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Integration of Notch 1 and Calcineurin/NFAT Signaling Pathways in Keratinocyte Growth and Differentiation Control

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Summary

The Notch and Calcineurin/NFAT pathways have both been implicated in control of keratinocyte differentiation. Induction of the p21$^{WAF1/Cip1}$ gene by Notch 1 activation in differentiating keratinocytes is associated with direct targeting of the RBP-Jκ protein to the p21 promoter. We show here that Notch 1 activation functions also through a second Calcineurin-dependent mechanism acting on the p21 TATA box-proximal region. Increased Calcineurin/NFAT activity by Notch signaling involves downregulation of Calcipressin, an endogenous Calcineurin inhibitor, through a HES-1-dependent mechanism. Besides control of the p21 gene, Calcineurin contributes significantly to the transcriptional response of keratinocytes to Notch 1 activation, both in vitro and in vivo. In fact, deletion of the Calcineurin B1 gene in the skin results in a cyclic alopecia phenotype, associated with altered expression of Notch-responsive genes involved in hair follicle structure and/or adhesion to the surrounding mesenchyme. Thus, an important interconnection exists between Notch 1 and Calcineurin-NFAT pathways in keratinocyte growth/differentiation control.

Introduction

Complex biological systems are intrinsically "robust," in that they are based on an interconnection of signaling pathways with built-in positive and negative feedback mechanisms for maintenance of normal cellular homeostasis (Barabasi and Oltvai, 2004). Notch signaling plays a key role in cell-fate determination and differentiation (Artavanis-Tsakonas et al., 1999). The biological endpoint of activation or suppression of this pathway is critically dependent on context-specific interactions with other signaling pathways.

The Notch gene family encodes evolutionarily conserved type-1 transmembrane receptors that are activated by ligand binding and proteolytic cleavage, with release of the Notch intracellular domain (Artavanis-Tsakonas et al., 1999). Upon translocation to the nucleus, Notch binds to a ubiquitous DNA binding protein of the CSL family (RBP-Jκ/CBF1 in mammals), converting it from a repressor into an activator of transcription (Artavanis-Tsakonas et al., 1999). The best characterized targets of Notch/RBP-Jκ-mediated activation are members of the HES and HERP families of bHLH transcriptional repressors (Iso et al., 2003). However, a number of other direct targets of Notch/RBP transcription have been identified, which can be induced by Notch activation in a cell-type-specific manner.

In primary mouse keratinocytes, where increased Notch signaling triggers cell cycle withdrawal and differentiation, Notch 1 activation induces p21$^{WAF1/Cip1}$ expression through an RBP-Jκ-dependent mechanism with the RBP-Jκ protein binding directly to the p21 promoter (Rangarajan et al., 2001). However, such a mechanism does not operate in other cell types where Notch signaling promotes growth and suppresses differentiation. Thus, besides direct promoter binding and activation, the induction of p21 expression by Notch/RBP-Jκ activation is likely to involve a cross-talk with other pathways implicated in growth/differentiation of keratinocytes.

Levels of extra- and intracellular calcium play a major role in keratinocyte growth/differentiation control (Dotto, 1999), and the calcium/Calmodulin-dependent phosphatase Calcineurin has been recently implicated in this process (Santini et al., 2001). Calcineurin is the only known serine/threonine phosphatase under calcium/calmodulin control (Crabtree and Olson, 2002). Among the proteins that are dephosphorylated as a consequence of Calcineurin activation are the nuclear factors of activated T cells (NFATs). Increased Calcineurin activity promotes the localization of NFATs to the nucleus, and its effect is counteracted by the phosphorylation of these factors by a number of both constitutive and inducible kinases such as GSK3, CK1, p38, and JNK1 (Crabtree and Olson, 2002). Such a complexity of regulation is reflected by the fact that induction of NFAT-dependent transcription by Calcineurin activation is not immediately associated with increases in intracellular calcium levels, but requires a prolonged stimulus consistent with an oscillatory and accumulative mechanism of NFAT dephosphorylation and nuclear translocation (Crabtree and Olson, 2002).

Studies on the biological function of Calcineurin have

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been greatly facilitated by the use of the inhibitory drugs Cyclosporin A (CsA) and FK506 (Crabtree and Olson, 2002). Several endogenous Calcineurin inhibitors have also been reported (Shibasaki et al., 2002). Among these is Calcipressin (CALP1), also known as the *DSCR1* gene product, located in the Down Syndrome Critical Region of human chromosome 21 and mouse chromosome 16 (Rothermel et al., 2003). This protein binds directly to the CnA subunit and inhibits its activity (Rothermel et al., 2003). Importantly, Calcipressin gene expression is under direct positive control of Calcineurin/NFAT activity, so that this protein is thought to function as a feedback inhibitor of Calcineurin signaling, with an impact on T cell activation as well as the response to different stress stimuli in cardiac hypertrophy (Rothermel et al., 2003).

The function of Calcineurin has been elucidated in great detail in T cells, but has also been studied in the hematopoietic, neuronal, myogenic, and vascular systems (Crabtree and Olson, 2002). Calcineurin/NFAT activity has also been directly implicated in keratinocyte growth/differentiation control (Santini et al., 2001) and, in vivo, in control of the hair cycle (Gafter-Gvili et al., 2003). Molecular analysis of the role of this pathway in keratinocytes has focused on control of p21 gene transcription. Induction of $p21^{(WAF1/Cip1)}$ is one of the earliest regulatory events associated with keratinocyte differentiation, contributing to withdrawal from the cell cycle (Missero et al., 1995). In mouse primary keratinocytes, p21 expression is induced by increased extracellular calcium, and the responsive region of the p21 promoter maps to a 78 bp GC-rich region close to the TATA box, containing six Sp1/Sp3 binding sites (Prowse et al., 1997). Calcineurin induces activation of this promoter through the Calcineurin-dependent association of NFAT with the transcription factors Sp1/Sp3 (Santini et al., 2001).

We show here that Notch 1 activation induces p21 transcription not only through direct binding of the RBP-Jκ protein to the p21 promoter, but also through the calcium/Calcineurin-responsive TATA box-proximal region. Underlying this effect, induction of Calcineurin/NFAT activity by Notch signaling involves downregulation of Calcipressin, in opposition to positive control of this gene by Calcineurin/NFAT itself. Besides control of p21 expression, Calcineurin signaling plays a significantly broader role in the transcriptional response of keratinocytes to Notch 1 activation. In particular, inducible deletion of the CnB1 gene in the skin causes a cyclic alopecia phenotype that is linked to altered expression of several Notch-responsive genes involved in hair follicle structure and adhesion to the surrounding mesenchyme.

Results

Calcineurin-Dependent Induction of the $p21^{WAF1/Cip1}$ Promoter by Notch 1 Activation

We previously showed that Notch 1 activation induces p21 transcription in differentiating keratinocytes through a RBP-Jκ-dependent mechanism. However, while RBP-Jκ binds directly to the p21 promoter (Rangarajan et al., 2001), the minimal region of this promoter that responds to induction of differentiation is separate from the RBP-Jκ binding site and maps to a 78 bp region adjacent to the TATA box (Prowse et al., 1997). To assess whether Notch 1 activation may also induce activity of this minimal region of the p21 promoter, primary mouse keratinocytes were transfected with luciferase reporters for either the 2.4 kb region of the p21 promoter (pW2400), containing the RBP-Jκ binding site, or the 78 bp TATA box-proximal sequence (pW78) together with expression vectors for the intracellular domain of Notch 1 or Notch 2. As previously reported, activity of the 2.4 kb promoter was induced by expression of activated Notch 1 but not Notch 2 (Rangarajan et al., 2001; Figure 1A). Activated Notch 1 induced also the minimal p21 promoter to a surprisingly high extent, suggesting the possible existence of a repressor element in the longer promoter region (Figure 1B). Even with the minimal reporter, activated Notch 2 caused only a marginal induction. As for the full-length p21 promoter (Rangarajan et al., 2001), increased activity of the minimal promoter in differentiating keratinocytes was counteracted by expression of a dominant-negative RBP-Jκ mutant protein, indicating that an endogenous Notch-RBP-dependent mechanism is involved (Figure 1C). Induction of this promoter by activated Notch 1 was also RBP-Jκ dependent (Figure 1D).

