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Desmoglein 4 is Regulated by Transcription Factors Implicated in Hair Shaft Differentiation

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Abstract

The hair fiber is made of specialized keratinocytes, known as trichocytes, that primarily express hair keratins which are cemented by a multitude of keratin-associated proteins (KAPs). The hair keratins form the intermediate filament cytoskeleton of the trichocytes, which are linked to abundant cell-cell adhesion junctions, called desmosomes. Desmoglein 4 (DSG4) is the major desmosomal cadherin expressed in the hair shaft cortex where the hair keratins are highly expressed. In humans, mutations affecting either the hair keratins or *DSG4* lead to beaded hair phenotypes with features of monilethrix. In this work, we postulated that the regulatory pathways governing the expression of hair shaft components, such as hair keratins and *DSG4*, are similar. Therefore, we studied the transcriptional regulation of *DSG4* by transcription factors/pathways that are known regulators of hair keratin or KAP expression. We show that HOXC13, LEF1 and FOXN1 repress *DSG4* transcription and provide *in vitro* and *in vivo* evidence correlating the Notch pathway with the activation and/or maintenance of *DSG4* expression in the hair follicle.

Keywords

Desmoglein 4; Notch; Hair Follicle; Hox; Keratin; Differentiation

Introduction

In the mammalian hair follicle (HF), the hair shaft is produced during the anagen or growth phase of the hair cycle which continues in repeated cycles throughout life (Hardy, 1992). The hair shaft or hair fiber is the only part of the HF that protrudes above the skin surface and plays various physiological roles including protection of the skin and thermal insulation. The hair shaft consists of three concentric layers with a cuticle on the outside, a cortex, and a medulla

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on the inside (Fig 1A). In humans, the cortex makes up the bulk of the hair shaft, with the size and shape of the medulla varying greatly depending on hair type and ethnic background (Jave-Suarez et al., 2002). The hair shaft is surrounded by the inner root sheath (IRS) whose innermost layer is in direct contact with hair shaft cuticle cells, and is called the IRS cuticle. The IRS plays a pivotal and supportive role during hair shaft growth (Rogers, 2004). The outermost layers of the HF consist of the companion layer and the outer root sheath (ORS) which is produced during the downgrowth of the HF (Fuchs et al., 2001).

The production and assembly of the hair shaft layers are highly coordinated processes that involve the very rapid proliferation of the matrix cells in the HF bulb, followed by a gradual cellular differentiation program that takes place within the funnel-shaped precortex region (Fig 1A). It has been shown that the physical position of the matrix cells along the basement membrane separating the epithelial matrix cells from the mesenchymal dermal papilla cells along the proximo-distal axis determines, in part, their final differentiation fate in the concentric layers of the HF (Hardy, 1992;Langbein and Schweizer, 2005;Legue and Nicolas, 2005).

The major structural proteins found in the hair shaft are the hair keratins and keratin-associated proteins (KAPs) (Lee et al., 2006). Keratins constitute the intermediate filament cytoskeleton of both epidermal keratinocytes and the specialized hair shaft trichocytes, which is indispensable since these cells must withstand substantial and continuous mechanical stress (Rogers, 2004). To achieve this, the intermediate filaments or keratins form an intracellular network that links to the plaque proteins of cell-cell adhesion complexes called desmosomes. This intra- and inter-cellular network of keratins and desmosomes provides the hair fiber with its properties of a relatively high tensile strength as well as elasticity.

Desmosomes are calcium dependent cell junctions that are prevalent in tissues that are subjected to continuous mechanical stress such as the skin and heart (Bazzi and Christiano, 2007). The desmosomal cadherins, desmogleins (Dsg1–4) and desmocollins (Dsc1–3), are at the core of the adhesive interface of desmosomes. Of the desmogleins, desmoglein 4 (Dsg4) is highly expressed in the cortex region of the hair shaft, and its functional absence leads to localized autosomal recessive hypotrichosis (LAH) in humans and the *lanceolate hair* phenotype in rodents (*lah*) characterized by abnormal hair shaft differentiation and hair loss in these organisms (Fig 1B) (Kljuic et al., 2003; Bazzi and Christiano, 2007; Messenger et al., 2005; Wajid et al., 2007). Interestingly, some LAH patients present with an additional beaded hair phenotype resembling that of monilethrix patients, who have mutations in hair keratins (Schaffer et al., 2006; Shimomura et al., 2006; Zlotogorski et al., 2006).

The expression of hair shaft components is governed by a large number of transcription factors (e.g. LEF1, β-catenin, SMAD, NICD, FOXN1, MSX2 and HOXC13), many of which operate as downstream effectors of the major morphogenic pathways. For example, earlier studies have shown that the Wnt pathway with its downstream effector, Lef1, is one of the major regulators of the hair shaft differentiation process. Lef1 knockout mice completely lack vibrissae and display a significant reduction in pelage HFs (van Genderen et al., 1994)]. Another well-established regulator of hair keratin promoters in the precortex is Hoxc13. Similar to Lef1, Hoxc13 knockout mice lack hair fibers (Godwin and Capecchi, 1998). Foxn1 and Msx2 transcription factors have also been shown to regulate hair keratin expression and their knock out phenotypes resemble those of Lef1 and Hoxc13 (Mecklenburg et al., 2005; Ma et al., 2003). In addition, the knockout of Notch1 or γ-secretase, which is a presinilin-like protease that releases the active form of Notch (NICD), results in abnormal hair cortex differentiation and precortical swellings reminiscent of those observed in Dsg4 mutant mice (Pan et al., 2004).

Based on the close functional relationship between hair keratins and DSG4, as well as the similarity of the phenotypes that result when both genes are perturbed, we hypothesized that the transcription factors that regulate the expression of hair keratins and KAPs might also regulate the expression of *DSG4* (Bazzi et al., 2006). Here, we show that HOXC13, LEF1 and FOXN1 repress *DSG4* transcription in HaCa T keratinocytes *in vitro* and provide evidence that the Notch pathway indirectly regulates *DSG4* expression in the HF and epidermis.

Materials and Methods

Animals

The rodent models used in this study have been described previously: *Dsg4* −/− mice are known as $lah^{J-/-}$ (Sundberg et al., 2000; Kljuic et al., 2003) and rats are known as $lah^{IC-/-}$ (or *Iffa Credo*) (Bazzi et al., 2004), *presenilin 1* and *2* double conditional knockout (PSDCKO, genotype: Msx2-Cre +/−; *Psn1 flox/flox*; *Psn2* −/−) (Pan et al., 2004).

Histology

The histological staining procedures used in this study are either standard haematoxylin and eosin staining or Dane and Herman staining where indicated (Dane and Herman, 1963). The latter staining procedure of paraffin embedded tissue sections is based on haematoxylin, phloxine B, alcian blue, and orange G interspersed with washes in water. The slides are then permanently mounted and photographed with regular light microscopy.

Immunofluorescence

Fresh frozen sections of human HFs discarded after surgery were fixed in 4% PFA for 10 minutes (mins) at room temperature. While frozen sections of mouse back skin were fixed in methanol at −20°C for 15 mins followed by acetone for 1 min at −20°C. The sections were washed in 1XPBS, blocked in 10% of the appropriate serum, and incubated with the primary antibody overnight at 4°C. After washing in 1XPBS, 594 or 688 Alexafluor® conjugated secondary antibodies (Molecular Probes, Invitrogen, Carlsbad, CA, USA) were applied and the signal was visualized using an HRC Axiocam fitted onto an Axioplan2 fluorescence microscope (Carl Zeiss, Thornwood, NY, USA). The antibodies used were guinea pig anti-HOXC13 and anti-Ha1 (1:2000, a kind gift of Dr. Lutz Langbein), rabbit anti-FOXN1 (1;200, Santa Cruz), goat anti-LEF1 (1:100, Santa Cruz Biotchnology Inc., Santa Cruz, CA, USA), rabbit anti-IRS3a.1 (1;1000, a kind gift of Dr. Rebecca Porter), and mouse monoclonal anti-DSG4 clone 18G8 (1:10) (Bazzi et al., 2006). For mouse antibodies on mouse tissues the Mouse On Mouse (M.O.M) kit from Vectorlabs was used per the manufacturer's recommendations (Burlingame, CA, USA).

In Silico Promoter Analysis

For the analysis of the upstream region of *desmogelin 4* we used the VISTA website for comparative genomics (http://genome.lbl.gov/vista/index.shtml) (Loots et al., 2002), in addition to the AliBaba2.1 tool on the Gene-Regulation website (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). We also used a microsoft word search tool for known consensus binding sequences of transcription factors such as Lef1: (T/C)CTTTG(A/T)(A/T), HOXC13 : TT(A/T)AT(N)(A/G)(A/G), and RBPjK (GTGGGAA) considering all permutations.

