Inactivation of a Gαs–PKA tumour suppressor pathway in skin stem cells initiates basal-cell carcinogenesis

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Genomic alterations in GNAS, the gene coding for the Gαs heterotrimeric G protein, are associated with a large number of human diseases. Here, we explored the role of Gαs on stem cell fate decisions by using the mouse epidermis as a model system. Conditional epidermal deletion of Gnas or repression of PKA signalling caused a remarkable expansion of the stem cell compartment, resulting in rapid basal-cell carcinoma formation. In contrast, inducible expression of active Gαs in the epidermis caused hair follicle stem cell exhaustion and hair loss. Mechanistically, we found that Gαs–PKA disruption promotes the cell autonomous Sonic Hedgehog pathway stimulation and Hippo signalling inhibition, resulting in the non-canonical activation of GLI and YAP. Our study highlights an important tumour suppressive function of Gαs–PKA, limiting the proliferation of epithelial stem cells and maintaining proper hair follicle homeostasis. These findings could have broad implications in multiple pathophysiological conditions, including cancer.

Among the rapidly self-renewing tissues in the adult body, the epidermis is a unique system to study stem cell biology¹–⁵. Its integrity is highly dependent on resident self-renewing stem cells located in the basal layer of the stratified epidermis. Stem cells have to balance self-renewal and differentiation to maintain proper tissue homeostasis and respond to a variety of conditions, including tissue injury. This proper balance is achieved in part by a milieu of micro-environmental signals controlling stem cell fate decisions and their cellular responses.

G-protein-coupled receptors (GPCRs) are the largest family of cell-surface molecules involved in signal transduction, and play central roles in numerous physiological processes and pathological conditions⁶,⁷. However, our understanding of the functions of GPCRs and their linked heterotrimeric G proteins in stem cell biology in vivo is still largely incomplete. Here, by focusing on the role of Gαs on stem cell fate using the epidermis as a model system, we demonstrate that this Gα protein exerts a central role in coordinating self-renewal and differentiation in epithelial stem cells. Alone, conditional epidermal deletion of Gnas or inactivation of protein kinase A (PKA) in mice was sufficient to cause an aberrant expansion of the stem cell compartment, resulting in the rapid formation of basal-cell carcinoma (BCC)-like lesions. In contrast, expression of active Gαs caused hair follicle stem cell exhaustion and hair loss. Mechanistically, Gαs and PKA disruption promoted the concomitant cell autonomous activation of GLI and YAP. These findings support a central role for Gαs and PKA in stem cell fate decisions in mammals, and reveal a tumour suppressive mechanism by which the Gαs–PKA signalling axis limits the aberrant proliferation of epithelial stem cells and maintains hair follicle and skin homeostasis.

RESULTS
Gnas deletion in the skin is sufficient to induce BCC-like lesions
To explore the role of Gαs in stem cell fate we generated epidermal-specific Gnas knockout mice. Mice expressing a tamoxifen-inducible Cre driven by the keratin 14 promoter (K14CreER), which targets the epidermal stem cell compartment⁸, were crossed with mice carrying loxP sites surrounding Gnas exon one⁹ (Fig. 1a). Unexpectedly, all Gnas epidermal knockout mice (Gnas eKO) developed skin lesions characterized by thickening of the epidermis and hair loss, primarily on the ears, snout and paws, only a few weeks after Gnas excision (Fig. 1bc and Supplementary Fig. 1). Histologically, these lesions exhibited extensive proliferation of basaloïd cells, which formed clumps and islands that deeply invaded the underlying stroma (Fig. 1d). Tumours were morphologically similar to superficial and
nodular human BCCs (ref. 10; Fig. 1e), developing in body regions aligned with previous BCC mouse models

The epidermal basal identity of tumour lesions in Gnas eKO mice was confirmed by the expression of the basal marker cytokeratin 5 (CK5) and the stem cell marker p63 (Fig. 1f). Cells showed altered proliferation patterns and polarity, as reflected by Ki67 (Fig. 1g) and integrin α6 staining, respectively (Fig. 1h), and were positive for the hair follicle and BCC marker cytokeratin 15 (CK15; ref. 13; Fig. 1i) but negative for the differentiation marker loricrin (Fig. 1j). Increased thickness of the CK15+ skin layer (Supplementary Fig. 1c) and multiple additional markers reflected the expansion of the basal cells. Thus, deletion of Gnas from mouse epidermis is sufficient to induce rapid expansion of the stem cell compartment and development of lesions resembling BCC.

Transcriptional analysis in Gnas eKO mice uncovers the activation of Hedgehog GLI and Hippo YAP1 transcriptional networks

Gene ontology analysis of transcripts in the skin of Gnas eKO mice showed significantly increased expression of genes associated with epithelial development (Fig. 2a). Analysis of upregulated transcriptional signatures revealed alterations in multiple transcriptional networks (Supplementary Table 1) that were filtered by focusing on transcription factors with known functions in hair follicle and epidermal stem cell maintenance. These included GLI1, NFAT family genes, TP63, EZH2 and YAP1 (Fig. 2b). Interestingly, GLI transcription factors are the main drivers of human BCC development3,10,11,14. We confirmed the overactivation of the GLI transcriptional network by analysing the messenger RNA levels of Hedgehog-signalling members in Gnas eKO mice (Fig. 2c,d). Although GLI members and their transcriptional targets Ptc1 and Ptc2 were all upregulated, we did not detect increased mRNA levels of Sonic Hedgehog (Shh) or Indian Hedgehog (Ihh; Fig. 2c). This might indicate that the stimulation of GLI signalling in Gnas eKO mice is cell autonomous, and does not depend on the increased expression of these PATCH ligands.