Since induction of the minimal p21 promoter in differentiating keratinocyte is dependent on Calcineurin/NFAT activity, we tested whether this pathway is also required for induction by activated Notch 1. Treatment of keratinocytes with CsA in the same increasing amounts that are required to inhibit Calcineurin/NFAT activity (Santini et al., 2001) suppressed induction of the p21-proximal promoter by activated Notch 1 (Figure 1E). As a second independent approach, cells were cotransfected with an expression vector for VIVIT, a high-affinity Calcineurin binding peptide that competes with Calcineurin-NFAT interaction, thereby suppressing NFAT activation (Aramburu et al., 1999). Even in this case, induction of the p21 promoter by activated Notch 1 was significantly, even if not totally, suppressed (Figure 1F).

Calcineurin/NFAT Activity Is Induced by Increased Notch Signaling in Keratinocytes

The above results raised the possibility that Notch 1 activation in keratinocytes causes an increase in Calcineurin activity. NFAT activation provides a measure of endogenous Calcineurin activity (Crabtree and Olson, 2002). Accordingly, primary keratinocytes were transfected with a NFAT reporter (Zhu and McKeon, 1999) or a reporter specific for AP-1, with or without a vector for activated Notch 1. As shown in Figure 2A, activated Notch 1 induced activity of the NFAT reporter in a dose- and time-dependent manner (1 and 2 days), while, consistent with previous results (Chu et al., 2002; Talora et al., 2002), it caused some suppression of the AP-1 reporter. Similar inducing effects were exerted by activated Notch 1 on a second NFAT reporter, based on nine copies of the NFAT binding site from the IL-4 promoter (Parsons et al., 2003; Figure 2B).

Keratinocytes express all four NFAT family members (NFATc1 to -c4) (Al-Daraji et al., 2002; Santini et al., 2001; our unpublished observations), whose cytoplasmic versus nuclear localization is similarly regulated

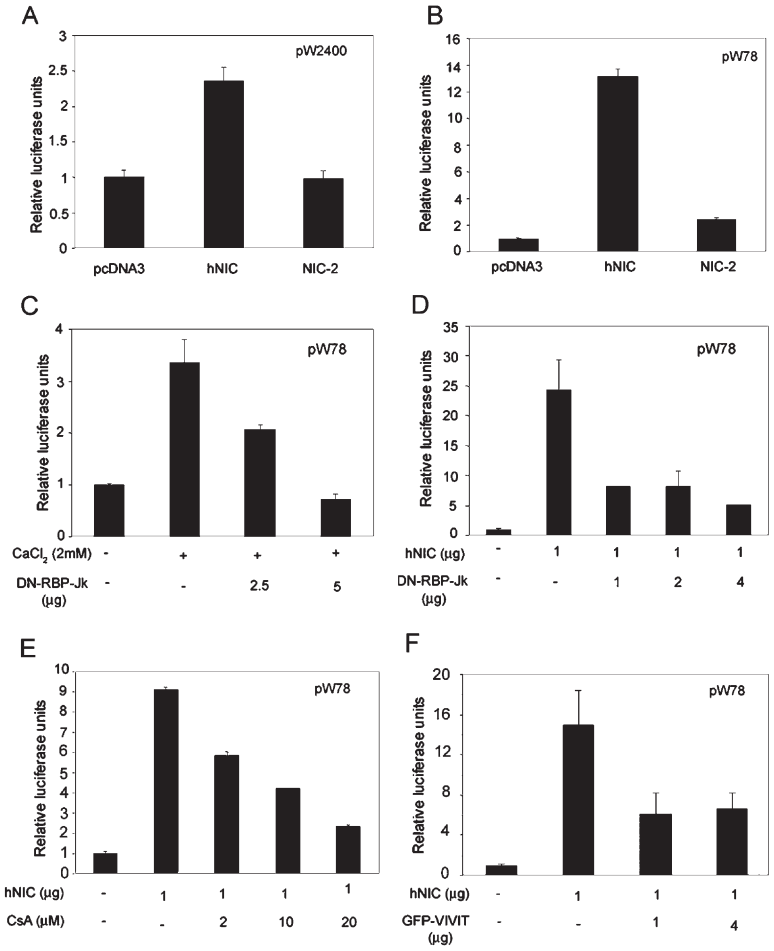

Figure 1. Induction of the Minimal p21 Promoter by Activated Notch 1 Is RBP-Jκ and Calcineurin Dependent

(A and B) Primary mouse keratinocytes were transfected with a luciferase with the 2.4 kb (pW2400) (A) or the 78 bp TATA box-proximal region (pW78) of the p21 promoter (0.5 μg) (B) plus/minus vectors (1 μg) for activated Notch 1 (hNIC) or Notch 2 (NIC-2). Promoter activity was measured 48 hr after transfection.

(C) Keratinocytes were transfected with pW78 and increasing amounts of a vector for dominant-negative RBP-Jκ (DN-RBP-Jκ) and kept under growing conditions or induced to differentiate with 2 mM $CaCl_2$ for the last 48 hr of the experiment (72 hr).

(D) Keratinocytes were transfected with pW78 and vectors for hNIC and DN-RBP-Jκ in increasing amounts as indicated.

(E) Keratinocytes were transfected with pW78 minus/plus the vector for hNIC. Cells were treated with Cyclosporin A (CsA) for the last 24 hr in increasing amounts as indicated.

(F) Keratinocytes were transfected with pW78 and vectors for hNIC and GFP-VIVIT in increasing amounts as indicated.

Error bars indicate SD.

(Crabtree and Olson, 2002). As an independent approach to monitor Calcineurin activity in response to Notch 1 activation, we examined the cellular localization of NFATc3 by immunofluorescence. We found that in keratinocytes, the endogenous protein assumes a prevalent nuclear localization as a consequence of Notch 1 activation (Figure 2C).

Induction of NFAT-dependent transcription by activated Notch 1 was inhibited by treatment with CsA (Figure 2D), consistent with Calcineurin activation being involved. Importantly, the increased NFAT activity in differentiating keratinocytes (Santini et al., 2001) as well as the induction by activated Notch 1 were suppressed by the concomitant expression of a dominant-negative RBP-Jκ mutant (Figure 2E). Finally, in contrast to keratinocytes, in mouse dermal fibroblasts, human 293T cells, or C2C12 myoblasts, activated Notch 1 caused a decrease rather than an increase of NFAT activity (Figure 2F), indicating that positive regulation of Calcineurin/NFAT activity by increased Notch signaling involves a cell-type-specific mechanism.

Increased Calcineurin/NFAT Activity by Notch 1 Activation Is Mediated by Downmodulation of Calcipressin through a HES-1-Dependent Mechanism

To investigate how Notch 1 activation can induce Calcineurin/NFAT signaling, we performed a global analysis of gene expression of primary keratinocytes infected with an adenovirus expressing activated Notch 1 (Ad-hNIC) versus a GFP control (Ad-GFP). We found that expression of the endogenous Calcineurin inhibitor Calcipressin (Rothermel et al., 2003) is reduced in keratinocytes as a consequence of increased Notch 1 activity. Real-time PCR analysis of RNA derived from these cells with primers specific for the common region of the gene confirmed this conclusion (Figure 3A). Immunoblot analysis showed that activated Notch 1 also causes downmodulation of the Calcipressin protein, and that this downmodulation is counteracted by shRNA-mediated knockdown of HES-1 (Ross et al., 2004), a main mediator of Notch-dependent transcriptional repression (Iso et al., 2003; Figure 3B).