Reporter Gene Assays

A ~3Kb and ~1Kb (~1.1 for the human) DNA fragments upstream of the translation initiation site (A in the first ATG is considered +1) of human and mouse *DSG4* were cloned into the

pGL3 basic luciferase reporter vector (Promega, Madison, WI, USA). The primers used were as follows:

DSG4 3Kb F: 5'-CTCTGGAAACAACTTGTGCT-3'

DSG4 1Kb F: 5'-AGTTTATTGATGTTGCTGCC-3'

DSG4 3Kb or 1Kb R: 5'-TCCTTTGGGTTTCTCTTGCA-3'

Dsg4 3Kb F: 5'-CGTGTAGGATATGTCCATCT-3'

Dsg4 1Kb F: 5'-CACACACCCTCATTTCTGTT-3'

Dsg4 3Kb or 1Kb R: 5'-TGCTTCGAGTCTCTCTTGG-3'

HaCa T keratinocytes were grown in DMEM with 10% FBS and 1% Penicillin-Streptomycin (Invitrogen) and seeded in six-well plates at a density of 3×10^5 /well. Twenty four hours after seeding, HaCaT cells were transfected with the indicated plasmids at 0.5ug/well each using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations. Total DNA was equalized per experiment by adding the same concentration of an appropriate empty vector. In addition, a β-galactosidase expressing construct was used as an internal control for transfection efficiency and normalization. Each treatment was run in triplicate and each experiment was reproduced two or three times. The cells were lysed 48 hrs after transfection and the signals were assayed using the appropriate substrates for luciferase (Steady Glo® luciferase assay system) and β-galactosidase (Promega) on a $20/20^n$ luminometer (Turner Biosystems, Inc., Sunnyvale, CA, USA) for luciferase and a Model 680 microplate reader (BioRad, Hercules, CA, USA) for β-galactosidase. Student's t-test was used to assess significance between two different treatment groups with a p-value of 0.05. Human β-catenin and LEF1 expression plasmids were generous gifts of Dr. Jan Kitajewski (Columbia University). MSX2 expression plasmid was a gift from Dr. Cheng-Ming Chuong (USC). HOXC13 expression plasmid was a gift of Dr. Lutz Langbein (DKFZ). Only HaCaT keratinocytes that displayed an intact differentiation program (i.e. expressed DSG4 mRNA and protein upon confluency) were used in this study.

Microarray Analysis

Backskins from six-day old $lah^{J-/-}$ and $lah^{J+/-}$ littermates were dissected and incubated in 0.25% dispase II (Invitrogen) at 4°C overnight. Following incubation, the epidermis was separated from the dermis and was minced using scissors and flash frozen in liquid nitrogen. Total RNA was isolated from the epidermal tissues using the RNeasy® Minikit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Triplicate RNA samples from three independent mice for each of WT and mutant mice were amplified once and labeled for hybridization on microarray chips (MOE430A) using the Affymetrix reagents and protocols (Affymetrix Inc., Santa Clara, CA, USA). The data output was normalized and analyzed using both GeneSpring GX 7.0 (Agilent Technologies Inc., Palo Alto, CA, USA) and GeneTraffic™ (Iobion Informatics, Stratagene, Agilent Technologies Inc.) commercial software packages which gave comparable results. The p-value cutoff was set to 0.05 and the significant fold difference was considered two-fold higher or lower than baseline (Bazzi et al., 2007).

Results

Desmoglein 4 is expressed in the hair cortex and IRS cuticle of rat and mouse hair follicles

We previously determined the expression of DSG4 within the hair cortex and IRS cuticle of the human anagen HF (Bazzi et al., 2006). Using a DSG4 specific antibody, mouse monoclonal antibody clone 18G8, we assessed the expression of mouse and rat Dsg4 in the HFs. We have also recently briefly reported the expression of mouse Dsg4 in pelage or back skin HF cortex

(Owens et al., 2008). Similar to the human expression pattern, we found that Dsg4 is expressed in the cortex and IRS cuticle of rat vibrissae follicles, as well as rat and mouse pelage follicles during the anagen phase of the hair cycle (Fig 2A–C,E,F,I,J). Importantly, the protein is absent from Dsg4 null mutant HFs in the *Iffa Credo* rat and *Lah*$^{J-/-}$ mouse (Fig 2D,G,H,K,L). Moreover, Dsg4 expression commences with hair cortex differentiation on day1 *post natum* and is maintained around the forming club hair in the catagen phase of the hair cycle in normal rat follicles (Fig 3). This pattern of expression is consistent with the sites of main defects in LAH humans and *lah* rodents with *desmoglein 4* mutations, which is abnormal hair shaft differentiation starting in the precortex region during anagen (Fig 1B).

Transcriptional profiling of Dsg4 null mouse epidermis

To gain mechanistic insight into how the perturbation of cell-cell adhesion or physical separation of HF keratinocytes in *Dsg4* −/− mouse skin leads to failure in cell positioning and/or signaling, we performed microarray analyses on epidermis isolated from day 6 anagen skin of *lah*$^{J-/-}$ mutant mice and *lah*$^{J+/-}$ littermates. Interestingly, we found that the major class of downregulated genes in mutant epidermis was comprised of hair keratins (Hb6 or KRT86, Ha3-1 or KRT33A), as well as KAPs, particularly those of the Krtap-16 family (Table 1, Krtap-16 family highlighted in red). Most of the down-regulated Krtap-16 family members in *Dsg4* −/− epidermis have been shown recently to be expressed in the HF cortex, coinciding with the expression domain of DSG4 (Pruett et al., 2004). Interestingly, many of the promoter regions of Krtap-16 genes are also potential downstream targets of Hoxc13 (Pruett et al., 2004).

HOXC13, LEF1 and FOXN1 are repressors of DSG4 expression

The functional relationship between hair keratins, KAPs and DSG4 during hair shaft differentiation led us to hypothesize that common transcription factors and regulatory pathways collectively control their expression. To place DSG4 into the context of a regulatory network, we investigated the upstream regulation of *Dsg4* transcription. Using *in silico* promoter analysis, we identified 10 and 19 putative binding sites for LEF1 (red in Supplementary Fig 1) and HOXC13 (green in Supplementary Fig 1), respectively, within 3Kb of the upstream region of human *DSG4.* Many of these sites were conserved in the corresponding mouse and rat *Dsg4* genomic regions (Supplementary Fig 1A,B). The high prevalence of these binding sites and their conservation between species suggests functional relevance. FOXN1 has an 11-base pair consensus binding sequence that includes an invariable 4 nucleotides (ACGC) core. While the statistical probability of finding this short 4 nucleotide core in a random 3 kb DNA sequence is relatively high compared to the HOXC13 and LEF1 consensus biniding sequences, it occurs only 3 times in the corresponding 3 kb DSG4 upstream segment.

In support of a functional relationship, using immunofluorescence analyses, we showed that DSG4 expression overlaps with the expression of HOXC13, LEF1 as well as FOXN1 in the precortex region of the human HF (Fig 4). HOXC13 is also co-expressed with DSG4 in the IRS cuticle (Fig 4A,B) and FOXN1 showed prominent expression in the differentiating cortex which is the major site of DSG4 expression (Fig 4C,D).

We next cloned the upstream regions (1Kb and 3Kb, “H” for human and “m” for mouse) of *DSG4* from both humans and mice and performed reporter gene assays using the luciferase system. When we transfected these reporter constructs into HaCaT keratinocytes, a non-tumorigenic human keratinocytes cell line, they showed an endogenous activity that was significantly higher than background levels (Fig 5A). This is consistent with our earlier findings that HaCaT keratinocytes express endogenous DSG4 when confluent (Bazzi et al., 2006).

We next examined the effect of each of the above mentioned three transcription factors on H3 transcriptional activity in HaCaT keratinocytes. We found that HOXC13 repressed H3 endogenous transcriptional activity to background levels in a similar manner (Fig 5B). This outcome was specific to HOXC13, since a mutant HOXC13 that lacked the DNA binding domain showed no effect on the H3 reporter construct activity (Fig 5B, Mut HOXC13). Moreover, FOXN1 repressed H3, while MSX2 had no significant effect on H3 reporter construct expression (Fig 5C). LEF1 expression also completely repressed the endogenous activity of all four constructs to pGL3 control levels (data not shown, e.g. the human 3Kb reporter construct H3 in Fig 5D). We obtained the same result upon transfecting both LEF1 and β-catenin together with H3 (data not shown).

Notch signaling is involved in DSG4 expression in vitro and in vivo

We next screened additional transcription factors that are expressed in the HF precortex or cortex in search of positive regulators of hair shaft differentiation. Our results showed that Notch (signaling through NICD), as well as the homeodomain gene Hoxc12, significantly activated H3 above endogenous levels (Fig 6A). Hoxc12 is expressed in the hair shaft cortex of mouse HFs coinciding with *Dsg4* expression (Shang et al., 2002). The finding that NICD activates *DSG4* transcription *in vitro* is intriguing given the striking similarity in the HF precortex defect in $lah^{J-/-}$ and *presenilin1* and *2* double conditional knockout (PSDCKO), that lack γ-secretase activity in MSX2 expressing tissues including some HFs (Pan et al., 2004), compared to WT follicles (Fig 6B–D). We then asked if Notch signaling regulates *Dsg4* expression *in vivo* and therefore examined Dsg4 expression in the HFs of PSDCKO animals. We found that Dsg4 is markedly reduced in the abnormal precortex and accumulates beneath the keratinizing swelling compared to normal cortical expression in WT HFs (Fig 6E,F).

