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# Jagged 1 is a β-catenin target gene required for ectopic hair follicle formation in adult epidermis

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The Wnt and Notch signalling pathways regulate hair follicle maintenance, but how they intersect is unknown. We show that Notch signalling is active in the hair follicle pre-cortex, a region of high Wnt activity, where commitment to hair lineages occurs. Deletion of jagged 1 (*Jag1*) results in inhibition of the hair growth cycle and conversion of hair follicles into cysts of cells undergoing interfollicular epidermal differentiation. Conversely, activation of Notch in adult epidermis triggers expansion of the base of the hair follicle, sebaceous gland enlargement and abnormal clumping of the follicles. In adult epidermis, the induction of new hair follicle formation by β-catenin is prevented by blocking Notch signalling pharmacologically or through *Jag1* deletion. Conversely, activation of both pathways accelerates growth and differentiation of ectopic follicles. β-catenin stimulates Notch signalling by inducing *Jag1* transcription. We conclude that the Notch pathway acts downstream of the Wnt/β-catenin pathway to determine epidermal cell fate.

**KEY WORDS: β-catenin, Notch, Jagged 1, Epidermis, Mouse**

## INTRODUCTION

Mammalian epidermis is maintained throughout adult life through the activity of stem cells that lie in specific locations (Owens and Watt, 2003). Epidermal stem cells self-renew and generate transit amplifying cells that exit the cell cycle after a few rounds of division and undergo terminal differentiation along the multiple lineages that constitute the hair follicle, interfollicular epidermis (IFE) and sebaceous gland. Several evolutionarily conserved pathways that regulate embryonic skin development are important in postnatal life, including the Wnt, hedgehog, Notch and Bmp pathways (Fuchs et al., 2004). It is now clear that varying the strength, timing and duration of an individual signal exerts different effects on the epidermal stem cell compartment (Lowry et al., 2005; Silva-Vargas et al., 2005). However, little is known about how multiple pathways intersect at the level of individual cells. In this report we have investigated the interaction between the Wnt and Notch signalling pathways in the skin.

A key component of the canonical Wnt pathway is β-catenin (Reya and Clevers, 2005). In the absence of Wnt, β-catenin is degraded in the cytoplasm. When Wnt binds to its cell surface receptors, the β-catenin destruction complex is inactivated, allowing β-catenin to enter the nucleus, where it binds to transcription factors of the Lef/Tcf family and activates transcription. Inhibition of Wnt signalling prevents hair follicle formation, whereas activation of β-catenin in postnatal epidermis induces de novo follicles (Alonso and Fuchs, 2003; Lo Celso et al., 2004). Depending on the level of β-catenin activation and cellular context, β-catenin can expand the epidermal stem cell compartment (Zhu and Watt, 1999) or trigger stem cells to become transit amplifying cells (Lowry et al., 2005) and form ectopic hair follicles in adult epidermis (Alonso and Fuchs, 2003; Silva-Vargas et al., 2005).

An essential aspect of Notch signalling is a proteolytic cleavage event mediated by γ-secretase activity (Artavanis-Tsakonas et al., 1999). On interaction of the Notch receptor with its ligands γ-secretase liberates the Notch intracellular domain ($N^{ICD}$). $N^{ICD}$ translocates to the nucleus and heterodimerises with RBP-Jκ (CSL, CBF1; Rbpsuh – Mouse Genome Informatics), thereby activating target gene transcription. In contrast to Wnt, Notch signalling is not essential for embryonic specification of hair follicles; however, Notch is required for subsequent hair follicle maintenance (Lin et al., 2000; Pan et al., 2004; Uyttendaele et al., 2004; Vauclair et al., 2005). Whereas β-catenin activation is linked to skin tumour formation (Alonso and Fuchs, 2003), Notch1 acts as a tumour suppressor (Nicolas et al., 2003) and promotes differentiation of keratinocytes in vitro (Lowell et al., 2000; Rangarajan et al., 2001).

Based on the gain- and loss-of-function studies of Wnt and Notch in mouse epidermis, it is likely that the pathways interact in controlling hair follicle differentiation. Several potential mechanisms would place Notch upstream of Wnt. Notch1 activation is reported to suppress epidermal expression of Wnt4 (Devgan et al., 2005). In *Drosophila*, the region of Notch C-terminal to the cdc10/ankyrin repeats associates with Armadillo/β-catenin and negatively regulates β-catenin transcriptional activity (Hayward et al., 2005). To elucidate potential Wnt/Notch crosstalk within the epidermis, we examined where Notch is active, the impact of Notch loss of function on β-catenin-induced hair follicle formation, and the consequences of simultaneously activating both pathways in the same cells.

## MATERIALS AND METHODS

### Mice

$Jag1^{flox/flox}$ mice (gift of J. Lewis) (Brooker et al., 2006) were crossed with K5Cre mice (gift of Jose Jorcano) (Ramirez et al., 2004), K14CreER mice (gift of Barry Stripp) (Hong et al., 2004) and K14ΔNβ-cateninER mice (D2 line) (Lo Celso et al., 2004). TNR mice, which contain a transgene that acts as a GFP reporter of Notch activity (Duncan et al., 2005), were the gift of N. Gaiano. K14ΔNLef1 mice were also examined (Niemann et al., 2002).

The $N^{ICD\Delta OP}$ER transgene consists of amino acids 1751-2290 of the mouse Notch1 intracellular domain ($N^{ICD\Delta OP}$) with an N-terminal FLAG sequence and a modified oestrogen receptor (ER) fused in frame at the C terminus (gifts of U. Just and P. Chambon) (Schroeder and Just, 2000). The

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$N^{ICD\Delta OP}$ER construct was subcloned into the *Bam*HI restriction site of the Keratin 14 (K14) promoter cassette provided by E. Fuchs (Vasioukhin et al., 1999) and injected into the pronucleus of (CBA×C57bl) F1 embryos. Potential founder lines were screened by PCR (forward: rabbit β-globin intron 5′-TACTCTGAGTCCAAACCGGGC-3′ and reverse: mouse Notch1 5′-CACTCGTTCTGATTGTCGTC-3′) and confirmed by Southern blotting. Founder lines 5199.15A,B+C (CA3, low copy number) and 5199.21A,B+C (CA4, high copy number) were established; both lines had a similar phenotype, the severity correlating with copy number.

Line 5199.21A,B+C was used to characterise the K14$N^{ICD\Delta OP}$ER phenotype. We examined 34 4OHT-treated transgenic mice, 23 acetone-treated transgenic mice and 18 4OHT-treated wild-type mice. The animals were analysed between 6 and 13 weeks of age.

To activate transgenes that contained an ER domain, mice were treated topically with 1-3 mg 4OHT (Sigma) as described (Lo Celso et al., 2004). Some mice were treated with 1 mg N-S-phenyl-glycine-t-butyl ester (DAPT, Sigma), dissolved in 1% DMSO/acetone. When mice were treated with DAPT and 4OHT, DAPT was applied 30 minutes before 4OHT.

### Histology and immunohistochemistry

Whole mounts, frozen sections and paraffin-embedded sections were prepared and immunolabelled as described previously (Braun et al., 2003; Lo Celso et al., 2004). Antibodies to the following proteins were used: CCAAT displacement protein (CDP, a gift of M. Busslinger, IMP, Vienna, Austria), Ki67 (Vector Laboratories), trichohyalin (AE15, a gift of T. T. Sun, NYU Medical Center, New York, USA), Jag1 (Santa Cruz Biotechnologies), keratin 14 (MK14, Covance), keratin 10 (MK10, Covance), β1 integrin (MB1.2, a gift of B. Chan, University of Western Ontario, London, Canada), oestrogen receptor (HC-20, Santa Cruz Biotechnologies), green fluorescent protein (GFP, Molecular Probes or Abcam), inner root sheath keratins (AE13; a gift of T. T. Sun), cleaved Notch1 (Val 1744, Cell Signaling Technologies or AB8925, Abcam) and β-catenin (Santa Cruz Biotechnologies). Haematoxylin or DAPI was used as nuclear counterstain.

In whole mounts of tail skin, $N^{ICD}$ was detected using the TSA Plus Amplification Kit (Perkin Elmer). Tail skin was incubated with primary antibody overnight, followed by an overnight incubation with an HRP-conjugated anti-rabbit secondary antibody (Amersham), reacted with fluorescein-conjugated tyramide and mounted with Mowiol.

### Luciferase assays

Spontaneously immortalised keratinocytes from wild type and K14ΔNβ-cateninER mice were grown on a feeder layer of J2-3T3 cells (Lo Celso et al., 2004). To measure Notch and β-catenin dependent transcription, $8\times10^4$ or $2\times10^5$ cells were plated per 35 mm well in KSFM. Cells were transfected using Lipofectamine 2000 (Invitrogen) with Hes1 luciferase, pCDNA3 mNotch1$^{ICD}$, pCDNA3 hNotch1 or TOPFlash luciferase. FOPFlash luciferase, pRL and pCI empty vectors served as controls. Post-transfection, cells were cultured in KSFM with or without 200 nmol/l 4OHT and/or 1 μmol/l DAPT. Forty-eight or 72 hours after transfection, luciferase levels were measured as previously described (Silva-Vargas et al., 2005). Statistical significance was determined with Student's *t*-test using QuickCalcs software (GraphPad Software).

