

## RESEARCH

# Hedgehog signaling in endocrine and folliculo-stellate cells of the adult pituitary

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## Abstract

Ubiquitous overactivation of Hedgehog signaling in adult pituitaries results in increased expression of *proopiomelanocortin* (*Pomc*), *growth hormone* (*Gh*) and *prolactin* (*Prl*), elevated adrenocorticotrophic hormone (Acth) production and proliferation of Sox2<sup>+</sup> cells. Moreover, ACTH, GH and PRL-expressing human pituitary adenomas strongly express the Hedgehog target *GLI1*. Accordingly, Hedgehog signaling seems to play an important role in pathology and probably also in homeostasis of the adult hypophysis. However, the specific Hedgehog-responsive pituitary cell type has not yet been identified. We here investigated the Hedgehog pathway activation status and the effects of deregulated Hedgehog signaling cell-specifically in endocrine and non-endocrine pituitary cells. We demonstrate that Hedgehog signaling is unimportant for the homeostasis of corticotrophs, whereas it is active in subpopulations of somatotrophs and folliculo-stellate cells *in vivo*. Reinforcement of Hedgehog signaling activity in folliculo-stellate cells stimulates growth hormone production/release from somatotrophs in a paracrine manner, which most likely is mediated by the neuropeptide vasoactive intestinal peptide. Overall, our data show that Hedgehog signaling affects the homeostasis of pituitary hormone production via folliculo-stellate cell-mediated regulation of growth hormone production/secretion.

## Key Words

- ▶ Hedgehog
- ▶ Smoothed
- ▶ Patched
- ▶ pituitary
- ▶ folliculo-stellate cells

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## Introduction

The pituitary gland is a key regulator of body homeostasis and responsible for signal exchanges between the hypothalamus and peripheral organs. Besides of the six different endocrine cell types (e.g. corticotrophs/adrenocorticotrophic hormone- (Acth), somatotrophs/growth hormone- (Gh), prolactin- (Prl), thyroid-stimulating hormone-, luteinizing hormone-, follicle-stimulating hormone-secreting cells), the anterior lobe (AL) of the pituitary consists of Sox2<sup>+</sup> (stem) cells and a meshwork of non-endocrine Sox2<sup>+</sup> folliculo-stellate cells (FSC). The latter ones are implicated in regulation

and maintenance of the endocrine cells by delivering paracrine factors (reviewed in [Cox \*et al.\* 2017](#)).

Hedgehog (Hh) signaling plays a major role in the development of the pituitary. However, its function in homeostasis and disease of the adult gland is far from clear. Under normal physiological conditions Hh signaling is inactive in most cells of adult tissues. Activation occurs upon binding of Hh ligands (e.g. mammalian Sonic, Indian or Desert Hh) to the receptor protein Patched1 (Ptch). This releases the inhibition of Smoothed (Smo), which results in

translocation of Smo into the primary cilium and nuclear translocation of transcription factors of the Gli family to induce target gene expression (e.g. *Gli1*, *Gli2* or *Ptch*) (reviewed in [Bangs & Anderson 2017](#)). Inactivation or overactivation of the pathway during pituitary organogenesis can lead to agenesis of the gland ([Roessler \*et al.\* 2003](#)), hypopituitarism and pituitary malformations ([França \*et al.\* 2010](#), [Flemming \*et al.\* 2013](#)) or hyperplasia of the pituitary ([Treier \*et al.\* 2001](#)), respectively. Several lines of evidence additionally point toward a regulative function of the pathway in stem cell maintenance and regenerative processes in the adult pituitary. Thus, our group described the enhanced proliferation of Sox2<sup>+</sup> cells in the AL after ubiquitous Hh signaling activation ([Pyczek \*et al.\* 2016](#)). Furthermore, other groups demonstrated that stem cells of the pituitary side population express the Hh signaling regulators *Ptch* and *Smo* ([Chen \*et al.\* 2009](#), [Vankelecom 2010](#)) and that regenerative processes induce the expression of the Hh signaling target genes *Gli1* and *Gli2* in these cells ([Gremeaux \*et al.\* 2012](#), [Willems \*et al.\* 2016](#)). Additionally, a regulatory function of Hh signaling in hormone-producing cells (e.g. corticotrophs) was proposed. Thus, Hh signaling regulates *Acth* expression in AtT-20 cells ([Vila \*et al.\* 2005a,b](#), [Pyczek \*et al.\* 2016](#)) and *ex vivo* activation of the pathway in the whole pituitary leads to elevated *Acth*, *Gh* and *Prl* expression ([Pyczek \*et al.\* 2016](#)).

Additionally, there is evidence that Hh signaling is involved in hormone secretion or formation of pituitary tumors. For example patients and mice with heterozygous *PTCH/Ptch* germline mutation occasionally develop acromegaly-like symptoms ([Kahn & Gordon 1967](#), [Codish \*et al.\* 1973](#), [Marcos \*et al.\* 1982](#), [Cramer & Niederdelmann 1983](#), [Bale \*et al.\* 1991, 1994](#), [Kimonis \*et al.\* 1997](#), [Wicking \*et al.\* 1997](#), [Hahn \*et al.\* 1998](#), [Lo Muzio \*et al.\* 1999](#)). However, although human ACTH, GH or PRL-expressing pituitary adenoma show very high expression of the HH signaling inducer SHH and the HH target gene *GLI1* ([Pyczek \*et al.\* 2016](#)), a direct link between Hh signaling (e.g. mutations or pathway overactivation) and tumor formation in the AL has not been confirmed.

