and EDA/EDAR/NF-κB pathways appear to play the earliest roles (Fuchs, 2007; Schmidt-Ullrich and Paus, 2005). Expression of several Wnt ligands and Wnt reporter transgenes is specifically elevated in developing hair follicles (DasGupta and Fuchs, 1999; Maretto et al., 2003; Reddy et al., 2001), and forced activation of β-catenin signaling promotes hair follicle fate in both embryonic and postnatal skin (Gat et al., 1998; Narhi et al., 2008; Zhang et al., 2008). Conversely, ectopic expression of the secreted Wnt inhibitor DKK1 in embryonic mouse epidermis prevents the initiation of hair follicle development and blocks patterned expression of all molecular placode markers, including Wnt ligands, suggesting the importance of an earlier acting, broadly expressed Wnt signal (Andl et al., 2002). Inefficient depletion of β-catenin from embryonic epidermis also blocks early stages of hair follicle development (Huelsken et al., 2001), although the precise stage of arrest remains unclear.

Binding of the A1 isoform of the Tumor Necrosis Factor (TNF)-family ectodomain Ectodysplasin (known as

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developing hair follicle placodes (Kumar et al., 2001; Schmidt-Ullrich et al., 2006; Yan et al., 2000). Loss-of-function mutations in these genes or suppression of NF-κB activity by ubiquitous expression of the transdominant super-repressor IκBαΔN block very early stages in the formation of primary and zigzag hair follicles, but do not affect awl or vibrissa follicle development (Schmidt-Ullrich and Paus, 2005, Schmidt-Ullrich et al., 2006). Transient primary preplacode structures are detected in the absence of EDA/EDAR/NF-κB signaling (henceforth referred to as EDAR signaling), but these fail to express *Shh* or *cyclin D1* and are not maintained (Schmidt-Ullrich et al., 2006).

Formation of a regular, patterned array of primary hair follicles is thought to occur via a reaction-diffusion mechanism based on competition between placode-promoting and placode-inhibitory morphogens (Jiang et al., 2004). Secreted Wnt inhibitors, such as DKK4, may contribute to array establishment by blocking the actions of placodal Wnts in adjacent interfollicular epidermis (Bazzi et al., 2007; Sick et al., 2006), while the EDAR-BMP mutual activation-inhibition system is suggested to stabilize a labile prepattern established by early Wnt/β-catenin signaling (Mou et al., 2006). However, it is unclear how the Wnt/β-catenin and EDAR signaling pathways intersect at the molecular level, and to what extent these pathways are interdependent. It is also not clear whether Wnt/β-catenin signaling operates solely within the ectoderm in its interactions with EDAR pathway components or whether Wnt indirectly controls such interactions via the dermis (Andl et al., 2002).

To address these questions we analyzed the effects of specific genetic manipulations of the Wnt/β-catenin pathway on the pattern of Wnt signaling activity, and on EDAR signaling and function. Conversely, we determined the effects of loss of EDAR signaling on Wnt pathway activity. The results of these experiments demonstrated an unexpected requirement for epithelial β-catenin in establishing patterned dermal Wnt activity, and revealed a complex interplay and interdependence between the Wnt and EDAR signaling pathways in primary hair follicle placode formation.

RESULTS

Wnt/β-Catenin Pathway Activation Is First Observed Broadly in the Dermis

To detect Wnt/β-catenin signaling pathway activity in embryonic skin, we utilized three independent Wnt reporter lines: *conductin*$^{+/lacZ}$ (*cond-lacZ*) mice in which β-galactosidase reporter gene expression is regulated by the endogenous promoter of the *conductin/axin2* gene, a direct target of canonical Wnt/β-catenin signaling (Jho et al., 2002; Yu et al., 2005); and *TOPGAL* and *BAT-gal* mice that carry transgenes containing three or seven copies of a consensus LEF1/TCF DNA-binding sequence, respectively, placed upstream of different minimal promoters and *lacZ* (DasGupta and Fuchs, 1999; Maretto et al., 2003). Wnt reporter activity is blocked by ectodermal Dkk1 expression, indicating that it is specific (Chu et al., 2004; Liu et al., 2007).

Cond-lacZ activity, assayed by X-gal staining or immunofluorescence for β-galactosidase, was present uniformly in the upper epithelium or dermis at E12.5–E13.5 (Figure S1B and data not shown), and the *Fzd10* Wnt receptor gene shows prominent expression in both surface epithelial and immediately underlying dermal cells (Figure S1C).

At E13.5 *cond-lacZ* activity remained in the upper dermis, and was focally elevated in the dermis and epithelium at sites of future pelage hair placode formation (Figure 1A, Stage 0). At E14.5 *cond-lacZ* activity persisted in the dermal condensate, and, within the epithelium, became elevated and restricted to cells in the center of the placode (Figure 1A). *TOPGAL* expression was also focally observed in the upper dermis at E13.5, but faded from this site at E14.5, appearing instead in developing Stage 0 and Stage 1 epithelial placodes. At E15.5 both *cond-lacZ* and *TOPGAL* were expressed in the center of the epithelial placode, and in the dermal condensate (Figure 1A, Stage 2). Lack of dermal X-Gal staining in Stage 1 hair follicles in *TOPGAL* embryos could be due to differences in promoter sensitivity to Wnt signaling between *TOPGAL* mice and *cond-lacZ* mice. *LacZ* expression in *cond-lacZ* mice reflects activity of an endogenous Wnt-responsive promoter and so may provide a more accurate readout of β-catenin signaling. Consistent with this, nuclear localized β-catenin, another indicator of β-catenin pathway activity (Clevers, 2006), is prominent in the upper dermis at E12.5 before becoming mostly restricted to dermal condensates at E13.5 (Figure 1B). Epithelial preplacodes displayed elevated β-catenin protein at this stage, but clear nuclear localization was not evident in the epithelium, possibly due to the prominent membrane staining in epithelial cells.

The β-catenin gene (*Ctnnb1*) is transcribed ubiquitously at low levels, including in the dermis, but shows specific transcriptional upregulation in certain tissues (Huelsken et al., 2001). In contrast to Wnt reporter gene activity and nuclear localized β-catenin in dermal cells, specific transcriptional upregulation of *Ctnnb1*, assayed by in situ hybridization or by X-gal staining of *β-cat*$^{lacZ/+}$ (*βcatlacZ*) knockin embryos (Huelsken et al., 2001), was confined to skin epithelial cells. Within the epithelium, β-catenin mRNA was expressed uniformly at E12.5, was locally upregulated in developing placodes at E13.5 (Figure S1D, stage 0), and remained elevated in specific subsets of hair follicle epithelial but not dermal cells throughout embryonic development and in adult life (Huelsken et al., 2001; Figure S1D and data not shown). Thus upregulation of β-catenin mRNA may contribute to signaling in hair follicle epithelial, but not dermal cells.

Patterning of Wnt/β-Catenin Activity in the Dermis Requires Epithelial β-Catenin

The most sensitive Wnt reporter examined here, *cond-lacZ*, shows patterned activity similar to that of nuclear localized β-catenin, and is observed earlier than other described preplacode markers, suggesting that its expression reflects early patterning events. As we could not temporally separate the appearance of patterned *cond-lacZ* activity in the dermis and epidermis at E13.5, patterned epithelial signaling could occur in response to patterned dermal signaling, or vice versa; alternatively these patterning events could be due to independent competitive interactions in dermis and epidermis. To begin to