Lesions in Gnas eKO mice seemed to originate from hair follicles, raising the possibility that BCC development might be due to an amplification of a subset of hair follicle stem cells. Indeed, several transcriptional regulators of hair follicle stem cell maintenance and proliferation12 were upregulated in Gnas eKO mice (Fig. 2e). Interestingly, GLI transcription factors are essential for hair follicle stem cell regeneration5,15 and the Hedgehog response gene Gli1 defines...
a subset of stem cells that can regenerate the hair follicle and migrate into skin wounds during healing. To track GLI1+ cells after Gnas deletion we took advantage of GLI reporter mice (GLI+; ref. 17). As previously described, in Gnas wild-type (WT) GLI+ mice, GLI1 is expressed almost exclusively in the isthmus and secondary hair germ of resting hair follicles (Fig. 2g). After Gnas deletion, however, GLI1+ cells expanded to the entire hair follicle and spread to the interfollicular epidermis (Fig. 2g). In advanced lesions, most of the epidermis was replaced by GLI1+ cells (Fig. 2g). These results confirmed the overactivation of the Hedgehog GLI signalling network following Gnas deletion and suggested that BCC-like lesions might arise from an expansion of GLI1+ hair follicle stem cells.

In the search for additional mechanisms leading to the massive hair follicle stem cell expansion after Gnas deletion, we next focused on YAP1, a transcriptional co-activator that maintains the self-renewal capacity and undifferentiated state of epidermal skin progenitor cells. Recent reports indicate that YAP1 activity is tightly regulated by GPCRs (ref. 19). Indeed, YAP1 transcriptional activity was significantly increased in Gnas eKO mice (Fig. 2b), concomitant with increased YAP1 nuclear localization (Fig. 2h,i). Furthermore, a YAP1 transcriptional signature differentiated mRNAs from Gnas eKO mice from control mice by unsupervised hierarchical clustering (Fig. 2j). This approach helped identify multiple YAP1 targets that were significantly upregulated in Gnas eKO skin (Supplementary Table 2). Human BCCs also show a significant increase in nuclear YAP1 expression in normal skin and BCC. n=3 normal and 6 BCC tissue sections. Data are presented as means ± s.e.m., and significance was calculated by ANOVA and Student’s t-test (NS P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001).

Gnas eKO triggers ectopic activation of GLI and YAP1

To further explore the role of GLI and YAP1 in stem cell expansion, we focused on early events triggered by Gnas deletion, before any phenotypic alterations were detected. Using whole mount of tail epidermis, we observed that shortly after Gnas excision (one day following tamoxifen treatment) GLI is ectopically activated in the base of the isthmus of...
Reduced expression of YAP1 or GLI1 resulted in a significant decrease in colony-forming efficiency of keratinocytes derived from WT and Gnas eKO mice two weeks after tamoxifen treatment. Cells from 2 mice of each genotype were plated in duplicate and colonies counted (cells from 2 WT mice and 2 Gnas eKO mice). Representative pictures of wells and quantification from clonogenic assays of keratinocytes isolated from WT and Gnas eKO mice. n = 3 technical replicates, one representative experiment of three is shown.

Figure 3 Gnas eKO triggers ectopic/de novo activation of GLI and YAP1. (a,b) Representative pictures of βGal staining of tail skin whole mounts from WT Glilz and Gnas eKO Glilz mice showing GLI1 cells one day after finishing the administration of tamoxifen. Arrows indicate the location of GLI1 epithelial stem cells. In b hair follicles were removed to facilitate visualization of GLI activation at the base of the isthmus of hair follicles. (c,d) Representative pictures of tail skin whole mounts from WT and Gnas eKO animals stained to show expression of YAP1 (green) and cytokeratin 15 (CK15, red), one day after finishing the administration of tamoxifen. (e) Representative pictures of wells and quantification of clonogenic assays of keratinocytes isolated from WT and Gnas eKO mice two weeks after tamoxifen treatment. n = 3 technical replicates, one representative experiment of three is shown. Inset panels in images show details at higher magnification. Hair follicles are delineated with a white dotted line. Data are presented as means ± s.e.m., and significance was calculated by ANOVA and Student’s t-test (**P < 0.01; ***P < 0.001).

Gnas eKO triggers an ectopic and cell autonomous increase in GLI and YAP1 activity, which may contribute to aberrant epidermal stem cell proliferation.