Discussion

The mechanisms governing the transcriptional regulation of hair shaft expressed genes are poorly understood. A major challenge in studying hair shaft cells is the lack of a cell culture model for their precursors. *In silico* studies backed by functional electromobility shift assays (EMSA) and/or reporter gene assays have been performed on a very limited number of hair keratin genes (Ha1 and Ha5) and KAPs (KAP 16-5) (Jave-Suarez et al., 2002; Pruett et al., 2004). Correlation of the overlap in expression patterns of transcription factors and genes in question between different HF compartments has proven to be a fruitful starting point for such studies. Here, we systematically screened a battery of transcription factors that are expressed in the precortex and cortex of the HF for possible regulatory roles on *Dsg4* regulation. We found that transcription factors previously shown to activate hair keratin expression, such as HOXC13, LEF1 and FOXN1, repress *DSG4* reporter activity in HaCaT keratinocytes (Fig 5). In contrast, our studies implicate Notch signaling through NICD, as well as Hoxc12 in *DSG4* transcriptional activation (Fig 6).

Our finding that NICD activates the H3 reporter above endogenous levels suggests a role for Notch signaling in the regulation of *DSG4* expression (Fig 6A). This is corroborated in vivo by the striking similarity in the HF phenotypes of *Dsg4*−/− or *lah* mutant mice and PSDCKO mice that lack γ-secretase activity and therefore a processed NICD (Fig 6C,D) (Pan et al., 2004;Bazzi et al., 2005). The decrease in Dsg4 expression in PSDCKO HFs also points to a role of NICD in Dsg4 expression regulation. It remains possible that the physical disruption of cell-cell contacts in *Dsg4*−/− HF precortex cells impairs Notch signaling by mechanical means. Since Notch signaling is mediated through the binding of a membrane-bound ligand and receptor on two adjacent cells, this could be an additional mechanism by which the phenotype in PSDCKO is reminiscent of *Dsg4*−/− HFs.

The crucial role of Notch signaling in epidermal differentiation is indisputable (Rangarajan et al., 2001; Blanpain et al., 2006). Notch signaling, through its downstream transcription factor RBPjK (which provides the DNA binding component for the NICD transcriptional activation domain), has been shown to act as a commitment switch from basal to suprabasal layers in the mouse epidermis (Blanpain et al., 2006). The upstream region of *DSG4* up to 3Kb harbors only one RBPjK consensus binding site at −2089 of the translation start site that is not conserved in mouse and rat. Repeated trials with chromatin immunoprecipitation (ChIP) using an anti-NICD antibody (Cell Signaling) did not show any binding of an NICD-containing complex to this RBPjK site (data not shown). In addition, the γ-secretase inhibitor DAPT {N-(N-[3,5-difluorophenacetyl]-L-alanyl)-S-phenylglycine t-butyl ester}, was unable to reduce the endogenous activity of H3 in HaCaT keratinocytes (data not shown) (Geling et al., 2002). These findings suggest that the regulation of *DSG4* by Notch signaling is indirect. This indirect regulation of *DSG4* by Notch is supported by the expression of DSG4 in the upper spinous and granular layers of the epidermis and the role of Notch in the earlier transition step from basal to spinous differentiation (Bazzi et al., 2006; Blanpain et al., 2006).

Cadherins in general, including both classical and desmosomal cadherins, are calcium-dependent cell-cell junctions. Classical cadherins are linked to the actin cytoskeleton of the cell while desmosomal cadherins are linked to the intermediate filament cytoskeleton. Classical cadherins and the actin cytoskeleton are believed to be organized prior to and provide a framework for the desmosmal cadherins and the intermediate filament cytoskeleton in an epithelial sheet (Yin and Green, 2004). The post-translational regulation of cadherin assembly and the role of calcium in this process is relatively well understood (Peinado et al., 2004; Denning et al., 1998). The transcriptional regulation of expression of classical cadherins such as E-cadherin is also a widely studied area mainly because of E-cadherin involvement in epithelial to mesenchymal transition (EMT) during cancer invasiveness and metastasis (Peinado et al., 2004). In contrast, the transcriptional regulation of desmosomal cadherins has been largely understudied. It has been shown that DSC expression is regulated by CCAAT/enhancer binding proteins (C/EBP) in the epidermis and that DSG1 is regulated by protein kinase C (PKC) (Smith et al., 2004; Denning et al., 1998). Apart from our recent studies on the transcriptional regulation of *Dsg4* by the BMP downstream effectors Smads, particularly Smad4, as well as the transcriptional regulation of *Dsc2* by *Hoxc13* and *Foxq1*, very little is known about desmosomal cadherin transcriptional regulation in the HF (Owens et al., 2008; Potter et al., 2006).

In summary, we report that *desmoglein 4* expression, activation and/or maintenance correlate with Notch signaling *in vitro* and *in vivo*, as well as Hoxc12 expression in the hair cortex. We also show that dsg4 is repressed by Wnt signaling downstream effectors (LEF1 and β-catenin) and other transcription factors such as HOXC13 and FOXN1. Our findings highlight the importance of fine tuning transcriptional regulation during the transition from proliferation to differentiation in the hair shaft precortex. Desmogleins and desmocollins reside in a highly conserved genomic cluster on chromosome 18 in humans, mice, and rats (Hunt et al., 1999). DSGs are transcribed in one direction and DSCs in the opposite direction, andhe expression of desmosomal cadherins also correlates with the differentiation state of the tissue (Bazzi et al., 2006; Kurzen et al., 1998). The mechanisms of coordinated transcriptional regulation of this genomic cluster during development, differentiation, skin homeostasis and when these processes go awry undoubtedly warrant further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

HF	hair follicle
DSG4/DSG4	human desmoglein 4 gene/protein
Dsg4/Dsg4	mouse or rat desmoglein 4 gene/protein
LAH	localized autosomal recessive hypotrichosis
lah	*lanceolate hair* rat or mouse
ORS	outer root sheath
IRS	inner root sheath
PSDCKO	*Presenilin 1* and *2* double conditional knockout