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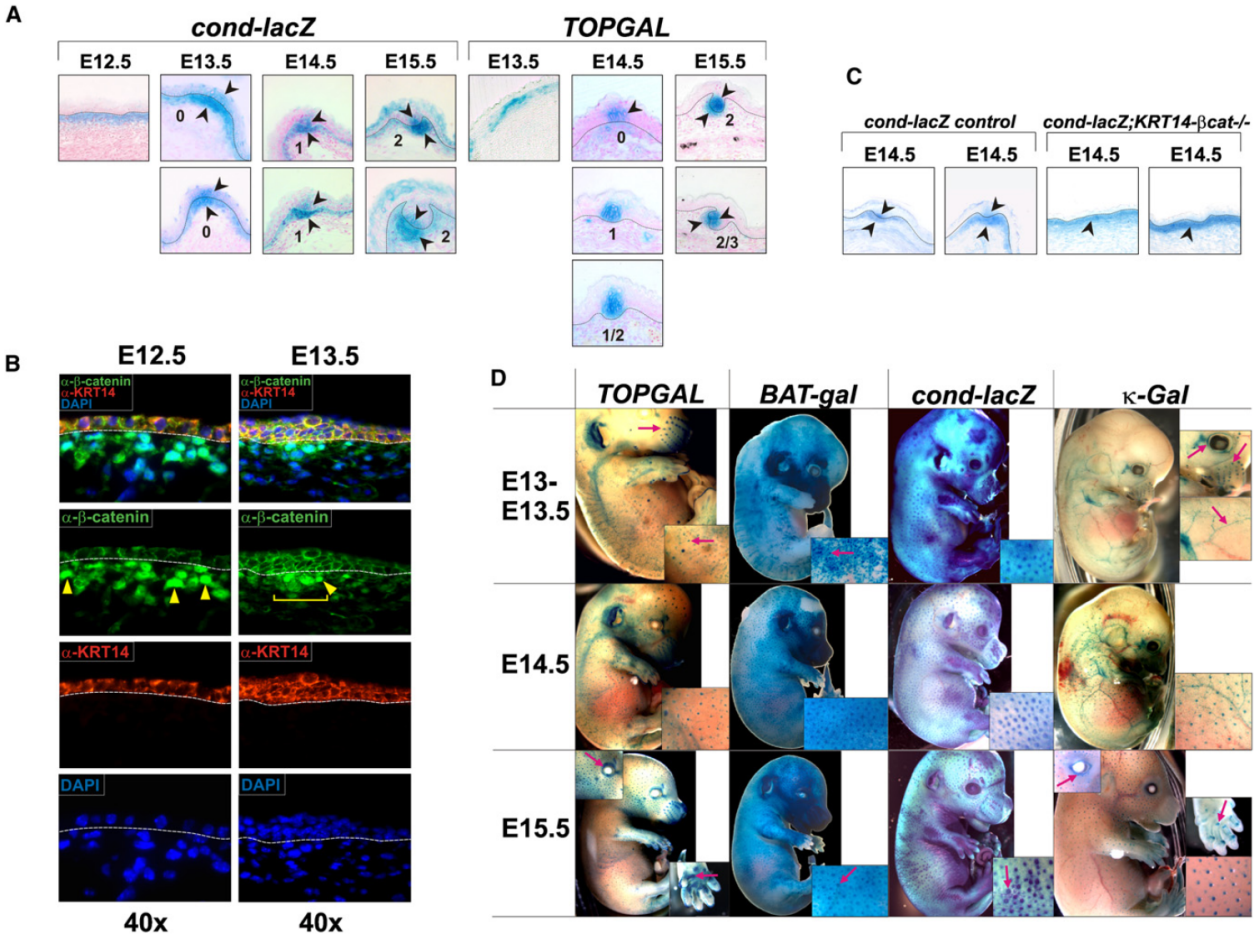

Figure 1. Wnt/β-Catenin and NF-κB Signaling in Embryonic Skin

(A) Technovit sections of X-Gal stained *cond-lacZ* and *TOPGAL* Wnt reporter embryos at E12.5–E15.5. Arrowheads indicate X-Gal staining. Dashed lines indicate the dermal-epidermal boundary. Numbers specify developmental stages of placodes.

(B) Immunofluorescence detection of β-catenin (green). α-KRT14 antibody (red) was used to mark the epidermis. Yellow arrowheads indicate nuclear β-catenin staining in the dermis. A yellow bracket indicates localized dermal nuclear β-catenin at E13.5. Dashed lines indicate the dermal-epidermal boundary.

(C) Technovit sections of E14.5 X-Gal stained *cond-lacZ* and *cond-lacZ;KRT14-βcat*$^{-/-}$ embryos. Arrowheads indicate X-Gal staining. Dashed lines indicate the dermal-epidermal boundary.

(D) Whole-mount X-Gal staining of Wnt reporter (*TOPGAL*, *BAT-gal*, *cond-lacZ*) and NF-κB reporter (*κ-Gal*) embryos at the time points indicated. Red arrows in insets indicate hair placodes, vibrissae, blood vessels, eyelids, and developing sweat glands in footpads.

KRT14-Cre line (Liu et al., 2007) (Figure S3). As expected, *cond-lacZ* expression was absent from the epithelium of *KRT14-Cre;Ctnnb1*$^{fl/fl}$*;cond-lacZ* (*KRT14-β-cat*$^{-/-}$*;cond-lacZ*) embryos at E13.5 and E14.5. Patterned dermal *cond-lacZ* expression was not observed in these mutants; instead, *cond-lacZ* staining was observed uniformly in the upper dermis, both at E13.5 and E14.5 (Figure 1C and data not shown). Thus, patterning of Wnt/β-catenin signaling in the dermis requires epithelial β-catenin.

Focal Wnt/β-Catenin Activity and β-Catenin mRNA Upregulation Are Observed Prior to the Onset of NF-κB

primary (guard) hair follicles start to develop, we compared β-galactosidase expression in *TOPGAL*, *BAT-gal*, and *cond-lacZ* Wnt reporter embryos and the NF-κB reporter line *(Igκ)*$_{3x}$*cona-lacZ* (*κ-Gal*) (Schmidt-Ullrich et al., 1996; Figure 1D). At E13.5, Wnt reporter activity appeared in a pattern corresponding to primary pelage hair follicle development (Figure 1D). By contrast, NF-κB reporter gene activity was not observed in the skin until E14.5 (Schmidt-Ullrich et al., 2006; Figure 1D). While differing reporter gene sensitivities may influence these data, these results suggest that localized Wnt/β-catenin pathway activation occurs approximately 24 hr before the onset of NF-κB signaling.

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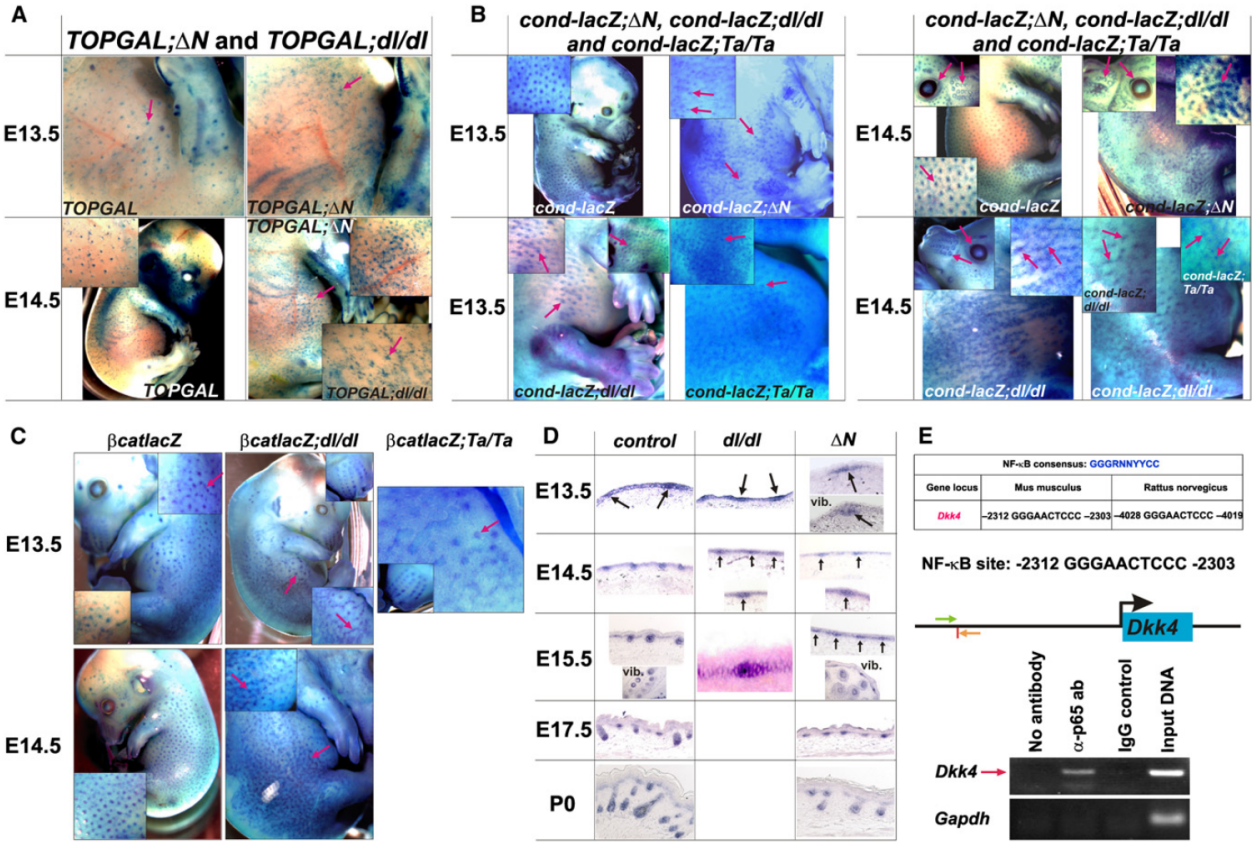

Figure 2. Wnt Activity and β-Catenin mRNA Upregulation in the Absence of EDAR Signaling

(A and B) X-gal stained E13.5 and E14.5 embryos of the following genotypes: control *TOPGAL*, *TOPGAL;ΔN,* and *TOPGAL;dl/dl* (A); and control *cond-lacZ*, *cond-lacZ;ΔN*, *cond-lacZ;dl/dl* and *cond-lacZ;Ta/Ta* (B). Arrows indicate hair placodes displaying Wnt reporter gene expression. Arrows in insets indicate hair placodes, eyelids, and vibrissae.

