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## Cutaneous Papillomavirus E6 oncoproteins associate with MAML1 to repress transactivation and NOTCH signaling

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### Abstract

Papillomavirus E6 oncoproteins associate with LXXLL motifs on target cellular proteins to alter their function. Using a proteomic approach, we found the E6 oncoproteins of cutaneous papillomaviruses Bovine Papillomavirus Type 1 (BE6) and HPV types 1 and 8 (1E6 and 8E6) associated with the MAML1 transcriptional co-activator. All three E6 proteins bind to an acidic LXXLL motif at the carboxy-terminus of MAML1 and repress transactivation by MAML1. MAML1 is best known as the co-activator and effector of NOTCH induced transcription, and BPV-1 E6 represses synthetic NOTCH responsive promoters, endogenous NOTCH responsive promoters, and is found in a complex with MAML1 in stably transformed cells. BPV-1 induced papillomas show characteristics of repressed NOTCH signal transduction, including suprabasal expression of integrins, talin, and basal type keratins, and delayed expression of the NOTCH dependent HES1 transcription factor. These observations give rise to a model whereby papillomavirus oncoproteins including BPV-1 E6 and the cancer associated HPV-8 E6 repress Notch induced transcription, thereby delaying keratinocyte differentiation.

### Keywords

CBF1; RBP-J; RBP-Jk; squamous; epithelial; transformation; cancer

### Introduction

Papillomaviruses are small encapsidated DNA viruses with double-stranded circular genomes that induce benign squamous epithelial neoplasms (papillomas) in vertebrates, and replicate within the differentiating cell layers of the papilloma. Although all papillomas are initially benign, some papillomas may evolve over time to produce malignancies, most notably human ano-genital and upper respiratory carcinomas (reviewed in (1)), and cotton tailed rabbit papillomavirus (CRPV, ((2)). The subset of viruses associated with anogenital

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mucosal cancers is referred to as “high risk” HPV types (the prototype is HPV-16), and the related mucosal viruses that do not cause malignancies are called “low risk” (the prototype is HPV-11). In addition, distinctly different sets of HPVs can cause cutaneous papillomas, including plantar warts caused by the prototype of that group, HPV-1 or commensal infections of normal skin (prototype is HPV-8), but in persons with the recessive disorder epidermodysplasia verruciformis, these papillomas can progress to squamous cell cancers (3).

Papillomaviruses must both manipulate and coordinate their life cycle to the life cycle of their host squamous epithelial cells. Two signaling pathways are critical in controlling this process. Integrins on basal epithelial cells engage extracellular matrix on the basement membrane and enable proliferation and repress keratinocyte differentiation; keratinocytes differentiate when detached from matrix (4) and differentiation can be suppressed using antibodies to beta-1 integrin in the suspended cells (5, 6). In cells that detach from the basement membrane, NOTCH signaling drives cell cycle withdrawal and differentiation (reviewed in (7, 8)). The NOTCH ligand Jagged2 is expressed in the basal layer; NOTCH 1 signaling in the spinous cell layer then drives early squamous epithelial differentiation as well as terminal epithelial differentiation (9) (10). Ectopic expression of activated NOTCH in the basal layer suppresses basal cell fate (11). Upon canonical NOTCH signaling, the NOTCH receptor is cleaved by the intramembrane gamma-secretase protease complex, liberating the NOTCH intracellular domain that then forms a complex with the RBP-J (also known as CBF1) DNA binding protein. This displaces a repressor-histone-deacetylase complex from RBP-J (CBF1) and recruits the Mastermind-like 1 (MAML1) co-activator to the NOTCH -CBF1 complex, thus converting the RBP-J (CBF1) complex from a transcriptional repressor to an activator (reviewed in (12)). The primary downstream effectors of the NOTCH -CBF1-MAML1 complex are a set of b-HLH transcriptional repressors including the HES and HEY families (reviewed in (13)). The N-terminus of MAML shows the highest homology between the four MAML proteins ((14) and references therein), contains a basic region that interacts with NOTCH (15), and an acidic region within the part of the NOTCH receptor that interacts with p300 (16, 17)). The C-terminus of MAML1 also contains an acidic domain important for MAML1 transcriptional activity in vivo (15). Interestingly, this acidic domain in MAML1 contains a LXXLL motif (amino acids 1009-1013).

The initial virus-induced papillomas have low copy numbers of episomal viral DNA in proliferative basal epithelial cells. After cell division in the basal cell layer, progeny cells are pushed up off the basement membrane into the spinous cell layer, and a subset of these spinous cells, under the influence of viral oncogenes, fail to differentiate but rather re-enter the cell cycle to amplify viral DNAs to high copy number (reviewed in (18, 19)). As cells with amplified viral DNA copy number move even higher within the stratified epithelium, a subset of the cells that have amplified the viral genomes express the two late gene capsid proteins within the granular cell layers, encapsidate viral DNA, and are finally shed from the surface. Thus in the papillomavirus infectious cycle, squamous cells must be both manipulated to stop differentiation so that viral genomic amplification can ensue, and then later allowed to differentiate so as to form an epithelium that can serve a barrier function for the host and couple this event to the expression of viral capsid proteins.

Three viral early open reading frames (ORFs) called E5, E6, and E7 encode proteins that stimulate cell proliferation, survival, and modulate keratinocyte differentiation. E6 proteins interact with target cellular proteins through docking on short acidic amphipathic helices often containing the sequence LXXLL. (20-22). Bovine Papillomavirus Type-1 (BPV-1) E6 (hereafter termed BE6) binds acidic LXXLL motifs in the clathrin AP1 adaptor complex (23) and the focal adhesion protein paxillin; these interactions with paxillin are required for cellular transformation by BE6, and in vivo competition with LXXLL motifs can block transformation by BE6 (22, 24-26). HPV-16 E6 (16E6) also binds to LXXLL motifs on the transcription factor IRF-3 (27) and the cellular E3 ubiquitin ligase E6AP (28); the complex formed by E6AP and 16E6 interacts with p53, thereby triggering the ubiquitin ligase activity of E6AP and the proteasome mediated degradation of p53 (28-33). The directly interacting binding motifs for other papillomavirus E6 oncoproteins have not yet been described.

We are interested in cellular proteins that associate with various E6 proteins through LXXLL interactions. We found association between the E6 oncoproteins of cutaneous viruses HPV-1 (1E6), HPV-8 (8E6) and BPV-1 (BE6) and the NOTCH co-activator MAML1, and found that BE6 represses NOTCH -induced transcription associated with squamous epithelial differentiation.