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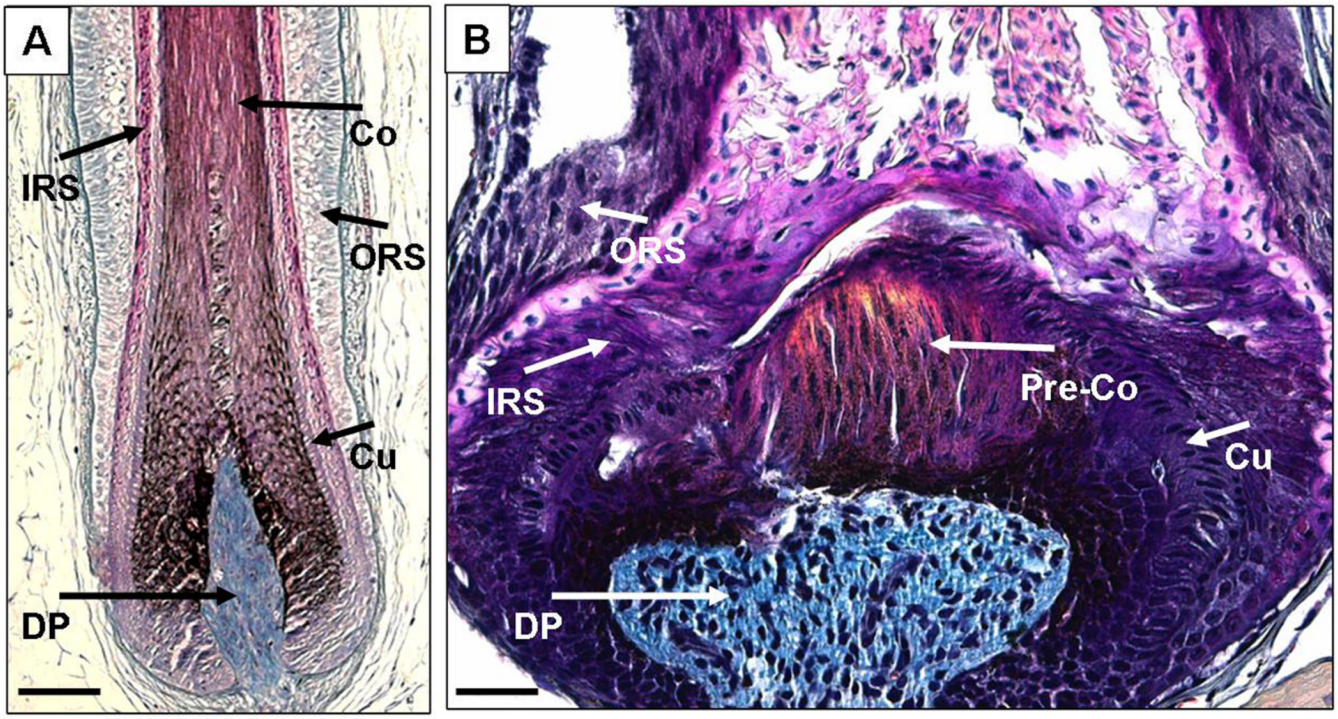
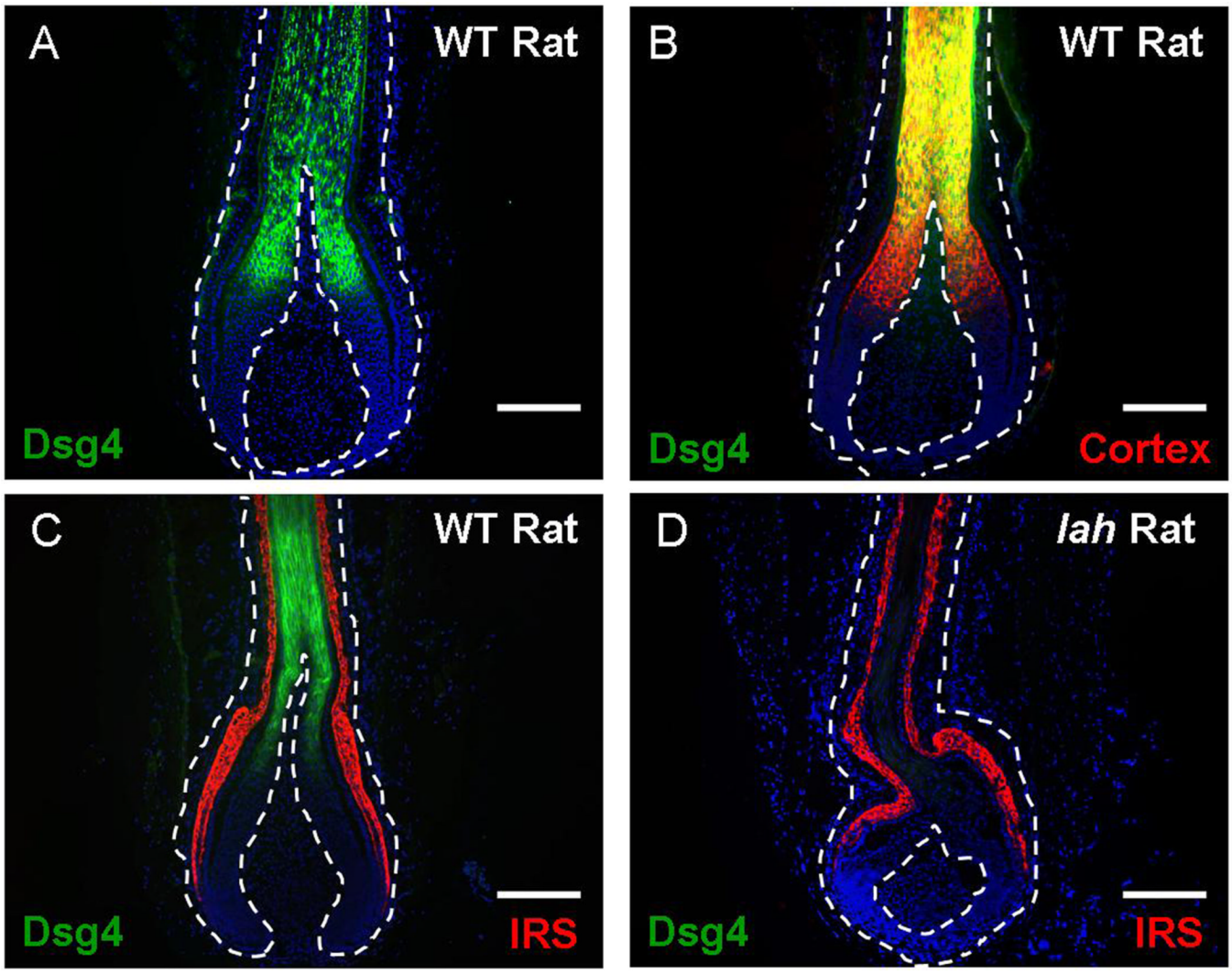
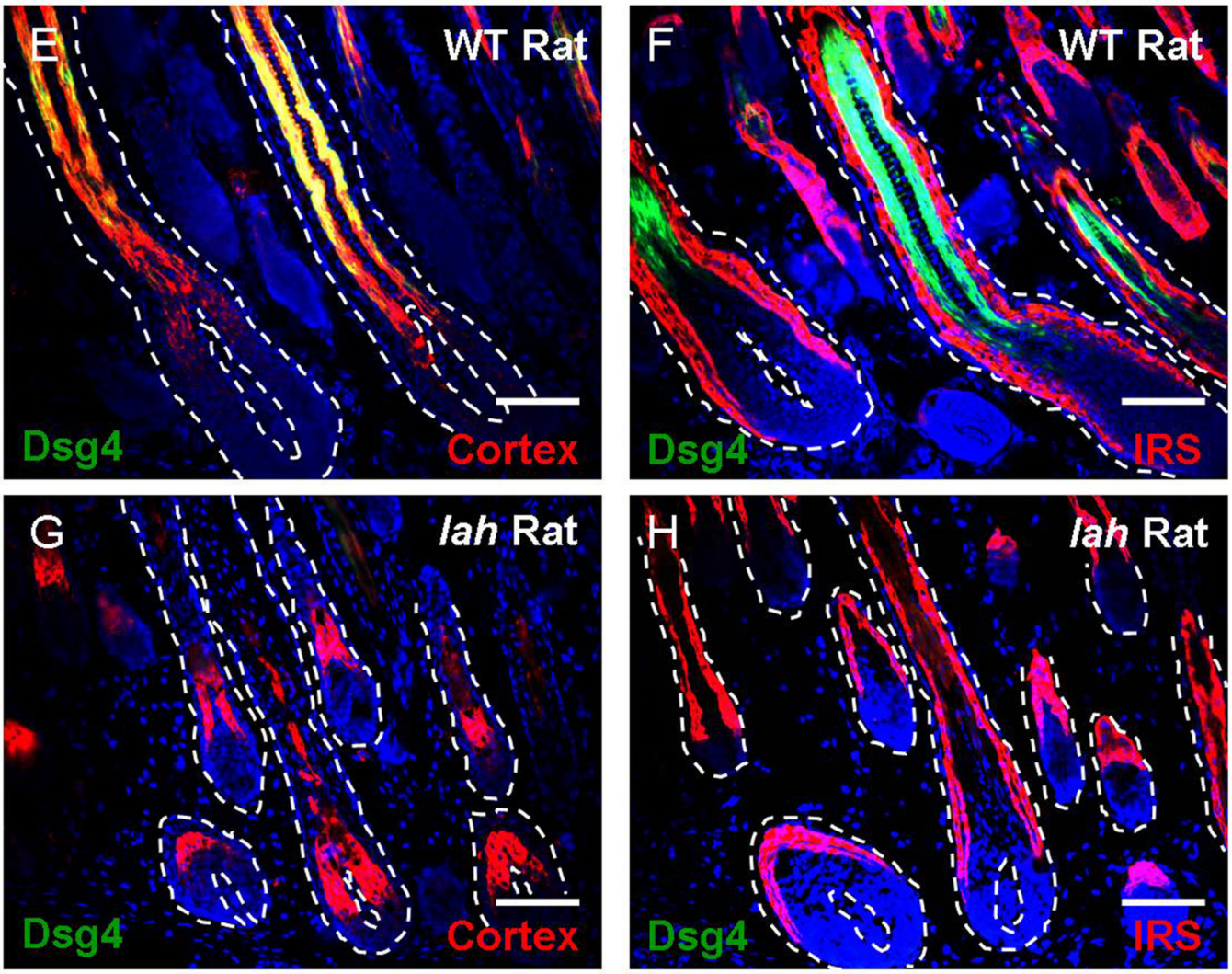


Figure 1.

Dane and Herman staining of a normal human HF (A) and a *Dsg4* −/− rat Vibrissae follicle (B). The dermal papilla is light blue. Note the abnormal keratinized mass at the tip of the abnormal precortex in (B) which is a hallmark of *Dsg4* mutations in rodents. Abbreviations: dermal papilla, DP; Outer root sheath, ORS; Inner root sheath, IRS; Cortex, Co; Pre-cortex, pre-Co; Cuticle, Cu. Scale bars: A, 100 μm; B, 40 μm.