Gαs restrains Hedgehog and YAP signalling through PKA

The absence of Gαs in the epidermis resulted in increased GLI and YAP1 signalling, suggesting that under normal conditions Gαs might limit the transcriptional activity of these stem cell regulators. Gαs controls a myriad of signalling networks, including cyclic AMP signalling, cellular metabolism and intracellular trafficking. In the search for potential mechanisms underlying the impact of Gnas deletion we focused on the protein kinase A (PKA), one of the main signalling effectors of Gαs downstream of cAMP activation. To limit PKA activity, we took advantage of the PKA inhibitor protein (PKI; ref. 26) and fused its PKA-interacting residues downstream of
GFP–PKI (GFP–PKI; Fig. 4a). Critical PKI residues were also mutated to disrupt binding to PKA (GFP–PKI4A) and used as a control (Fig. 4a). We also used a constitutively active mutant form of Gαs (GαsR201C; ref. 6) or increased the intracellular levels of cAMP by combining forskolin and 3-isobutyl-1-methylxanthine (IBMX), to confirm that GFP–PKI but not GFP–PKI4A blocks PKA signalling (Fig. 4b and Supplementary Fig. 2a,b).

These experimental tools demonstrated that the activation of PKA by Gαs exerts a remarkable negative impact on the transcriptional activity of GLI1 and YAP1 (Fig. 4c,d), the latter in line with recent reports.7,28. Furthermore, PKA could phosphorylate GLI1 (Fig. 4e). This phosphorylation is known to regulate GLI transcriptional activity,29, suggesting that Gαs may control GLI directly through PKA, independently of Hedgehog signalling. Indeed, PKA inhibition by GFP–PKI was alone sufficient to significantly increase GLI1 transcriptional activity in 293 (Fig. 4c) and NIH3T3 cells (Fig. 4f). This effect seems to be independent from the Hedgehog-signalling protein smoothened (SMO), because GLI1 activation by GFP–PKI was only partially reduced by the SMO inhibitor cyclopamine (Fig. 4f).

To further explore this possibility we used keratinocytes derived from WT or Gnas eKO mice expressing the Gli2 reporter. GLI transcriptional activity induced by SAG was abolished in Gnas WT Gli2 keratinocytes by cyclopamine treatment (Fig. 4g,h). Instead, in Gnas eKO Gli2 keratinocytes, GLI activity was only partially diminished by cyclopamine (Fig. 4g,h), and increasing the intracellular levels of cAMP had a stronger effect (Fig. 4g,h)
Figure 5 PKA mediates cAMP-induced inactivation of YAP1 through LATS and NF2. (a) Western blot analysis of YAP1 regulatory molecules in non-confluent HACAT cells treated with cAMP-raising agents, forskolin + IBMX (FI), for the indicated times. (b) Representative pictures of non-confluent HACAT cells treated or not with forskolin + IBMX (FI) for 2 h and stained to show expression of YAP1 (green) and nuclei (blue). (c,d) RNA-interference experiments of the indicated YAP1 regulatory molecules. Cells were transfected with the indicated siRNAs for 48 h and then treated (FI) or not (C) with forskolin + IBMX for 2 h and stained to show expression of YAP1 (c); or collected for the indicated western blot analysis (d). For c, the proportion of cells per field showing YAP1 localization in the nucleus or cytoplasm was quantified. n=3 independent experiments, 4 fields with 30 to 50 cells were counted per condition per experiment. Data are presented as means ± s.e.m., and significance was calculated in the nucleus fraction by Student’s t-test against the control of the same group (NS P > 0.05; ∗∗P < 0.01; and ∗∗∗P < 0.001). Full images of blots are shown in Supplementary Fig. 6.

Supplementary Fig. 4). Moreover, increased CAMP levels (but not cyclopamine) repressed the transcriptional activity of YAP1 in Gnas eKO keratinocytes, as judged by the levels of its transcriptional target Ctgf (Supplementary Fig. 4). Interestingly, deletion of Gnas also sensitized cells to Hedgehog pathway activation by SAG (Fig. 4g,h), supporting that Gnas acts to restrain Hedgehog signalling.

PKA mediates G\textsubscript{\textalpha} inactivation of YAP1 through LATS

YAP1 activity is mostly regulated post-transcriptionally through the inhibitory components of the Hippo pathway, which converge on the activation of LATS kinases that phosphorylate YAP1, inducing its cytoplasmic retention\textsuperscript{30,31}. Using a human keratinocyte cell line (HACAT) in which YAP1 is active under non-confluent conditions, stimulation of cAMP led to an increase in YAP1 phosphorylation and LATS1 activation, the latter revealed by its phosphorylation at the activation loop (Ser 909; Fig. 5a). The induction of cAMP resulted in the phosphorylation of LKB1 and the Hippo core components MST1/2 (Fig. 5a), both of which have been shown to activate LATS and inhibit YAP1 (refs 30–32). cAMP elevation also resulted in cytoplasmic retention of YAP1 in non-confluent cell cultures (Fig. 5b).

To investigate how PKA represses YAP1 activity, we used the cytoplasmic retention induced by cAMP as readout in RNA-interference experiments. Among the Hippo pathway components, only knockdown of LATS1/2 rescued the inhibitory effect of increased cAMP (Fig. 5c), suggesting that LATS kinases mediate PKA-induced YAP1 inhibition, at least in part independently of the core Hippo pathway components MST1/2 and SAV. Interestingly, LKB1 knockdown did not protect YAP1 from cAMP-mediated inhibition, but knockdown of the NF2 tumour suppressor partially rescued this effect (Fig. 5c). NF2 organizes Hippo signalling at the plasma membrane and coordinates the activation of LATS kinases and YAP1 phosphorylation\textsuperscript{33}. Indeed, knockdown of LATS1/2 and NF2 abolished the cAMP-induced phosphorylation of YAP1, whereas knockdown of MST1/2, SAV and LKB1 had no effect (Fig. 5d). NF2 knockdown also blocked the increased LATS1 phosphorylation induced by cAMP (Fig. 5d), supporting that NF2 contributes to the PKA-induced activation of LATS.