To assess whether downmodulation of Calcipressin by activated Notch 1 can account for the observed increase of Calcineurin/NFAT activity, keratinocytes were transfected with the NFAT reporter plus/minus vectors for activated Notch 1, shRNA for HES-1, or Calcipressin. Induction of NFAT-dependent transcription by activated Notch 1 was suppressed substantially, even if not completely, by HES-1 shRNA expression, and a similar reduction of Notch 1 effects was caused by increased Calcipressin expression (Figures 3C and 3D). As with the NFAT reporter, induction of the minimal p21 TATA box-proximal region by activated Notch 1 was

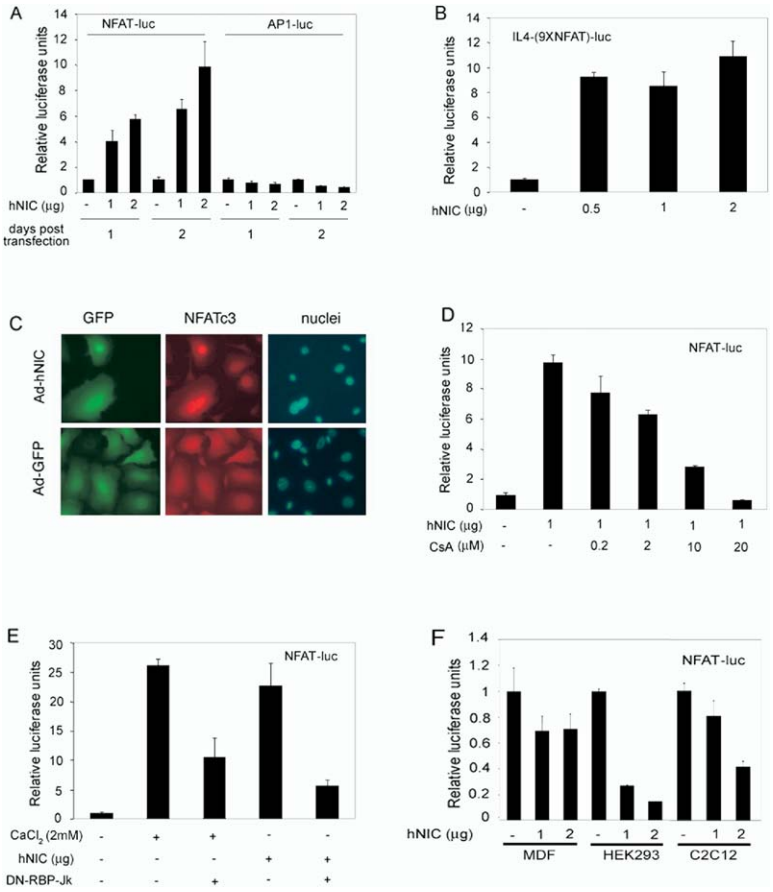

Figure 2. Calcineurin/NFAT Activity Is Induced by Increased Notch 1 Signaling in Keratinocytes

(A) Keratinocytes were transfected with a reporter with a minimal SV40 promoter linked to four NFAT binding sites (NFAT-luc) or a reporter for AP-1 (AP1-luc, Stratagene) plus/minus the hNIC vector in the indicated amounts. Luciferase activity was measured 1 and 2 days after transfection.

(B) Keratinocytes were transfected with a reporter plasmid with nine NFAT binding sites of the Interleukin 4 promoter [IL4-(9XNFAT)-luc] and the hNIC vector in increasing amounts. Luciferase activity was measured 48 hr later.

(C) Keratinocytes were infected at low multiplicity for 17 hr with adenoviruses expressing activated Notch 1 and GFP (Ad-hNIC) or GFP alone (Ad-GFP). Cells were analyzed by immunofluorescence with an antibody against NFAT-c3 followed by Rhodamine-conjugated secondaries. GFP fluorescence was used for identification of infected cells and Hoechst staining was used for nuclear detection. The white arrows indicate NFAT-C3 nuclear localization.

(D) Primary keratinocytes were transfected with NFAT-luc plus/minus the hNIC vector. Cells were treated with Cyclosporin A (CsA) in increasing amounts as indicated for the last 24 hr prior to termination of the experiment (72 hr).

(E) Keratinocytes were transfected with NFAT-luc plus/minus the DN-RBP-Jκ vector and were either kept under growing condition or induced to differentiate with 2 mM $CaCl_2$ for 48 hr. Alternatively, keratinocytes were transfected with the NFAT-luc reporter plus/minus DN-RBP-Jκ and hNIC vectors as indicated.

(F) Mouse primary dermal fibroblasts, HEK293, and C2C12 cells were transfected with NFAT-luc and increasing amounts of hNIC vector as indicated.

Error bars indicate SD.

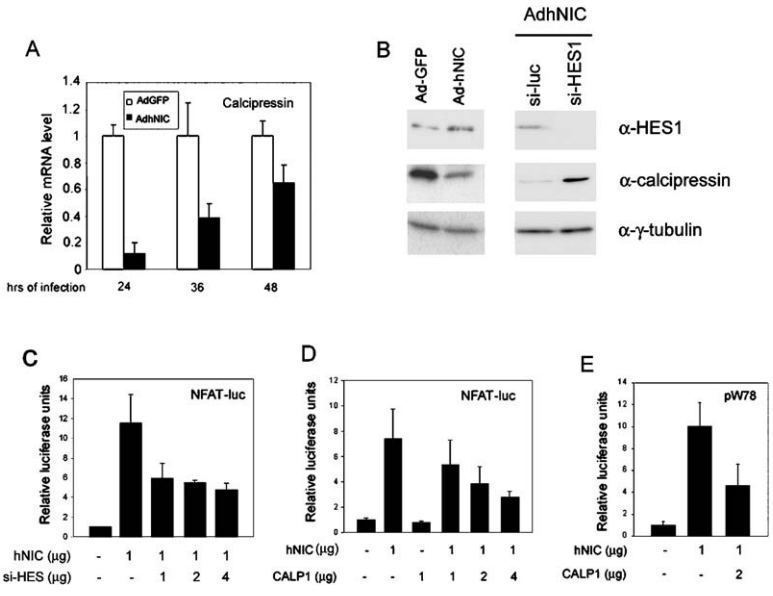

Figure 3. Induction of Calcineurin/NFAT by Activated Notch 1 Involves Downmodulation of Calcipressin Expression through a HES-1-Dependent Mechanism

(A) Primary keratinocytes were infected with the Ad-GFP or Ad-hNIC viruses for the indicated times. Calcipressin mRNA levels were determined by real-time RT-PCR with primers specific for the common region of the Calcipressin gene, and values were normalized for GADPH mRNA levels and expressed as arbitrary units.

(B) Keratinocytes were infected with the Ad-hNIC virus in parallel with the Ad-GFP control (left) or after infection with retroviruses expressing siRNA for mouse HES-1 (Ross et al., 2004) or luciferase control (right). Total cell extracts were analyzed by immunoblotting with antibodies against HES-1 and Calcipressin. Reprobing with antibodies against γ-tubulin was used as equal loading control.

(C) Keratinocytes were transfected with NFAT-luc plus/minus hNIC and siHES-1 vectors in increasing amounts as indicated.

(D and E) Keratinocytes were transfected with either NFAT-luc (D) or pW78 (E) plus/minus vectors for hNIC and Calcipressin (CALP1) in increasing amounts as indicated. Promoter activity was measured 48 hr after transfection.

Error bars indicate SD.

suppressed by Calcipressin to a significant extent (Figure 3E).