(C) X-gal stained E13.5 and E14.5 *βcatlacZ*, *βcatlacZ;dl/dl*, and *βcatlacZ;Ta/Ta* embryos.

(D) In situ hybridization for β-catenin mRNA using sagittal skin sections of control, *dl/dl*, and *ΔN* embryos at the time points indicated. Arrows indicate developing placodes.

(E) *Dkk4* is an NF-κB target gene. (Upper panel) The upstream promoter of *Dkk4* contains a consensus NF-κB DNA-binding site, located at −2303 – −2312 in mouse *Dkk4* (vertical red line). Green and orange arrows indicate the positions of ChIP primers in the mouse *Dkk4* promoter. (Lower panel) ChIP using wild-type E14.5 epidermal extracts, anti-p65 antibody (α-p65 ab) or IgG control, and *Dkk4* or control *Gapdh* primers.

Schmidt-Ullrich et al., 2006). At E14.5 and E15.5 similar numbers of X-gal stained spots were observed in *TOPGAL* and *κ-Gal* embryos, corresponding to guard hair placodes. In *cond-lacZ* and *BAT-gal* embryos additional spots were visible. These may correspond to the locations of secondary hair follicle preplacodes, reflecting greater sensitivity of the *cond-lacZ* and *BAT-gal* compared with *TOPGAL* reporter genes; alternatively they may represent transient placodal structures that are not maintained.

Similar to expression of Wnt reporter transgenes, elevated focal *Ctnnb1* transcriptional activity was observed 1 day before the onset of NF-κB reporter gene expression (Schmidt-Ullrich et al., 2006; Figure S1D). At subsequent stages, elevated *Ctnnb1* transcriptional activity and NF-κB reporter gene expression coincided, and were confined to hair follicle epithelial cells, first

Focal Wnt/β-Catenin Signaling Occurs in the Absence of EDA/EDAR/NF-κB Signaling

The overlapping patterns of Wnt and NF-κB reporter gene expression and β-catenin transcriptional elevation in hair follicle epithelial cells are consistent with direct crosstalk between these signaling pathways. However, the earlier appearance of patterned Wnt reporter activity suggests that Wnt/β-catenin signaling may be activated independently of NF-κB. To test this, we crossed *TOPGAL*, *cond-lacZ*, and *βcatlacZ* mice with *Eda*$^{-/-}$ (*tabby*) (*Ta/Ta*) (Mikkola et al., 1999) and *Edar*$^{-/-}$ (*downless*) (*dl/dl*) (B6C3FE-a/a-Edar$^{dl\text{-}J}$, Jackson Laboratories #000210) (Headon and Overbeek, 1999) mice, or mice with suppressed NF-κB activity (*c*^{IκBαΔN}) (*ΔN*) (Schmidt-Ullrich et al., 2001) (Figures 2A and 2B). Localized Wnt reporter gene activity was detected in *dl/dl*, *Ta/Ta*, and *ΔN* embryos between E13.5 and E14.5.

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in Figures 2A and 2B). *Ctnnb1* expression at E14.5 showed similar ill-defined borders and string-like structures in *dl/dl* and *Ta/Ta* mutants (Figures 2C and 2D). Interestingly, at E13.5 irregular X-gal positive foci and string-like structures were observed in some control embryos, as well as in *dl/dl* and *Ta/Ta* mutants, suggesting that these structures represent an early stage in placode formation. These data indicate that initial localized upregulation of both *Ctnnb1* transcription and Wnt/β-catenin signaling activity is independent of NF-κB signaling. However, subsequent refinement of the pattern of Wnt/β-catenin activity into placodes with well-defined borders requires activation of the EDAR pathway.

The secreted Wnt inhibitor and direct Wnt target gene *Dkk4* has been suggested to engage in negative feedback signaling to regulate placode size and spacing (Bazzi et al., 2007; Sick et al., 2006). To determine whether Eda/Edar/NF-κB signaling might coregulate *Dkk4*, we carried out chromatin immunoprecipitation (ChIP) assays with extracts of E14.5 embryonic epidermis, using primers that amplify a region of the *Dkk4* promoter containing a perfect consensus NF-κB binding site that is conserved between mouse and rat (Figure 2E). These experiments demonstrated that NF-κB complexes bind directly to the *Dkk4* promoter (Figure 2E). In line with this, *Dkk4* expression was strongly reduced, although not absent, in *ΔN* and *dl/dl* embryonic skin compared with littermate controls (Figure S2A), and was enhanced in *KRT14-Eda-A1* embryos that constitutively express EDA in the epidermis (Mustonen et al., 2003) compared with littermate controls (Figure S2B), consistent with independently obtained data from another group (Fliniaux et al., 2008). Thus *Dkk4* expression is regulated by NF-κB as well as by Wnt signaling, providing a possible mechanism for the failure of refinement of Wnt active patches in NF-κB pathway mutant ectoderm.

β-Catenin Is Required within the Skin Epithelium for Activation of EDA/EDAR/NF-κB Signaling

Forced constitutive expression of the secreted Wnt/β-catenin pathway inhibitor DKK1 in embryonic mouse surface ectoderm blocks patterned expression of *Edar* (Andl et al., 2002), suggesting that NF-κB signaling may require Wnt/β-catenin signaling in order to be activated. To test this, we generated *KRT5-rtTA; tetO-Dkk1* embryos in which epidermal *Dkk1* expression can be induced by placing the pregnant mothers on oral doxycycline, efficiently blocking Wnt/β-catenin signaling in epithelial and immediately underlying dermal cells by E11.5 (Chu et al., 2004). Localized upregulation of *Edar* and an additional placode marker, *Wnt10b*, was absent as expected in induced E14.5 *KRT5-rtTA;tetO-Dkk1* embryos (Figure 3A). Expression of *Eda* is downregulated at sites of primary placode formation in E14.5 wild-type embryos (Laurikkala et al., 2002). We found that ectopic *Dkk1* blocked this patterned downregulation (Figure 3B). To determine whether inhibition of Wnt/β-catenin signaling affected epithelial NF-κB signaling, we X-gal stained *KRT5-rtTA;tetO-Dkk1;κ-Gal* embryos and their littermates that had been treated with doxycycline from E0.5. NF-κB reporter gene expression was maintained in the blood vessels of induced *KRT5-rtTA;tetO-Dkk1;κ-Gal* embryos at E14.5 and E16.5, but its activity was completely absent in the skin epithelium (Figure 3C).

In contrast with the effects of DKK1, a diffusible molecule, prior data suggested that patterned expression of *Edar* is maintained following late depletion of β-catenin in the surface ectoderm (Huelsken et al., 2001). These data raise the possibility that DKK1 might affect EDAR signaling indirectly, by inhibiting dermal β-catenin signaling and subsequent production of secreted dermal factor(s) expressed in response to β-catenin activation. To re-examine this question, we utilized *KRT14-β-cat*$^{-/-}$ embryos generated using our early-acting *KRT14-Cre* line (Liu et al., 2007; Figure S3). In contrast with previously reported data (Huelsken et al., 2001), patterned expression of *Edar* was completely absent from the surface ectoderm of *KRT14-β-cat*$^{-/-}$ embryos at E14.5 (Figure 3D). Similarly, patterned upregulation of the *Wnt10b* placode marker, and patterned downregulation of *Eda*, were not observed in *KRT14-β-cat*$^{-/-}$ embryos (Figures 3D and 3E), and NF-κB reporter expression was completely absent from the skin of *KRT14-β-cat*$^{-/-}$;*κ-Gal* embryos at E14.5 and later stages (Figure 3F and data not shown). While these data do not rule out functions for β-catenin signaling in the dermis, or indirect effects of epithelial β-catenin signaling in activating the EDAR pathway, they demonstrate unequivocally that β-catenin is required within ectodermal cells for activation of Eda/Edar/NF-κB signaling.