Altogether, our data and those from other labs strongly suggest that Hh signaling plays a role in pathology and probably in function of the adult pituitary gland, especially in corticotrophs, somatotrophs, lactotrophs and/or Sox2<sup>+</sup> cells. However, it never has been analyzed whether pituitary endocrine cells and/or other cell types are Hh responders under physiological conditions. Moreover, the fact that Hh signaling is a key player in tumorigenesis and obviously also plays a role in pituitary adenoma

substantiates the efforts to unravel the Hh-responsive cell type/s in the normal adult pituitary gland.

Here, we investigated the Hh signaling activation status of the adult pituitary gland on cellular level and studied the impact of a deregulated pathway in endocrine and non-endocrine pituitary cells using *in vivo* and *in vitro* approaches. By investigating mouse models for lineage tracing and for conditional cell-specific deregulation of Hh signaling we demonstrate that the Hh pathway does not play a role in corticotrophs in the adult pituitary gland. However, subpopulations of somatotrophs and FSC of the adult pituitary gland express the surrogate marker for active Hh signaling *Gli1* and descend from *Gli1*-expressing cells. Remarkably, we show here for the first time that activation of Hh signaling in FSC induces *Gh* release from somatotrophs in a paracrine manner, which most likely is mediated by the neuropeptide vasoactive intestinal peptide (*Vip*).

## Materials and methods

### Mice

All experiments using animals were performed in compliance with all German legal and ethical requirements and have been approved by the Lower Saxony State Office for Consumer Protection and Food Safety (file number 33.9-42502-04-15/1787). The following mouse strains were used in the study: *Ptch*<sup>1<sup>tm1Hahn</sup></sup> (*Ptch*<sup>flox/flox</sup> ([Uhmann \*et al.\* 2007](#)), JAX stock # 012457), *Smo*<sup>tm2Amc</sup> (*Smo*<sup>flox/flox</sup> ([Long \*et al.\* 2001](#)), JAX stock # 004526), *Tg(Pomc-cre/ERT2)*<sup>#Jke</sup> (*PomcCreERT2* ([Berglund \*et al.\* 2013](#)) a kind gift from J K Elmquist), *Gli1*<sup>tm3(cre/ERT2)Alj</sup> (*Gli1CreERT2* ([Ahn & Joyner 2004](#)), JAX stock #007913), *Gt(ROSA)26Sortm9(CAG-tdTomato)Hze* (*tdT* ([Madisen \*et al.\* 2010](#)), JAX stock #007905) and *Tg(S100b-EGFP)*<sup>11Lgrv</sup> (*S100b-EGFP* ([Vives \*et al.\* 2003](#)) a kind gift from C Legraverend and P Mollard).

*Ptch*<sup>flox/flox</sup>, *PomcCreERT2*, *tdT* and *S100b-EGFP* strains were maintained on C57BL/6 and *Smo*<sup>flox/flox</sup> and *Gli1CreERT2* mice on a 129/Sv background. Both genders of transgenic mice were used. No sex-specific differences were observed. Genotyping of the mice was conducted by PCR on genomic DNA isolated from tail or ear biopsies using primer pairs recommended by the donating investigators ([Vives \*et al.\* 2003](#), [Berglund \*et al.\* 2013](#)) or by The Jackson Laboratory (<https://www.jax.org/jax-mice-and-services>). For CreERT2-mediated homozygous deletion of *Ptch* or *Smo* *Ptch*<sup>flox/flox</sup> or *Smo*<sup>flox/flox</sup> mice, respectively, were bred to the respective CreERT2-deleter mouse strain. For lineage-tracing experiments the

CreERT2-deleter strains were crossed to *tdT* mice that in some experiments additionally carry the *S100b-EGFP* transgene. The CreERT2-activity of the transgenic mice was induced by five single intraperitoneal injection (i.p.) of 1 mg tamoxifen on 5 consecutive days at an animal age of 8 weeks (Uhmman *et al.* 2007). Untreated mice without the respective CreERT2-recombinase gene and solvent-treated mice carrying the floxed alleles and the respective CreERT2-recombinase genes served as controls. For lineage-tracing experiments mice were analyzed after the first tamoxifen application as indicated in the respective figure legends. Body weight and blood samples of *PomcCreERT2 Ptch<sup>fllox/fllox</sup>*, *PomcCreERT2 Smo<sup>fllox/fllox</sup>* and the respective control mice were taken weekly or every second week, respectively, up to 250 days after the first tamoxifen/solvent application when the mice were sacrificed (Fig. 1B). Measurements of blood glucose and serum hormone levels are described in the Supplementary methods (see section on [supplementary materials](#) given at the end of this article). The number of analyzed animals is given in Supplementary Table 1 or in the respective figure legends.

## Compounds

If not otherwise stated all compounds were obtained from Sigma-Aldrich, Darmstadt, Germany. Beta-Ala-Lys-N(epsilon)-aminomethylcoumarin acetate ( $\beta$ -Ala-Lys-N( $\epsilon$ )-AMCA) was obtained from Carbosynth (Berkshire, UK) and Smoothened Agonist (SAG) from Cayman Chemical (Ann Arbor, USA).  $\beta$ -Ala-Lys-N( $\epsilon$ )-AMCA was dissolved in HBSS (0.952 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 5.36 mM KCl, 0.411 mM KH<sub>2</sub>PO<sub>4</sub>, 0.812 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 136.7 mM NaCl, 0.385 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM D-glucose·H<sub>2</sub>O, 10 mM HEPES). SAG was dissolved in dimethyl sulfoxide (DMSO). The preparation of tamoxifen/ethanol/sunflower oil for *in vivo* application has been previously described (Uhmman *et al.* 2007).