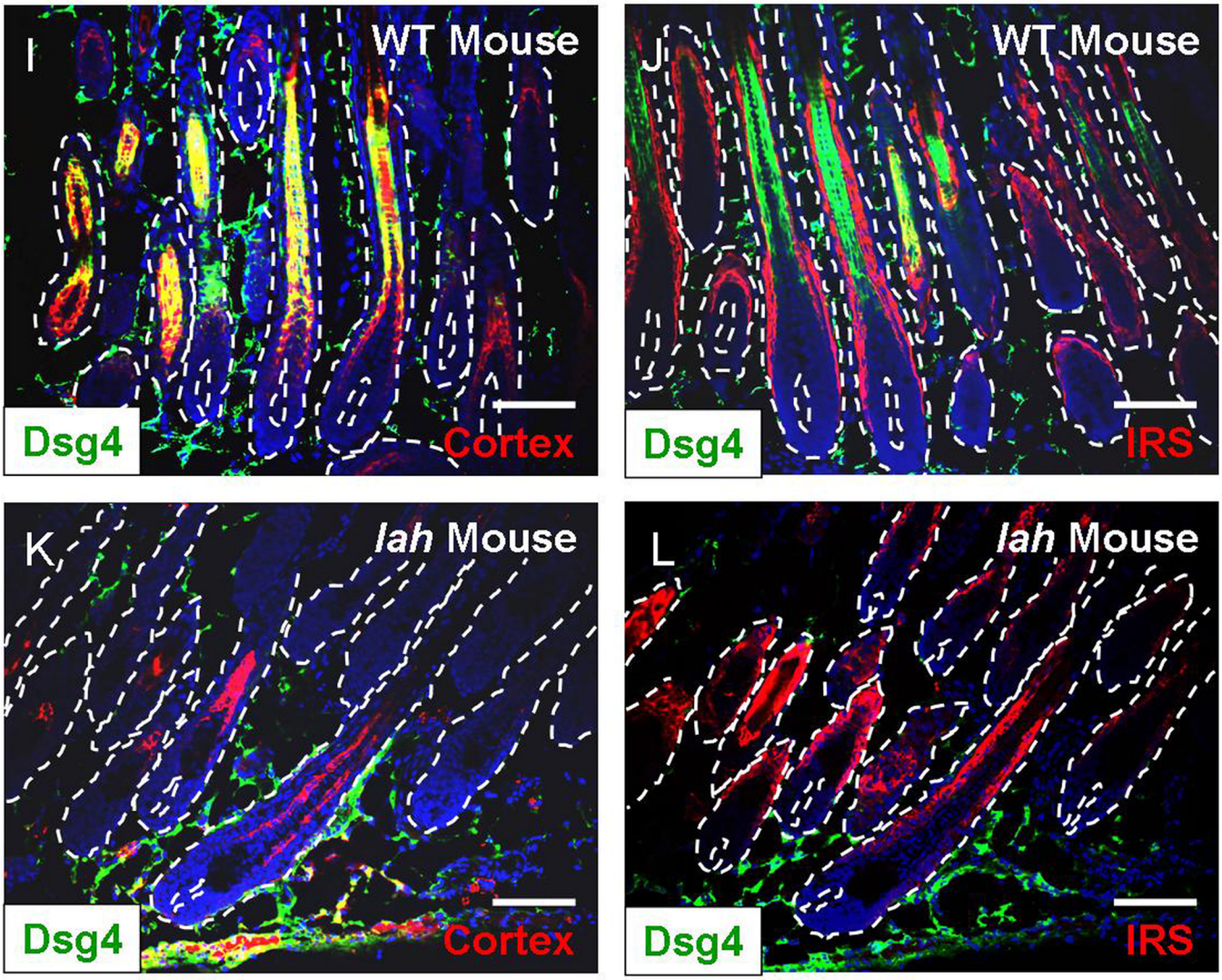


Figure 2.

Dsg4 is expressed in the cortex and IRS cuticle of rat vibrissae follicles and rat and mouse pelage follicles during anagen but absent from *Dsg4*−/− follicles. (A–L) Dsg4 is in green, the cortex hair keratin Ha1 is in red (B,E,G,I,K), the IRS keratin IRS3a.1 is in red (C,D,F,H,J,L), DNA counterstain DAPI is in blue. (A–D) Rat vibrissae follicles. (E–H) Rat pelage follicles. (I–L) mouse pelage follicles. The green staining in the dermis is background staining since the anti-Dsg4 is mouse monoclonal antibody used on mouse tissue. White dotted line in this and subsequent figures represents the basement membrane region. Scale bars: A–D, 800 μm; E–H, 100 μm; I–L, 150 μm.

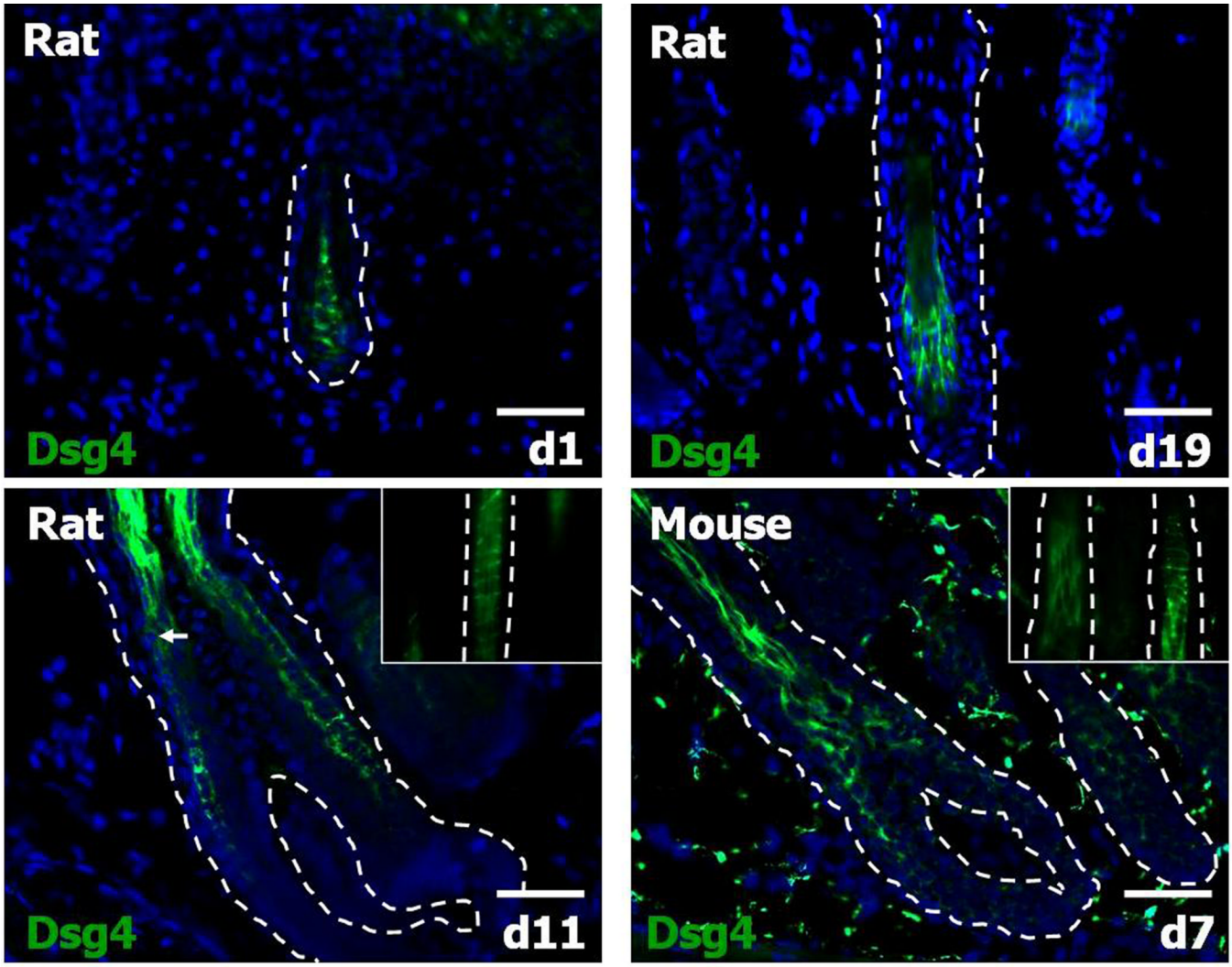


Figure 3.
Dsg4 is expressed in the forming cortex on day 1 (d1) and is more prominent in the cortex in mid to late anagen (d11) in rat pelage follicles. Dsg4 is still expressed in the receding hair cortex and around the forming club hair during catagen on d19 in rat pelage follicles. Cortical staining of Dsg4 is also evident in mid anagen mouse pelage follicles on d7 (see insets). Scale bars: Rat, 70 μm, Mouse, 50 μm.