Edar Is a Potential Direct Target of β-Catenin Transcriptional Complexes

As our results indicate that epithelial Wnt/β-catenin is required for activation of EDAR signaling, we asked whether the broad expression of *Edar* that is observed in the surface ectoderm prior to placode induction (Headon and Overbeek, 1999; Schmidt-Ullrich et al., 2006) is β-catenin-dependent. Real-time PCR assays revealed a more than 5-fold reduction in *Edar* transcript levels in both *Dkk1*-expressing and β-catenin deleted skin at E13.5, one day before patterned *Edar* upregulation is observed in controls (Figures 4A and 4B). Thus, initial uniform expression of *Edar* depends on β-catenin signaling within the surface ectoderm. In line with previous data identifying *Eda* as a direct Wnt/β-catenin target (Durmowicz et al., 2002; Laurikkala et al., 2001), *Eda* transcripts were also reduced in *Dkk1*-expressing and β-catenin deleted skin at E13.5 (Figures 4A and 4B).

As expected from in situ hybridization data (Figures 3A and 3D), *Edar* expression was strongly reduced in *Dkk1*-expressing and β-catenin deleted skin compared with control skin at E14.5 and E15.5 (Figures 4A and 4B). Consistent with downregulation of *Eda* at sites of placode formation in control embryos, *Eda* levels were increased or similar to controls in *Dkk1*-expressing and β-catenin deleted skin at these stages (Figures 4A and 4B). Thus while initial uniform *Eda* expression is regulated at least in part by β-catenin signaling, other factors likely control its expression at later time points. A similar temporal shift in the regulatory relationship between Wnt/β-catenin and *Eda* has been described in chick feather bud development (Houghton et al., 2005). In line with this conclusion, mutation of ectodermal β-catenin to a constitutively active form (Zhang et al., 2008) caused significantly increased *Edar* expression but did not

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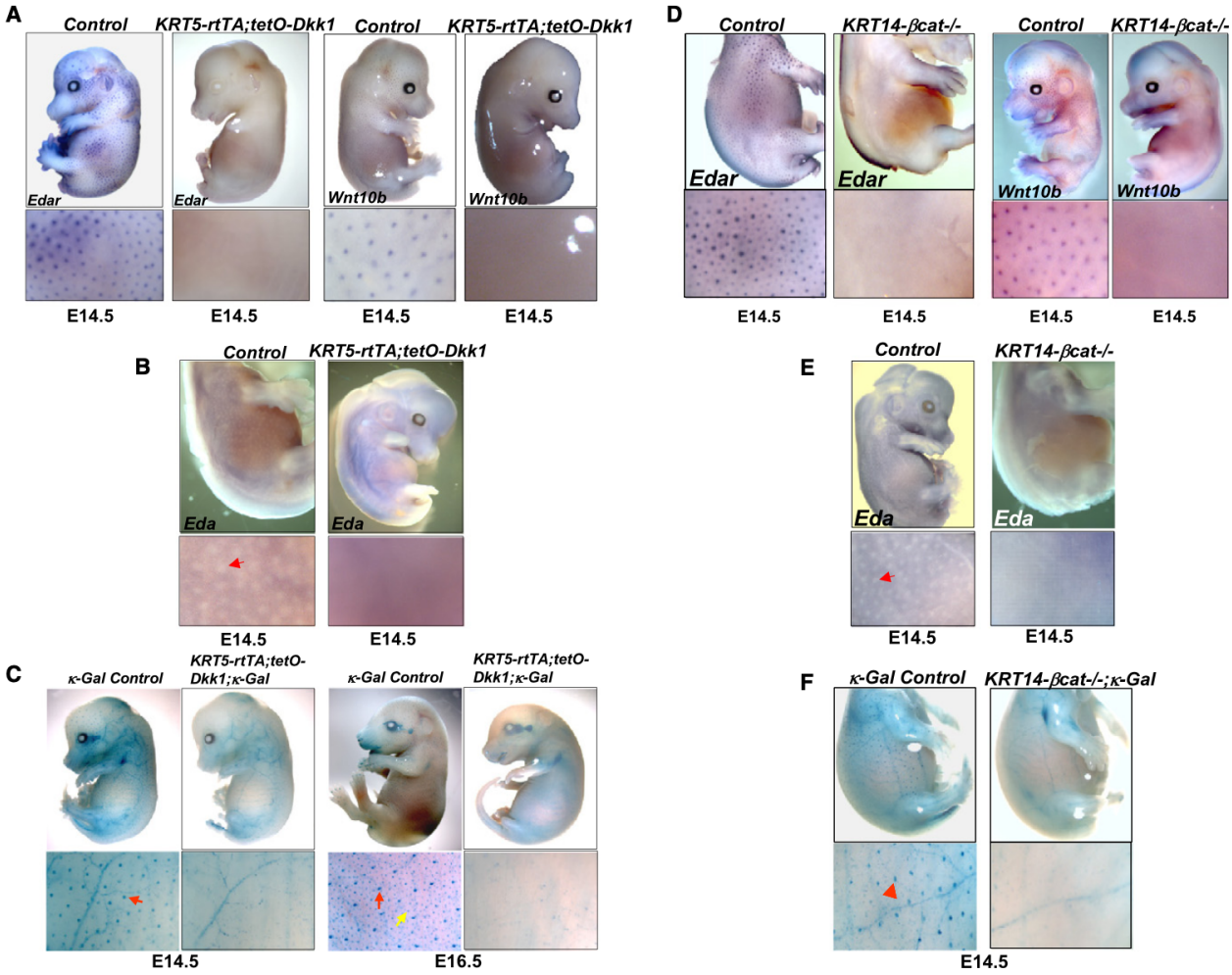

Figure 3. Wnt/β-Catenin Pathway Activity Is Required for EDA/EDAR/NF-κB Signaling

(A and B) Whole-mount in situ hybridization of E14.5 littermate control and *KRT5-rtTA;tetO-Dkk1* embryos doxycycline-treated from E0.5, using *Edar* and *Wnt10b* (A) and *Eda* (B) probes.

(C) Whole-mount X-gal staining of E14.5 and E16.5 *κ-Gal* control and *KRT5-rtTA;tetO-Dkk1;κ-Gal* embryos doxycycline-treated from E0.5. Red arrows indicate primary hair placodes. Yellow arrow indicates a secondary hair placode.

(D and E) E14.5 *KRT14-Cre;Ctnnb1*$^{fl/fl}$ (*KRT14-βcat*$^{-/-}$) and control littermate embryos hybridized with the probes indicated.

(F) Whole-mount X-gal stained E14.5 *KRT14-Cre;Ctnnb1*$^{fl/fl}$*;κ−Gal* (*KRT14-βcat*$^{-/-}$*;κ-Gal*) and control littermate *κ-Gal* NF-κB reporter embryos. Red arrowhead indicates hair placode.

transcripts at E14.5 and E15.5, and that this increase was completely blocked by ectopic *Dkk1* (Figure 4D).