## Cell culture

GH3 (CCL-82.1, January 2016) and AtT-20 cells (CCL-89, July 2014) were obtained from ATCC and grown in Ham's F12-K Medium (Gibco, Life Technologies) supplemented with 15% Horse serum and 2.5% heat-inactivated FBS or in Ham's F12-K Medium (Gibco) supplemented with 15 horse serum and 2.5% FBS, respectively. TtT/GF cells were obtained from RIKEN BRC (RCB1279, September 2019) and cultured in DMEM/HamF12 (Gibco) supplemented with 10% Horse serum and 2.5% FBS. Starvation medium resembles the growth medium but contains 0.5% Horse

serum and 0.125% FBS (heat inactivated for GH3 cells). Routinely, all cell lines were tested for mycoplasma contamination by using Mycoplasma Detection Kit (minerva biolabs, Berlin, Germany). Identity of the cells was analyzed by marker gene expression analyses and immunofluorescent stainings against marker proteins as shown in Supplementary Fig. 3. Passage numbers between 15 and 30 of cell lines were used for the respective experiments.

Detailed information about SAG treatment, preparation of conditioned medium, medium transfer experiments, measurements of supernatant hormone/neuropeptide levels and BrdU incorporation analysis are given in Supplementary methods.

## Detection of recombination of the *Ptch<sup>fllox</sup>* and the *Smo<sup>fllox</sup>* loci

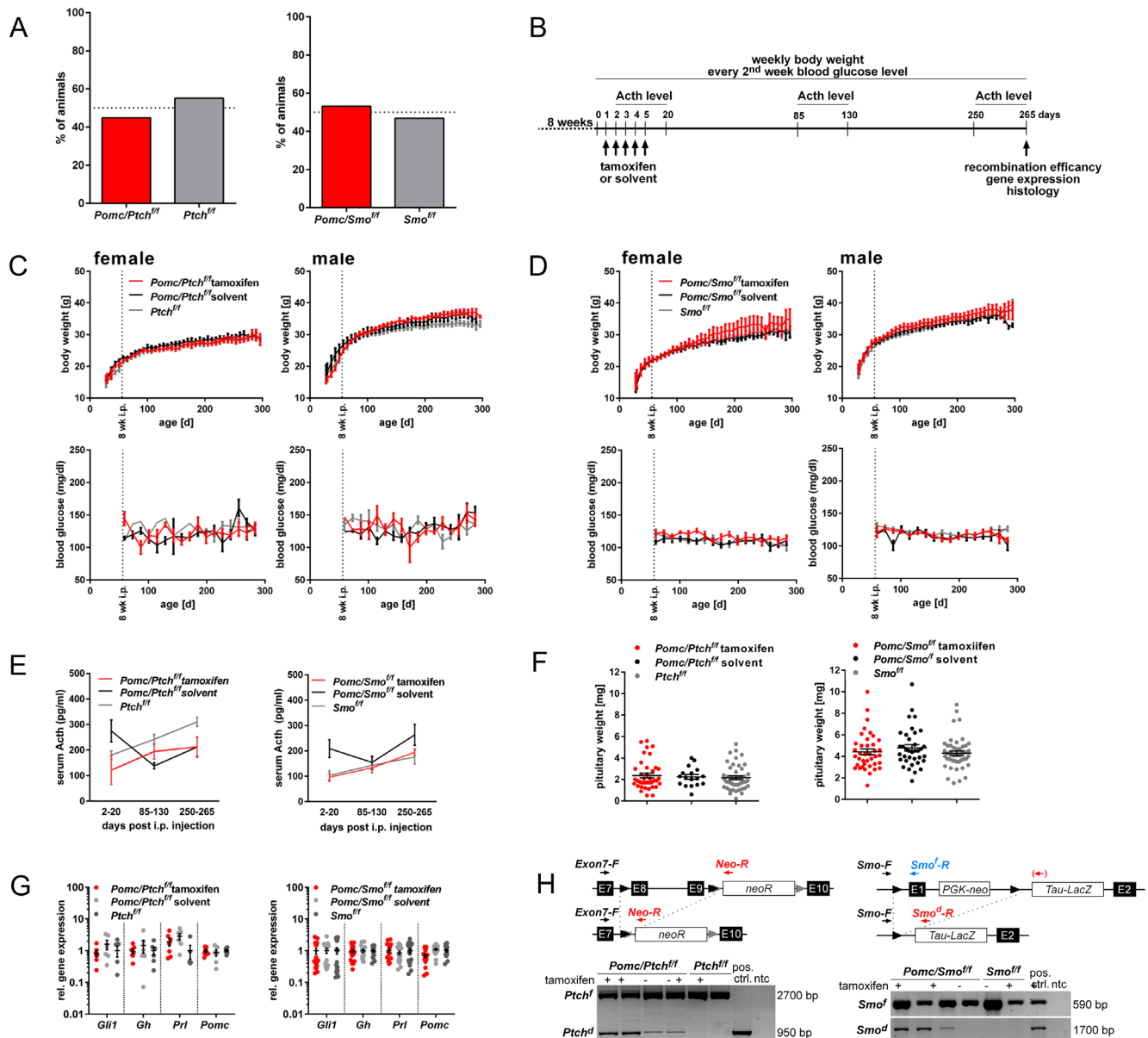
Isolation of genomic DNA from pituitary glands was performed as previously described (Pyczek *et al.* 2016). For PCR-based detection of the CreERT2-mediated recombination at the *Ptch<sup>fllox</sup>* or *Smo<sup>fllox</sup>* locus the primer pairs indicated in Fig. 1H and G were used. The sequences of the primers are given in Supplementary Table 2.