## Results

### E6 oncoproteins associate with MAML1

We examined host proteins that associate with BE6, 16E6, and 1E6 by in vitro binding to chitin-binding-domain fusions to the E6 oncoproteins, and discovered that peptides from MAML1 were present in the BE6 and 1E6 but not 16E6 pull down samples (data not shown). MAML1 has an acidic LXXLL peptide at its carboxy-terminus that is remarkably similar to the BE6-binding sites on paxillin and E6AP (Supplemental Fig. 2), so we hypothesized that this would be the site of E6 association. HA-tagged E6 proteins were co-transfected with FLAG-tagged MAML1 or FLAG-MAML1 deleted of the last 10 amino acids, eliminating the acidic LXXLL motif, termed hereafter as MAML1<sup>Δ</sup>. Immune precipitated MAML1 associated with BE6, 1E6 and 8E6, but minimally with 16E6 and not with 11E6 (Fig. 1). Immune precipitated MAML1<sup>Δ</sup> was greatly decreased for association with the E6 proteins, but not completely devoid of association. Interestingly, co-expression of MAML1 containing the LXXLL motif resulted in higher expression levels of BE6, 16E6, 1E6, and 8E6 compared to co-expression with MAML1<sup>Δ</sup> deleted of the LXXLL motif (Fig. 1), suggesting that co-expression of even weakly associating E6 LXXLL-binding partners stabilizes the expression of E6. Because transient over-expression or epitope tagging of E6 could alter its protein associations, we determined if BE6 could associate with MAML1 in BE6 transformed cells. We found it difficult to transduce primary keratinocytes with native BE6; we obtained few colonies, and could not detect BE6 expression in the resulting cells. Therefore, murine C127 cells, which are transformed by BE6, were retrovirally transduced first with FLAG-MAML1, FLAG-MAML1<sup>Δ</sup> or vector, drug selected (G418 resistance), and then transduced with native BE6 and puromycin selected. Fig. 2 shows that BE6 is in a complex with MAML1 but not MAML1<sup>Δ</sup> in stably transformed cells, and that BE6 expression and MAML1 expression levels are similar with and without BE6.

Earlier work on the association of BE6 with paxillin had established a close correlation between the interaction of BE6 and BE6 mutants with paxillin and transformation by BE6 and the BE6 mutants (34). The strong similarity between the acidic LXXLL carboxy-terminal motif of MAML1 and paxillin LXXLL motifs suggests that transformation by BE6 will also closely correlate with association with MAML1; indeed, in screening our extensive collection of E6 mutants, we have found very similar interactions between the MAML1 and paxillin LXXLL motifs with BE6, and no mutants that clearly discriminate between the two LXXLL binding motifs (Supplemental Fig. 3).

### **E6 association with MAML1 represses MAML1 transcriptional activation**

MAML1 contains two acidic regions associated with transactivation activity, the carboxy-terminal of which binds BE6 (MSDLDDLGS) and a more generally central acidic region (aa 75-305) (15). We hypothesized that E6 association with the carboxy-terminal MAML1 LXXLL motif would repress MAML1 transactivation. GAL4-MAML1 or GAL4-MAML1 were co-transfected with the untagged E6 proteins. As predicted, E6 proteins that bound MAML1 in Fig.1 also repressed GAL4-MAML1 transactivation in Fig. 3. The greatest repression was by BE6. E6 proteins showed weak repression activity against GAL4-MAML1 which is deleted of the LXXLL binding motif, although BE6 did consistently modestly repress GAL4-MAML1. 16E6, which bound poorly to MAML1 in Fig. 1, showed no ability to repress GAL4-MAML1. Interestingly, GAL4-MAML1 activity at higher E6 expression levels was similar to the activity of GAL4-MAML1 deleted of the LXXLL motif, indicating that the predominant activity of the E6 proteins is to repress a transactivation function of MAML1 that resides at the carboxy-terminus LXXLL motif. Although not shown in Fig. 3, 11E6 also failed to repress GAL4-MAML1 transactivation. Because BE6 was the most potent in this assay and because of our access to BE6 mutants, antibodies and productively infected bovine papillomas (below), we focused further studies on this protein.

### **BE6 represses canonical NOTCH induced transcription**

We determined if BE6 could repress NOTCH-induced transactivation. We transfected a luciferase reporter plasmid containing 4 copies of a RBP-J (also known as CBF1) DNA binding site upstream of a minimal promoter (CBF1-Luc) or an identical reporter plasmid containing mutated CBF1 binding sites (CBF1(mt)-Luc) into CV1 cells. The activity of this reporter in the absence of NOTCH signaling was low, presumably reflecting basal or low endogenous NOTCH signaling in CV1 cells. Upon co-transfection of an activated NOTCH Intracellular Domain (NID), luciferase activity was activated (presumably from the association of NID with endogenous MAML1) and transactivation was further activated to about 10 fold above basal level upon co-transfection of NID and MAML1 (Fig. 4A). Co-transfection of low amounts of BE6 (10ng) repressed activity by about 60%. Interestingly, BE6 also repressed reporter activity arising from co-transfected of NID and MAML1, indicating that an additional mode of repression may exist. No significant repression of the CBF1(mt)-Luc reporter by BE6 was observed (data not shown).

Although the CBF1-luc reporter is specific for NOTCH associated transcriptional activation, it is not an endogenous NOTCH responsive reporter. We therefore repeated the Fig. 4A

experiment using HES1 promoter luciferase reporter (HES1-luc). HES1 is a member of the HES family of b-HLH transcription factors that are the transcriptional targets and effectors of Notch signaling (13). Fig. 4B with the HES1-Luc reporter shows results very similar to CBF1-Luc in transient transfections. In order to determine the effect of E6 upon HES-1 RNA levels under stable expression conditions, human diploid fibroblasts were retrovirally transduced with BE6, 8E6 or 16E6; HES1 RNA levels determined by qRT-PCR showed repression by BE6 and 8E6 but not by 16E6 (Fig. 4C). In order to compare the effects of BE6 and 16E6 upon NOTCH dependent transcription, BE6 or 16E6 was titrated together with activated NOTCH, MAML1 and either CBF1-Luc or CBF1(mt)-Luc reporters (Fig. 5). BE6 repressed NOTCH induced transcription significantly at 60 fold less plasmid input than 16E6.