Two potential LEF/TCF-binding sites were identified in the *Edar* promoter region, with the site most proximal to the transcription start site being best conserved between humans and mice (Figure 4E), suggesting *Edar* as a direct Wnt target. Chromatin IP (ChIP) using primers to amplify this region of the mouse *Edar* promoter and antibody to β-catenin revealed binding of β-catenin complexes to this site in vivo in wild-type E14.5 epidermal extracts, and reduced binding in *Dkk1* transgenic

Forced Expression of EDA or Activated EDAR Fails to Rescue Primary Hair Follicle Formation or NF-κB Activity in Embryos with Impaired Ectodermal Wnt/β-Catenin Signaling

Epidermal EDA overexpression results in continuous de novo embryonic hair follicle formation (Mustonen et al., 2003; Zhang et al., 2003), and expression of a constitutively active EDAR receptor causes a 40% increase in hair placode numbers (Mou et al., 2006). These data raise the possibility that forced EDAR signaling might override the requirement for Wnt/β-catenin

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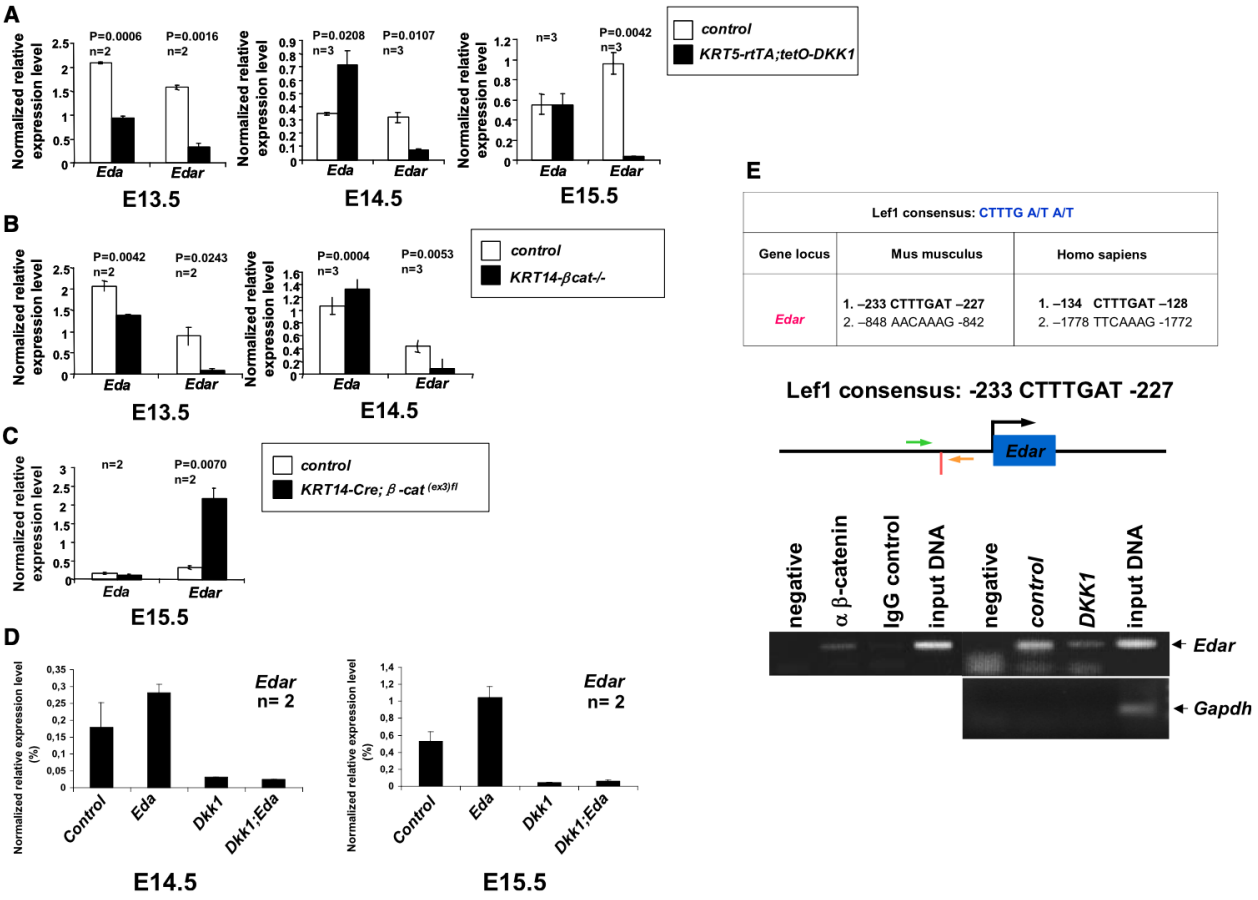

Figure 4. *Edar* Is a Direct β-Catenin Target

(A–C) Epidermal *Eda* and *Edar* mRNA expression assayed by real-time PCR in E13.5–E15.5 littermate controls and embryos with forced epidermal *Dkk1* expression (*KRT5-rtTA;tetO-Dkk1*, [A]), epidermal β-catenin deficiency (*KRT14-βcat*$^{-/-}$, [B]), or carrying an epidermal β-catenin gain-of-function mutation (*KRT14-Cre;β-cat*$^{(ex3)fl}$, [C]). Results are mean ± SEM. Two or three embryos were analyzed for each genotype.

(D) *Edar* mRNA expression assayed by real-time PCR in epidermis from doxycycline-treated control, *KRT14-Eda-A1* (*Eda*), *KRT5-rtTA;tetO-Dkk1* (*Dkk1*), and *KRT5-rtTA;tetO-Dkk1;KRT14-Eda-A1* (*Dkk1;Eda*) embryos at E14.5 and E15.5. Two embryos were analyzed for each genotype.

(E) (Upper panel) Two conserved LEF/TCF binding sites in murine and human *Edar* promoters. (Middle panel) Vertical red bar indicates the position of the proximal LEF/TCF site in murine *Edar*; arrows indicate the locations of primers. (Lower panels, left) ChIP using wild-type E14.5 dorsal epidermal extracts, primers for *Edar* promoter, and anti-β-catenin antibody (α β-catenin) or IgG control. (Lower panels, right) ChIP using dorsal epidermal extracts from doxycycline-treated E14.5 control or *KRT5-rtTA;tetO-Dkk1* (*DKK1*) embryos, anti-β-catenin antibody, and primers for *Edar* promoter (upper right) or *Gapdh* negative control (lower right).

To test whether forced epidermal expression of *Eda* can rescue the effects of Wnt/β-catenin inhibition or loss of ectodermal β-catenin, we generated *KRT5-rtTA;tetO-Dkk1* embryos and *KRT14-β-cat*$^{-/-}$ embryos that also carried a *KRT14-Eda-A1* transgene (Mustonen et al., 2003). The placode marker *Shh* and NF-κB reporter expression were elevated in *KRT14-Eda-A1* and *KRT14-Eda-A1;κ-Gal* embryos, respectively (Figures S4A and S4B). Overexpression of *Eda* was confirmed by whole-mount in situ hybridization (Figure S4C). Doxycycline-treated *KRT5-rtTA;tetO-Dkk1;KRT14-Eda-A1* embryos displayed a total absence of hair follicle development, similar to the phenotype of *KRT5-rtTA;tetO-Dkk1* littermates (Figure 5A), and whole-mount in situ hybridization and histological analysis of patterning and organization of 2006), was also absent in *KRT5-rtTA;tetO-Dkk1;K14-Eda-A1* skin (Figure S4B). Similarly, *KRT14-β-cat*$^{-/-}$*;KRT14-Eda-A1* embryos lacked all histological signs of hair follicle development (Figure 5D) and patterned expression of placode markers (Figure 5E and Figure S4D). Thus, forced expression of *Eda* was not sufficient to rescue hair follicle development in embryos lacking ectodermal Wnt/β-catenin activity.

Lack of *Edar* expression in the absence of Wnt/β-catenin signaling could explain the failure of expression of *Eda* alone to rescue hair placode formation. To test this we utilized *KRT14-LMP-Edar* transgenic mice in which a ligand-independent LMP1-EDAR fusion protein is expressed at low levels in basal