## RNA isolation and quantitative real-time PCR analyses

Gene expression analyses of murine tissue samples and *in vitro* cultured cells, RNA-isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR) analyses were conducted as previously described (Pyczek *et al.* 2016). All primer pairs, except those for amplification of 18S rRNA serving for normalization of the amount of sample cDNA, were intron-flanking and are summarized in Supplementary Table 3. Each cDNA was measured in triplicates.

## Transcriptome analyses

For transcriptome analyses of three biological replicates of RNA from TtT/GF cells treated with either 100 nM SAG or solvent (see previous description) were analyzed. RNA quality control (Fragment Analyzer, Agilent Technologies), cDNA library preparation (TruSeq® RNA Sample Preparation v2; Illumina, San Diego, USA) and RNA sequencing (HiSeq 4000; Illumina) were performed at the NGS Service Facility for Integrative Genomics, Institute of Human Genetics, University Medical Center Göttingen, Germany. For detailed description see

**Figure 1**

*In vivo* deregulation of Hh signaling in Pomc-expressing cells of the adult pituitary gland. (A) Percentual birth rates, (B) experimental setup, (C and D) body weight (top), blood glucose level (bottom), (E) Acth serum levels, (F) pituitary weight, (G) pituitary gene expression levels, (H) genomic loci before and after recombination (top), recombination analysis on genomic pituitary DNA (bottom) of tamoxifen- or solvent-treated *Pomc/Ptch<sup>fl/fl</sup>* and *Ptch<sup>fl/fl</sup>* or *Pomc/Smo<sup>fl/fl</sup>* and *Smo<sup>fl/fl</sup>* mice. Analyzed animal numbers are given in Supplementary Table 1, no gender-specific differences in serum Acth levels, pituitary weight or gene expression levels were observed (data not shown). Gene expression levels were normalized to 18S rRNA expression and to the respective gene expression levels of solvent-treated controls. Each circle in F and G indicates one biological replicate (bars: mean  $\pm$  s.e.m.), circles in C–E represent mean  $\pm$  s.e.m. No significant differences were detected by using non-parametric Holm–Sidak method or Mann–Whitney tests.

Supplementary methods. RNAseq data were deposited in the gene expression omnibus, accession: GSE153550.

### Western blot and histological analyses

Immunohistological and immunofluorescent antibody stainings of paraffin and cryosections have been

described previously (Pyczek *et al.* 2016). For detailed description of protein isolation, Western blot analysis, paraffin, cryotome and vibratome sections, immunofluorescent stainings of adherent or non-adherent cells, combined RNAScope/immunofluorescent staining and  $\beta$ -Ala-Lys-N(e)-AMCA incubation see Supplementary methods.

Used antibodies, antibody dilutions and antigen retrieval procedures are summarized in Supplementary Table 4.

## Statistics

Statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software Inc., San Diego, USA). The used statistical tests are given in the respective figure legends.

## Results

### Deregulation of Hh signaling in Pomc-expressing cells has no impact on homeostasis of adult pituitary glands

Constitutive activation of Hh signaling by a Rosa26-CreERT2-driven homozygous deletion of *Ptch* in *ex vivo* cultured adult pituitaries lead to an increase in *Pomc*, *Gh* and *Prl* expression and enhanced BrdU-incorporation of Sox2<sup>+</sup> pituitary cells (Pyczek *et al.* 2016). Since the Rosa26-CreERT2-deleter recombines the *Ptch*<sup>fllox</sup> locus in virtually every pituitary cell, these experiments did not allow for the determination of the specific/individual phenotype-triggering cell type. Therefore, we first tested whether Hh signaling directly regulates Acth expression in corticotrophs *in vivo*. For this purpose, we bred *Ptch*<sup>fllox/fllox</sup> or *Smo*<sup>fllox/fllox</sup> to *PomcCreERT2* mice, which express the tamoxifen-inducible CreERT2-recombinase under the control of the murine *proopiomelanocortin* (*Pomc*, encodes for the Acth precursor polypeptide) promoter (Berglund *et al.* 2013). To verify inducibility, specificity and potential leakiness of the deleter strain we furthermore generated *PomcCreERT2* R26-tdTomato (*Pomc/tdT*) mice. Each mouse cohort was subdivided into two groups that received tamoxifen or solvent at an age of 8 weeks.