Inhibition of PKA in the skin is sufficient to phenocopy Gnas eKO

As PKA mediates the inhibitory effects of G\textsubscript{\textalpha} on GLI and YAP1, we next challenged the possibility that reduction in PKA activity might initiate BCC development. We generated mice expressing GFP–PKI and GFP–PKI4A under the control of the \textsuperscript{1}Tet-responsive element and bred them with mice expressing the reverse tetracycline-activated transactivator \textsuperscript{r}TA2 under the control of the cytokeratin 5 promoter (K5rtTA), targeting the epidermis and its stem cell compartment\textsuperscript{34,35} (Fig. 6a).
Figure 6 Inactivation of PKA is sufficient to initiate BCC formation. (a) Schematic representation of the animal model used to target the inducible expression of the PKA inhibitor protein (GFP–PKI) to the basal epidermal stem cell compartment. (b) Histological analysis of WT and K5rtTA Tet–GFP–PKI mice. K5rtTA Tet–GFP–PKI skin shows basaloid cells growing in the stroma resembling micronodular and superficial BCCs. (c) Representative pictures of the skin of K5rtTA Tet–GFP–PKI4A and K5rtTA Tet–GFP–PKI mice stained to show expression of GFP (green), α6 integrin (red) and nuclei (blue). (d–f) Representative pictures of the skin of K5rtTA Tet–GFP–PKI animals stained to show expression of the stem cell marker p63 (green) and the basal progenitor marker cytokeratin 5 (CK5, red); the hair follicle marker cytokeratin 15 (CK15, red) and nuclei (blue) (e), and the proliferation marker Ki67 (red) and nuclei (blue) (f). (g) Representative picture of βGal staining of the skin of K5rtTA Tet–GFP–PKIGlilz mice showing activation of GLI. (h) Representative picture of YAP1 immunohistochemistry staining of the skin of K5rtTA Tet–GFP–PKI showing nuclear localization of YAP1 in skin lesions. (i) qRT–PCR analysis of mRNA levels of Yap1 and the YAP1-regulated gene Ctgf, and transcriptional regulators and markers essential for hair follicle stem cell maintenance and proliferation in primary cultures of keratinocytes from K5rtTA Tet–GFP–PKI mice compared with cultures from WT littermates. n = 3 independent cultures from 3 WT and 3 K5rtTA Tet–GFP–PKI mice. Data are presented as means±s.e.m., and significance was calculated by ANOVA and Student’s t-test (NS P > 0.05; ** P < 0.01; and *** P < 0.001). Inset panels in images show details at higher magnification. The location of the basal membrane is indicated with a white dotted line.

When GFP–PKI (but not GFP–PKI4A) was expressed in the epithelium in response to doxycycline, mice rapidly developed extensive lesions in the skin, revealing histological features similar to human BCC that were indistinguishable from those of Gnas eKO mice (Fig. 6b,c). Skin and basal epithelial growths were positive for GFP (Fig. 6c), indicating the expression of GFP–PKI and GFP–PKI4A.
Staining for CK5 and p63 confirmed the basal epithelial identity of these cells (Fig. 6d). Lesions were also positive for the hair follicle and BCC marker CK15 and showed altered proliferation patterns by Ki67 staining (Fig. 6e,f). Using Gli1 reporter mice, we observed an expansion of GLI+ cells after GFP–PKI expression (Fig. 6g). Lesions were also positive for nuclear YAP1 (Fig. 6h). Furthermore, YAP1 was activated in K5rtTA Tet–GFP–PKI keratinocytes, as measured by increased levels of the YAP1 transcriptional targets Ctgf and Cyr61, as well as transcriptional regulators of hair follicle stem cell maintenance and proliferation (Fig. 6i).

**Overactivation of Gαs induces epithelial stem cell differentiation and depletion**

To investigate the impact of activating Gαs in the stem cell compartment, we next developed doxycycline-inducible mice expressing GαsR201C (Tet–GαsR201C) and bred them with K5rtTA mice (Fig. 7a). GαsR201C expression was rapidly induced in keratinocytes derived from K5rtTA/Tet–GαsR201C mice after doxycycline treatment (Fig. 7b). Remarkably, the persistent expression of active Gαs in mouse epidermis resulted in progressive hair loss (Fig. 7c) in both males and females. Hair follicles in K5rtTA/Tet–GαsR201C mice terminally differentiated into keratinized cyst structures (Fig. 7d), suggesting that Gαs activation in the skin induces the nearly complete differentiation and exhaustion of hair follicle stem cells. Supporting this hypothesis, CD34+ hair follicle stem cells were depleted in K5rtTA/Tet–GαsR201C mice (Fig. 7e). Gαs activation also led to a reduction in the basal progenitor markers p63 and CK5 (Fig. 7f) and decreased cell proliferation (Ki67 staining, Fig. 7g), particularly in the hair follicles. Furthermore, expression of GαsR201C resulted in a significant reduction in the colony-forming...
efficiency of epidermal cells (Supplementary Fig. 5a,b). By using whole-mount tail epidermis, we observed that GαsR201C induced the cytoplasmic retention of YAP1 and a decrease in the hair follicle stem cell marker CK15 (Fig. 7h and Supplementary Fig. 5c). Finally, cultured keratinocytes from K5rtTA/Tet–GαsR201C mice showed increased expression of differentiation markers (Fig. 7i), and a concomitant reduction in hair follicle stem cell markers and GLI and YAP1 transcriptional activity (Fig. 7j). Collectively, these results support the emerging concept that Gαs acts as a key regulator of epithelial stem cell fate in the skin.