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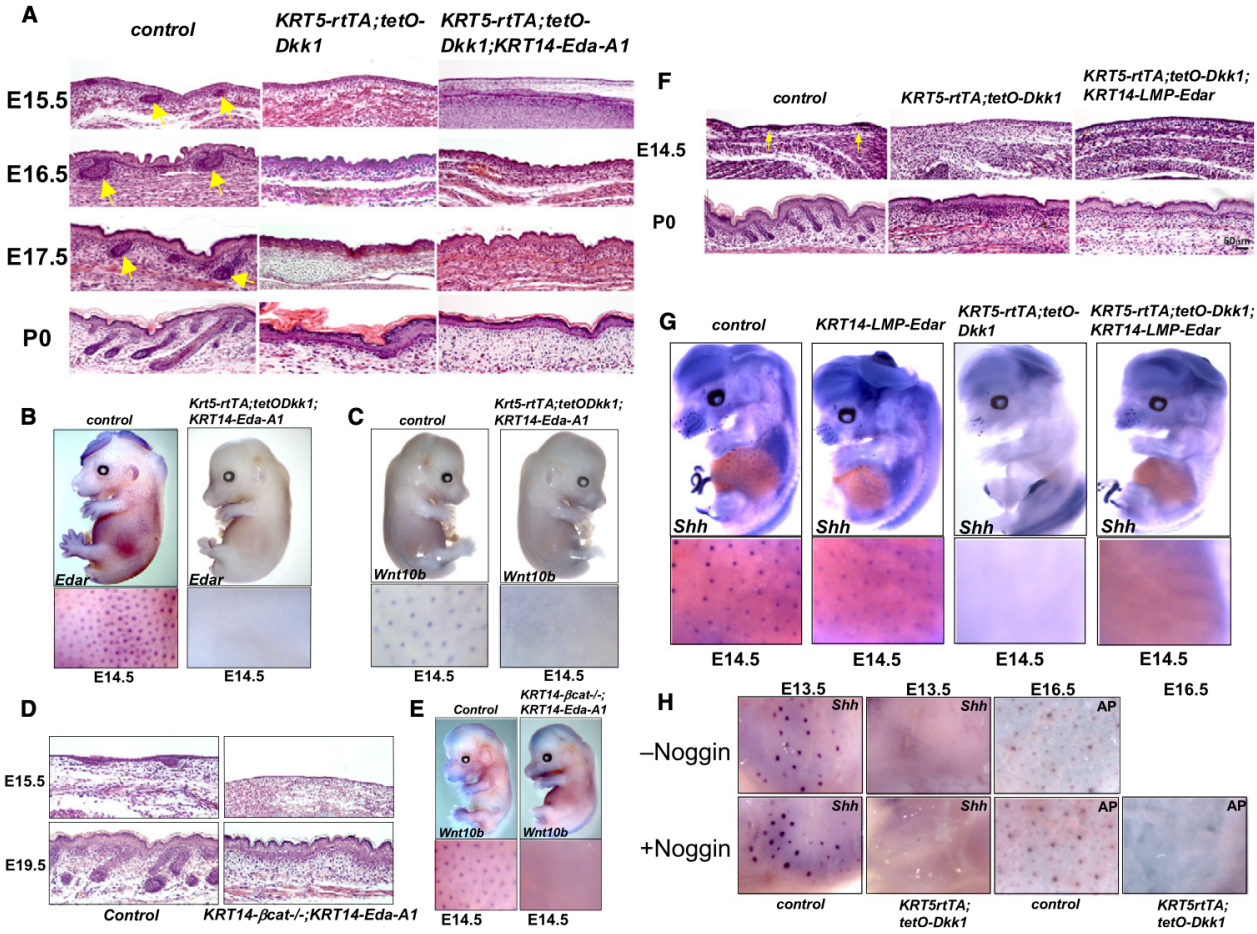

Figure 5. EDA, Activated EDAR, or Noggin Fail to Rescue Primary Hair Follicle Development in Wnt-Inhibited Skin

(A) Hematoxylin/eosin (H&E)-stained sections of skin from doxycycline-treated *KRT5-rtTA;tetO-Dkk1*, *KRT5-rtTA;tetO-Dkk1;KRT14-Eda-A1* and control littermate embryos. Yellow arrows indicate developing hair follicles in controls.

(B and C) In situ hybridization of doxycycline-treated E14.5 *KRT5-rtTA;tetO-Dkk1;KRT14-Eda-A1* and control embryos for *Edar* (B) and *Wnt10b* (C).

(D) H&E stained sections of *KRT14-Cre;Ctnnb1*$^{fl/fl}$*;KRT14-Eda-A1* (*KRT14-βcat*$^{-/-}$*;KRT14-Eda-A1*) and control littermate skin at E15.5 and E19.5.

(E) In situ hybridization of E14.5 *KRT14-βcat*$^{-/-}$*;KRT14-Eda-A1* and control embryos for *Wnt10b*.

(F) H&E stained E14.5 and P0 skin sections of the indicated genotypes.

(G) Whole-mount in situ hybridization of doxycycline-treated E14.5 *KRT14-LMP-Edar*, *KRT5-rtTA;tetO-Dkk1* and *KRT5-rtTA;tetO-Dkk1;KRT14-LMP-Edar* and control embryos, using *Shh* probe.

(H) Treatment of skin explants from doxycycline-treated E13.5 and E16.5 littermate control and *KRT5-rtTA;tetO-Dkk1* embryos with or without Noggin for 24 hr. Hair follicle induction was monitored by in situ hybridization for *Shh* (E13.5) or staining with alkaline phosphatase (AP) to reveal dermal condensates (E16.5).

enlarged, irregular placodes at E18.5 (Figure S5A). In *Eda* null (*Ta/Ta*) mutant animals and in *downless-Sleek* mice that carry a dominant negative mutation in *Edar*, expression of LMP1-EDAR rescues primary hair follicle and tooth development (Tucker et al., 2004; Figure S5B, and data not shown), confirming that LMP1-EDAR acts as a constitutively active receptor. Patterned activation of NF-κB signaling, and formation of primary hair follicle placodes, assayed by histological analysis and whole-mount in situ hybridization for *Shh* at E14.5, were completely absent in *KRT5-rtTA;tetO-Dkk1;KRT14-Edar-LMP*

sufficient to generate patterned NF-κB signaling activity, or hair follicle placode development, in the absence of Wnt/β-catenin signaling.

Exogenous Noggin Fails to Rescue Hair Follicle Formation in Embryonic Skin with Impaired Wnt/β-Catenin Signaling

EDAR signaling directs the expression of BMP antagonists that counteract the placode inhibitory effects of BMPs, and the BMP inhibitor Noggin can partially rescue primary hair follicle

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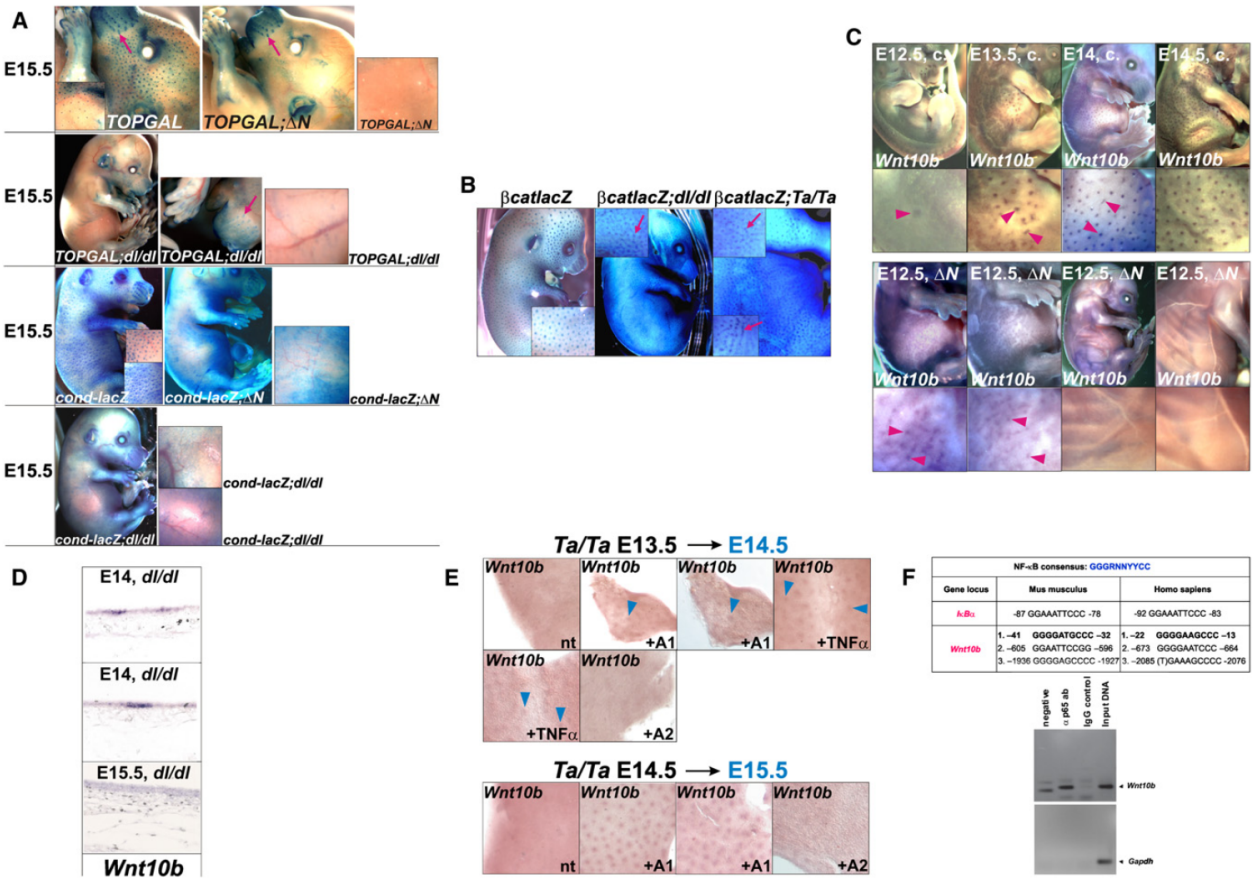

Figure 6. At Later Stages of Primary Hair Follicle Development EDA/EDAR/NF-κB Signaling Is Required for Maintenance of Wnt/β-Catenin Activity and *Wnt10b* Expression, but Not for Patterned β-Catenin mRNA Upregulation, and *Wnt10b* Is a Potential Direct NF-κB Target

(A) *TOPGAL* and *cond-lacZ* Wnt reporter expression analyzed by X-Gal staining in control, *ΔN*, and *dl/dl* backgrounds at E15.5.