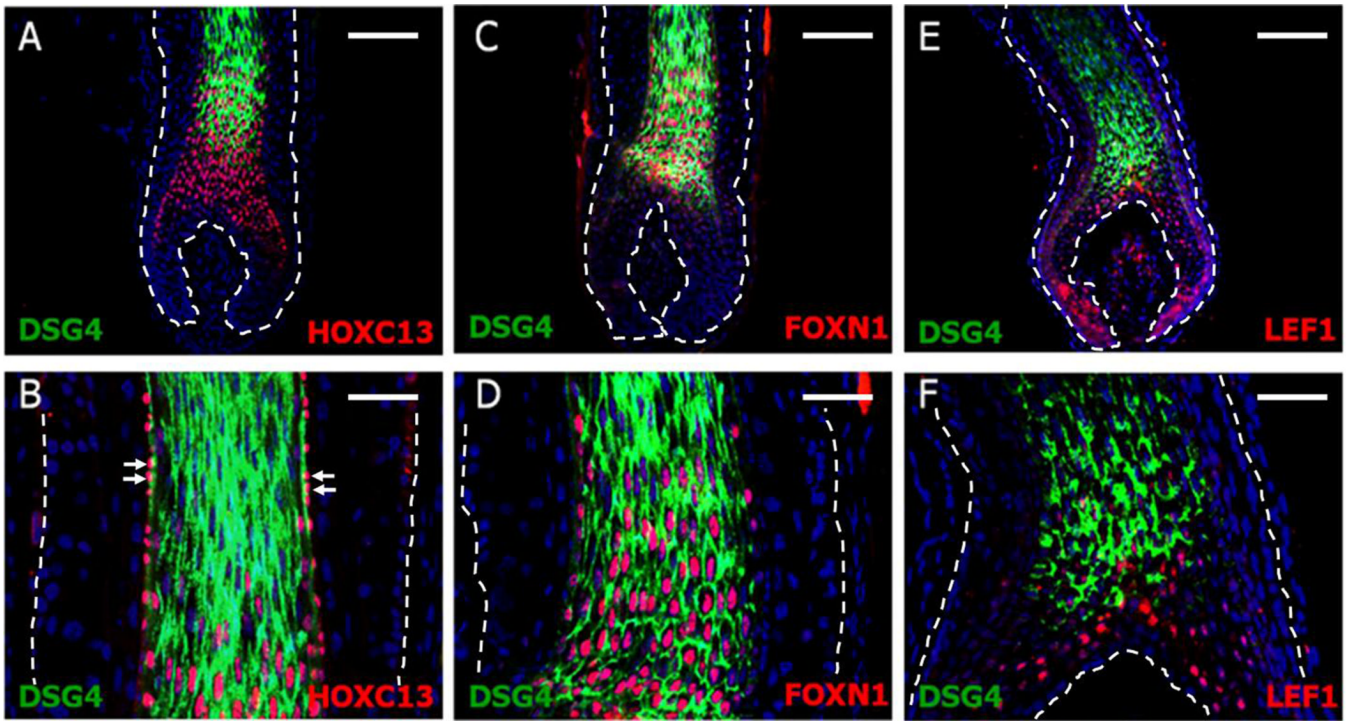
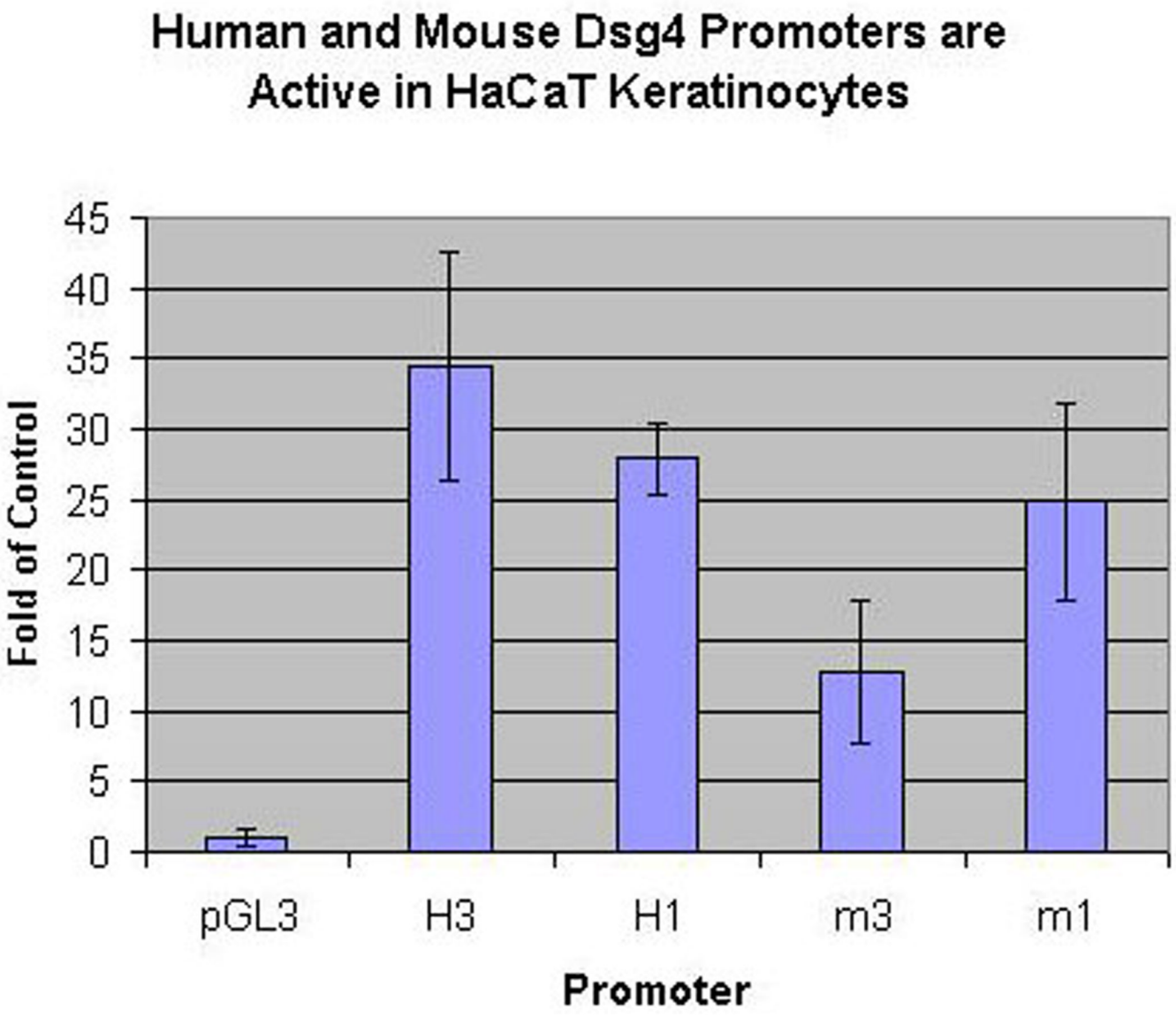
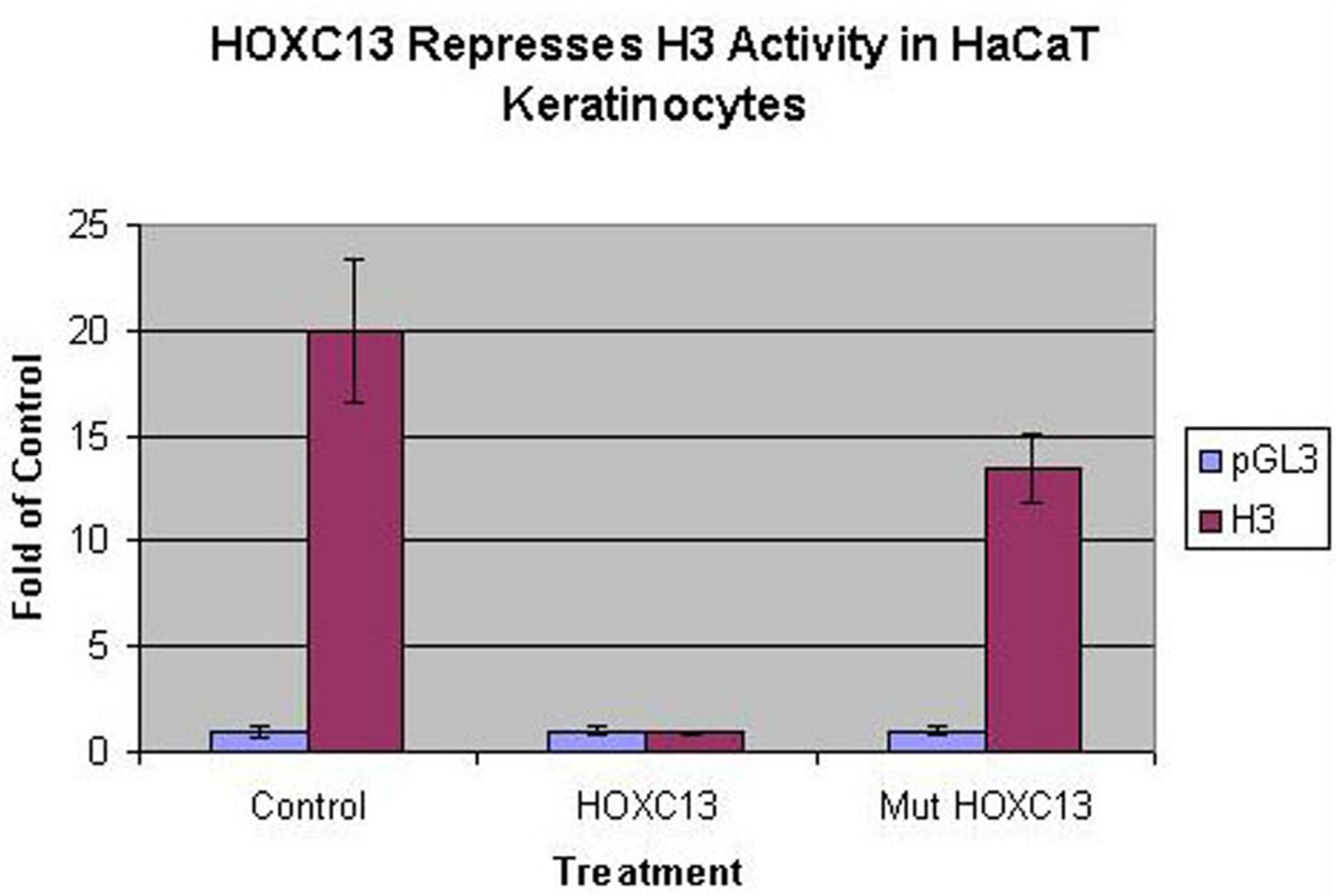
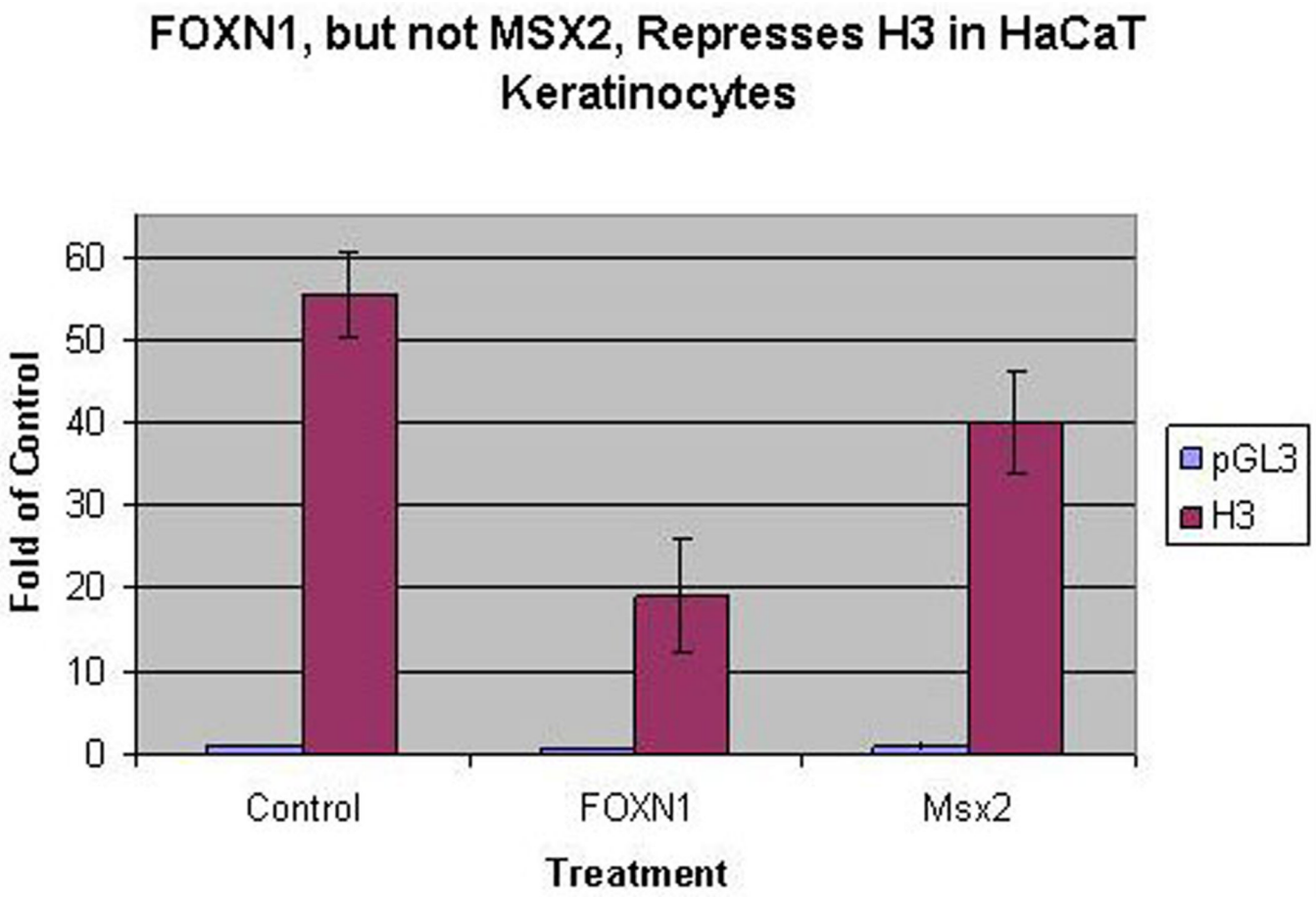