The Calcipressin gene (*DSCR1*) comprises seven exons. Two different promoters drive the expression of two major transcripts with exons 1 and 4 as alternative first exons, while exons 5 to 7 are common (Rothermel et al., 2003). The exon 4-proximal promoter contains 15 consensus NFAT binding sites and is positively controlled by Calcineurin/NFAT signaling (Rothermel et al., 2003). Sequence analysis revealed that the 2.5 kb upstream region of the distal exon 1 promoter contains four high-affinity HES-1 binding sites and one low-affinity binding site (N box and class B sites, respectively) (Iso et al., 2003), while only one low-affinity binding site (class B) is present in the proximal exon 4 promoter. RT-PCR analysis with primers specific for differently initiated transcripts of the Calcipressin gene showed that transcription from the exon 1 promoter was stably downregulated by activated Notch 1 expression (Figure 4A), while transcription from the proximal exon 4 promoter was significantly repressed at 24 hr, but returned to basal levels after 48 hr of activated Notch 1 expression (Figure 4B). Increased HES-1 expression caused a similar downmodulation of Calcipressin, with much greater reduction of transcription from the exon 1 than exon 4 promoters (Figure 4C). By contrast, no downmodulation was observed after overexpression of HEY-1 and HEY-2 (Figure 4D), two members of the related HERP family of transcriptional repressors (Iso et al., 2003) that are also induced by Notch 1 activation in keratinocytes.

Chromatin immunoprecipitation (ChIP) analysis of keratinocytes overexpressing HES-1 showed that this protein can bind to the HES binding sites present in both distal and proximal promoters (Figure 4E). Antibodies suitable for the immunoprecipitation of the endogenous HES-1 protein for ChIP analysis are not available. However, HES-1 binds to a specific transcriptional corepressor, Tle-1 (Stifani et al., 1992), which can be readily immunoprecipitated by existing antibodies. ChIP analysis with these antibodies revealed that activated Notch 1 expression induced association of the Tle-1 protein to the HES-1 binding regions of the exon 1 promoter, while no such association was detectable at the exon 4 promoter (Figure 4F).

To assess whether Calcipressin expression is controlled by activation of endogenous Notch signaling, keratinocytes were cocultured for 72 hr with either control mouse NIH3T3 fibroblasts or fibroblasts expressing the full-length ligand Jagged 1 (Small et al., 2001). As expected, HES-1 was induced in keratinocytes cocultured with Jagged 1-expressing fibroblasts (Figure 4G). In the same cells, expression of the Calcipressin gene from the exon 1 promoter was downmodulated, while that from the exon 4 promoter was slightly increased. Levels of the common region of Calcipressin transcripts did not show any variations, as predicted for cells having reached an equilibrium between transcription from the two promoters (Figure 4G). As a complementary approach, we evaluated levels of Calcipressin transcripts in keratinocytes with a deletion of the RBP-Jκ gene. Loss of RBP-Jκ caused a significant increase in exon 1 promoter transcription, with little or no effects on steady-state levels of common or exon 4-specific transcripts (Figure 4H).

The Calcineurin Signaling Pathway Is a Mediator of the Response of Keratinocytes to Notch 1 Activation

While the above studies focused on the connection between the Notch 1 and Calcineurin signaling pathways and its consequences on control of the p21 promoter, both pathways have a much broader role in keratinocyte growth/differentiation control (Rangarajan et al., 2001; Santini et al., 2001). Therefore, we set to determine to which extent Calcineurin signaling contributes to the general response of keratinocytes to Notch 1 activation.

The Calcineurin B1 (CnB1) subunit is essential for enzymatic activity in non-germ cells, and its functional inactivation is incompatible with life (Crabtree and Olson, 2002). Therefore, we bred mice harboring the CnB1 gene flanked by loxP sites (Neilson et al., 2004) with transgenic mice that express the Cre recombinase fused to a RU486-inducible progesterone receptor (CrePR1), driven by the keratin 5 promoter specific for the basal layer of the epidermis and the outer root sheath (ORS) of hair follicles (Zhou et al., 2002). To obtain CnB1-deficient cells, primary keratinocytes derived from CnB1loxP gene mice plus/minus the K5-CrePR1 transgene were treated in culture with RU486 for 3 days. Immunoblot analysis indicated that CnB1 expression was reduced by >95% in cells harboring the K5-CrePR1 transgene versus negative controls (Figure 5A). Functionally, promoter activity assays showed that in keratinocytes with induced deletion of the CnB1 gene, activation of the NFAT reporter by both calcium treatment and activated Notch 1 was drastically suppressed (Figure 5B).

Upon Adeno-Notch 1 infection, the induction of HES-1 and HEY-1 occurred to a significantly lesser extent in the CnB1 knockout keratinocytes, and p21 mRNA levels in these cells were not increased (Figures 6A–6C). To evaluate the global transcriptional response to Notch 1, cells with or without the CnB1 gene and plus/minus activated Notch 1 were used for cRNA probe preparation and hybridization to gene arrays. ~10% of genes that were induced or suppressed >3-fold by activated Notch 1 expression in wild-type keratinocytes were modulated much less in the CnB1-deficient cells (see Supplemental Table S1 available with this article online). Expression of several genes was confirmed by RT-PCR analysis. Among the ones differentially downmodulated by activated Notch 1 in $CnB1^{+/+}$ versus $CnB1^{-/-}$ cells were those for "late" terminal differentiation markers, such as loricrin and small proline rich-like proteins (Figures 6D and 6E), that, unlike the "early" markers of the intermediate epidermal layers (keratin 1, involucrin), are under negative Notch control in keratinocytes (Rangarajan et al., 2001). Among the genes differentially induced by activated Notch 1 in $CnB1^{+/+}$ versus $CnB1^{-/-}$ keratinocytes were those for Nestin, an intermediate filament protein and neuronal stem cell marker previously reported to be also expressed in skin (Li et al., 2003; Figure 6F), and Collagen 6a1 (Col6a1) and PRELP, two proteins involved in extracellular matrix and basement membrane composition, respectively (Grover and Roughley, 2001; Sabatelli et al., 2001; Figures 6G and 6H).

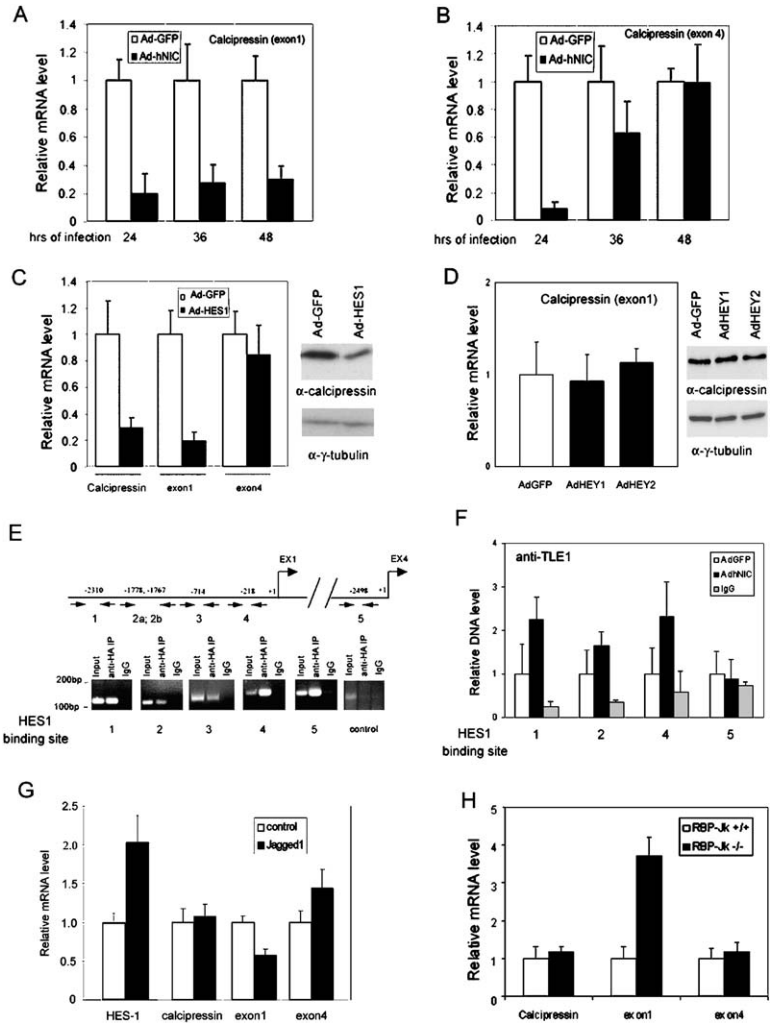

Figure 4. Expression of the Calcipressin Gene Is Differentially Regulated at Exon 1 versus Exon 4 Promoters by Notch 1/HES-1 Activation