(B) Whole-mount X-gal staining of E15.5 *β-catenin*$^{+/lacZ}$ (*βcatlacZ*), *β-catenin*$^{+/lacZ}$;*dl/dl* (*βcatlacZ;dl/dl*), and *β-catenin*$^{+/lacZ}$;*Ta/Ta* (*βcatlacZ;Ta/Ta*) embryos as indicated. Patches of β-catenin expression appear fused and form strings in *downless* and *tabby* mutant embryos (red arrows), as also observed in embryos of these genotypes at E13.5 and E14.5 (Figure 2C).

(C) Whole-mount in situ hybridization of control (c) and *ΔN* embryos at the time points indicated using *Wnt10b* probe. Arrowheads indicate focal expression.

(D) In situ hybridization for *Wnt10b* using sagittal sections of control and *dl/dl* skin at E14.0 and E15.5.

(E) Whole-mount in situ hybridization of E13.5 (upper panels) and E14.5 (lower panels) *Ta/Ta* skin explants using *Wnt10b* probe. Explants were treated for 24 hr with recombinant Fc-EDA-A1 (+A1), Fc-EDA-A2 (+A2), TNFα (+TNFα), or were untreated (nt).

(F) (Upper panel) Conserved NF-κB binding sites in the human and murine *Wnt10b* promoters at the positions indicated. A verified NF-κB DNA-binding site in the IκBα promoter is listed for comparison. (Lower panels) ChIP using E14.5 dorsal skin extracts, primers that amplify a region encompassing the two proximal NF-κB consensus sequences in murine *Wnt10b*, and anti-p65 or IgG control antibodies. Primers amplifying a *Gapdh* promoter fragment were used as a negative control.

development in Wnt/β-catenin-inhibited skin, we treated skin explants from control and induced *KRT5-rtTA;tetO-Dkk1* transgenic E13.5 and E16.5 embryos with recombinant Noggin for 24 hr. Exogenous Noggin increased hair follicle induction, monitored by in situ hybridization for *Shh*, and by an enzymatic assay for the dermal condensate marker alkaline phosphatase, in wild-type control skin (Botchkarev et al., 1999; Figure 5H) but was unable to restore primary (E13.5) or secondary (E16.5) placode development in skin from induced *KRT5-rtTA; tetO-Dkk1* embryos (Figure 5H). Thus, in the absence of Wnt/β-

Maintenance of Wnt/β-Catenin Activity at Later Stages of Primary Hair Follicle Development Requires EDAR Signaling

Sites of β-catenin and NF-κB signaling activity overlap after the initiation stage of hair follicle development, suggesting that these pathways may also interact later in morphogenesis. To determine whether Wnt/β-catenin activity is maintained at later stages in the absence of EDAR signaling, we examined Wnt reporter gene expression in offspring of matings between *TOPGAL* and *cond-lacZ* Wnt reporter mice and *ΔN*, *dl/dl*, or *Ta/Ta* mice at

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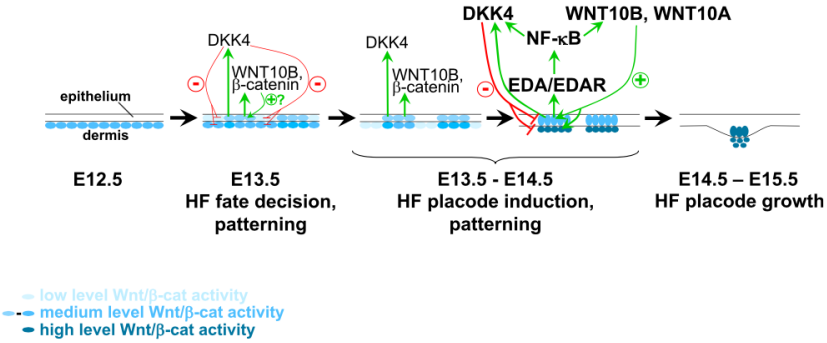

Figure 7. Model for Interactions of Wnt/β-Catenin and EDAR Signaling Pathways in Primary Hair Follicle Development

Wnt activity lies both upstream and downstream of EDAR signaling. At early stages of hair follicle development an irregular prepattern of Wnt activity is established in the epithelium and is required for patterned inhibition of Wnt signaling in interplacodal regions, possibly via the actions of secreted Wnt-induced inhibitors, such as DKK4. Reinforcement of signaling in preplacodes involves elevation of β-catenin and *Wnt10b* transcription. Expression of *Eda* and *Edar* requires Wnt signaling. Maintenance of Wnt signaling and elevated *Wnt10a*, *Wnt10b*, and *Dkk4* expression at later stages requires EDA/EDAR/NF-κB activity. HF, hair follicle.

placodes requires EDAR activity, suggesting that NF-κB may regulate the expression or activity of Wnts or components of the canonical Wnt pathway.

Wnt10b Is a Potential Direct NF-κB Target Gene

In contrast to Wnt reporter gene expression, focal upregulation of *Ctnnb1* transcription was maintained in the absence of EDAR signaling at E15.5 (Figure 6B). We therefore asked whether Wnt ligand expression was affected by loss of EDAR signaling. In situ hybridization for *Wnt10b* in E12.5–E15.5 wild-type embryos revealed that focal expression appeared first at E13.5 in an irregular pattern similar to that seen for Wnt reporter expression in some embryos at this stage. By E14.0, this pattern had resolved into a regular array (Figure 6C). By contrast, in *ΔN* embryos, *Wnt10b* expression was very weak at E14.0, with an irregular pattern. By E14.5 patterned *Wnt10b* expression was absent from *ΔN* skin (Figure 6C and Figure S6A), and also disappeared from *dl/dl* mutant skin after E14.0 (Figure 6D). These data suggest that the initial irregular pattern of *Wnt10b* expression is NF-κB-independent, but that enhancement, refinement, and maintenance of expression requires NF-κB activity. Similarly, patterned expression of *Wnt10a* and *Lef1* was absent in trunk skin of *ΔN* embryos at E14.5 and E15.5 (Figure S6B), consistent with previous identification of *Wnt10a* as a potential NF-κB target in murine B cells (Krappmann et al., 2004). Development of secondary awl hair and vibrissae follicles is unaffected by loss of EDAR signaling, and these displayed normal expression of *Wnt10b*, *Wnt10a*, and *Lef1* in *ΔN* embryos (Figures S6A and S6B and data not shown).

To examine whether EDAR signaling can promote *Wnt10b* expression in primary hair follicle development, skin explants from E13.5 or E14.5 *Ta/Ta* embryos were treated for 24 hr with recombinant Fc-Eda-A1 or TNFα, that stimulate NF-κB transcriptional activity, or with Fc-Eda-A2 isoform, which is incapable of activating NF-κB in vivo (Schmidt-Ullrich et al., 2006). Fc-Eda-A1 and TNFα were able to maintain *Wnt10b* expression in hair placodes of *Ta/Ta* skin, while untreated and Fc-Eda-A2-treated explants lacked patterned *Wnt10b* expression (Figure 6E). Similarly, *Wnt10b* expression was expanded in E14.5 embryonic skin by forced expression of Eda from a *K5Tet1.Eda-A1* transgene (Figure S6C). We identified th

epidermis revealed binding of the p65 subunit of NF-κB to a region of chromatin encompassing the two sites most proximal to the transcription start site of murine *Wnt10b* (Figure 6F). These data identify *Wnt10b* as a potential direct target of NF-κB in primary hair follicle development. Our results further suggest that the requirement for NF-κB signaling in maintenance of Wnt/β-catenin signaling at later stages of primary hair follicle development may be due in part to NF-κB-dependent expression of *Wnt10b* and its close relative *Wnt10a*.