Strikingly, the cyst formation observed on K5rtTA/Tet–GαsR201C mice resembles the skin phenotype of mice in which β-catenin is lost or repressed. However, by measuring the levels of Axin2 mRNA, a well-known marker of β-catenin function, we could not find any differences in β-catenin pathway activation (not shown). One possible connection is that in β-catenin KO skin SHH signalling is lost. Similarly, increased Gαs signalling blocks the transcriptional activity of GLI, suggesting that in both cases, suppression of Hedgehog signalling might result in hair follicle progenitor cells losing their follicular differentiation and instead exhibiting squamous epidermal characteristics, forming hair follicle-derived cysts. Indeed, formation of epidermal-like cyst structures has been observed by conditional disruption of SHH signalling in the skin.

**DISCUSSION**

We demonstrate here that Gαs and PKA signalling function as non-canonical regulators of GLI and YAP1 transcriptional networks, controlling the proliferation and differentiation of epithelial stem cells and maintaining proper hair follicle and skin homeostasis (Fig. 8a).

PKA signalling can limit the activity of the Hedgehog pathway by an incompletely understood mechanism. Here we show that Gαs controls GLI activity directly through PKA, independently of SHH or SMO, and that repression of Gαs or PKA alone is sufficient to induce a cell autonomous increase on GLI-dependent transcription. We also present evidence that repression of Gαs or PKA results in the parallel activation of the YAP1 transcriptional network. Recent evidence indicates that PKA can block YAP1 through the activation of LATS kinases. Our experiments extend these previous findings, establishing that PKA mediates the inactivation of YAP1 downstream of Gαs through LATS by a mechanism that involves the NF2 tumour suppressor protein. In this regard, NF2 can be directly phosphorylated by PKA and this phosphorylation renders NF2 in an open conformation, known to be required for its interaction with LATS (ref. 33).

SHH and SMO signalling can increase YAP1 activity by upregulating its mRNA levels. However, we did not observe any increase in *Yap1* or *Shh* mRNAs on Gαs or PKA inactivation, and inhibition of SMO signalling by cyclopamine did not have any effect on the transcriptional activity of YAP1. On the other hand, YAP1 can induce expression of GLI1 mRNA (ref. 46), but YAP1 activation might not be sufficient to trigger Hedgehog signalling. Indeed, skin-specific knockout of Hippo signalling components resulting in increased YAP1 activity or overexpression of active mutants of YAP1 does not result in the development of BCC-like lesions. Moreover, activation of GLI and YAP1 signalling occurs in both overlapping and distinct anatomical locations after Gnas deletion. Thus, although an interplay between YAP1 and GLI may occur, the available information points towards the concomitant parallel activation of YAP1 and GLI, both initiated downstream from PKA inhibition (see Fig. 8). Of interest, although the evidence presented indicates that this activation of GLI and YAP1 might be the main cause of BCC development in our mouse models, several additional transcriptional networks were also upregulated after *Gnas* excision (Supplementary Table 1). The elucidation of their precise intervening molecular mechanisms and potential cooperating roles in BCC development warrants further investigation.

Deregulation of the expression and activity of heterotrimeric G proteins and GPCRs is frequently observed with human malignancies. Activating mutations in *GNAS* promote aberrant growth of human thyroid and pituitary tumours and are found in multiple other neoplasms. In contrast to its well-established tumour...
promoting role, our findings suggest that Gs and its downstream effector PKA function as part of a tumour suppressive pathway in the skin. The disruption of this signalling axis is sufficient to promote rapid stem cell expansion and BCC formation, at least in part by relieving the restraining effect of Gs and PKA on YAP1 and GLI (Fig. 8). These findings raise the possibility that Gs and PKA might function as tumour suppressor genes in hyperproliferative diseases driven by GLI and YAP1 activation. In line with this, inactivating genomic alterations in GNAS have been found in SHH-driven medulloblastoma, and ablation of Gnas in neural progenitors promotes medulloblastoma formation in mice with increased SHH signalling, suggesting that the tumour suppressive function of Gs–PKA may have broad implications in multiple pathophysiological conditions.