Because transient transfections can give rise to artifacts, and because CV-1 cells are not the natural host cells for papillomaviruses, we introduced into keratinocytes a self-inactivating lentiviral reporter with four copies of CBF1 binding sites (activating a minimal CMV early promoter); after integration into the chromosome, luciferase activity should be responsive to Notch-induced transcriptional activation only. This has some advantages, since cross-talk from other transcription factors could aberrantly activate NOTCH -induced cellular genes through non- NOTCH mechanisms. After stable integration of the NOTCH -reporter, the resulting cells were transduced with replication defective lentiviruses expressing 16E6, BE6, or empty vector. Fig. 6 shows that while 16E6 did not repress luciferase activity, BE6 significantly repressed luciferase activity.

Impaired NOTCH signaling causes delayed differentiation and ablated notch signaling causes failure of differentiation in squamous epithelia. In particular, proteins that are normally only expressed in the basal layer are observed in the suprabasal cell layers, particularly integrins (9). We hypothesize that BE6 repression of NOTCH -induced transcription should result in delayed differentiation, manifested as a delay in the expression of NOTCH -dependent HES1, and suprabasal expression of beta1-integrin. We further hypothesize that NOTCH dependent transcription should be present in at least a subset of cells so as to maintain epithelial integrity and terminal differentiation that is linked to papillomavirus capsid expression. Fibropapillomas from Bovine Papillomavirus infected cows were examined for basal cell markers (beta-1 integrin, paxillin and talin, Fig. 7) and expression of MAML1 and the NOTCH transcriptional target HES1 (Fig. 8). Beta1-integrin, and paxillin are expressed in the suprabasal layers of bovine fibropapillomas but are restricted to the basal layer of adjacent skin as we have previously reported (35). Like beta1-integrin and paxillin, the basal protein talin (36) was also suprabasally expressed in the fibropapilloma (Fig. 7). MAML1 was expressed in the nucleus of all cell layers of the normal and papilloma epidermis, although nuclear staining was less distinct in the basal layer, especially in the fibropapilloma (Fig. 8). HES1 expression was faint in normal epidermis and hair follicles, but was suprabasal as previously described (Fig. 8A) (10). However, in the fibropapilloma, HES1 expression was absent in the first suprabasal layer, and unapparent in much of the papilloma. Focally, strong HES1 expression was seen about 4 or more cell layers above the basement membrane, indicating a sporadic and delayed expression of HES1 in the papilloma (Fig. 8B). Taken together these observations suggest

that BE6 repression of NOTCH signaling results in absent and or delayed expression of HES1 and delayed keratinocyte differentiation.

## Discussion

Saturation mutagenesis screens in *Drosophila* identified zygotic lethal mutations that generated a neurogenic state, including NOTCH, Enhancer of split, Delta and Mastermind (mam). Although Mastermind (mam) was cloned by p-element tagging (37), the mammalian homolog of mam, MAML1, was cloned from a yeast two hybrid hunt using a gal4-fusion to HPV-16E6 (15). In that study, although MAML1 interacted with 16E6 by yeast two-hybrid (similar to our lexA-16E6 fusions in yeast, data not shown), no consistent physiologic consequence of 16E6 expression on MAML1 transactivation in vivo was reported in that study or observed in our study, indicating that the association was only observed in yeast. In our study, the association between mucosal E6 proteins and MAML1 was similarly weak (Fig. 1), but in contrast, the interaction between cutaneous E6 proteins and MAML1 was robust, and 1E6, the cancer-associated 8E6, and BE6 all repressed GAL4-MAML1 transactivation, while mucosal 16E6 did not (Fig. 3). This likely reflects subtle differences between the preferences of mucosal and cutaneous E6 proteins for LXXLL docking peptides on the target cellular proteins, reflecting the different biology between mucosal and cutaneous epithelium.

NOTCH signaling between adjacent cells affects the developmental choices of neighboring cells. One characteristic feature of NOTCH signaling is that modest differences in NOTCH signaling have developmental consequences, classically revealed in that heterozygous mutations of NOTCH, NOTCH ligands, and components of the NOTCH signaling complex result in haplo-insufficiency syndromes (reviewed in (13)). Complete disruption of NOTCH signaling in the squamous epithelium of transgenic mice by tissue specific NOTCH deletion (9, 38), squamous epithelial gamma secretase deletion to block the cleavage of NOTCH (39), epithelial deletion of the CBF1 (RBP-J) -binding subunit of the NOTCH transcription complex (10, 40), or expression of a dominant negative MAML1 in squamous epithelium (41) all result in loss of differentiation and squamous cell carcinomas. This demonstrates that all three components of the NOTCH transcription effector complex act together as a tumor suppressor in normal mouse squamous epithelium, and the critical role of MAML1 in this process. Thus, the association of cutaneous E6 oncoproteins to repress MAML1 as a co-activator and thereby manipulate NOTCH signaling is biologically plausible.

However, Notch signaling in different tissues can drive either terminal differentiation or cellular proliferation, making this subject complex, especially in the context of a viral infection that co-express other viral early gene products in a temporally and spatially controlled manner. In T cell acute lymphocytic leukemia and chronic lymphocytic leukemia *NOTCH1* contains activating mutations (42, 43), while in diffuse large B cell lymphoma *NOTCH2* is mutated (44), all with mutations that result in constitutively activated NOTCH signaling. These observations correlate with the finding that in some cell types, NOTCH signaling is necessary for the maintenance of stem cell populations.

In contrast to the above, in other tissues Notch signaling functions as a tumor suppressor with loss of function recently reported in myeloid leukemia (45). NOTCH tumor suppression is perhaps best illustrated in cutaneous squamous epithelia as noted above, where chronic administration of gamma-secretase inhibitors or ablation in mice of the NOTCH signaling components results in squamous cell cancers. As this manuscript was prepared, two independent reports demonstrated a high frequency of amino-terminal mis-sense mutations of *NOTCH1* receptors in both HPV positive and negative human head and neck squamous cell cancers (46, 47). *NOTCH1* mutations were the most frequently mutated gene after *TP53*. *NOTCH1* mutations were both missense and nonsense types upstream of the intracellular transactivation domain, with two separate different inactivating mutations of each allele in some cases. Underlying the centrality of NOTCH signaling in head and neck cancers, additional mutations were found that connect to the NOTCH pathway. First, activating mutation of the *FBXW7* gene whose protein targets NOTCH1 for degradation were found (46) and would be predicted to result in lower NOTCH 1 expression (48); second, nonsynonymous point mutations in *NOTCH2* or *NOTCH3* were also found (47). Since Notch signaling generates the NOTCH-RBPJ-MAML1 active transcription complex, these observations correlate with and enhance the significance of MAML1 targeting by cutaneous papillomavirus E6 proteins.