DISCUSSION

The Wnt/β-catenin and NF-κB signaling pathways play critical roles in development, homeostasis, and cancer (Clevers, 2006; Courtois, 2005; Naugler and Karin, 2008). However, how Wnt and NF-κB pathway components interact in the complex network of biological communication that regulates these processes remains unclear. Here we have used an accessible and well-characterized developmental system to dissect the precise temporal relationship and molecular crosstalk by which these key signaling pathways intersect to initiate development of a complex miniorgan, the primary hair follicle. Our results reveal a mandatory role for Wnt/β-catenin signaling within the surface ectoderm in priming keratinocytes to become follicular keratinocytes. This finding is consistent with the observed induction of ubiquitous hair placode formation in embryos carrying an activating mutation in surface ectodermal β-catenin (Narhi et al., 2008; Zhang et al., 2008). Our data support a model in which Wnt/β-catenin and EDA/EDAR/NF-κB signals engage in a complex interplay following the initial adoption of placode fate by surface ectodermal cells in response to activated Wnt signaling (Figure 7).

Tissue recombination experiments suggest that a dermal signal initiates hair follicle placode development (Hardy, 1992); however, whether this signal is uniform, or is generated by clustered mesenchymal cells that form a prepattern, has been unclear. We identified *cond-lacZ* expression as a very early marker of skin patterning that is initially expressed broadly in the upper dermis and becomes patterned in both epithelium and dermis at least 1 day before the morphological appearance

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than the dermis. Specific inhibition and activation of the Wnt/β-catenin pathway in upper dermal cells will be required to determine the functional relevance of dermal β-catenin signaling for epithelial patterning and hair follicle development.

Irrespective of the contribution of dermal Wnt signaling, we show here that Wnt/β-catenin signaling within epithelial cells is required for activation of EDA/EDAR/NF-κB signaling, and subsequent molecular and morphological events essential for hair follicle development. We find that, in addition to its known role in regulating *Eda* expression (Durmowicz et al., 2002; Laurikkala et al., 2001), Wnt/β-catenin also directly regulates expression of *Edar*. One role of EDAR signaling is to suppress placode-inhibitory BMP signals (Mou et al., 2006; Pummila et al., 2007). However, we find that neither exogenous Noggin, nor forced activation of EDA or ligand-independent EDAR, can rescue hair follicle development in the absence of Wnt signaling. These data indicate that expression of additional Wnt targets is required. As secondary (awl) hair follicle morphogenesis requires Wnt/β-catenin but is independent of NF-κB signaling, some of these additional targets may also be utilized in secondary hair follicle development. Our data further suggest that formation of the "messy" prepattern established by focal activation of β-catenin signaling at E13.5 is necessary to produce a molecular context in which downstream patterning events can proceed.

While focal Wnt/β-catenin signaling occurs in the absence of EDAR signaling, we show that refinement of the pattern of Wnt/β-catenin activation is dependent on activity of the Edar pathway in primary placode induction. In the absence of NF-κB signaling, the borders of Wnt reporter-positive cell patches are irregular, and these patches sometimes appear to be fused, or occur in string-like shapes. Thus, consistent with previous suggestions (Mou et al., 2006), NF-κB signaling plays a critical role in refining the pattern of hair placode borders. Our data, and those from another group (Fliniaux et al., 2008), indicate that expression of the secreted Wnt inhibitor DKK4 is regulated by NF-κB as well as by LEF/TCF/β-catenin, suggesting a possible mechanism by which NF-κB signaling indirectly limits Wnt activity and refines placode borders. Competition between short-range or cell-autonomous placode promoting signals (such as WNT10B and β-catenin) and longer range inhibitory signals (DKK4) acting downstream of initial irregular Wnt activation is consistent with a reaction-diffusion model for establishment of a regular array of placodes (Sick et al., 2006).

Although initially activated, Wnt signaling and focal expression of *Wnt10b* and its close relative *Wnt10a* are not maintained in the skin in the absence of NF-κB signaling. Our data implicate *Wnt10b* as a potential direct target of the EDAR pathway in primary placode induction, and suggest that NF-κB-dependent maintenance of *Wnt10b* and *Wnt10a* expression could explain in part the eventual disappearance of Wnt pathway activity in the absence of NF-κB signaling. *Wnt10a* and *Wnt10b* display overlapping expression in developing hair follicles, suggesting partial functional redundancy (Reddy et al., 2001). Consistent with this, while the precise role of *Wnt10b* in hair follicle development is unclear, loss-of-function mutations in human *WNT10A*

Unlike Wnt/β-catenin signaling activity, upregulated *β-catenin-lacZ* expression is maintained in EDA/EDAR/NF-κB pathway mutants at E15.5. Patterned upregulation of β-catenin mRNA expression is absent in embryos with forced epithelial expression of the Wnt inhibitor Dkk1, indicating that initiation of this transcriptional activity depends on Wnt signaling (Andl et al., 2002). However, our results suggest that, once established, upregulated *Ctnnb1* transcription may occur independently of both Wnt and EDAR signaling.

The Wnt/β-catenin and EDAR pathways are activated and have important functions in tooth and sweat gland development, suggesting that similar interacting mechanisms to those described here may be relevant to the development of other ectodermal appendages. While β-catenin signaling plays a critical role in diverse skin cancers (Malanchi et al., 2008; Yang et al., 2008), possible interactions of the Wnt/β-catenin and NF-κB signaling pathways in these conditions have not been fully explored and would be an interesting subject for future studies.

EXPERIMENTAL PROCEDURES

Generation of Mouse Lines

Mice or embryos were genotyped by PCR of genomic DNA. Mice mated into *dl/dl* or *Ta/Ta* backgrounds were bred to homozygosity for the *dl/dl* or *Ta/Ta* mutations. To induce *Dkk1* expression in *KRT5-rtTA tetO-Dkk1* double transgenic embryos, pregnant female mice were placed on doxycycline chow (1 mg/kg, Bio-serv, Laurel, MD) from E0.5. All aspects of animal care and experimental protocols were approved by the Berlin Animal Review Board (Reg. 0261/02) or the University of Pennsylvania IACUC Committee.

Histology, Immunofluorescence, X-Gal Staining, and In Situ Hybridization

Immunofluorescence of paraffin-sectioned tissue, whole-mount X-Gal staining for detection of β-galactosidase activity, and whole-mount and section in situ hybridization were performed as described previously (Andl et al., 2006; Chu et al., 2004; Schmidt-Ullrich et al., 1996, 2001, 2006). Detailed methods and probe sequences are provided in the Supplemental Experimental Procedures.

Quantitative Real-Time PCR

Dissected dorsal skin was dispase-treated (BD Bioscience, Sparks, MD) to separate epidermis and dermis. RNA was extracted using RNeasy Mini Kit (QIAGEN, Inc, Valencia, CA). Real-time PCR primers are detailed in the Supplemental Experimental Procedures. Reactions were performed in triplicate using SYBR green on an MJ Opticon II thermocycler (Bio-Rad, Hercules, CA). Relative expression levels were standardized using β-actin as an internal control. Data were analyzed using the Opticon III program. Statistical significance was calculated using Student's t test.

Embryonic Skin Culture

Embryonic back skin explants were cultured for 24 hr on Millipore filters at 37°C in DMEM, 10% FCS, 1 mM sodium pyruvate, and 100 units/ml penicillin/streptomycin using Falcon center-well organ culture dishes and fine metal grids (Goodfellow), or on Transwell Permeable Supports in DMEM, 10% FBS, in Corning 12-well Transwell plates. Where indicated, Fc-EDA-A1 or Fc-EDA-A2 (0.1–0.5 μg/ml; Gaide and Schneider, 2003), TNFα (25 ng/ml), or recombinant mouse Noggin (1000 ng/ml; R&D Systems, MN), were added to the culture medium.

Chromatin Immunoprecipitation (ChIP) Assays

The TRANSFAC programs "patch" and "Alibaba2" (http://www.gene-regulation.com/index.html) and Motif Search (http://motif.genome.jp/)

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Charlottesville, VA). Epidermal cells were dissociated, fixed in 1% formaldehyde for 15 min at RT, sonicated, and incubated overnight at 4°C with anti-β-catenin antibody (clone 14, BD Bioscience, San Jose, CA), anti-p65 (C-20, SC-372, Santa Cruz Biotechnology, Santa Cruz, CA), or control IgG, followed by addition of Protein A agarose Beads. Purified DNA was subjected to semi-quantitative PCR with primers described in the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental Data include six figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00213-5/.

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