Finally, GPCRs are essential for the maintenance of the epidermal stem cell compartment. Thus, we can postulate that Gs and its yet to be defined coupled receptors may preserve the integrity and function of the epidermis and its resident self-renewing stem cells, ensuring proper tissue homeostasis and protecting against cancerous growth. We believe that this study will prompt the in-depth analysis of alterations in Gs and Gs-coupled receptors, and perhaps Gs-coupled receptors that counteract Gs signalling, in future cancer genomic studies, in light of the unique stem cell regulatory and tumour suppressive functions of Gs and PKA.

\section*{METHODS}

Methods and any associated references are available in the online version of the paper.

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\section*{COMPETING FINANCIAL INTERESTS}

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DNA constructs. pCMX-GAL4T4ED and pGAL4.23.5×GAL4-binding UAS promoter luciferase have been described previously22. Human YAP1 was cloned by PCR from pDsRed Monomer CI-1AP2 (ADDGENE, Plasmid 19057) with a C-terminal HA tag into pCEFL (pCEFL GLI1HA) or with a C-terminal GAL4 DNA-binding domain followed by a HA tag into pCEFL (pGAL4 GLAGLI). The PKA inhibitor (PKI) was cloned by inserting the 24 amino acids after the initial methionine of the coding sequence of human PKI downstream of GFP (GFP–PKI). For use as a control, the phenylalanine and arginine residues of the PKI peptide were replaced with alanine to disrupt binding to PKA. Human GLI1 was cloned by PCR from pBluescript KS GLI1 (ADDGENE, Plasmid 16419) with a C-terminal 2xFlag tag into pCEFL (pCEFL GLI1HA) from Sigma. Cell lines. All animal studies were carried out according to NIH-Intramural Animal Care and Use Committee (ACUC)-approved protocols, in compliance with the Guide for the Care and Use of Laboratory Animals. Mice carrying a tamoxifen-inducible Cre-mediated recombination system driven by the human keratin 14 promoter (K14CreER mice) were from The Jackson Laboratory (Stock Number 005107, Stock Tg(Krt14-cre/ERT2)20Efu/JK14Cre). Epithelial-specific Gnas knockouts were obtained by crossing K14CreER mice with mice carrying loxP sites surrounding Gnas exon 1 (ref 9,56). Gli reporter mouse was described previously23 and they were obtained from the Jackson Laboratory (Stock Number 013254, Stock TgGli1m2Alj/J). Ngn2 mice carrying the cytokeratin 5 promoter in the reverse tetracycline transactivator (rtTA) (SrtTA) have been described previously24. For the generation of Tet–GFP–PKI, Tet–GFP–PKI4a and Tet–Go, R021C transgenic mice, the GFP–PKI, GFP–PKI4a and Go, R021C coding sequences were cloned downstream of the Tet–responsive element (TetO7) in a modified pBSRV vector25. The fragment containing the expression cassette was isolated by PmeI digestion from vector DNA and purified for micro-injection into FVB/N mouse fertilized oocytes. Founder mice were identified for the presence of the transgene by screening genomic DNA from tail biopsies using a PCR reaction. The presence of wild-type, floxed and recombined Gfko was analysed as previously described26. The presence of the Gfko–GFP and GFP–PKI4a transgenes was determined with the following primers: forward sequence 5'–CTAGAGTCTCCGTCTGGCAGA–3' and reverse sequence 5'-CTTGCGAGTTAGAGGCATGTA–3', band approximately 1.100 bp. The presence of the Go, R021C transgene was determined with the following primers: forward sequence 5'-CTAGAATTTCGTGTCTGCGA–3' and reverse sequence 5'-GCATTTTGTGTTCTGCTCTC–3', band approximately 620 bp. PCR reactions were performed with the following conditions: 95 °C for 1 min, followed by 3 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min, and a final cycle with 10 min of extension at 72 °C. The investigators were not blinded to allocation of samples to treatments and outcomes and assessment. Both male and female mice were used in the studies. Treatment was started between weeks 6 to 10 after birth. No statistical method was used to predetermine sample size. No randomization was used and all experiments were conducted using littermate controls. Doxycycline was administered in the food grain-based pellets (Bio-Serv) at 60 mg kg⁻¹. Tumorxifen (1 mg per mouse, per day, in corn oil) was applied by gavage to one-month-old mice for targeting control siRNA D-001206-13; from IDT DsiRNA Duplexes human MST1 siRNA M-046247-01-0005 from IDT DsiRNA Duplexes human NF2 siRNA L-003917-0005 from IDT transfected one day after plating and in all cases cells were treated/collection two days after plating and HACAT cells were 10 to 14 days and processed and quantified as previously described27. Free medium (KSFM; Invitrogen) supplemented with antibiotics was used as the wash solution (2 mM MgCl₂, 0.1% Nonidet P40, 0.5 mg ml⁻¹; 10 mM KCl, 60 mM NaCl, 0.1% Nonidet P40, in PBS). Tissues were stained with anti-GFP antibody (Cell Signaling; clone no. 100G7E; catalogue no. 61055-1), anti-α-tubulin (Cell Signaling; clone no. 14C10; catalogue no. 2188; 1:2,000), anti-c-myc (Cell Signaling; clone no. 9E10; catalogue no. 4970; 1:1,000), or antiTARGETfluorescent nuclear stain (Fig. 1f). Cells were fixed with 4% paraformaldehyde, 0.2% glutaraldehyde, in PBS for 15 min on ice on each side. Then tissue was washed twice for 20 min each with β-gal wash solution (2 mM MgCl₂, 0.1% Nomidet P40, in PBS). Tissues were stained overnight at room temperature, in the dark, on a rolling platform in β-gal stain solution (5 mM β-gal wash solution; 10 mM Fe(CN)₆⁻, 10 mM KFe(CN)₆, 3H₂O, 2 mM MgCl₂, 0.1% Nomidet P40, 0.5 mg ml⁻¹; x-gal in DMF, all diluted in PBS). Afterwards, staining solution was removed and tissues were washed twice by 20 min each in β-gal wash solution. Tissues were directly visualized or stored in ethanol 70% and processed for paraffin sectioning and stained with Neutral red or processed by cryosectioning. Immunoblot analysis. Western blot assays were performed as described previously28 and repeated at least 3 independent times. Antibodies used were: anti-β-gal (Cell Signaling; clone no. 14C10; catalogue no. 2188; 1:2,000), anti-α-tubulin (Cell Signaling; clone no. 9E10; catalogue no. 4970; 1:1,000), anti-α-catenin (Cell Signaling; clone no. DM1A; catalogue no. 3873; 1:2,000), non-phospho-PAK substrate (RRXS/T) antibody (Cell Signaling; clone no. 100G7E; catalogue no. 61055-1, 1:1,000), non-phospho-PAK substrate antibody (Cell Signaling; 61055-1). Tail skin whole mounts and β-gal stain. Whole mounts were prepared and stained with antibodies (see Immunofluorescence section below) as previously described29. For β-gal staining, back and ear skin were shaved in the area of dissection and skin was peeled off while cutting the attached subcutaneous layers. Skin and whole mounts were fixed in 2% paraformaldehyde, 0.2% glutaraldehyde, in PBS for 15 min on ice on each side. Then tissue was washed twice for 20 min each with β-gal wash solution (2 mM MgCl₂, 0.1% Nomidet P40, in PBS). Tissues were stained overnight at room temperature, in the dark, on a rolling platform in β-gal stain solution (5 mM β-gal wash solution; 10 mM Fe(CN)₆⁻, 10 mM KFe(CN)₆, 3H₂O, 2 mM MgCl₂, 0.1% Nomidet P40, 0.5 mg ml⁻¹ x-gal in DMF, all diluted in PBS). Afterwards, staining solution was removed and tissues were washed twice by 20 min each in β-gal wash solution. Tissues were directly visualized or stored in ethanol 70% and processed for paraffin sectioning and stained with Neutral red or processed by cryosectioning. doi:10.1038/ncb31614
Luciferase assays and immunopurification. To measure YAP1 activity, cells in 12- or 24-well plates were co-transfected overnight with GAL4TEAD (0.25 μg cm⁻²), UASLuc (0.1 μg cm⁻²) plus the DNA constructs indicated in the figure: GFP (0.4 μg cm⁻²), YAP1 (0.2 μg cm⁻²), Go, R201C (0.2 μg cm⁻²), GFP-PKI (0.5 μg cm⁻²). GFP-PKI4A (0.5 μg cm⁻²). The next day, cells were serum-starved overnight and then luciferase activity was measured using a Dual-Glo Luciferase Assay Kit (Promega) and a Microtiter plate luminometer (Dynex Tech.). To measure GAL4GLI activity, cells were co-transfected with GAL4GLI (0.3 μg cm⁻²) and a Microtiter plate luminometer (Dynex Tech.). To measure YAP1 activity, cells were co-transfected with YAP1 (0.2 μg cm⁻²) and a Microtiter plate luminometer (Dynex Tech.). For immunopurification, cells were co-transfected with YAP1 (0.2 μg cm⁻²) plus the DNA constructs indicated in the figure at the same concentration as for GFP activity and processed as indicated above. CRE assays were performed as described previously. Luciferase normalization was performed in every case by co-transfecting a Renilla luciferase vector (0.025 μg cm⁻²; Promega). Immunopurification was performed as previously described