In contrast to head and neck squamous cell cancers where loss of NOTCH1 is associated with progression of disease, many studies on the role of notch signaling in HPV-positive cervical cancer show tumor-promoting roles for NOTCH1 with enhanced expression of cleaved and nuclear NOTCH1 in invasive cancers (49-51), cooperative transformation between activated NOTCH1 and the papillomavirus oncoproteins (52), and reduced cell proliferation upon knockdown of NOTCH1 expression in cervical cancer cell lines (53-57). Studies conflicting with these results showed that re-expression of active NOTCH1 fragment repressed the transcription of the HPV E6 and E7 genes, resulting in the restoration of p53 expression and the arrest of cell proliferation (58-61); however another study has attributed both the repression of cell proliferation and E6/E7 transcription to non-physiologic overexpression of the NOTCH1 active fragment (62). The different manifestations of *NOTCH1* signaling or knockdown in cervical compared to head and neck cancers clearly requires further study.

Papillomaviruses must both manipulate keratinocyte differentiation to enable vegetative viral DNA amplification in the spinous cell layers, but also enable keratinocyte terminal differentiation in the corneal layer in order to ensure a competent epithelial barrier, since loss of barrier function would predictably result in microbial infections and immune cell infiltration of the papilloma. Indeed, papillomaviruses have evolved to couple the expression of their capsid proteins to terminal differentiation of keratinocytes, underscoring the importance of epithelial integrity to the long-term relationship with the host. Thus, one would expect E6 to not simply ablate NOTCH signaling, but to modulate NOTCH signaling during the viral life cycle, and perhaps, to restore or enhance NOTCH signaling in the upper cell layers of the papilloma. BE6 does not ablate NOTCH signaling, which would result in loss of epithelial integrity, but rather negatively modulates NOTCH signaling in cultured cells. While BE6 binds to the carboxy-terminal transactivation LXXLL motif of MAML1, it

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does not fully repress MAML1 transactivation as might occur if BE6 was recruiting a repression function such as a histone deacetylase. Thus, the BE6-MAML1 association is not a complete switch, but rather a modulation. The NOTCH + CBF1 complex interacts with GCN5, PCAF (63, 64) and p300 (65) in addition to MAML proteins, so how E6 proteins alter the role of these factors in Notch-induced transcription remains to be determined. Future studies will be directed at the normal cellular interactions at the MAML1 carboxy-terminus and how BE6 might displace or remodel the complex of proteins associated with MAML1. It will also be of interest in the future to determine the role of BE6 in combination with the other early region products and the presence or absence of integrin signaling in the manipulation of NOTCH signaling in keratinocytes.

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MAML1 is a co-activator for p53 transactivation (66), MEF2C (67), and is a NOTCH - independent co-activator together with beta-catenin of cyclinD1 expression in colon cancer cells (68). Thus, while we have shown that BE6 can repress notch induced signaling, it may impact additional pathways through its association with MAML1.

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NOTCH signaling is so central to keratinocyte differentiation that one would predict that other papillomaviruses will also modulate NOTCH signaling directly or indirectly, although they may not act in the same way as BE6. It is possible, for example, that other E6 proteins could even activate NOTCH signaling through association with MAML1 in order to promote terminal keratinocyte differentiation, or that repression of NOTCH signaling could occur through indirect means. The high-risk E6 oncoproteins target the degradation of p53; p53 has been proposed to activate transcription of the *NOTCH1* gene, and repression of NOTCH1 expression by siRNA together with expression of oncogenic ras is sufficient to produce squamous cell carcinomas in human keratinocytes (69). It is possible that in our experiments where keratinocytes were not subjected to stress to induce p53, that the effect of 16E6 upon NOTCH signaling in this context was not observed, or it could be that a different experimental approach would reveal an effect of 16E6. NOTCH1 and p63 are also involved in reciprocal inhibition; NOTCH signaling represses p63 expression in suprabasal layers. Interestingly, the high risk E7 oncoprotein could block this through the repression of mir203, whose primary target is p63 (70). It will be interesting in the future to determine how oncoproteins from other HPV types manipulate NOTCH signaling and keratinocyte differentiation and how the timing of NOTCH signaling may be utilized in the viral life cycle.

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## Materials and Methods

### Cells and cell Culture

CV1 and 293T human diploid lung fibroblasts were obtained from ATCC (Manassas, Virginia). NIKS (normal immortalized keratinocytes) were cultured on mitomycin C treated feeder cells as described (71). Primary human keratinocytes were obtained from newborn foreskins and cultured in KSFM media (Invitrogen). Replication defective murine retroviruses expressing BE6 or MAML1 were based upon pLXSN or pBabe-puro. E6 lentiviral expression was from the lentiviral plasmids pCDH1-EF1\_MCS-IRES-GFP. Gal4 fusions to full length MAML1 (aa 1- 1016) or MAML1 deleted of the C-terminal LXXLL motif (aa 1-1006) were cloned into pBIND. NOTCH reporter plasmids pCBFRE-Luc



(Addgene plasmid 26897) and pCBFRE(mt)-Luc (Addgene plasmid 2689), contain four copies of CBF Notch binding sites or 4 mutated binding sites respectively from EBV upstream of pSve minimal promoter (72). The NOTCH reporter plasmid pGreenFire-Notch (SB Biosciences) is a self-inactivating lentiviral plasmid expressing a luciferase reporter downstream of four NOTCH responsive RBP-J binding sites (GTGGGAACGGCATTGTAGCG) and a minimal CMV early reporter; upon integration the NOTCH -responsive reporter is the only intact promoter. Lentiviruses and retroviruses were packaged by transient transfection and lentiviruses concentrated by PEG precipitation and then re-suspension in keratinocyte growth media (73), and lentiviruses titered by infection of HEK-293T cells with dilution series of packaged lentiviruses and scoring for GFP positive cells 48 hours later.

### qRT-PCR

Total RNA was extracted using RNazol (Invitrogen), equalized, and pRT-PCR performed using iQ SYBR-green supermix kit (BioRad) and a BioRad iCycler according to the manufacturer's recommendations. Threshold levels were normalized to levels obtained using primers to GAPDH and actin. Primer design was from Biowww.net (<http://biowww.net>) and (74-76) and sequences are listed in the supplemental data.

### Luciferase reporter assays

CV-1 and HEK-293T cells were transfected using polyethylenimine. All transfections included a constant amount of co-transfected beta-galactosidase expression plasmid used to normalize luciferase results for transfection variability.

### Yeast expression

Modified LexA-based yeast 2-hybrid assays (77) were performed as previously described (35).