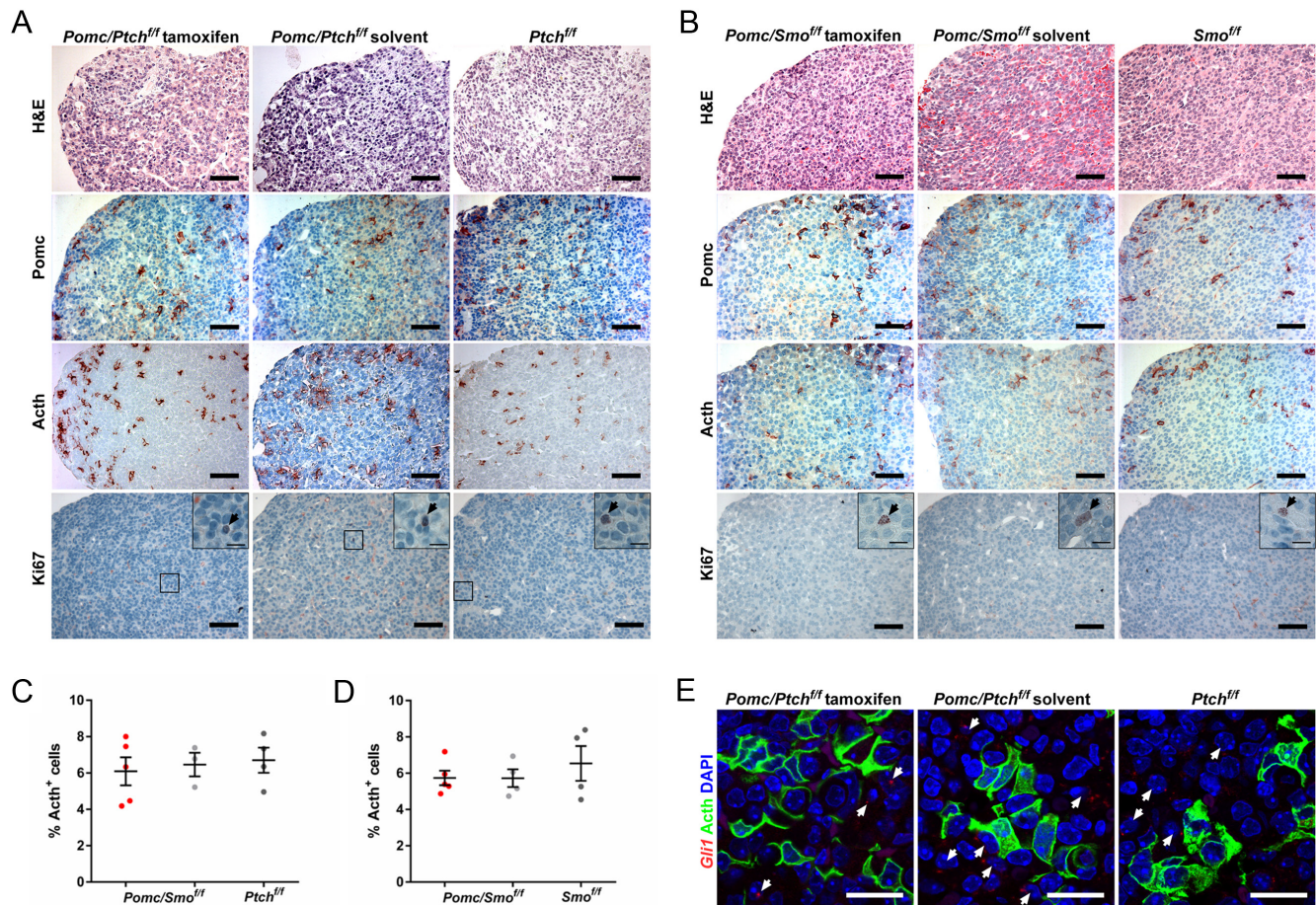
As judged by the amount of tdT<sup>+</sup> cells isolated from adult pituitaries of solvent-treated *Pomc/tdT* mice (experimental setup see Supplementary Fig. 1A), the *Pomc/tdT* reporter was highly or mildly leaky in the intermediate lobe or the AL, respectively (Supplementary Fig. 1B). However, tamoxifen-application strongly increased the number of tdT<sup>+</sup> cells in the AL within 7 days after CreERT2-induction and the cells were trackable until 250 days after tamoxifen injection without any reduction of labeled cell numbers (Supplementary Fig. 1B). Double immunofluorescence analyses furthermore verified tdT expression in Pomc- and Acth- but not in Gh- or Prl-expressing cells in both

tamoxifen- and solvent-treated *Pomc/tdT* mice (Supplementary Fig. 1B) indicating a cell-specific expression of the *PomcCreERT2*-transgene in corticotrophs. Thus, we expected that under normal physiological conditions the *PomcCreERT2*-deleter allows for a long-term observation of genetically modified *Pomc*-expressing cells and crossed the *PomcCreERT2*-transgene with *Ptch*<sup>fllox/fllox</sup> or *Smo*<sup>fllox/fllox</sup> mice. Irrespective of the leakiness of the deleter, *PomcCreERT2* *Ptch*<sup>fllox/fllox</sup> (*Pomc/Ptch*<sup>flf</sup>) and *PomcCreERT2* *Smo*<sup>fllox/fllox</sup> (*Pomc/Smo*<sup>flf</sup>) mice were born at a Mendelian ratio (Fig. 1A) and did not show any obvious developmental abnormalities without tamoxifen application. Similarly, (experimental setup see Fig. 2B) none of the *Pomc/Ptch*<sup>flf</sup> and *Pomc/Smo*<sup>flf</sup> mice showed signs of a deregulated hormone status (e.g. alopecia, weight loss/gain (Fig. 1C and D), abnormal blood glucose levels (Fig. 1C and D), abnormal serum Acth levels (Fig. 1E) or increased pituitary weight (Fig. 1F)) 250 days after CreERT2-induction. Neither Hh signaling activity nor *Gh*, *Prl* or *Pomc* expression levels were altered (Fig. 1G) albeit *Ptch*<sup>fllox</sup> or *Smo*<sup>fllox</sup> loci were efficiently recombined in *Pomc/Ptch*<sup>flf</sup> and *Pomc/Smo*<sup>flf</sup> mice (Fig. 1H). In addition, no histological abnormalities were observed in *Pomc/Ptch*<sup>flf</sup> and *Pomc/Smo*<sup>flf</sup> pituitaries (Fig. 2A and B, respectively). Thus, the distribution of hormone-releasing cells was normal and the *Pomc/Ptch*<sup>flf</sup> or *Pomc/Smo*<sup>flf</sup> pituitaries did not show any signs of hyperplasia (Fig. 2A) or hypoplasia (Fig. 2B), respectively, compared to the controls. Moreover, the percentage of Acth<sup>+</sup> cells was not altered in tamoxifen-treated *Pomc/Ptch*<sup>flf</sup> (Fig. 2C) or *Pomc/Smo*<sup>flf</sup> mice (Fig. 2D). Finally, combined RNAScope/immunofluorescent analyses revealed that *Gli1* transcripts are not expressed in Acth-expressing cells, neither in tamoxifen-treated *Pomc/Ptch*<sup>flf</sup> mice nor in the controls (Fig. 2E).