### RT-PCR

Spontaneously immortalised mouse keratinocytes were plated for 48 hours in KSFM (Gibco) and then treated with cycloheximide (25 μmol/l) for 30 minutes. Cells were incubated for a further 4 hours in medium containing cycloheximide and 200 nmol/l 4OHT (Sigma) or acetone. Total RNA was isolated from cells using TriReagent (Helena Bioscience), and subjected to reverse transcription using Superscript reverse transcriptase (Invitrogen). cDNAs were amplified by PCR using the following oligonucleotide pair as *Jag1* primers: 5′-TCCAGGTCTTACCACCGAAC-3′ and 5′-GGACGCCT-CTGAACTCTGAC-3′. Mouse β-actin primers were described previously (Giangreco et al., 2004).

### *Jag1* promoter analysis

We used TESS software to analyse the promoter sequence of *Jag1*. This software is freely available online at: http://www.cbil.upenn.edu/cgi-bin/tess/tess.

For ChIP assays, spontaneously immortalised mouse keratinocytes were plated without feeders for 48 hours in complete medium and then treated with 200 nmol/l 4OHT for 4 hours. $1\times10^6$ cells were used per immunoprecipitation and processed using a ChIP assay kit (Upstate) according to the manufacturer's instructions. Immunoprecipitations were performed using a rabbit antibody to β-catenin (Santa Cruz Biotechnologies) and an isotype-matched control antibody with an overnight incubation at 4°C. Immunoprecipitated DNA was amplified using the following PCR primer pairs specific for the putative Tcf/Lef binding sites or irrelevant sequence in the mouse *Jag1* promoter: 4827-4334: 5′-GCCAAACCAACGCTTAACAT-3′; 5′-CATAGCCAGAGGCAGACACA-3′; 3580-3157: 5′-TCCATCCC-CCAATTAAGACA-3′; 5′-TGATGCCTCAATTCCCTTTC-3′; 2958-2553: 5′-TTCAGGGGTGATCAAGGAAG-3′; 5′-GAGCCAGCCACCTGAG-TTAC-3′; 2618-2139: 5′-TTTCACGAAGCCCAGATTGT-3′; 5′-ATT-TTCCAGTGTGCCCAGTC-3′; 1778-1359: 5′-TAGAAGGGTTGAG-GCGCTAA-3′; 5′-CTTTCCCGGAGTCAGACTTG-3′; 831-370: 5′-CCTTCCAGGTTCCTTTCTCC-3′; 5′-CCTCTCGGCTTTCTTTCCTT-3′.

## RESULTS

### Notch signalling in adult epidermis

We used a transgenic GFP reporter of Notch activity (TNR mice) (Duncan et al., 2005) to determine where Notch was active in adult epidermis (Fig. 1A-C). GFP was detected in the pre-cortex of growing (anagen) hair follicles, the cuticle of the inner root sheath, the outer root sheath and the dermal papilla (Fig. 1A). The pre-cortex is the zone at the base of the follicle in which selection of most of the hair follicle lineages takes place (Legue and Nicolas, 2005). As previously reported (Pan et al., 2004; Vauclair et al., 2005), the pre-cortex was positively labelled with an antibody to $N^{ICD}$ (Fig. 1D,E), confirming that it is a region of Notch signalling activity. The same region is known to express Lef1 and to be a site of β-catenin reporter activity (DasGupta and Fuchs, 1999). Notch-GFP reporter activity, and positive staining for $N^{ICD}$, was also detected in small clusters of cells of the basal layer of interfollicular tail epidermis and throughout the suprabasal layers (Lowell et al., 2000) (Fig. 1B,C; data not shown).

The Notch ligand, Delta1, is expressed during embryonic hair follicle development but not in postnatal skin (Favier et al., 2000; Powell et al., 1998). In adult skin jagged 1 was expressed in the pre-cortex and matrix (the proliferative zone below the pre-cortex) of growing hair follicles, in a stripe of cells along the outer root sheath, and in clusters of cells in the IFE (Fig. 1A,F,G). Therefore, the locations of jagged 1 expression corresponded to the locations of Notch activity in postnatal epidermis. Jagged 1 expression and Notch activity were elevated in regions of the epidermis where cells were undergoing commitment to terminal differentiation and thus appear to be associated with transit amplifying cells rather than stem cells (Legue and Nicolas, 2005; Lowell et al., 2000).

### Consequences of inhibiting or activating the Notch pathway

To examine the role of Jag1 in adult mouse skin, we crossed Jag1$^{flox/flox}$ mice (Brooker et al., 2006) with K5Cre mice (Fig. 1H,P). As expected, Jag1 protein was not detected in K5Cre Jag1$^{flox/flox}$ epidermis (Fig. 1H). In addition, hair follicle staining with an antibody to $N^{ICD}$ (activated Notch) was reduced on deletion of *Jag1* (Fig. 1I,J). The K5Cre Jag1$^{flox/flox}$ mice appeared normal at birth but started to lose their hair and whiskers at 5 weeks and were completely bald at 7.5 weeks. Subsequent hair growth was sparse and the hairs were short, resembling the Notch1 deletion phenotype (Vauclair et al., 2005) (Fig. 1P).

To activate the Notch pathway in keratinocytes, we used a truncated form (amino acids 1751-2290) of the intracellular domain of mNotch1 ($N^{ICD\Delta OP}$) coupled to a modified form of the human oestrogen

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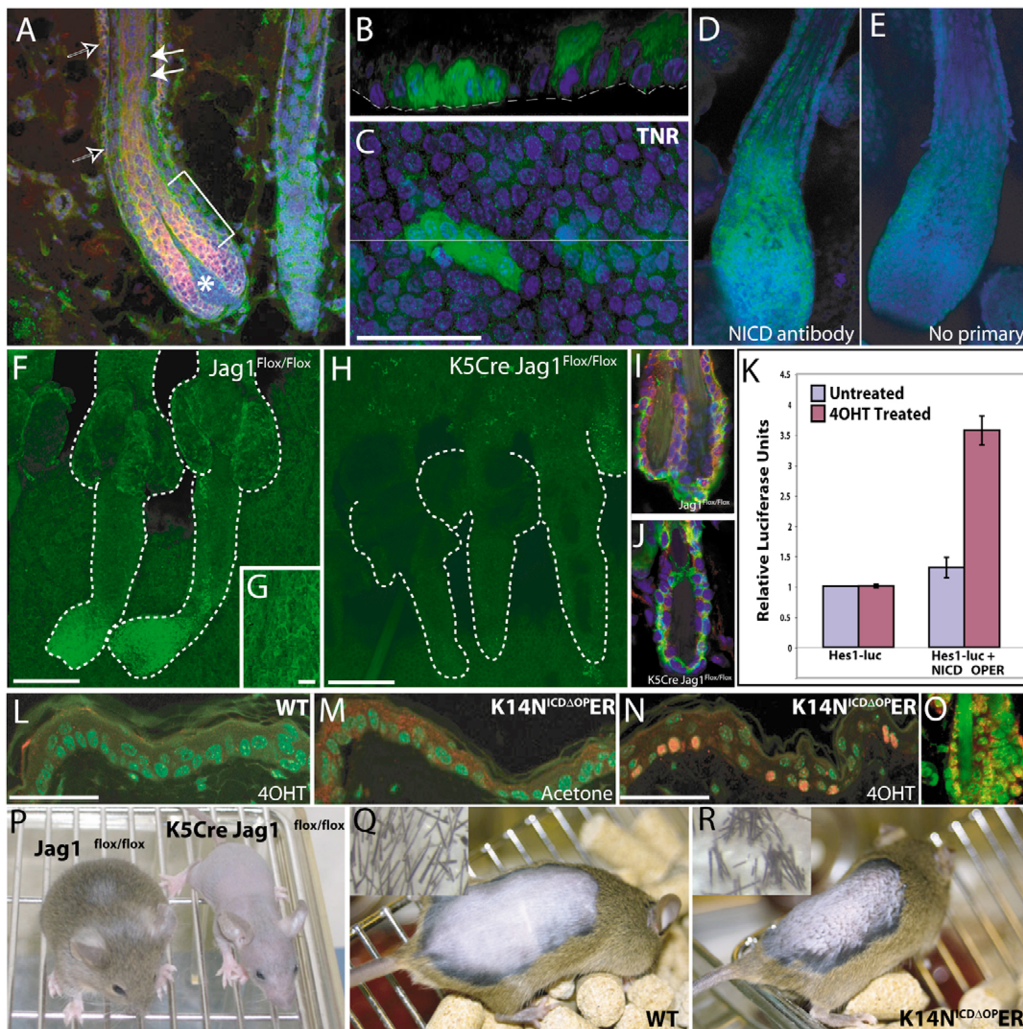