### Antibodies, western blots and immune fluorescence analysis

Transfected mammalian cells were lysed in SDS, equilibrated for protein content (Biorad assay kit), proteins resolved by SDS-PAGE electrophoresis and transferred to PVDF membranes. Antibody sources: FLAG epitope, tubulin (Sigma); rabbit anti- MAML1 and HES1 (Bethyl Antibodies), monoclonal antibody to beta1 integrin (Developmental Studies Hybridoma Bank); paxillin and talin (Transduction Laboratories, BD Biosciences). Western Blot images were captured with a Alpha Innotech CCD video camera and images adjusted for contrast in NIH ImageJ software.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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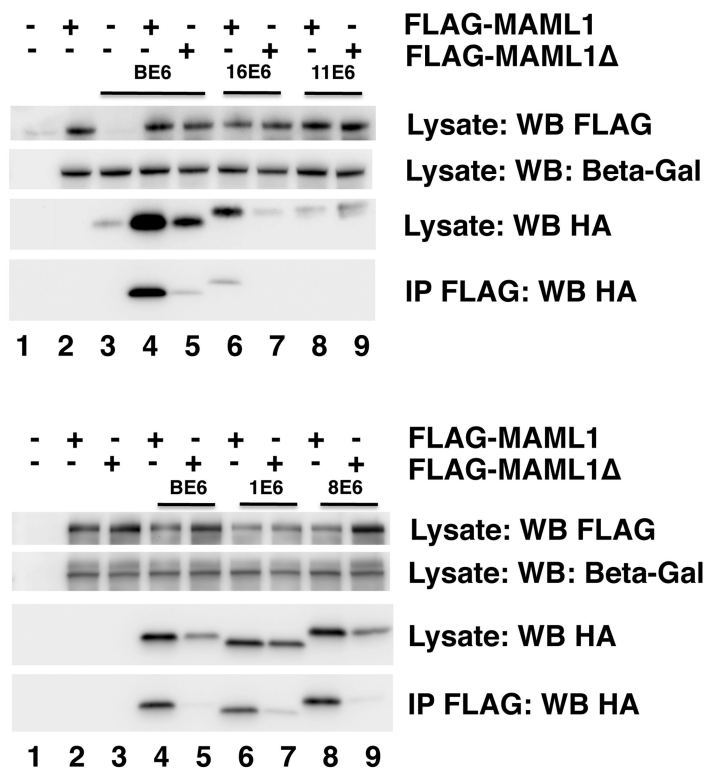
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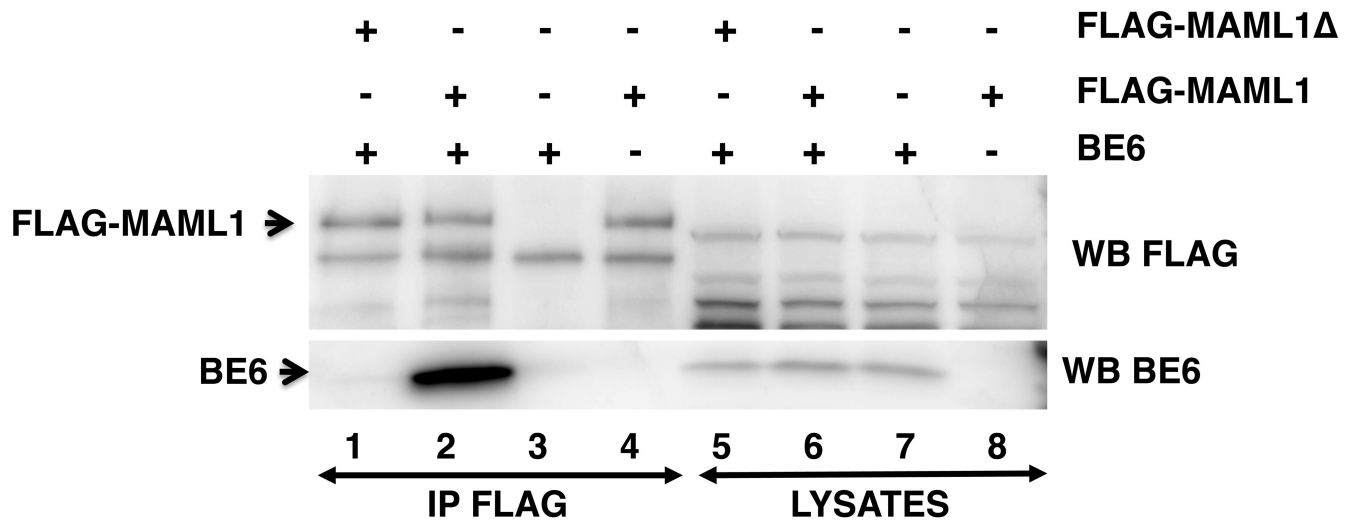
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**Fig. 1. Cutaneous E6 proteins associate with MAML1**