Figure 4.

DSG4 expression (green) overlaps with that of HOXC13 (A,B), FOXN1 (C,D), and LEF1 (all is red) in the precortex and sometimes cortex of the human hair follicles. Note that DSG4 and HOXC13 are co-expressed also in the IRS cuticle (arrows in B) and that DSG4 and FOXN1 overlap in the differentiating cortex (D). DAPI counterstain is in blue. Scale bars: A–E, 200 μm; B–F, 80 μm.







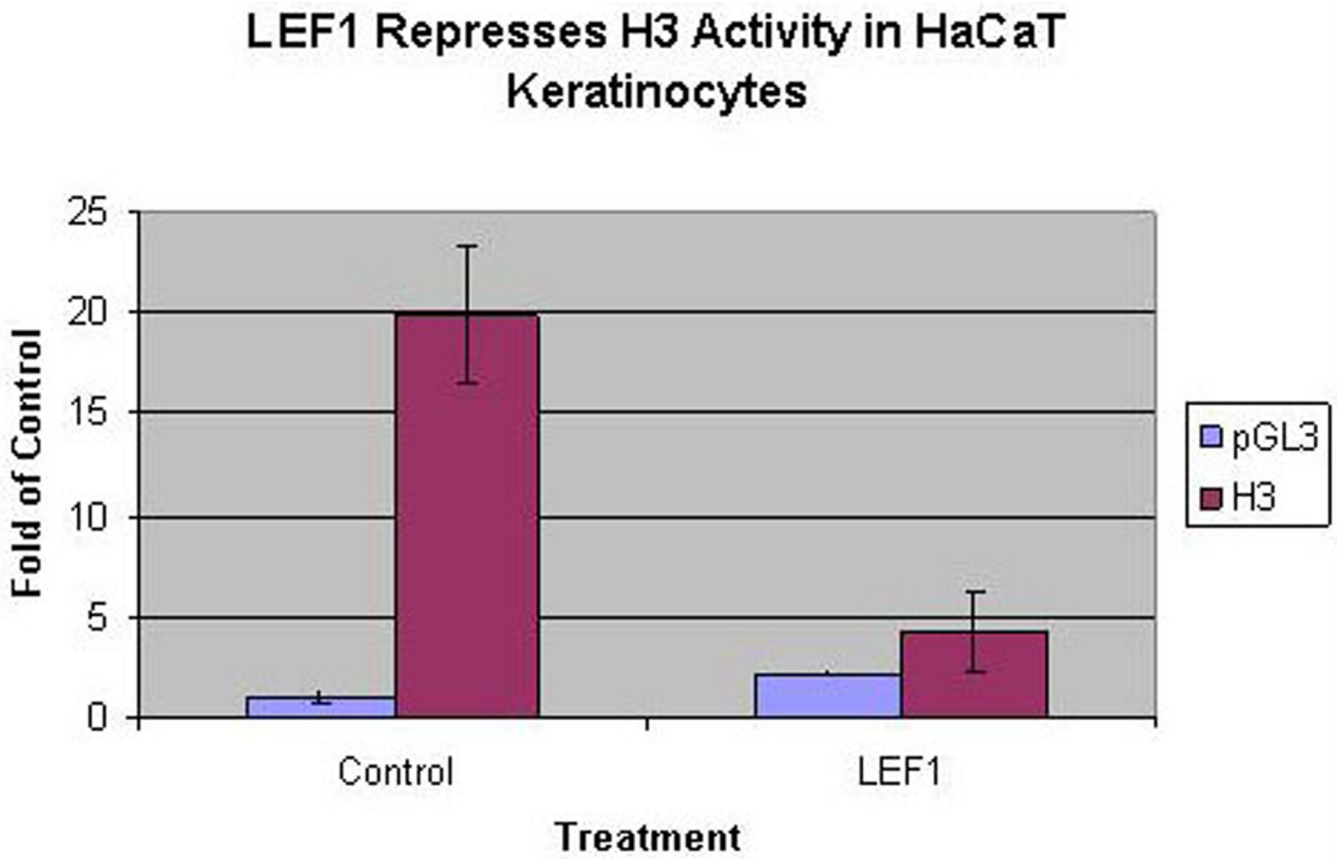
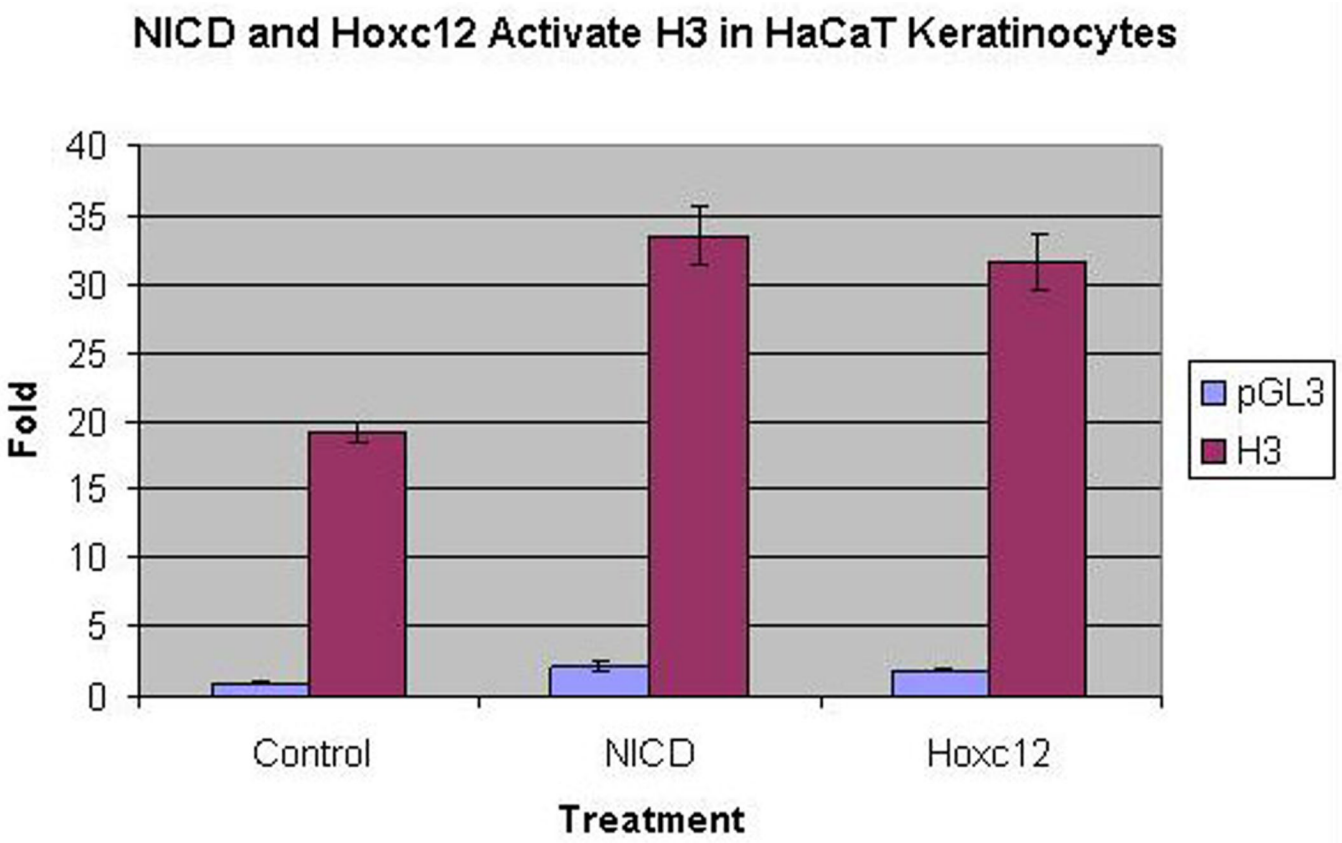