(A and B) Keratinocytes were infected with the Ad-GFP and Ad-hNIC viruses for the indicated times and analyzed by real-time RT-PCR with primers specific for exon 1 (A) and exon 4 (B) transcripts of the Calcipressin gene. Results were normalized for GADPH mRNA levels.

(C) Keratinocytes were infected with an HES-1-expressing adenovirus (Ad-HES1) or Ad-GFP control for 24 hr. Total RNA was analyzed by real-time RT-PCR with primers for common or exon 1- and exon 4-specific transcripts of the Calcipressin gene (left). Total cell extracts were analyzed by immunoblotting with antibodies against calcipressin and γ-tubulin as equal loading control (right).

(D) Keratinocytes were infected with adenoviruses expressing the mouse HEY-1 (Ad-HEY1) or HEY-2 (AdHEY2) or Ad-GFP control for 24 hr and analyzed as in the previous panels.

(E) Keratinocytes were infected with the adenovirus expressing HA-tagged HES-1 (Ad-HES1) and processed for chromatin immunoprecipitation with a monoclonal anti-HA antibody and purified mouse IgGs as nonimmune control. PCR amplification of five distinct regions of the Calcipressin gene containing HES-1 binding sites, as illustrated in the map above, were performed along with PCR of the CnB1 coding region, which was used as negative control. Unprecipitated chromatin preparations were used as "input" control. The consensus HES-1 binding sites present at the indicated nucleotide positions (relative to the translation initiation codon) are as follows: (1) CACAAG, (2a) CACAAG, (2b) CACAAG, (3) CACGAG, (4) CACGTG, (5) CATGTG. Sites 1, 2a, 2b, and 3 fully match the sequence of high-affinity HES-1 binding sites (N boxes), while sites 4 and 5 match the sequence of low-affinity HES-1 binding sites (class B sites) (Iso et al., 2003).

(F) Keratinocytes were infected with Ad-GFP or Ad-hNIC and processed for chromatin immunoprecipitation with an anti-Tle1 antibody and purified rabbit IgGs as nonimmune control. Real-time RT-PCR of four distinct regions of the calcipressin gene (as indicated in [E]) was performed and results were normalized for "input" levels of unprecipitated chromatin DNA.

(G) Keratinocytes were cocultured for 2 days with mytomicin-treated NIH3T3 cells stably transfected with an expression vector for full-length Jagged 1 or empty vector control. After removal of NIH 3T3 cells, keratinocytes were used for total RNA preparation and real-time RT-PCR analysis with primers specific for HES-1, and for the common and exon 1- and exon 4-containing transcripts of the Calcipressin gene. Values were normalized for GADPH levels.

(H) Primary keratinocytes derived from homozygous RBP-Jκ-loxP mice were infected with a Cre-expressing adenovirus (Ad-Cre) or Ad-GFP control to generate cells with (RBP-J$\kappa^{+/+}$) or without (RBP-J$\kappa^{-/-}$) the RBP-Jκ gene. Efficient deletion of the RBP-Jκ gene was verified by PCR analysis with primers specific for the loxP target region. Total RNA from the same cells was analyzed by real-time RT-PCR with primers specific for the common, exon 1- and exon 4-containing transcripts of the Calcipressin gene.

Error bars indicate SD.

Loss of Calcineurin Function in the Skin Leads to a Cyclic Alopecia Phenotype together with Downmodulation of Notch-Responsive Genes

To evaluate the consequences of CnB1 deletion in the skin, newborn CnB1loxP mice plus/minus the K5-CrePR1 transgene were treated topically with RU486 for 3 days. Efficient deletion of the floxed CnB1 gene and protein in the skin in vivo was verified by PCR of genomic DNA (Figure 5C), Western blotting, and immunofluorescence (Figures 5D and 5E). Mice with an induced CnB1 deletion showed initially normal skin and hair follicles, with all four types of hair (guard, awl, auchene, and zig-zag). However, beginning at day 21-22 postnatal (during the first telogen phase), CnB1$^{-/-}$ mice started to lose hair in the head-to-tail direction with hair loss being promptly followed by regrowth of new hair (Figure 7A). After restoration of the fur, a new wave of alopecia occurred around day 40–43, followed again by robust hair growth. In older mice, the pattern of hair loss and regrowth eventually resulted in a patchy alopecia as predicted from the age-related loss of synchrony in the mouse hair cycle (Koch et al., 1998).

Histological analysis of CnB1$^{-/-}$ mice revealed that hair shedding occurred in the telogen phase of the hair

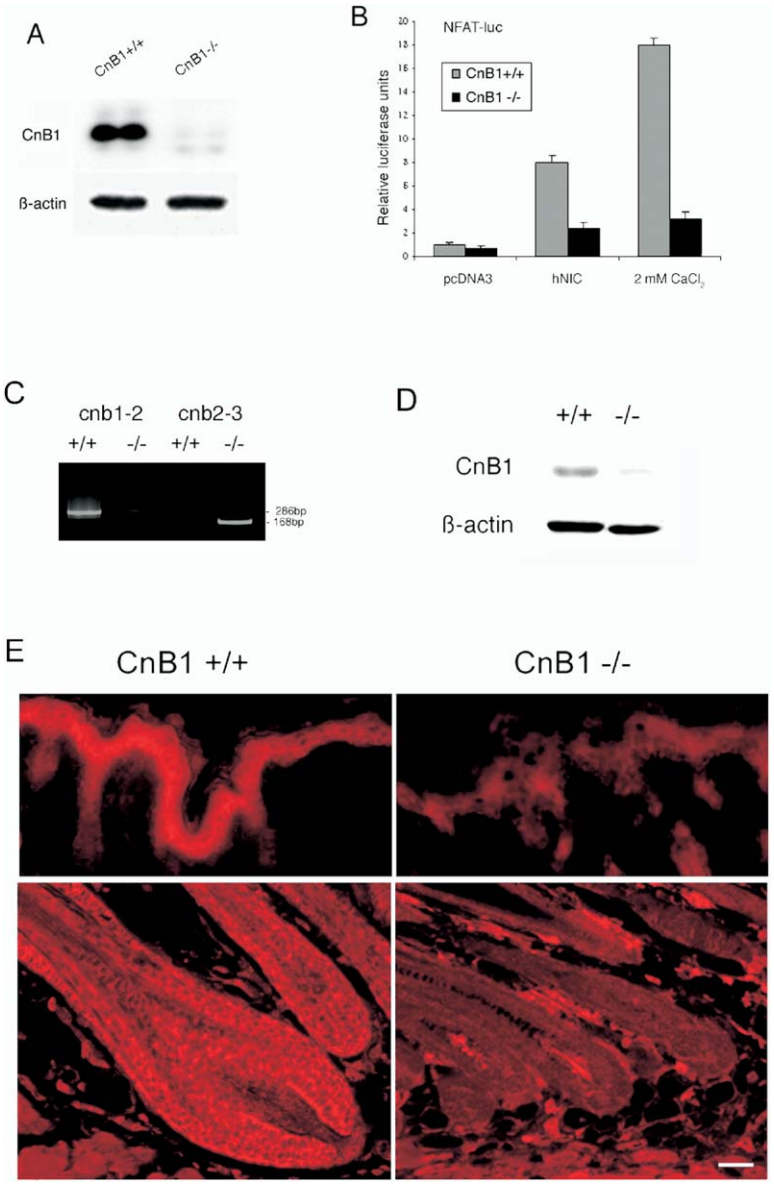

Figure 5. Keratinocyte-Specific Deletion of the CnB1 Gene Is Associated with Block of Notch 1- and Calcium-Induced NFAT Activation