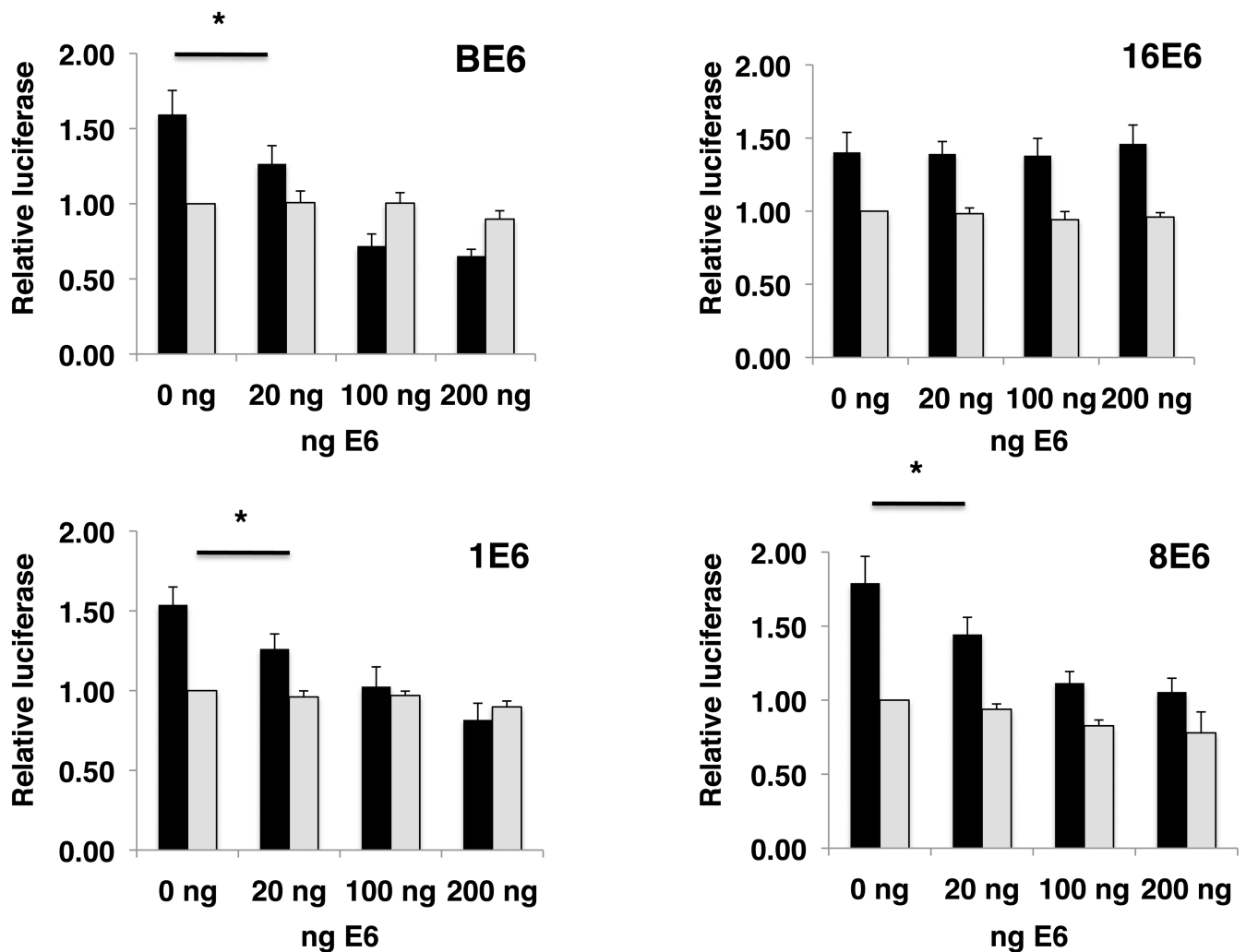
Plasmids for FLAG-tagged MAML1 and MAML1 $\Delta$  were transfected together with HA-tagged E6 and beta-galactosidase into HEK-293T cells as indicated in the figure and harvested by lysis in NP40 lysis buffer 24 hours later. Clarified lysates were immune precipitated with mouse anti-FLAG coupled to agarose beads and western blots probed with rabbit anti-HA and anti-FLAG antibody.



**Fig. 2. BE6 and MAML1 are in a complex in stably transduced cells**

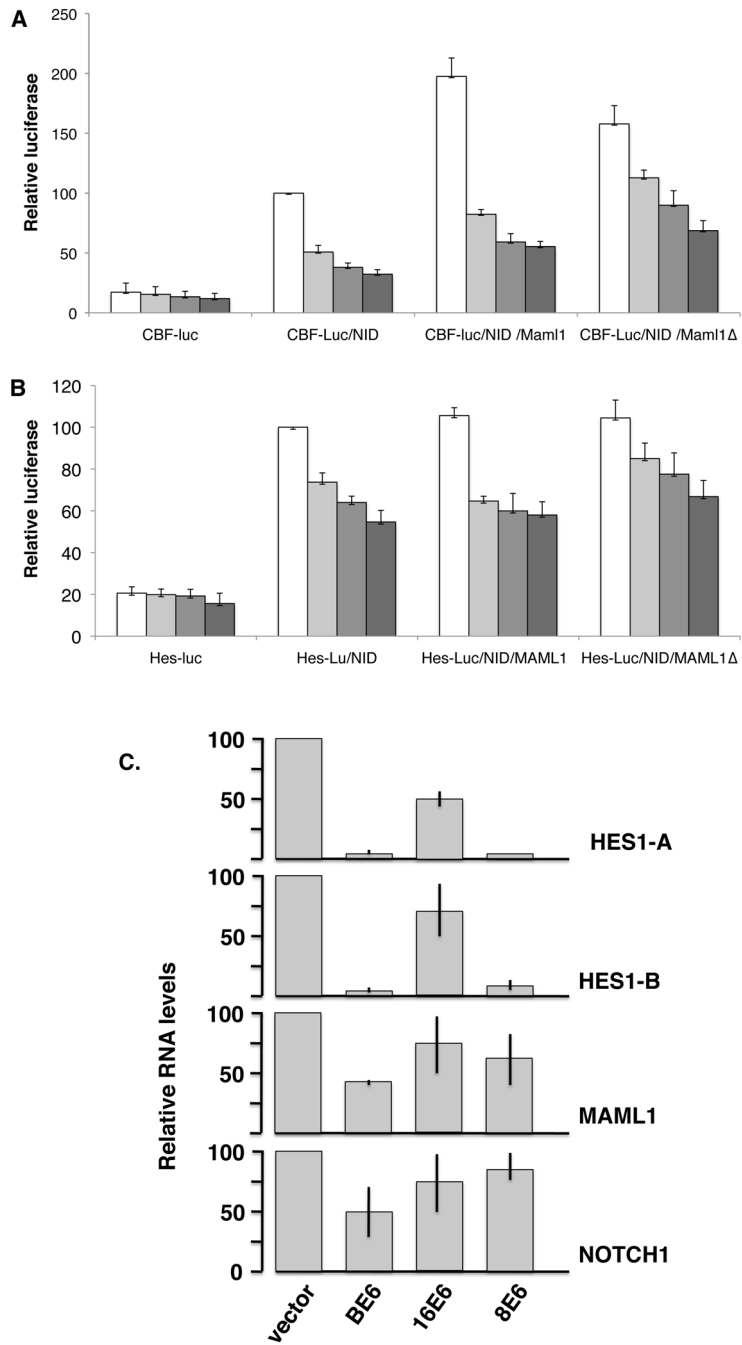
Murine C127 cells were sequentially retrovirally transduced and selected for FLAG-MAML1, and FLAG-MAML1, and then drug selected cells transduced with BE6 retrovirus with puromycin selection; resulting cell lines ( $1.5 \times 10^7$  cells per sample) were lysed with NP40 lysis buffer and immune precipitated with anti-flag antibody beads, blots probed with rabbit anti-BE6 and then re-probed with rabbit anti-FLAG. In the exposure shown, FLAG-MAML1 in the lysate samples is too faint to be seen in comparison to the signal in the immune precipitated sample.