Figure 5.

The endogenous activity of *Dsg4* upstream region reporter constructs is repressed by some transcription factors in HaCaT keratinocytes. (A) ~3Kb and ~1kb upstream of human (H1,3) and mouse (m1,3) show endogenous reporter construct activity in HaCaT keratinocytes compared to the pGL3 basic vector control. (B) HOXC13, but not a mutant version (Mut HOXC13) that is not capable of binding DNA, also represses H3 in the same cells. (C) FOXN1, but not MSX2, also represses H3 although to a lesser extent than HOXC13 and LEF1. (D) LEF1 represses H3 activity in HaCaT keratinocytes to background level (also H1, m3 and m1, not shown). Error bars, standard deviation in figures 5 and 6. Student t-test was used to assess significance between control and treated groups with a p-value cutoff of 0.05 in figures 5 and 6.



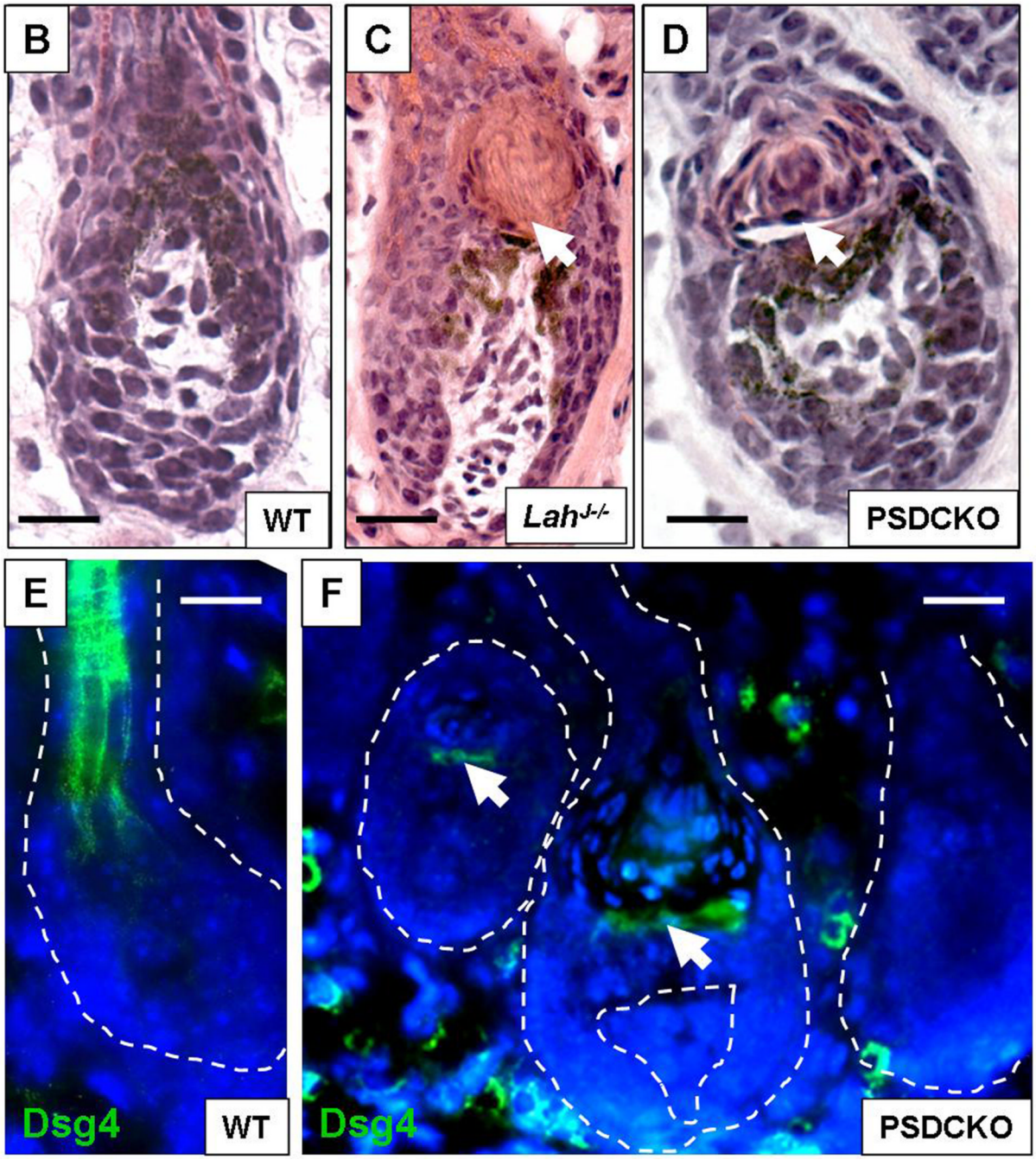


Figure 6.

Notch is a potential activator of Dsg4 expression *in vitro* and *in* vivo. (A) NICD, the downstream effector of Notch signaling, and Hoxc12 increase H3 reporter construct activity in HaCaT keratinocytes. (B–D) The precortical abnormal mass of keratinization is common to *lah* (arrow in C) and PSDCKO (arrow in D and F) mutant follicles compared to WT follicles (B). (E,F) Dsg4 expression (green) is markedly downregulated in PSDCKO follicles (F) compared to normal HFs (E). DAPI counterstain is in blue. Scale bars: B–F, 40μm.

Affy ID	Down in *lah*$^{J-/-}$	Gene Description
1427179_at	−14	keratin complex 1, acidic, gene 3
1422209_s_at	−14	keratin associated protein 9-1
1425430_at	−15	keratin associated protein 16-5
1449387_at	−15	keratin 33A
1422298_at	−15	RIKEN cDNA 5530401N06 gene
1427290_at	−16	keratin complex 2, basic, gene 19
1430509_at	−19	keratin associated protein 9-1
1419707_at	−20	keratin associated protein 14
1427365_at	−21	keratin complex 2, basic, gene 10 (Hb6)
1427842_at	−21	keratin associated protein 6-3
1421691_at	−22	keratin associated protein 16-7
1426438_at	−22	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3
1420452_at	−25	keratin associated protein 5-2
1427211_at	−28	keratin associated protein 8-1
1418742_at	−28	keratin complex 1, acidic, gene 4
1426203_at	−31	keratin associated protein 16-4
1451774_at	−32	keratin associated protein 4–10
1425655_at	−35	keratin associated protein 16-5
1425431_at	−39	keratin associated protein 16-3
1449919_at	−44	keratin associated protein 6-2
1425872_at	−50	keratin associated protein 6-1
1427801_at	−56	keratin associated protein 16-2
1419507_at	−59	keratin associated protein 15
1449560_at	−61	Keratin, high sulfur B2 protein
1427549_s_at	−62	keratin associated protein 16-3
1425237_at	−76	keratin associated protein 16-8
1451859_at	−96	keratin associated protein 6-1
1420415_at	−100	cDNA sequence AYO26312
1421689_at	−104	keratin associated protein 8-2
1420751_at	−106	keratin associated protein 6-1