**Fig. 1. Modulation of Notch activity in mouse epidermis.** (**A**) Anti-GFP (green) and anti-Jag1 (red) immunostaining with DAPI (blue) counterstain in a section of back skin anagen hair follicle of a TNR mouse. The bracket represents the pre-cortex, open arrows the outer root sheath, solid arrows the inner root sheath and asterisk the dermal papilla. (**B**,**C**) Tail epidermal whole mount of TNR mouse labelled with anti-GFP (green) antibody and DAPI (blue). (B) An optical transverse section at the position of the white line through *z*-stack images in C. The dashed line in B represents the basal epidermal surface. (**D**,**E**) Single plane images of wild-type tail epidermal whole mounts labelled with antibody to activated Notch (NICD) (D) or secondary antibody alone (E) (green) with DAPI (blue) counterstain. (**F-H**) Whole mounts of Jag1$^{flox/flox}$ (F,G) and K5Cre Jag1$^{flox/flox}$ (H) epidermis immunolabelled with anti-Jag1 antibody. The dashed lines indicate hair follicles and sebaceous glands. (G) Jagged 1 expression in the IFE. (**I**,**J**) Sections of Jag1$^{flox/flox}$ (I) and K5Cre Jag1$^{flox/flox}$(J) hair follicles labelled with antibodies to keratin 14 (green) and cleaved Notch1 (red) and DAPI counterstain (blue). (**K**) Mouse keratinocytes transiently transfected with Hes1 luciferase reporter (Hes1-luc) alone or in combination with N$^{ICD\Delta OP}$ER transgene and either treated with 4OHT (red bars) or untreated (blue bars). Data are expressed as average light units ±s.d., relative to the Renilla luciferase control. (**L-O**) Anti-ER immunostaining (red) with DAPI counterstain (green) of back skin from wild-type (L) or K14N$^{ICD\Delta OP}$ER transgenic (M-O) mice treated with acetone (M) or 4OHT (L,N,O) for 1 hour. Scale bars: 50 μm in B,C,I,J,L-N; 100 μm in A-H. (**P-R**) Gross phenotype of 7.5-week-old K5Cre Jag1$^{flox/flox}$ and Jag1$^{flox/flox}$ littermates (P), and wild-type (Q) and K14N$^{ICD\Delta OP}$ER (R) mice treated with 4OHT for 14 days. Insets in Q and R are higher magnification images of back skin.

receptor (ER). This activatable form of Notch lacks the C-terminal region (OPA and PEST domains) but contains the RAM/ankyrin repeats that mediate interaction with RBP-Jκ and transcription of Notch target genes (Schroeder and Just, 2000). In vitro, mouse keratinocytes transiently transfected with the N$^{ICD\Delta OP}$ER construct activated a Notch-responsive Hes1 luciferase reporter only in the presence of 4-hydroxytamoxifen (4OHT) (Fig. 1K).

We generated mice expressing N$^{ICD\Delta OP}$ER under the control of the K14 promoter (Fig. 1M-O,R). When sections of epidermis from transgene-negative mice were stained with an antibody to the ER, no staining was detected (Fig. 1L). In K14N$^{ICD\Delta OP}$ER transgenic mice treated with acetone, the ER epitope was detected in the cytoplasm of cells in the regions of known K14 promoter activity: the basal layer of the IFE, the outer root sheath of the hair follicle and the periphery of the sebaceous glands (Fig. 1M; data not shown). In addition there was weak suprabasal staining in some regions of the IFE, possibly due to the long half-life of the protein in the absence of 4OHT (Fig. 1M). In transgenic mice treated with 4OHT, ER expression was, as predicted, predominantly nuclear in transgene-positive cells in the IFE, sebaceous gland and hair follicle, and no suprabasal nuclear staining was observed (Fig. 1N,O; data not shown).

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Whereas *Jag1* deletion resulted in hair loss (Fig. 1P), Notch activation resulted in clumping of hair follicles, which was readily observed macroscopically (Fig. 1Q,R). At 6.5-10 weeks, the hair follicles of wild-type mice were in the resting (telogen) phase of the hair cycle and 4OHT treatment did not stimulate hair re-growth (Fig. 1Q). After 14 days of 4OHT treatment, K14N$^{ICD\Delta OP}$ER transgenic mice did not enter anagen, but their follicles were abnormally clustered, and the hairs were not uniformly oriented (Fig. 1R). The patchy phenotype was not due to low Notch activity, because it was seen in both homozygous and heterozygous transgenic mice (data not shown), and did not reflect inefficient induction of the transgene, as evidenced by the nuclear localisation of N$^{ICD\Delta OP}$ER (Fig. 1N,O). The fact that the mice failed to exhibit uniform hair re-growth in response to 4OHT treatment demonstrates that activation of Notch, unlike β-catenin (Lo Celso et al., 2004), is not sufficient to induce anagen.

## Notch signalling is required for differentiation of hair follicle lineages

We analysed the histology of Jag1$^{flox/flox}$, K5Cre Jag1$^{flox/flox}$ and K14N$^{ICD\Delta OP}$ER back skin and performed immunolabelling with antibodies to differentiation markers. As controls, K14N$^{ICD\Delta OP}$ER mice were treated with acetone, and Jag1$^{flox/flox}$ and wild-type mice were either untreated or treated with acetone or 4OHT. All controls were indistinguishable from untreated wild-type skin (Fig. 2A,B; data not shown).

K5Cre Jag1$^{flox/flox}$ back skin was examined at 5 weeks (Fig. 2C). In contrast to Jag1$^{flox/flox}$ follicles, which were in anagen (Fig. 2A), the K5Cre Jag1$^{flox/flox}$ follicles were in the resting (telogen) state. Seven and a half-week-old mice that were bald (Fig. 1P) had normal sebaceous glands, but developed epidermal cysts (Fig. 2D). The hair follicles failed to enter anagen and were thinner than normal (Fig. 2E). Conversely, the IFE was thicker in *Jag1*-null than wild-type epidermis (Fig. 2B,E). At 10 weeks, when control epidermis was once more in telogen (Fig. 2B), some hair regrowth was observed in K5Cre Jag1$^{flox/flox}$ mice, but the hairs were sparse and short (data not shown). This correlated with the presence of epidermal cysts and abnormal hair follicles (Fig. 2E), and resembled the phenotype of mice with epidermal deletion of γ-secretase, *Notch1* or both *Notch1* and *Notch2* (Pan et al., 2004; Vauclair et al., 2005).

We next performed histological analysis of the skin of 10- to 13-week-old 4OHT-treated K14N$^{ICD\Delta OP}$ER mice. This confirmed that the hair follicles were unevenly spaced (Fig. 1R), with multiple follicles often in close proximity (Fig. 2F). The patchy distribution of the follicles might be caused by changes in the interfollicular epidermal surface area between follicles, although this remains to be investigated. Similar to 10-week-old Jag1$^{flox/flox}$ mice, which had a wild-type phenotype, the follicles in 4OHT-treated transgenic mice were mainly in telogen (Fig. 2B,F). This supports the conclusion that Notch is not sufficient to induce hair follicle growth.

In treated K14N$^{ICD\Delta OP}$ER transgenic mice that were of an age (13 weeks) at which hairs had re-entered anagen, enlarged hair follicle bulbs and sebaceous glands were found (Fig. 2G,H). In many cases the enlarged bulbs appeared to have detached from the rest of the follicle, forming cysts resembling massive hair bulbs with aberrant hair shafts (Fig. 2H).

In order to characterise the *Jag1* deletion and Notch activation phenotypes further, we looked at expression of markers for the different epidermal lineages. K10, a marker of IFE terminal differentiation (Niemann et al., 2002), was expressed in the suprabasal layers of normal IFE (Fig. 3A). We observed increased numbers of K10-positive layers in the thickened K5Cre Jag1$^{flox/flox}$ IFE from back and tail skin compared with controls (Fig. 3B; data not shown). *Jag1*-null cysts were filled with cornified material (Fig. 2E) and were K10-positive (Fig. 3D). Although the IFE of 4OHT-treated K14N$^{ICD\Delta OP}$ER mice was also thicker than wild-type epidermis (Fig. 2F-H), expression of K10 was lost in large areas and there was a corresponding increase in the number of K14-positive