(A) Primary keratinocytes from CnB1-loxP ($CnB1^{+/+}$) or CnB1-loxP/K5-CrePR1 ($CnB1^{-/-}$) mice were treated for 3 days with 50 ng/ml RU486. Total cell extracts were analyzed by immunoblotting with an antibody against CnB1 or β-actin as equal loading control.

(B) Keratinocytes prepared as in (A) were transfected with NFAT-luc and either cotransfected with the hNIC vector or treated with 2 mM $CaCl_2$ for 48 hr. Luciferase activity was measured 72 hr after transfection. Error bars indicate SD.

(C) Genomic DNA isolated from the epidermis of adult CnB1-loxP ($CnB1^{+/+}$) or CnB1-loxP/K5-CrePR1 ($CnB1^{-/-}$) mice, treated with RU486 soon after birth, was analyzed by PCR with primers for the region of the CnB1 gene targeted for deletion (cnb1-2), or for the novel region generated by Cre-mediated recombination (cnb2-3).

(D) Protein extracts from the epidermis of RU486-treated $CnB1^{+/+}$ and $CnB1^{-/-}$ adult mice were analyzed by immunoblotting for the CnB1 protein.

(E) Immunofluorescence analysis with anti-CnB1 antibodies of the interfollicular epidermis (top) and hair follicles (bottom) of RU486-treated $CnB1^{+/+}$ and $CnB1^{-/-}$ mice (at 10 days of age). Scale bar equals 30 μm.

cycle. Hair follicles of $CnB1^{-/-}$ mice at this stage were characterized by a disorganization of the outer root sheath keratinocytes and lack of the expected adherence to the hair shaft, as observed instead in the wild-type controls (Figure 7B). In parallel with these alterations, club hair could be easily plucked from knockout mice by gentle pulling and residues of club hair material could be found at the bottom of the empty hair canal (Figure 7B, right panel, arrow). Hair loss in the $CnB1^{-/-}$ mice was followed by entry into anagen several days earlier than the controls (Supplemental Figure S1).

To gain insights into this phenotype and assess whether it involves altered expression of Notch-responsive genes, we prepared RNA from full-thickness back skin of $CnB1^{-/-}$ and $CnB1^{+/+}$ littermates, as well as from hair follicles isolated by laser capture microdissection (Figure 7C). As predicted from the in vitro results, levels of HES-1 and p21 expression were significantly lower in the CnB1-deficient skin and hair follicles compared to the wild-type (Figures 7D and 7E). To obtain a more comprehensive analysis of gene expression, RNA from laser-captured hair follicles, at a time when histological differences between mice of the two genotypes have not yet occurred (day 13 postnatal), was subjected to linear polymerase amplification followed by microarray hybridization. We found that ~200 genes were differentially expressed in $CnB1^{+/+}$ versus $CnB1^{-/-}$ hair follicles, and of these, as in the cultured cells, 10% were Notch responsive (Supplemental Table S2). The latter group of genes included several that were already identified by the analysis of cells in culture, as well as others related to cytoskeleton, cell attachment, and basement membrane and extracellular matrix composition. Real-time PCR analysis confirmed that expression of Nestin, Col6a1, and PRELP, which are induced by activated Notch 1 in $CnB1^{+/+}$ but not $CnB1^{-/-}$ keratino-

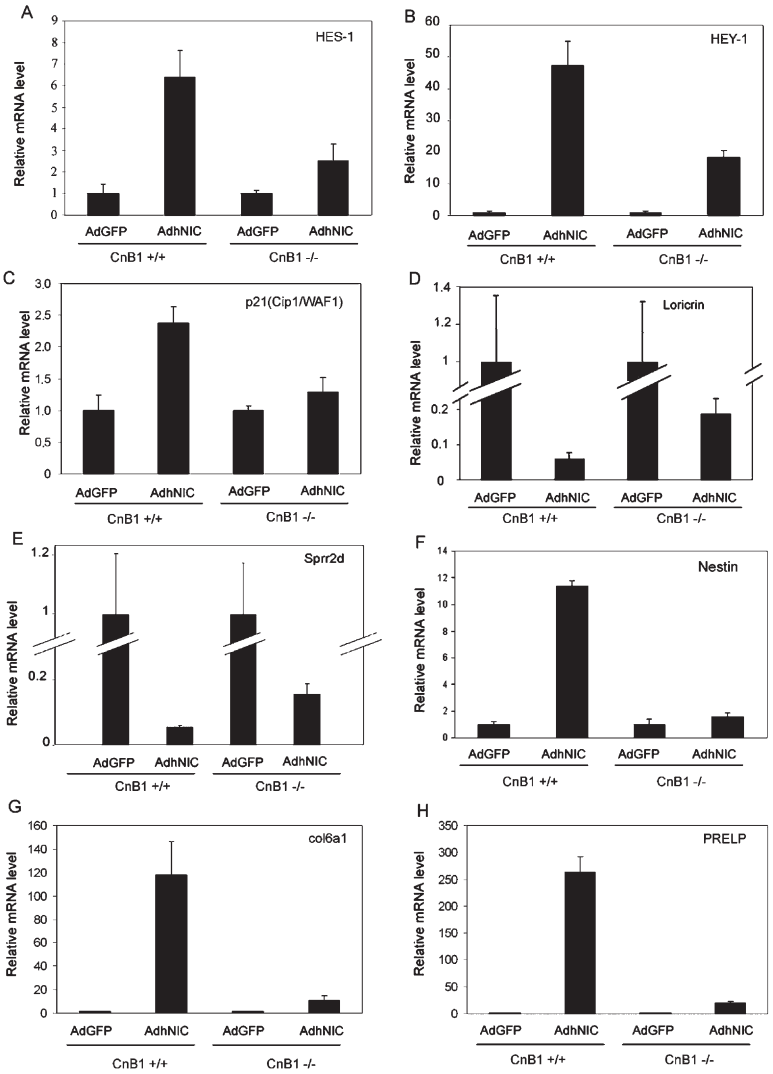

Figure 6. Notch 1-Responsive Genes Are Differentially Regulated in CnB1$^{+/+}$ versus CnB1$^{-/-}$ Keratinocytes

Primary keratinocytes from CnB1-loxP (CnB1$^{+/+}$) or CnB1-loxP/K5-CrePR1 (CnB1$^{-/-}$) mice were infected with Ad-GFP or Ad-hNIC. Real-time RT-PCR was performed with primers for the indicated genes and results were normalized for GAPDH mRNA levels. Relative expression levels of each gene in the Ad-hNIC versus Ad-GFP infected keratinocytes are indicated. Error bars indicate SD.

cytes (Figures 6F–6H), are also expressed in skin and hair follicles of CnB1$^{+/+}$ at significantly higher levels than in CnB1$^{-/-}$ mice (Figures 7F–7H). Syndecan-4 is another keratinocyte-expressed molecule that mediates attachment of cells to the extracellular matrix and response to growth factors (Saoncella et al., 2004). We found that expression of this gene is also under positive Notch 1 control and, as for the others, is expressed at much higher levels in skin and hair follicles of CnB1$^{+/+}$ than CnB1$^{-/-}$ mice (Figure 7I and Supplemental Table S2).