Together, *Ptch* or *Smo* depletion in *Pomc/Ptch*<sup>flf</sup> and *Pomc/Smo*<sup>flf</sup> pituitaries do not result in changes of Hh signaling activity or in the development of pathological phenotypes. These data show that a homozygous deletion of *Ptch* or *Smo* in *Pomc*-expressing cells has no impact on homeostasis of corticotrophs or other pituitary cells *in vivo*. These results are surprising, because *ex vivo* depletion of *Ptch* in whole pituitaries results in upregulation of *Pomc* (Pyczek *et al.* 2016).

### Somatotrophs and folliculo-stellate cells but not corticotrophs of the adult pituitary gland express *Gli1*

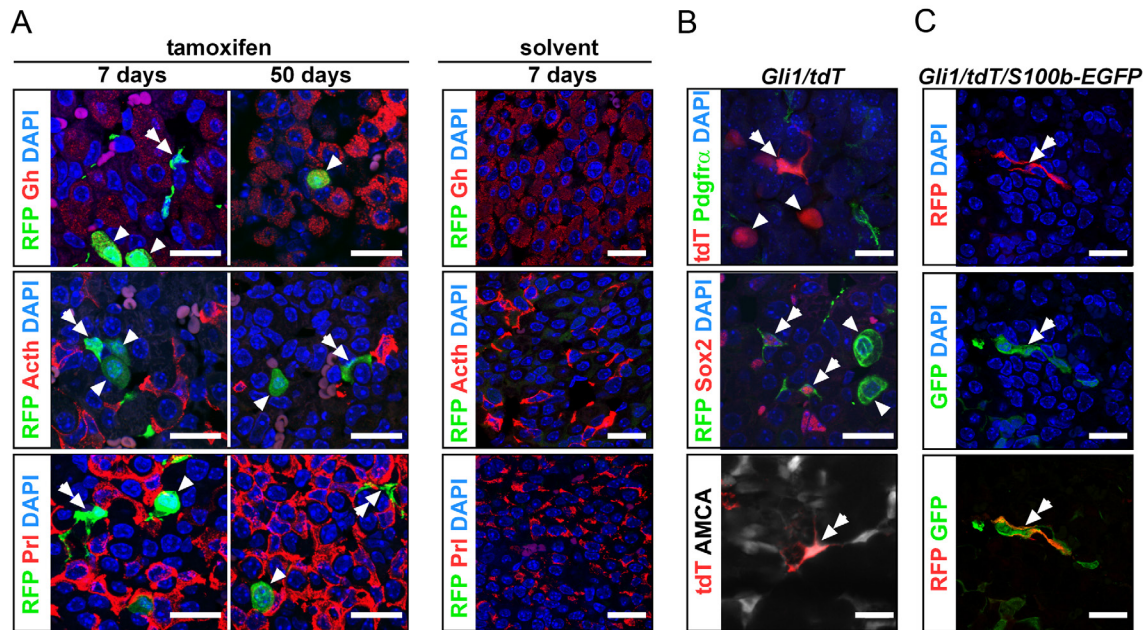
Because the above-mentioned *in vivo* experiments clearly excluded a direct impact of Hh signaling at least on corticotrophs, we hypothesized that Hh signaling might regulate hormone release in an indirect manner.

**Figure 2**

*In vivo* deregulation of Hh signaling in *Pomc*-expressing cells has no impact on the hormone expression pattern or cellular proliferation of the adult pituitary gland. (A and B) Representative (immune) histological analyses and (C and D) percentage of Acth<sup>+</sup> cells of pituitaries of tamoxifen- or solvent-treated (A and C) *Pomc/Ptch<sup>fl/fl</sup>* and *Ptch<sup>fl/fl</sup>* and (B and D) *Pomc/Smo<sup>fl/fl</sup>* and *Smo<sup>fl/fl</sup>* mice 265 days post tamoxifen or solvent treatment. (E) Representative *Gli1* RNAScope/anti-Acth antibody stainings of pituitaries of tamoxifen- or solvent-treated *Pomc/Ptch<sup>fl/fl</sup>* and *Ptch<sup>fl/fl</sup>* mice 265 days post-tamoxifen or solvent treatment (for RNAScope control stainings please see Fig. 4A). Analyzed animal numbers are given in Supplementary Table 1. Boxes: zoomed areas. Black arrows: Ki67<sup>+</sup> cells. White arrows: *Gli1*<sup>+</sup> cells. Scale bars: 50  $\mu$ m (A and B), 10  $\mu$ m (A insets, B insets, E). Circles in C and D represent mean  $\pm$  s.e.m. No significant differences were detected by using non-parametric Holm-Sidak method or Mann-Whitney tests.

To shed light on this, we performed *Gli1* lineage tracing experiments by generating *Gli1CreERT2 R26-tdTomato* (*Gli1/tdT*) mice and visualized the pituitary progeny of Gli1<sup>+</sup> cells under normal physiological conditions (same experimental setup as for *Pomc/tdT* mice, Supplementary Fig. 1A). Leakiness of the *Gli1CreERT2*-deleter strain was excluded by simultaneously investigated pituitary glands of solvent-treated *Gli1/tdT* mice (Fig. 3A). Remarkably, analyses of tamoxifen-treated *Gli1/tdT* adult pituitaries showed that two morphologically different pituitary cell types in the AL were marked by tdT reporter expression and thus developed from Gli1-expressing cells: one cell population with a round (Fig. 3A, arrow heads) and another with a stellate-shaped morphology (Fig. 3A, double arrows).