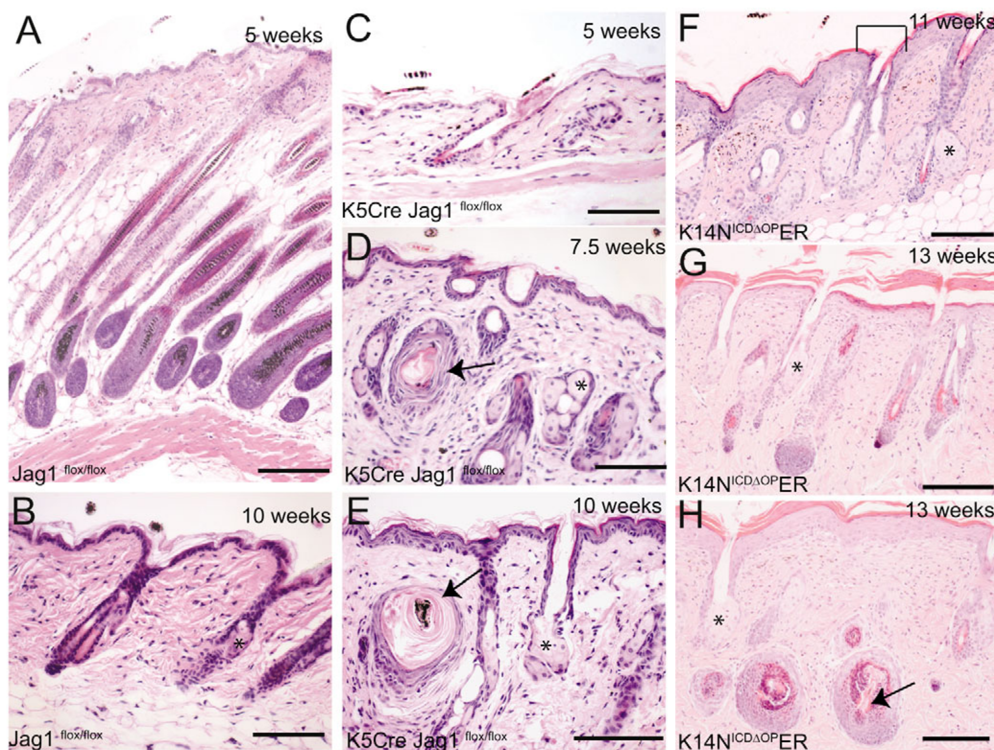


**Fig. 2. Histological analysis of K5Cre Jag1$^{flox/flox}$, K14CreER Jag1$^{flox/flox}$ and K14N$^{ICD\Delta OP}$ER skin.** (**A-H**) H&E stained sections of back skin of Jag1$^{flox/flox}$ mice aged 5 (A) and 10 (B) weeks, K5Cre Jag1$^{flox/flox}$ mice aged 5 (C), 7.5 (D) and 10 (E) weeks, and 4OHT-treated (21 days) K14N$^{ICD\Delta OP}$ER mice aged 11 (F) and 13 (G,H) weeks. Arrows indicate epidermal cysts, asterisks mark sebaceous glands, and bracket denotes hair follicle doublet. Scale bars: 100 μm in A-E; 200 μm in F-H.

cell layers (Fig. 3C; data not shown). The loss of K10 in K14N$^{ICD\Delta OP}$ER epidermis did not reflect a failure of IFE differentiation, because the cornified layers appeared normal (Fig. 3C). Instead, loss of K10 correlated with epidermal hyperproliferation, as judged by an increased number of Ki67-positive cells and expression of keratin 17 (data not shown).

We also examined expression of CDP, a marker of the companion layer and hair follicle bulb, and the inner root sheath marker, trichohyalin (Niemann and Watt, 2002). Expression of CDP (Fig. 3H-K,Q,U) and trichohyalin (Fig. 3L-O) was lost in K5Cre Jag1$^{flox/flox}$ epidermis, and whole-mount labelling of tail epidermis (Braun et al., 2003) confirmed that the hair follicles were thinner than controls (Fig. 3P,T). *Jag1* null cysts also lacked expression of CDP, trichohyalin and inner root sheath keratins (data not shown). By contrast, the enlarged hair bulbs in K14N$^{ICD\Delta OP}$ER epidermis were CDP-positive, and expressed trichohyalin and the inner root sheath keratins detected with antibody AE13 (Lo Celso et al., 2004) (Fig. 3E-G).

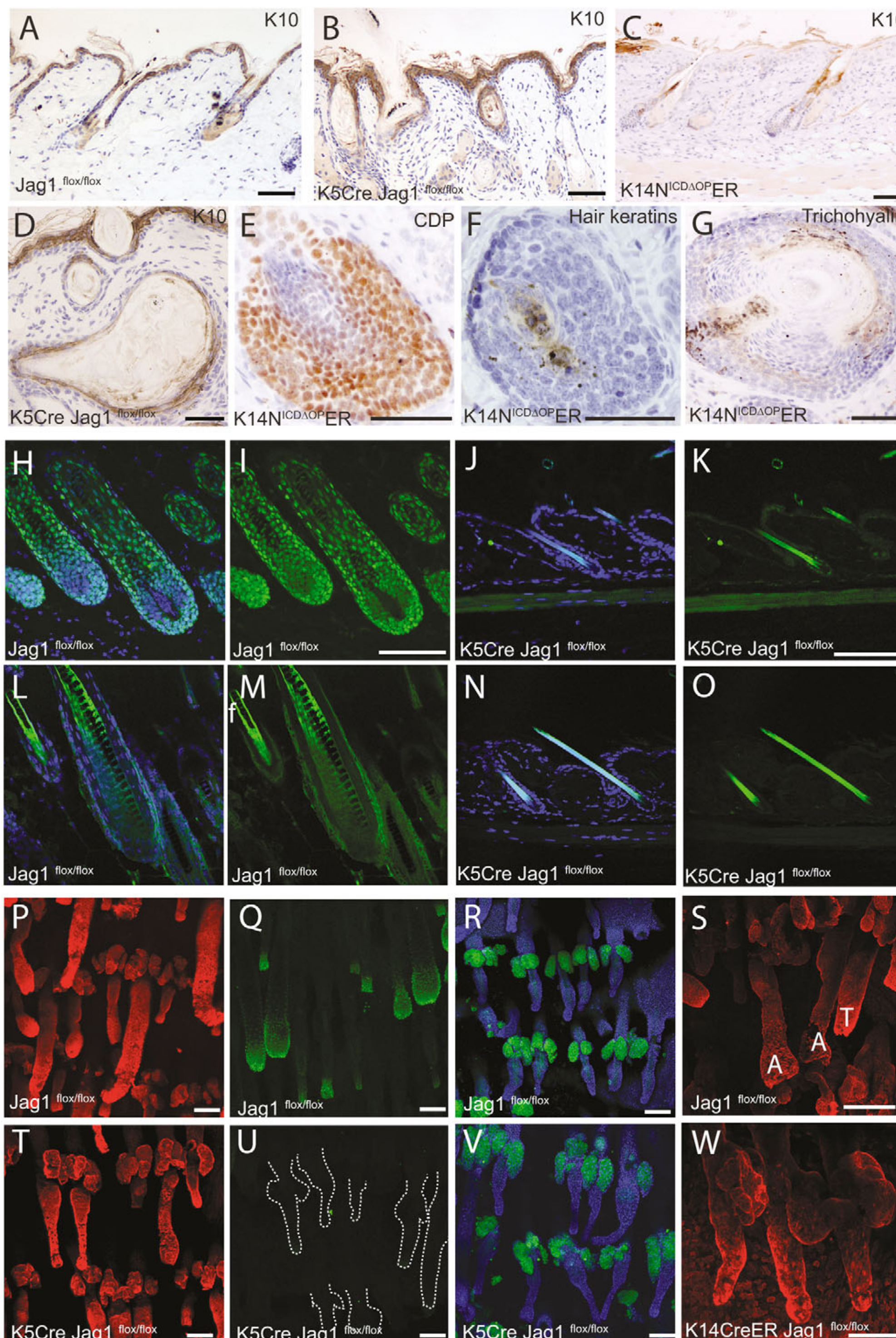


**Fig. 3. Analysis of differentiation markers in K5Cre Jag1$^{flox/flox}$, K14CreER Jag1$^{flox/flox}$ and K14N$^{ICD\Delta OP}$ER skin.** (**A-O**) Back skin of Jag1$^{flox/flox}$ (A,H,I,L,M), K5Cre Jag1$^{flox/flox}$ (B,D,J,K,N,O) and 4OHT-treated K14N$^{ICD\Delta OP}$ER (C,E,F,G) mice stained with antibodies to K10 (A-D), CDP (E,H-K), hair keratins (AE13) (F) or trichohyalin (G,L-O). (H,J,L,N) Sections DAPI counterstained (blue). (**P-W**) Whole mounts of Jag1$^{flox/flox}$ (P-S), K5Cre Jag1$^{flox/flox}$ (T-V) and K14CreER Jag1$^{flox/flox}$ (W) tail epidermis immunolabelled for K14 (P,T,S,W) and CDP (Q,U) or stained with Nile Red (green) and DAPI (blue) (R,V). Dashed lines outline the hair follicles and sebaceous glands. Scale bars: 50 μm in D; 100 μm in A-C,E-W. A, anagen follicles; T, telogen follicles.

Whereas Notch activation in 4OHT-treated K14N$^{ICD\Delta OP}$ER epidermis resulted in enlarged sebaceous glands (Fig. 2F), *Jag1* deletion did not affect the size of the sebaceous glands. However, when visualised by Nile Red staining of tail epidermal whole mounts, *Jag1*-null sebaceous glands were somewhat irregular in shape (Fig. 3R,V).

To examine whether there was an ongoing requirement for Jag1 in adult epidermis, we crossed Jag1$^{flox/flox}$ mice with K14CreER mice and deleted *Jag1* by topical application of 4OHT (Fig. 3S,W). Mice were treated with 4OHT or acetone for 3 weeks, starting when they were 8 weeks old. At 11 weeks, 54% of hair follicles in control, acetone-treated tail epidermis were entering anagen, as evaluated by strong CDP expression and the morphology of follicles in epidermal whole mounts (data not shown; Fig. 3S). By contrast, following deletion of *Jag1*, the number of anagen follicles in 11-week-old mice was reduced to 6.5% (Fig. 3W). We conclude that there is an ongoing requirement for Jag1 during the postnatal hair cycles.