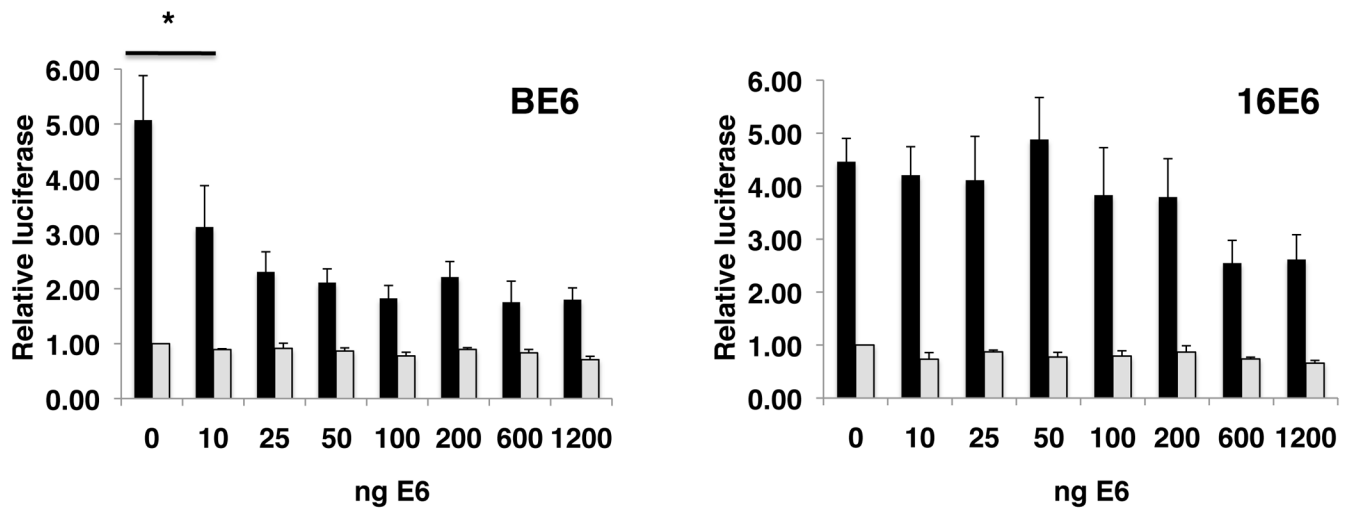
**Fig. 3. E6 proteins repress transactivation by MAML1**

Plasmids for GAL4 fusions to full length MAML1 (black bars) or LXXLL deleted MAML1 (grey bars) were co-transfected with plasmids for the indicated amounts of native E6 proteins, a luciferase reporter and internal control lacZ expression plasmid into HEK-293T cells that were harvested 24 hours later and assayed for lacZ activity and luciferase activity. Results shown on the vertical axis are relative luciferase activity and are the average and standard deviation of five separate experiments, normalized first to lacZ activity and then to GAL4-MAML1 activity in the absence of E6 (expressed as 100). Error bars are standard deviations. Asterisk denotes  $P < 0.01$  by student t-test.



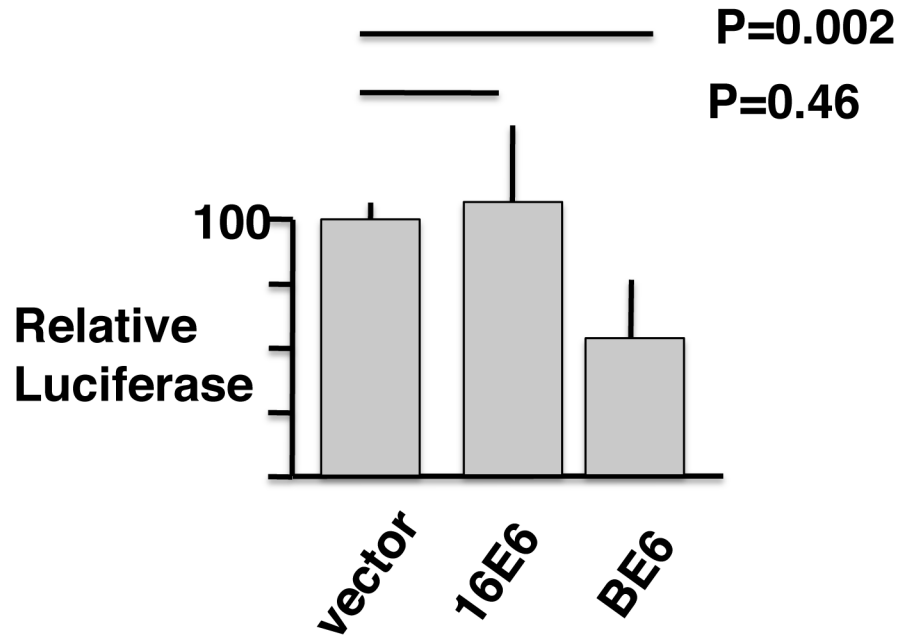
**Fig. 4. Repression of Notch dependent transcriptional activation by BE6**  
 CV-1 cells were transfected and analyzed for luciferase and beta-galactosidase activity 48 hrs later. A. Cells were co-transfected with 600 ng of a luciferase reporter with 4 copies of CBF1 (also termed RBP-J) binding sites upstream of a minimal SV40 early promoter (CBFRE1-Luc, abbreviated as CBF-Luc in the figure (72)) and as indicated, 300 ng Notch intracellular domain (NID) and 120 ng MAML1 or 120 ng MAML1Δ. White bars had no BE6, and increasingly darker grey bars were transfected with 25, 200, or 600 ng of BE6 expression plasmid, balanced with empty vector. pCMV-lacZ was co-transfected as an

internal transfection control. Results shown are relative luciferase activity, and are the average and standard deviation of five separate transfection experiments, with results normalized to lacZ expression and CBFRE1-Luc(mt) to normalize for variation in transfection efficiency. B. CV-1 cells were transfected with the HES1-luciferase reporter and expression plasmids as indicated in the figure and as described in part A. C. Cutaneous E6 proteins repress endogenous HES1 RNA expression. Human diploid lung fibroblasts were retrovirally transduced with the indicated vector or E6 genes and total RNA from drug-selected cells analyzed by qRT-PCR as detailed in the methods. Bars show the average of duplicate experiments with the lines showing the range of values obtained, normalized to vector control cells.