Immunofluorescence and immunohistochemistry. Sections were processed and stained as previously described. Immunohistochemical analysis of YAP1 in human BCC was done on a tissue array (US Biomax BC21014). The following antibodies were used: mouse Ki67 (Dako; clone no. MB15; catalogue no. M7249; 1:50), YAP1 (Cell Signaling; clone no. D8H1X; catalogue no. 14074; 1:500), p63 (Santa Cruz; clone no. 4A4; catalogue no. sc4331; 1:100), cytokeratin 5 (Covance; catalogue no. PRB-160P; 1:500), cytokeratin 15 (Covance; catalogue no. PCK-153P; 1:200), pan-cytokeratin (Dako; catalogue no. Z0622; 1:500), loricrin (Covance; catalogue no. PRB-160P; 1:500), integrin α6 (BD-Pharmingen 555734; 1:200), GFP (Cell Signaling 2956; 1:200), anti-mouse CD34 (eBioscience 14-0341-85; 1:50). Nuclei were stained with Hoechst 33342 (Invitrogen). Tissue section images were taken with a Zeiss Axio Imager Z1 microscope equipped with an Apotome device (Carl Zeiss) using a Zeiss Plan APOCHROMAT 20×/0.80a objective and Zen 2012 software (Carl Zeiss). Whole-mount fluorescent images were taken using an inverted Zeiss LSM 700 confocal microscope, coupled to Zen software 2012 (Carl Zeiss). 3D maximum projections were made with Zen software 2012 (Carl Zeiss). Final images were bright contrast adjusted with Zen 2012 (Carl Zeiss) or PowerPoin