The phenotypes of the K5Cre Jag1$^{flox/flox}$ and the K14N$^{ICD\Delta OP}$ER mice suggest that Notch signalling is required for, and promotes, differentiation of the mature hair follicle lineages, consistent with the observation that γ-secretase is required to maintain the inner root sheath cell fates (Pan et al., 2004). The similarities between epidermis lacking *Notch1* (Vauclair et al., 2005) and epidermis lacking *Jag1* (Fig. 2) identify jagged 1 as the key ligand for Notch signalling in postnatal epidermis.

### Notch activity is required downstream of β-catenin for ectopic hair follicle formation

Unlike β-catenin activation, Notch activation did not promote anagen or induce ectopic follicles, and this would tend to place Notch downstream of β-catenin. To investigate how the two pathways interacted, we examined microarrays of genes induced in response to β-catenin activation in K14ΔNβ-cateninER mice (Silva-Vargas et al., 2005). *Jag1* and the downstream Notch targets, Hes and Hey family members (Iso et al., 2003), were upregulated upon ectopic β-catenin activation (Table 1). The array results were confirmed by RNA in situ hybridisation (data not shown).

**Table 1. Fold upregulation of Notch family members in the skin of K14ΔNβ-cateninER mice**

| mRNA | Average fold upregulation in 7-day treated skin* |
|---|---|
| *Hes5* | 8.6 |
| *HeyL* | 3.7 |
| *Hey2* | 2.5 |
| *Jag1* | 2.1 |
| *Hes1* | 1.6 |

The relative mRNA levels of *Jag1* and the Notch targets *Hes1*, *Hes5*, *Hey1* and *HeyL* were compared in K14ΔNβ-cateninER mice treated for 7 days with 4OHT. Microarray analysis was performed as previously described (Silva-Vargas et al., 2005). *$P$<0.05.

To test the hypothesis that Notch acts downstream of β-catenin, we used two different approaches. First, we treated K14ΔNβ-cateninER mice with 4OHT in combination with the γ-secretase inhibitor, N-S-phenyl-glycine-t-butyl ester (DAPT) (Cheng et al., 2003). Second, we generated triple transgenic mice by crossing Jag1$^{flox/flox}$ mice, K14CreER mice (Hong et al., 2004) and K14ΔNβ-cateninER mice (Lo Celso et al., 2004).

We confirmed that DAPT inhibited Notch activation in vivo by performing western blots on protein lysates of mouse skin treated topically for 1 or 2 hours with DAPT or the vehicle control (Fig. 4A). At each time point the level of N$^{ICD}$ was reduced in treated, compared with untreated, skin. The effect was, however, transitory, as 4 hours after DAPT application the level of N$^{ICD}$ was indistinguishable from untreated control skin (data not shown).

We also tested the effects of DAPT in cultured keratinocytes by monitoring activation of the Hes1 luciferase reporter. DAPT inhibited Hes1 activation by full-length Notch1 (Fig. 4B), but not

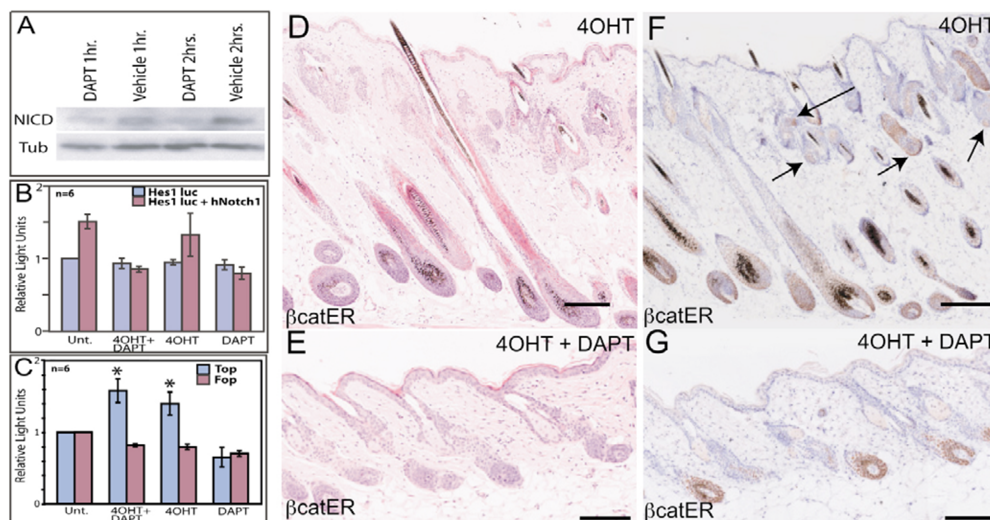


**Fig. 4. Pharmacological inhibition of Notch signalling impairs the ability of β-catenin to induce new hair follicles.** (**A**) Lysates from total skin of wild-type mice treated with 1 mg DAPT or vehicle alone were analysed by western blotting with antibodies to activated Notch (NICD) and β-tubulin. (**B**,**C**) K14ΔNβ-cateninER mouse keratinocytes were cultured with or without 4OHT, DAPT or 4OHT + DAPT for 72 hours, then co-transfected with (B) Renilla luciferase (pRL) and Hes1 luciferase (Hes1-Luc), with or without full-length Notch1 (hNotch1) or (C) with pRL and TOPFLASH or FOPFLASH luciferase reporters. Data are expressed as average light units ±s.d., relative to the Renilla luciferase control. *$P$<0.011. $n$, number of replicate samples. (**D-G**) H&E (D,E) and anti-CDP (F,G) stained sections of back skin from K14ΔNβ-cateninER mice treated with 4OHT (D,F) or 4OHT + DAPT (E,G). Arrows indicate ectopic hair follicles. Scale bars: 100 μm.

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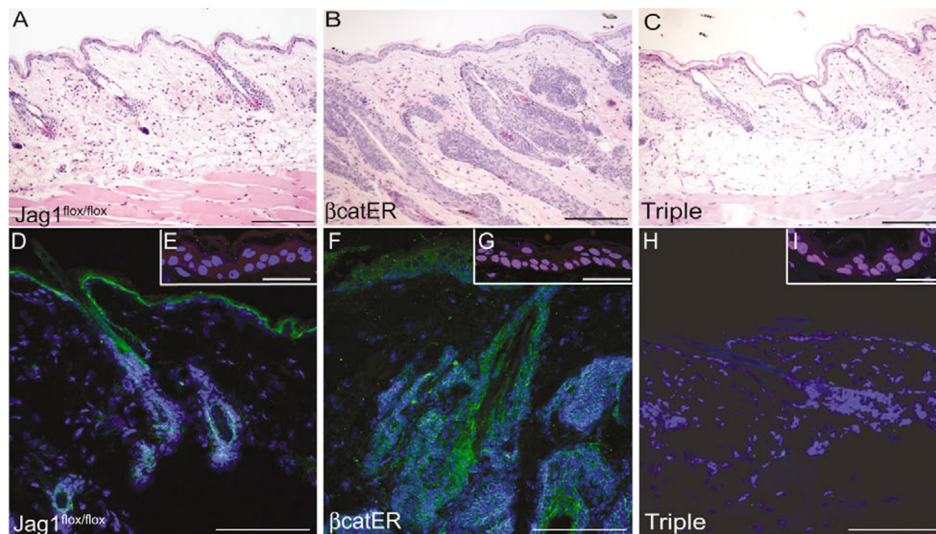
$N^{ICD}$ (data not shown). By contrast, DAPT treatment had no effect on activation of the TOPFLASH reporter in K14ΔNβ-cateninER keratinocytes treated with 4OHT (Fig. 4C).

K14ΔNβ-cateninER mice were treated with acetone, 4OHT or 4OHT plus DAPT for 15 days. In agreement with previous observations (Lo Celso et al., 2004), hair follicles in 4OHT-treated K14ΔNβ-cateninER mice entered anagen and de novo follicles formed from preexisting follicles, sebaceous glands and the IFE (Fig. 4D,F), while hair follicles in wild-type or acetone-treated transgenic animals (data not shown) remained in telogen. In K14ΔNβ-cateninER epidermis treated with 4OHT and DAPT, the onset of anagen of existing hair follicles and de novo hair follicle formation were severely delayed (Fig. 4E,G). Moreover, ectopic expression of CDP was reduced in K14ΔNβ-cateninER mice treated with DAPT (Fig. 4F,G).

In the $Jag1^{flox/flox}$×K14CreER×K14ΔNβ-cateninER triple transgenics, application of 4OHT deleted *Jag1* and activated β-catenin in cells in which the K14 promoter was active. Immunostaining of back skin sections with antibodies to jagged 1 and β-catenin confirmed that activation of β-catenin and *Jag1* deletion occurred in the same cellular compartments (Fig. 5D-I). Nuclear β-catenin was detected in the IFE of triple transgenics (Fig. 5I) and K14ΔNβ-cateninER single transgenics (Fig. 5G), but not in the $Jag1^{flox/flox}$ controls (Fig. 5E).