Only one previous report pointed to the possible expression of Nestin in hair follicles (Li et al., 2003), and expression of Col6a1 and PRELP in these structures to our knowledge was not previously analyzed. Immunofluorescence with the corresponding antibodies confirmed the hair follicle localization of these proteins and their decrease in CnB1$^{-/-}$ mice versus CnB1$^{+/+}$ (Figure 7J).

Thus, even in vivo, a number of Notch-responsive genes are dependent for their expression on Calcineurin function which, as discussed further below, can contribute to the cyclic alopecia phenotype caused by deletion of the CnB1 gene.

Discussion

Like Notch, Calcineurin signaling represents another important pathway involved in keratinocyte differentiation control. Rather than proceeding in a parallel fashion, we have shown here that the two pathways are linked, with Notch functioning as an upstream activator of Calcineurin/NFAT signaling through a mechanism that depends in part on decreased expression of Calcipressin, an endogenous Calcineurin inhibitor. Calcineurin signaling in turn plays a significant role in mediating the transcriptional response of keratinocytes to Notch 1 activation, with an impact in vivo on cell and tissue regulation.

Important homeostatic mechanisms exist in the cell that limit activation of Calcineurin activity (Crabtree and Olson, 2002). Notch 1 activation in keratinocytes does not lead to significant elevations of intracellular calcium levels (our unpublished observations). Rather, one mechanism whereby increased Notch signaling leads to Calcineurin activation involves downmodulation of Calcipressin, an endogenous Calcineurin inhibitor (Rothermel et al., 2003). The Calcipressin gene is tran-

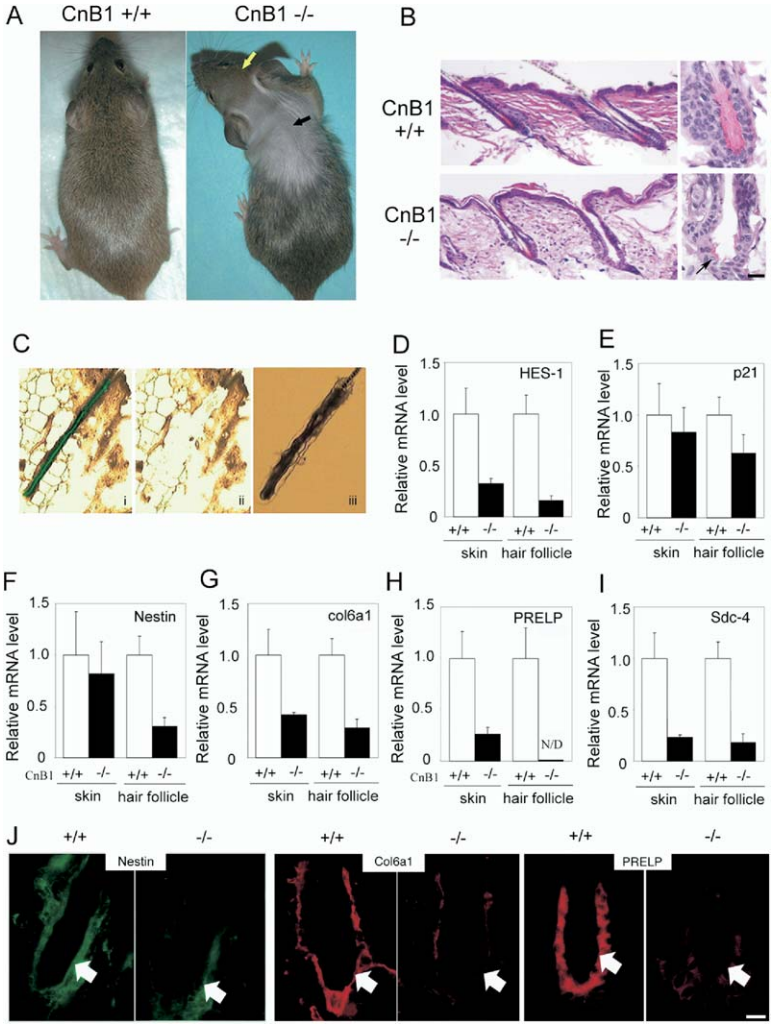

Figure 7. In Vivo Keratinocyte-Specific Deletion of the Calcineurin B1 Gene Causes a Cyclic Alopecia Phenotype Associated with Decreased Expression of Notch 1-Responsive Genes

(A) Phenotypic comparison between CnB1-loxP (CnB1$^{+/+}$) and CnB1-loxP/K5-CrePR1 (CnB1$^{-/-}$) littermates at 9 weeks of age that were topically treated with RU486 soon after birth to induce deletion of the CnB1 gene. Note the wave of hair loss in the CnB1$^{-/-}$ mouse running in the head-to-tail direction (lower arrow), followed by regrowth of new hair (upper arrow).

(B) Histological analysis of middorsal skin of CnB1$^{+/+}$ (top) and CnB1$^{-/-}$ (bottom) littermates at 9 weeks of age, during the second telogen phase. Left panels: note in the CnB1$^{-/-}$ mouse hair follicles with lack of tight adherence to the shaft and/or loss of hair. Right panel: note in the CnB1$^{-/-}$ mouse disorganization of outer root sheath keratinocytes and, in the empty hair canal, residues of club hair material (arrow). Scale bars equal 45 and 15 μm for left and right panels, respectively.

(C) Representative laser capture microdissection, showing the histological section before (i) and after (ii) the procedure, as well as the captured hair follicle (iii).

(D–I) Real-time RT-PCR with primers specific for the indicated genes (Sdc-4: Syndecan 4) was performed on CnB1$^{+/+}$ mice at day 13 postnatal. Results were normalized for GADPH mRNA levels and reported as relative expression levels. Error bars indicate SD.

(J) Immunofluorescence analysis for the indicated proteins in telogen hair follicles of CnB1$^{+/+}$ and CnB1$^{-/-}$ littermates at 12 weeks of age. Arrows point to the junction between outer root sheath and shaft. Scale bar equals 10 μm.

scribed into various differentially spliced mRNAs, specifically the exon 1- and exon 4-containing mRNAs. The resulting Calcipressin isoforms share a similar ability to bind and suppress Calcineurin activity (Rothermel et al., 2003). However, their regulation of expression is substantially different. The internal promoter driving expression of exon 4 transcripts contains repeated NFAT binding sites. Positive regulation of this isoform by increased Calcineurin/NFAT activity was demonstrated both in culture and in vivo (Rothermel et al., 2003) providing a negative feedback mechanism for Calcineurin/NFAT activity (Rothermel et al., 2003). Other mechanisms involved in control of Calcipressin expression have not yet been explored. HES-HERP family members are among the best understood downstream targets of Notch activation and mediators of transcriptional repression (Iso et al., 2003). In spite of their similar biochemical properties, these transcription factors can differ significantly in biological function and downstream targets. In the present context, we have found that knockdown of HES-1 expression by RNA interference is sufficient to counteract the negative effects of activated Notch 1 on Calcipressin levels. Conversely, increased HES-1 but not HEY-1 or HEY-2 expression negatively controls expression of the Calcipressin gene, more specifically transcription from the exon 1 promoter. This promoter is characterized by the presence of several HES-recognition sequences to which the HES-1 protein can bind. While binding of the endogenous HES-1 protein in response to Notch 1 activation could not be assessed, due to lack of appropriate antibodies, we found recruitment of endogenous Tle1, a HES-1-associated repressor and mammalian homolog of *Drosophila* Groucho (Stifani et al., 1992). Further evidence that the exon 1 promoter of the Calcipressin gene is under control of endogenous Notch signaling is provided by downmodulation of transcription at this site in keratinocytes after Jagged 1 ligand stimulation and its increased transcription after abrogation of Notch-dependent modulation of gene expression (by deletion of the RBP-Jκ gene). Thus, our data are overall consistent with a dual and opposite regulation of the Calcipressin gene by the Notch 1/HES-1 and Calcineurin/NFAT pathways. As increased Notch signaling suppresses transcription of the Calcipressin gene from the exon 1 promoter, the resulting increase in Calcineurin/NFAT activity leads to progressive activation of exon 4 promoter transcription. By direct functional assays, we

found that induction of the Calcineurin/NFAT pathway by Notch 1 is counteracted to a significant extent by HES-1 knockdown or overexpression of Calcipressin. However, this suppression is not complete, indicating that additional mechanisms besides downregulation of Calcipressin are also involved.