Statistical analysis. All analyses were performed in triplicate or greater and the means obtained were used for ANOVA or independent t-tests. Statistical analyses, variation estimation and validation of test assumptions were carried out using the Prism 5 statistical analysis program (GraphPad). Asterisks denote statistical significance (nonsignificant or NS, P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001). All data are reported as mean ± standard error of the mean (s.e.m.).
Supplementary Figure 1  Validation of effective Gnas deletion and epithelial thickness.  

**a**, Representative genotyping in mice treated or not with tamoxifen to show Gnas deletion. To distinguish wild-type (GnasWT), floxed (Gnasloxp) and excised (Gnasloxp excised) alleles PCR was performed using primers surrounding the loxP site as described in the Methods section. Presence or absence of the K14CreER transgene was determined by PCR using specific primers.  

**b**, qRT–PCR analysis of the expression of Gnas in keratinocytes from WT and Gnas eKO mice shows a decrease in Gnas mRNA levels. Data from one representative experiment of three are shown.  

**c**, Quantification of thickness of the cytokeratin 15+ skin layer reflecting the expansion of the basal stem cell compartment. n=7 sections from 3 different mice for each genotype. Data are presented as means±s.e.m., and significance was calculated by Student's t-test (NS P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001).
Supplementary Figure 2. Activation of YAP1 in the skin at short times after Gnas deletion. a, Representative pictures of interfollicular epidermis of tail skin whole mounts from WT and Gnas eKO animals stained to show expression of YAP1 (green), cytokeratin 15 (CK15, red) and nuclei (blue), one day after finishing the administration of tamoxifen. b, Representative pictures of skin sections from WT and Gnas eKO animals showing expression of YAP1 (green), cytokeratin 15 (CK15, red) and nuclei (blue), one day after finishing the administration of tamoxifen. Location of the basal membrane is indicated with a white dotted line.
Supplementary Figure 3 PKA inhibitor protein (PKI) can block Gαs and PKA signaling. a, b, 293 cells were transfected with either GFP-PKI4A and GFP-PKI and then treated with forskolin for 30 min (a) or were co-transfected with GαsR201C for 24hs (b). PKA activity was detected with anti-phospho-PKA substrate antibody that detects proteins containing a phospho-serine/threonine residue with arginine at the -3 and -2 positions (a, b) and by phosphorylation of the PKA regulatory subunit II (pRSII) (b). GFP-PKI was detected by an anti-GFP antibody and GαsR201C by an EE tag antibody. Full images of blots are shown in Supplementary Fig. 6.
Supplementary Figure 4 Forskolin treatment but not cyclopamine can block GLI and YAP1 activation in Gnas eKO keratinocytes. qRT–PCR analysis of mRNA levels of GLI-regulated genes Ptch1 and Ptch2, and Yap1 and the YAP1-regulated gene Ctgf in keratinocytes from WT and Gnas eKO mice treated with the indicated drugs for 48 hs. Data from one representative experiment of three are shown. FI: forskolin+IBMX. Data are presented as means.
Supplementary Figure 5 Overactivation of Gαs in keratinocytes leads to reduced clonogenic capacity and cytoplasmic retention of YAP1. **a**, Representative pictures of wells and quantification of clonogenic assays of keratinocytes isolated from control and active Gαs mice 5 months into doxycycline treatment. n=3 technical replicates, one representative experiment of three is shown. **b**, Representative pictures of colonies of keratinocytes from control and active Gαs mice. **c**, Details at higher magnification from Fig. 7h. Hair follicles from tail skin whole mounts in control (K5rtTA) and active Gαs mice (K5rtTA tet-GαsR201C) treated with doxycycline for 2 months. Staining shows expression of YAP1 (green) and cytokeratin 15 (CK15, red). Data are presented as means±s.e.m., and significance was calculated by ANOVA and Student’s t-test (NS P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001).
Supplementary Figure 6 Full images of Western blots indicating the section that was selected for the corresponding figure.
Supplementary Figure 6 continued

Related to Figure 5d-LATS siRNA

Related to Figure 5d-MST siRNA
Related to Figure 5d-SAV siRNA

Related to Figure 5d-LKB1 siRNA

Supplementary Figure 6 continued
Related to Figure 5d-NF2 siRNA

Supplementary Figure 6 continued
Related to Figure 7

![Related to Figure 7](image1)

Related to Supplementary Fig. 3a

![Related to Supplementary Fig. 3a](image2)

Related to Supplementary Fig. 3b

![Related to Supplementary Fig. 3b](image3)

*Supplementary Figure 6 continued*
Supplementary Table Legends

**Supplementary Table 1** Upregulated transcriptional signatures in Gnas eKO mice generated by the use of IPA (Ingenuity® Systems).

**Supplementary Table 2** YAP1 upregulated targets, reported as fold change expression levels by gene expression analysis in Gnas eKO skin.