When triple transgenics mice were treated with 4OHT for 21 days, the follicles remained in telogen and showed no ectopic hair follicle formation (Fig. 5A-C). The phenotype of the triple transgenics was more severe than that of the DAPT-treated mice (Fig. 4E,G), and we attribute this to the transient inactivation of Notch achieved with the pharmacological inhibitor.



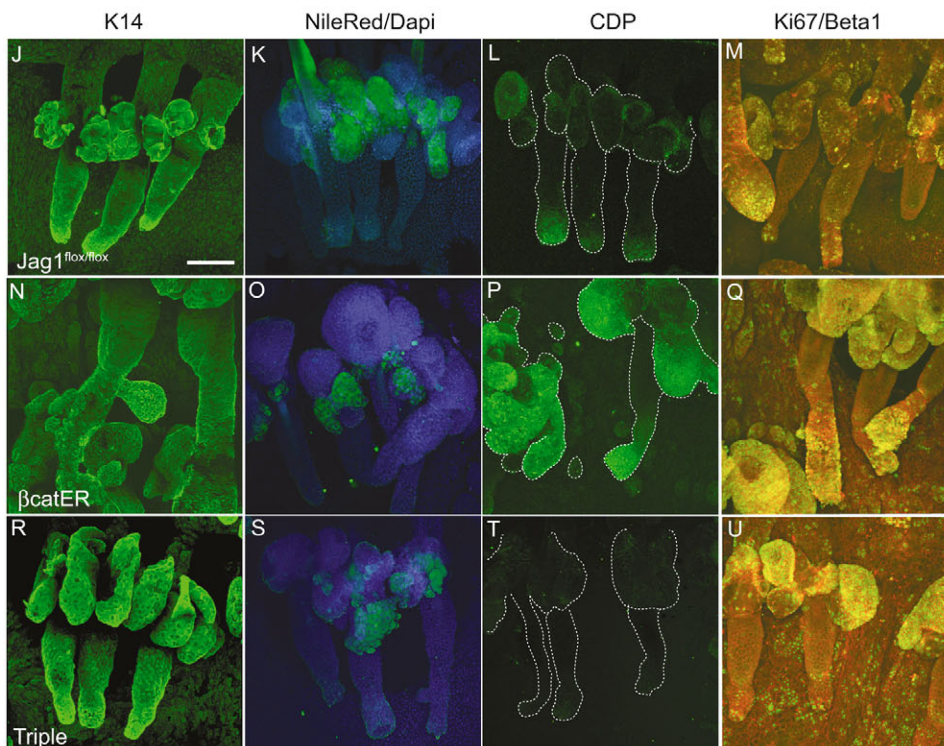




**Fig. 5. Deletion of *Jag1* blocks the ability of β-catenin to induce new hair follicles.** (**A-I**) Back skin sections of 4OHT-treated $Jag1^{flox/flox}$ (A,D,E), K14ΔNβ-cateninER (B,F,G) and triple transgenic mice (C,H,I). Sections were stained with H&E (A-C) or with antibodies to jagged 1 (D,F,H) and β-catenin (E,G,I) with DAPI counterstain (blue). E,G,I show nuclear pool of β-catenin in IFE. Scale bars: 50 μm in E,G,I; 100 μm in A-C,D,F,H. (**J-U**) Whole mounts of tail epidermis from 4OHT-treated $Jag1^{flox/flox}$, K14ΔNβ-cateninER and triple transgenic mice labelled with antibodies to K14 (J,N,R), CDP (L,P,T), β1 integrin (red) and Ki67 (green) (M,Q,U), or stained with Nile Red (green) and DAPI (K,O,S). Dashed lines outline hair follicles and sebaceous glands. Scale bar: 100 μm.

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Our results demonstrate that Notch signalling is required downstream of β-catenin for anagen onset and ectopic hair follicle formation.

The effects of deleting *Jag1* while simultaneously activating β-catenin were examined further by preparing tail epidermal whole mounts from Jag1$^{flox/flox}$ (wild-type phenotype), K14ΔNβ-cateninER and triple transgenics treated with 4OHT for 21 days (Fig. 5J-U). As in back skin (Fig. 5C), β-catenin-induced ectopic hair follicle formation was completely blocked by removal of *Jag1* (Fig. 5J,N,R). Nile Red staining showed that the reduction in differentiated sebocytes characteristic of K14ΔNβ-cateninER epidermis (Lo Celso et al., 2004) was prevented in triple transgenics, although sebaceous gland morphology was slightly disturbed relative to Jag1$^{flox/flox}$ epidermis (Fig. 5K,O,S). Ectopic expression of CDP was inhibited in the triple transgenics (Fig. 5P,T). In triple transgenics, consistent with the failure of the follicles to enter anagen, CDP expression was also absent from the bulb (Fig. 5L,T).

Pharmacological inhibition of hedgehog signalling blocks ectopic hair follicle formation in K14ΔNβ-cateninER epidermis by inhibiting β-catenin-induced proliferation (Silva-Vargas et al., 2005). By contrast, inhibition of Notch signalling in the triple transgenics did not prevent β-catenin-induced proliferation in the sebaceous glands and IFE, as evaluated by Ki67 staining (Fig. 5M,Q,U). We observed clusters of highly proliferative cells in the IFE and in the sebaceous glands in K14ΔNβ-cateninER mice and triple transgenics. There was a reduction in proliferation in the hair follicle bulb, but that correlated with the failure of the triple transgenic follicles to enter anagen (Panteleyev et al., 2001) (Fig. 5U). We conclude that while Notch, like hedgehog, is required downstream of β-catenin for ectopic hair follicle formation, its role differs from hedgehog in that it is not primarily a proliferative stimulus.

## Co-operation between Notch and β-catenin in promoting ectopic hair follicle formation

If Notch acts primarily to promote differentiation of the hair follicle lineages downstream of β-catenin, simultaneous activation of β-catenin and Notch should give rise to ectopic follicles that are more mature than those resulting from activation of β-catenin alone. To examine this, we crossed K14ΔNβ-cateninER and K14N$^{ICDΔOP}$ER mice to generate double transgenics. In response to 4OHT, hair follicles entered anagen in both the double and the K14ΔNβ-cateninER single transgenics, while hair follicles in 4OHT-treated wild-type skin remained in telogen (Fig. 6A-C).

We determined the number of patches of CDP expression per existing follicle as a means of quantitating ectopic hair follicle formation, as described previously (Fig. 6D-F) (Silva-Vargas et al., 2005). There were more follicles in double transgenics than in single K14ΔNβ-cateninER transgenics at 8 days of 4OHT treatment ($P$<0.003); however, by 15 days there was no significant difference ($P$<0.5; Fig. 6J). Thus, although there were initially more ectopic follicles in the double transgenics, there was no significant difference at the later time point. One possible explanation for this result is that Notch activation lowers the threshold of β-catenin activation required to initiate, but not to maintain, an ectopic follicle.

At all time points the ectopic follicles were longer and there were more Ki67-positive cells in double than in single transgenic epidermis (Fig. 6G-I). As previously reported (Lo Celso et al., 2004), β-catenin-induced follicles lacked the inner root sheath marker, trichohyalin (Fig. 6K). However, ectopic follicles in the double transgenics were frequently trichohyalin positive and thus more mature (Fig. 6L). We conclude that Notch activation promotes hair follicle differentiation in the presence of both endogenous (Fig. 3) and ectopic β-catenin (Fig. 6) signalling.

To confirm that β-catenin activation stimulates Notch activity, we crossed K14ΔNβ-cateninER and Notch reporter mice (TNR) and examined expression of GFP as a readout of Notch activity. As controls we also examined GFP expression in TNR single transgenics and in 4OHT-treated, K14N$^{ICDΔOP}$ER/TNR double transgenics. As shown in Fig. 1A-C, GFP expression was detected in TNR single transgenic mice in a subset of interfollicular basal and suprabasal cells and regions of the hair shaft (Fig. 6M,O). In 4OHT-treated K14N$^{ICDΔOP}$ER×TNR double transgenic mice GFP was expressed in all sites where the K14 promoter is active: throughout the basal layer of the IFE, along the hair follicle outer root sheath and at the periphery of the sebaceous glands (Fig. 1M-O and Fig. 6N,P). In K14ΔNβ-cateninER×TNR double transgenic mice, GFP expression was upregulated in the existing hair follicles and in ectopic follicles, but not in phenotypically normal IFE (Fig. 6Q). This confirms that Notch signalling is activated in response to β-catenin activation in hair follicles.