Double immunofluorescence analyses demonstrated that the round cell type was positive for Gh and negative for Prl and Acth/*Pomc*, thus representing somatotrophs (Fig. 3A, arrow heads). The stellate-shaped cell type did neither express Gh, Prl, Acth (Fig. 3A) nor *Pdgfra* (Fig. 3B), but was positive for Sox2 (Fig. 3B) and beta-Ala-Lys-N(epsilon)-aminomethylcoumarin acetate ( $\beta$ -Ala-Lys-N(epsilon)-AMCA) (Fauquier *et al.* 2002) uptake, resembling the phenotype of FSC (Fig. 3B). To further reinforce this assumption, we additionally examined pituitaries of tamoxifen-induced *Gli1/tdT/S100b-EGFP* mice, in which the progeny of Gli1<sup>+</sup> cells and cells that express the FSC marker S100b are marked simultaneously. Indeed, this approach revealed that tdT<sup>+</sup> stellate-shaped pituitary cells express EGFP (Fig. 3C) indicating that FSC represent progenies of Gli1<sup>+</sup> pituitary

**Figure 3**

Somatotrophs and FSC of the adult pituitary gland descent from *Gli1*<sup>+</sup> cells. Representative fluorescence analyses of adult (A and B) *Gli1/tdT* and (C) *Gli1/tdT/S100b-EGFP* pituitaries 7 or 50 days after *in vivo* tamoxifen or solvent application (A), 14 days (B bottom, C), 15 days (B top) or 22 days (B middle) days after tamoxifen injection. Similar experimental setup as shown in Fig. 1. Analyses were conducted on pituitaries of at least three animals per cohort. Arrow heads: somatotrophs; double arrows: FSC. Scale bars: 10  $\mu$ m.

cells. Moreover, combined RNAScope/immunofluorescent analyses (Fig. 4A, B, C, D, E and F) and subsequent quantification of *Gli1*<sup>+</sup> and *Gli2*<sup>+</sup> cells (Fig. 4G, H, I and J) verified that 33% (s.e.m. 1.5%) or 38% (s.e.m. 3.9%) of all somatotrophs (Fig. 4G and H) and 31% (s.e.m. 3.2%) or 34% (s.e.m. 7.2%) of *S100b-EGFP*<sup>+</sup> FSC (Fig. 4I and J) express *Gli1* or *Gli2* transcripts, respectively, and thus show active Hh signaling. The distribution and number of *Gli1*<sup>+</sup> somatotrophs and *Gli1*<sup>+</sup> FSC and their offspring did not grossly vary between different age-matched animals.

Taken together these data demonstrate that Hh signaling is active in a constant subpopulation of somatotrophs and FSC in the adult pituitary gland and thus most likely has a function in these two pituitary cell populations.

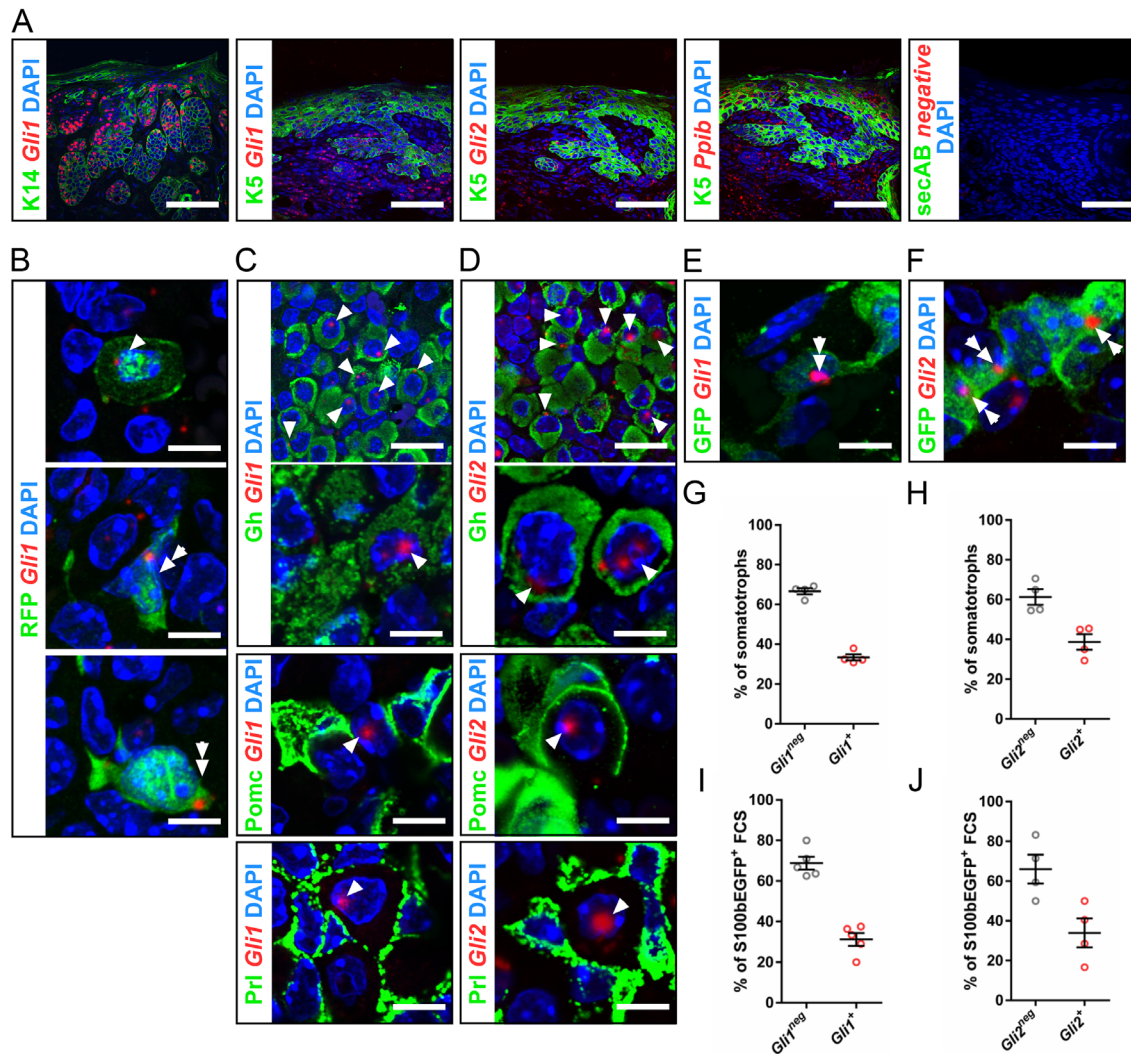
### Hh signaling is active in the folliculo-stellate cell line TtT/GF, but not in the somatotroph cell line GH3 or in the corticotroph cell line AtT-20