The functional consequence of the interconnection between Notch and Calcineurin on gene expression is best illustrated, in molecular terms, at the level of the p21 promoter. While endogenous RBP-Jκ binds to this promoter upstream of the NFAT-Sp1-Sp3 complex (Rangarajan et al., 2001; Santini et al., 2001), we have shown here that transcriptional activation of the p21 promoter by endogenous Notch 1-RBP-Jκ is dependent on Calcineurin-NFAT activity (as abrogated by either CsA treatment or CnB1 deletion). Thus, the most likely scenario is that p21 promoter activation is dependent on the concomitant upstream binding of Notch/RBP-Jκ and downstream of NFAT/Sp1-Sp3, with positive regulation of NFAT activity by activated Notch 1 as the most functionally significant aspect for control of p21 expression.

This coregulated mode of gene expression by Notch and Calcineurin is likely to extend to several other genes that we have found to be under concomitant positive control of the two pathways. In fact, the promoter regions of the HES-1 and HEY-1 genes contain several fully conserved NFAT binding sites as well as sites for MEF2 (Supplemental Table S3), with which NFATs have been shown to associate (Blaeser et al., 2000; Youn et al., 2000). Thus, the concomitant binding of RBP-Jκ and NFAT factors could explain why the maximal induction of the HES-1 and HEY-1 genes by activated Notch 1 is Calcineurin-NFAT dependent. From a more functional perspective, it can be proposed that increased Calcineurin-NFAT activity by Notch 1 activation serves as a positive feedback mechanism to reinforce induction of these "classical" Notch target genes. Even the promoter regions of other Notch-responsive genes implicated in the in vivo phenotype of the CnB1$^{-/-}$ mice contain multiple RBP-Jκ, NFAT, and MEF2 binding sites (Supplemental Table S3). The concomitant presence of these sites is potentially significant as, by computer-assisted analysis of 5 kb promoter regions of all known murine genes, we found that only <1% of genes contain this specific combination of sites.

In vivo, keratinocyte-specific deletion of the CnB1 gene results in interesting skin alterations, with a cyclic alopecia phenotype reported so far only for three other knockout mouse models, namely for a key cell-cell adhesion component (Desmoglein-3) (Koch et al., 1998), a lysosomal protease (Cathepsin-L) (Roth et al., 2000), and a developmentally relevant transcription factor (Msx-2) (Braz et al., 2003). Deletion of the CnB1 gene in keratinocytes does not affect expression of these genes (our unpublished observations). Rather, we have identified a number of other genes under combined Notch 1-Calcineurin control, whose decreased expression can contribute to the balding phenotype. In particular, Nestin is an intermediate filament protein that shares a low degree of sequence homology with other intermediate filaments (Lendahl et al., 1990). While the precise function of Nestin remains to be elucidated, its decreased expression may result in a weakening of the intermediate filament network and contribute to the disorganization of the outer root sheath keratinocytes characteristic of the CnB1$^{-/-}$ mice.

A significant number of genes under the combined control of Notch and Calcineurin, whose expression is reduced in the CnB1$^{-/-}$ hair follicles, might affect keratinocyte adhesion to the surrounding matrix. Overall, decreased expression of these molecules can be an important contributing factor for the "loosening" of hair follicle structures and surrounding connective tissue, with a consequent alteration of the outer root sheath, which ultimately results in reduced adherence and loss of hair. Importantly, mice with abrogation of Notch signaling by deletion of the genes for Notch 1 and 2, γ-secretase (Pan et al., 2004) or RBP-Jκ (Yamamoto et al., 2003), exhibit a more complex hair follicle phenotype characterized by follicular atrophy and epidermal cyst formation, consistent with the fact that genes under Notch and Calcineurin control are only partially overlapping.

Loss of the Notch 1 gene or its decreased expression promote keratinocyte tumor development (Nicolas et al., 2003; Talora et al., 2002), and pharmacological inhibition of Calcineurin function has been similarly linked to an increase in epithelial tumors in both experimental (Hojo et al., 1999) and clinical (Paul et al., 2003) situations. Thus, future studies will have to assess to what extent the interplay that we have uncovered between the Notch and Calcineurin/NFAT pathways impinges also on long-term control of skin homeostasis and carcinogenesis.

Experimental Procedures

Cell Culture

Conditions for cell preparations and transient transfections were as previously described (Rangarajan et al., 2001) using the Dual-Luciferase Reporter Assay System (Promega).

For coculture experiments, NIH3T3 cells expressing full-length Jagged 1 (Jag1) (Small et al., 2001) or empty vector control were treated with mytomicin for 2 hr and plated at 4:1 ratio on top of sparsely plated keratinocytes. After 2 days, NIH3T3 cells were selectively removed by mild trypsin treatment and RNA was purified from keratinocytes.

For siRNA knockdowns, primary keratinocytes were infected with retroviruses expressing siRNA for HES-1 (Ross et al., 2004) or luciferase together with a puromycin resistance marker, and puromycin selection (1.5 μg/ml) was applied 72 hr after infection and continued for 5 days. The antibiotic-resistant infected cells (>50% of initial population) were subsequently infected with Ad-hNIC for 24 hr as previously described (Rangarajan et al., 2001).

Plasmids, Adenoviruses, Antibodies, and Oligonucleotide Primers

List of all reagents used in these studies is provided as Supplemental Data.

Chomatin Immunoprecipitation and Analysis of Gene Expression

Chomatin immunoprecipitation and global analysis of gene expression by microarray hybridization (Affymetrix Gene Chip Mouse 430A 2.0) were carried out as previously described (Okuyama et al., 2004; Rangarajan et al., 2001).

In Vivo Deletion of the CnB1 Gene and Laser Capture Microdissection

2-day-old CnB1loxP (CnB1$^{+/+}$) or CnB1loxP/K5-Cre (CnB1$^{-/-}$) mice were treated topically on the back with RU486 (1 mg/ml in ethanol

70%) once a day for 3 consecutive days to induce CnB1 deletion (Zhou et al., 2002). The middorsal skin of the animals was harvested on postnatal day P13. Samples were quickly embedded in OCT medium and frozen in liquid nitrogen using a special technique to obtain longitudinal cryosections through the HF (Muller-Rover et al., 2001). Reagents and protocols used in the laser capture procedures were from Arcturus (Mountain View, CA). Approximately 50 follicles from 9 μm-thick frozen sections were captured for each sample using an AutoPix Automated Laser Capture Microdissection System. RNA was extracted with the PicoPure RNA isolation kit and subject either to two rounds of linear amplification using the RiboAmp RNA amplification kit or to one round of amplification and then used as a template for the BioArray High-Yield RNA Transcript Labeling kit (ENZO Life Science) to obtain biotinylated probes, as per Affymetrix's guidelines.

Supplemental Data

Supplemental Data include one figure and three tables and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/8/5/665/DC1/.

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