## *Jag1* is a β-catenin target gene

We next examined the mechanism by which the Notch pathway is upregulated by β-catenin activation. We found that jagged 1 protein (Fig. 7A,B) and mRNA (data not shown) were upregulated when K14ΔNβ-cateninER epidermis was treated with 4OHT. When 4OHT treatment was stopped, ectopic follicles regressed (Silva-Vargas et al., 2005) and jagged 1 expression was downregulated, becoming confined to the bulb of preexisting follicles (Fig. 7C). When 4OHT treatment was restarted, jagged 1 expression was again increased (Fig. 7D,E). This shows a very tight coordination of β-catenin activation and jagged 1 expression. Although *Jag1* is positively regulated by Notch (Ross and Kadesch, 2004), in K14ΔNβ-cateninER epidermis treated simultaneously with 4OHT and DAPT there was increased jagged 1 expression, even though ectopic hair follicle formation and Notch activation were inhibited (Fig. 7F,G).

Further evidence that β-catenin might directly regulate *Jag1* came from examining the skin of K14ΔNLef1 mice, in which inhibition of β-catenin signalling results in conversion of hair follicles into cysts of IFE with associated sebocytes (Niemann et al., 2002). Jagged 1 expression was undetectable in the epidermis of 3-month-old K14ΔNLef1 transgenics, an age at which the follicles had converted into epidermal cysts (Fig. 7H-J). Moreover, jagged 1 expression was already lost in tail epidermis of K14ΔNLef1 mice by 8 weeks of age (Fig. 7L,M).

When keratinocytes were transiently transfected with the full-length Notch1 intracellular domain (mNotch1$^{ICD}$) and a luciferase reporter of Notch activity (Hes1 luciferase), activation of β-catenin by 4OHT in K14ΔNβ-cateninER cells resulted in a significant increase ($P$<0.01) in luciferase activity compared with 4OHT-treated wild-type cells (Fig. 7K). As this effect was more pronounced at 72 hours post-transfection than at 24 hours, we speculate that the increase in luciferase activity is dependent on endogenous jagged 1 production.

Analysis of the sequence of the mouse *Jag1* promoter revealed five putative Tcf/Lef-binding sites with the same consensus sequence (Fig. 7N). Comparison with the human and rat *Jag1* promoters revealed the presence of, respectively, three and six putative binding sites with the same consensus sequence (CCTTTG). Their respective locations on the promoter suggest functional conservation between species. Our results are in good

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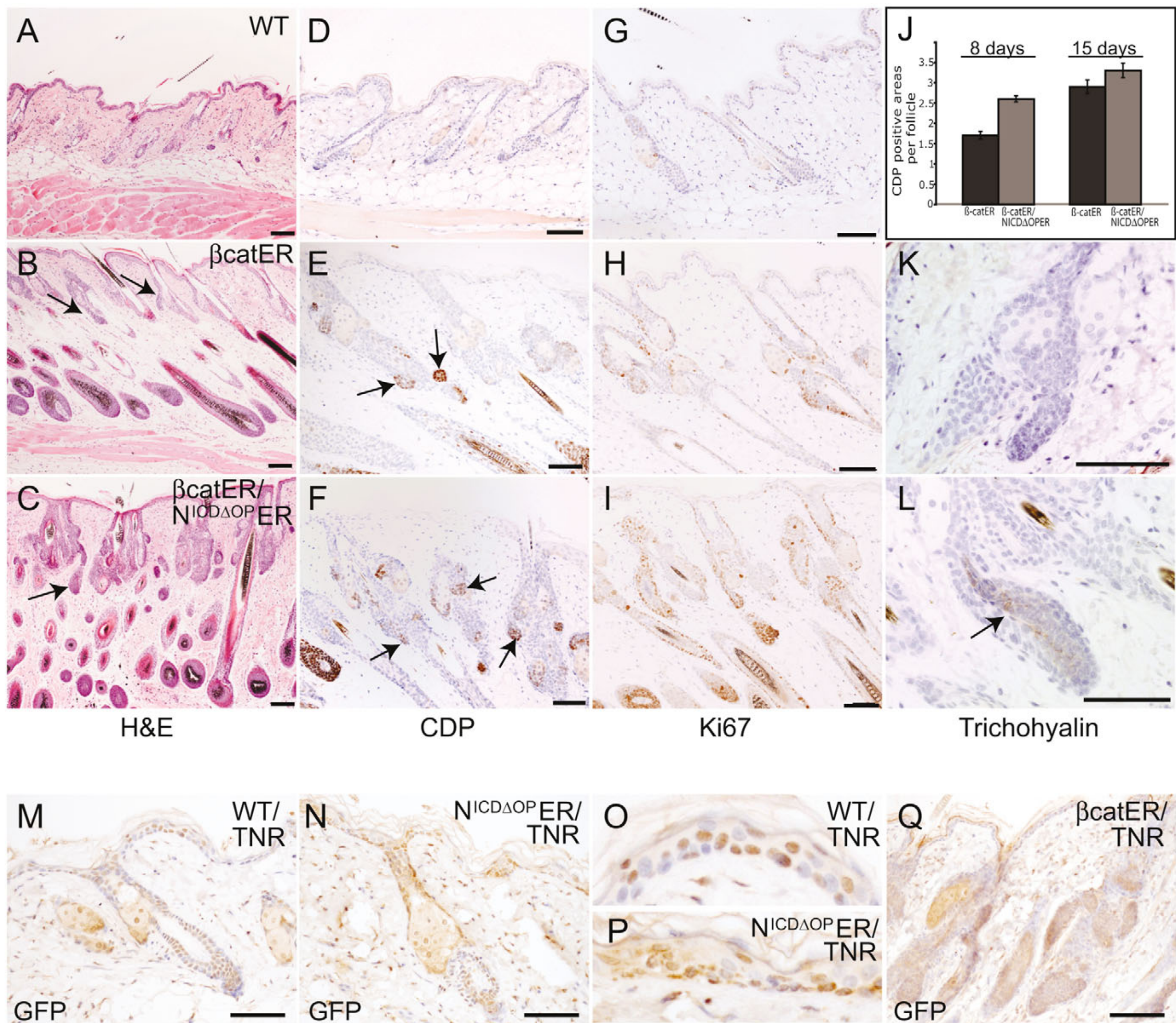


**Fig. 6. Notch activation enhances β-catenin-induced ectopic hair follicle formation and maturation.** (**A-L**) Back skin of wild-type (A,D,G), K14ΔNβ-cateninER (βcatER) (B,E,H,K) and K14ΔNβ-cateninER×K14N$^{ICDΔOP}$ER double (βcatER/N$^{ICDΔOP}$ER) (C,F,I,L) transgenic mice treated with 4OHT for 8 days. Sections were stained with H&E (A-C) or with antibodies to CDP (D-F), Ki67 (G-I) or trichohyalin (J-L). Arrows mark new follicles arising from preexisting follicles. (J) Quantitation of number of CDP positive areas (±s.e.m.) per existing follicle in K14ΔNβ-cateninER single or K14ΔNβ-cateninER×K14N$^{ICDΔOP}$ER double transgenics treated with 4OHT for the number of days shown. (**M-Q**) Epidermis of TNR mice crossed with wild-type (WT;M,O) or K14N$^{ICDΔOP}$ER (N,P) or K14ΔNβ-cateninER (Q) mice stained with antibodies to GFP (brown). Scale bars: 100 μm.

agreement with those of Katoh and Katoh (Katoh and Katoh, 2006), who have reported conservation of Tcf/Lef-binding sites within the promoter of mammalian *Jag1* orthologues.

*Jag1* mRNA was upregulated in K14ΔNβ-cateninER, but not wild-type, keratinocytes treated with 4OHT in the presence of cycloheximide, providing evidence that Jag1 is upregulated by β-catenin at the transcriptional level (Fig. 7O). *Jag1* was confirmed as a β-catenin target gene by performing a ChIP assay using an anti-β-catenin antibody and wild-type and K14ΔNβ-cateninER mouse keratinocytes (Fig. 7P). ChIP PCR bands were observed using different pairs of oligonucleotides that covered the five putative Tcf/Lef-binding sites identified in Fig. 7N. All the binding sites were also occupied by β-catenin in wild-type cells, suggesting that the activation of *Jag1* by β-catenin occurs in response to endogenous Wnt signalling and not solely as a consequence of β-catenin overexpression. As Dll1 is transcriptionally regulated by β-catenin during embryonic development (Galceran et al., 2004; Hofmann et al., 2004), we conclude that β-catenin regulates expression of both the embryonic and postnatal epidermal ligands of Notch.