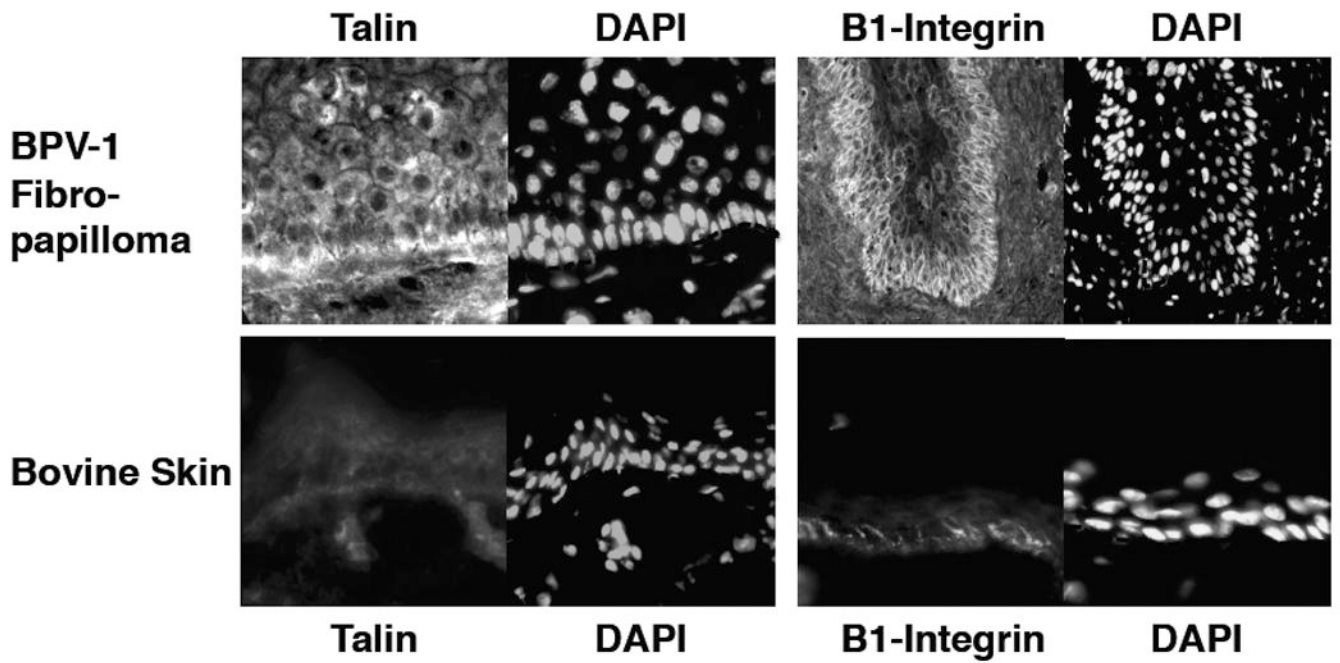
**Fig. 5. BE6 but not 16E6 repressed Notch dependent transcription**

Monkey CV-1 cells were transfected with 600 ng of either CBFRE1-Luc, (black bars) or by CBFRE(mt)-Luc, (grey bars), 300 ng of an expression plasmid for Notch1 intracellular domain, 120 ng of a MAML1 expression plasmid, and from 10 ng to 1.2 ug of the indicated E6 protein expression plasmid. Results shown on the vertical axis are relative luciferase activity, and are the average and standard deviation of seven separate experiments, normalized to the CBF1-Luc(mt) reporter in the absence of E6, set to 1.0. Asterisk denotes  $P < 0.01$  by student t-test.



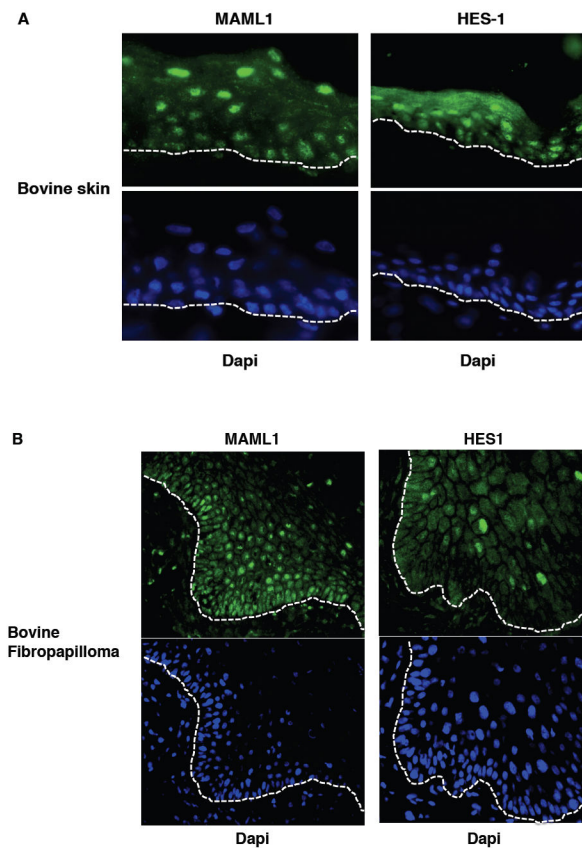
**Fig. 6. BE6 represses basal Notch signaling from chromosomal reporters**

Human keratinocytes were transduced at an MOI of 5 with a self-inactivating lentiviral Notch-responsive lentiviral reporter, and then passaged for 2 weeks. Feeder cells were then detached, and the keratinocytes transduced overnight with either empty lentiviral vector, lentiviral vector expressing 16E6, or lentiviral vector expressing BE6 at an MOI of 3, then feeder cells were added the next morning and the cells harvested for luciferase assay 3 days later. Results shown on the vertical axis are the average and standard deviation of four experiments.



**Fig. 7. Bovine fibropapillomas express basal cell markers suprabasally**

Frozen sections from BPV-1 induced fibropapillomas and adjacent normal skin were stained with monoclonal antibodies to talin or  $\beta$ 1-integrin as indicated and nuclei counter-stained with DAPI. Normal and papilloma images were captured with the same exposure time. A dashed white line shows the dermal-epidermal boundary.



**Fig. 8. Expression of MAML1 and HES1 in bovine skin and bovine fibropapillomas**  
 Frozen sections of a BPV-1 induced fibropapilloma that also included normal skin at the margin of the tumor were stained as indicated with rabbit anti-MAML1 or HES1 (green) and the DNA counter-stain DAPI (blue). A dashed white line shows the dermal-epidermal boundary. Normal and papilloma images were captured with the same exposure time. A. Expression of MAML1 and HES1 in normal skin. B. Expression of MAML1 and HES1 in the fibropapilloma.