## DISCUSSION

Our results show that in the epidermis, as in the haematopoietic system (Duncan et al., 2005) and intestine (Fre et al., 2005), the Wnt and Notch signalling pathways are closely linked. Both pathways must be active in order to maintain postnatal hair growth, and if either is blocked hair follicles convert into cysts of IFE (Fig. 2)

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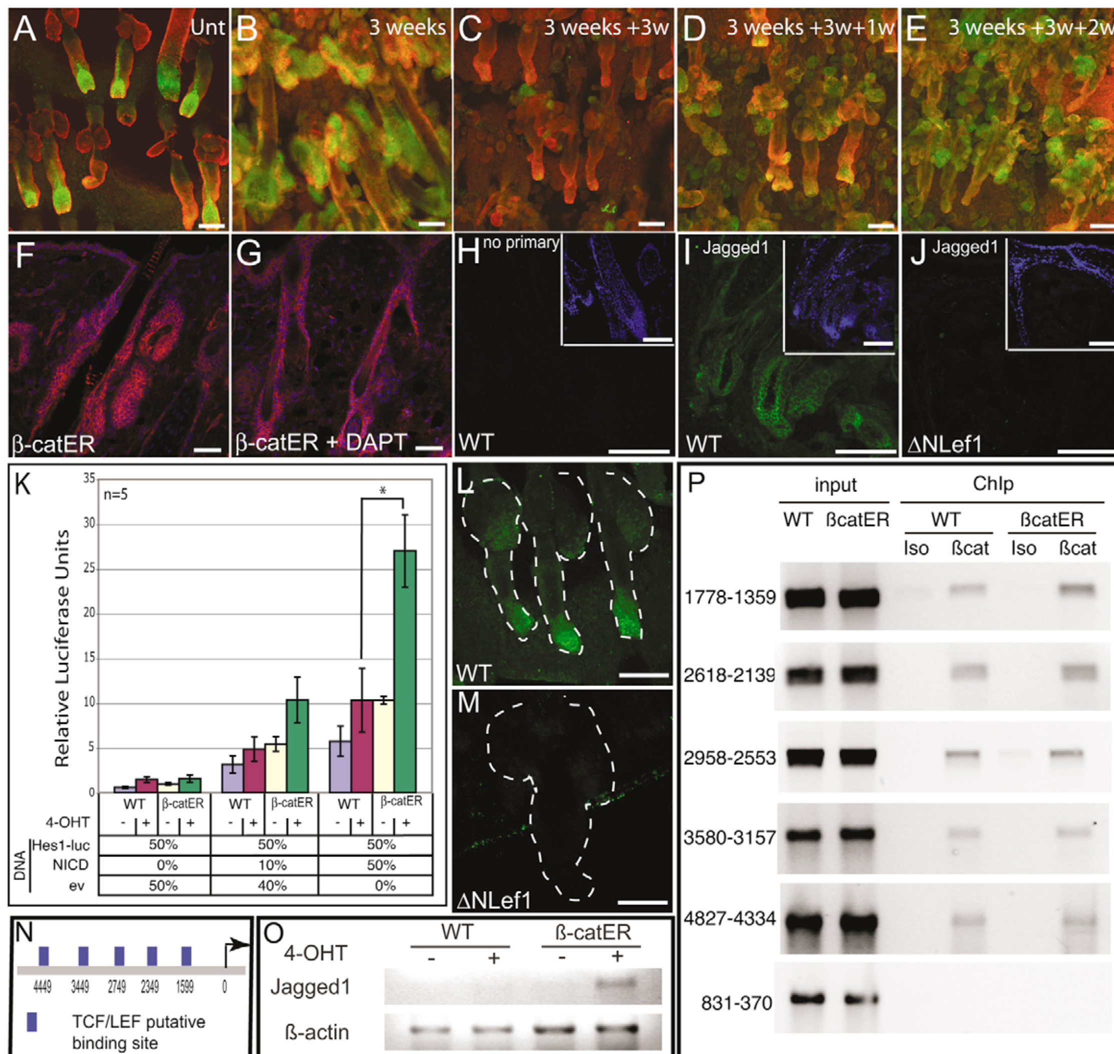


**Fig. 7. *Jag1* is a direct target gene of β-catenin.** (**A-E**) Whole mounts of K14ΔNβ-cateninER tail epidermis labelled with antibodies to K14 (red) and jagged 1 (green). Mice were untreated (A) or treated for 21 days with 4OHT and either examined immediately (B) or after 21 days without 4OHT treatment (C). Following 21 days without treatment, mice received 4OHT for 7 days (D) or 14 days (E). (**F**,**G**) Immunostaining for jagged 1 (red) in back skin sections from K14ΔNβ-cateninER mice treated with 4OHT (F) or 4OHT + DAPT (G). Scale bars: 100 μm (A-G). (**H-J**,**L**,**M**) Immunostaining for jagged 1 (green) in back skin sections (H-J) and tail epidermal whole mounts (L,M) of wild-type (H,I,L) and K14ΔNLef1 (J,M) mice. H is a control with no primary antibody. DAPI (blue) is shown at lower magnification (F-J). Dashed lines in L,M indicate hair follicles and sebaceous glands. Scale bars: 100 μm in H-J,L,M. (**K**) Wild-type or K14ΔNβ-cateninER mouse keratinocytes were cultured with or without 4OHT for 72 hours, then co-transfected with pRL (Renilla luciferase) and different ratios of empty vector (ev), mNotch1$^{ICD}$ (NICD) and Hes1 luciferase (Hes1-Luc). Data are expressed as average light units ±s.d., relative to the renilla luciferase control. *$P<0.01$. *n*, number of replicate samples. (**N**) Location of putative Tcf/Lef-binding sites in the mouse *Jag1* promoter. (**O**) RT-PCR of *Jag1* and β-actin transcripts from wild-type and K14ΔNβ-cateninER mouse keratinocytes treated with acetone or 4OHT in the presence of cycloheximide. Three independent experiments were analysed with consistent replicates and consistent results among experiments. (**P**) Wild-type and K14ΔNβ-cateninER mouse keratinocytes were fixed and subjected to chromatin immunoprecipitation using anti-β-catenin or control antibodies. Primers surrounding the putative Tcf/Lef-binding sites or an irrelevant region of the *Jag1* promoter (831-370) were used for the PCR analysis.

(Huelsken et al., 2001; Merrill et al., 2001; Niemann et al., 2002; Vauclair et al., 2005; Yamamoto et al., 2003). When both pathways are activated simultaneously, there are initially more ectopic follicles than when only β-catenin is active. However, the increase is only temporary, suggesting that Notch activation may lower the threshold of β-catenin activity required to initiate, but not to maintain, an ectopic follicle (Lowry et al., 2005; Silva-Vargas et al., 2005). Overall, our data suggest that the effect of Notch activation is to enhance the differentiated characteristics of β-catenin-induced ectopic follicles, rather than to increase follicle number.

While Notch and Wnt signalling are both important in the hair follicle, their roles are distinct. β-catenin activation induces anagen of postnatal follicles, but Notch activation is not sufficient to drive anagen. Activation of Wnt signalling is essential for hair follicle formation in the embryo, whereas Notch signalling is not required (Andl et al., 2002; Pan et al., 2004), demonstrating that crosstalk between the pathways is essential only in adult epidermis.

We also observed contrasting effects of β-catenin and Notch activity in the IFE. β-catenin activation does not alter proliferation or differentiation of the IFE (Lo Celso et al., 2004). By contrast,

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Notch activation resulted in a pronounced increase in proliferation and in the total number of IFE layers. Although a histologically normal cornified layer formed, keratin 10 expression was lost in large patches of the IFE. The mechanism by which Notch signalling influences the IFE remains to be explored, but it may involve signalling between cells that differ in Notch ligand expression (Lowell et al., 2000) (Fig. 1G), or Notch activation (Ross and Kadesch, 2004) (Fig. 1B,C and Fig. 6M-P).

Our studies place β-catenin upstream of Notch in the epidermis and show that *Jag1* transcription is regulated by β-catenin signalling. Based on our observations and the known sites of epidermal Wnt expression and activity (DasGupta and Fuchs, 1999; Niemann and Watt, 2002; Reddy et al., 2001), we propose that in the hair follicle pre-cortex β-catenin/Lef1 activity induces Jag1 expression, which then activates Notch in the pre-cortex and underlying dermal papilla cells (O'Shaughnessy et al., 2004; Rendl et al., 2005) to promote differentiation of hair follicle lineages (Yamamoto et al., 2003).

Although Notch is an epidermal tumour suppressor (Nicolas et al., 2003), deletion of *Jag1* does not have a major effect on epidermal proliferation (Fig. 5). Notch therefore differs from hedgehog signalling, which is also downstream of β-catenin, because hedgehog acts primarily to promote proliferation in adult and embryonic hair follicles (Oro and Higgins, 2003; Silva-Vargas et al., 2005). Thus, the mechanism by which β-catenin triggers de novo hair follicle formation in adult epidermis involves hedgehog-mediated proliferation and jagged 1-mediated cell fate specification.

Epidermal self-renewal and differentiation involve both positive and negative regulatory networks (Fuchs et al., 2004). In the case of the β-catenin/Notch interaction, two potential mechanisms for limiting signalling, to maintain normal follicle growth and differentiation, have already been identified. These are the inhibition of Wnt4 expression by the Notch1 target gene p21 (Devgan et al., 2005), and negative regulation of β-catenin through its association with the Notch C terminus (Hayward et al., 2005). It seems likely that activation of the two pathways is governed by feedback mechanisms in the epidermis.

In conclusion, we are only just beginning to appreciate the complex interplay between individual signalling pathways that control adult epidermal stem cell renewal and lineage selection. The Wnt and Notch pathways not only intersect with one another, but also with other signalling pathways, including integrins, Eph receptors and Bmps (Hasson et al., 2005; Kluppel and Wrana, 2005). The effects of signal strength and duration, and of synergy and antagonism between pathways, must also be considered. Wnt and Notch pathways are part of a network of multiple intersecting signalling pathways that control the epidermal stem cell compartment.

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