Next, we studied whether the aforementioned *in vivo* data also apply to pituitary cell lines. For this purpose, we used the folliculo-stellate cell line TtT/GF, the somatotroph cell line GH3 and the corticotroph cell line AtT-20 and studied the expression of cell-specific marker genes, the basal Hh signaling activity as well as the responsiveness to Hh

signaling activation. The results revealed that TtT/GF cells grow with a stellate-shaped morphology (Supplementary Fig. 2A) and express high levels of the FSC markers Sox2 (Supplementary Fig. 2A), *S100b*, *Vegfa*, *Mif* and *Fst* (Supplementary Fig. 2B), whereas GH3 or AtT-20 cells express Gh and *Ghrhr* (Supplementary Fig. 2C, D and E) or *Pomc* and *Acth*, respectively (Supplementary Fig. 2F and G). In contrast to GH3 and AtT-20 cells, TtT/GF cells furthermore express robust *Gli1* levels (Supplementary Fig. 3A) and show unambiguous Smo localization to primary cilia (Supplementary Fig. 3B, C and D), indicating basal Hh signaling activity. In addition, Smoothened Agonist (SAG)-treatment elevates the basal *Gli1* and *Gli2* transcription in TtT/GF (Supplementary Fig. 4A) but not in GH3 (Supplementary Fig. 4B) or AtT-20 cells (Supplementary Fig. 4C). This indicates that TtT/GF, but not GH3 or AtT-20 cells, are responsive to Hh signaling stimulation.

### Supernatant of Hh-stimulated TtT/GF folliculo-stellate cells induces Gh production in somatotroph GH3 cells, but has no impact on the corticotroph AtT-20 cell line

Since FSC were responsive to Hh signaling activation, we hypothesized that active Hh signaling might indirectly influence hormone release in Gh- or Acth-expressing



**Figure 4**

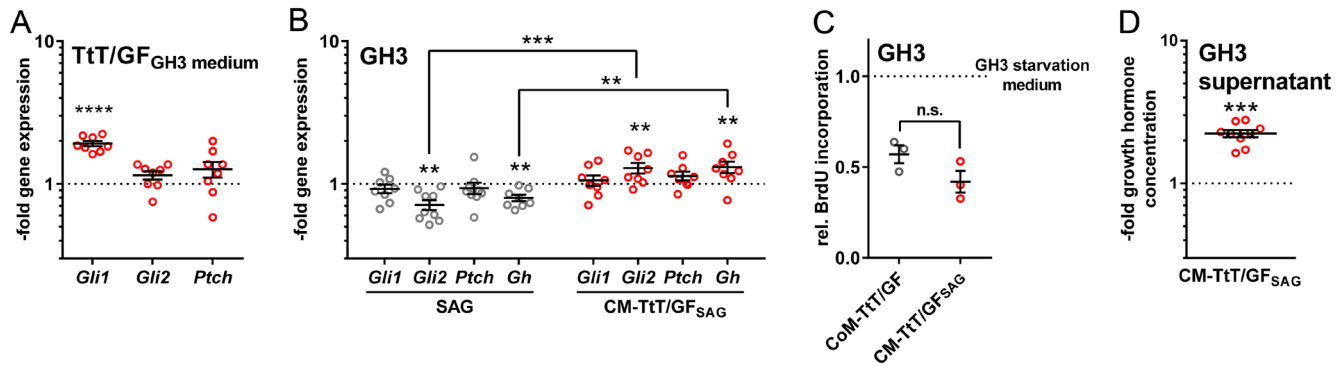
Somatotrophs and FSC of the adult pituitary gland express *Gli1* and *Gli2*. (A, B, C, D, E and F) Representative fluorescence analyses and (G, H, I and J) quantification of *Gli1*<sup>+</sup> (G) and *Gli2*<sup>+</sup> (H) somatotrophs and of *Gli1*<sup>+</sup> (I) and *Gli2*<sup>+</sup> FSC (J) of combined *Gli1* or *Gli2* transcripts detection using RNAScope technique and immunofluorescent stainings of murine basal cell carcinomas (A) (used as positive control for high *Gli1* and *Gli2* expression levels) and adult pituitary glands of tamoxifen-injected *Gli1/tdT* (B), C57BL/6N wildtype (C, D, G, H) and *S100b-EGFP* mice (E, F, I, J). Analyses were conducted on pituitaries of at least three animals per cohort. Arrow heads: somatotrophs; double arrows: FSC. secAB, secondary antibody only; negative, negative control probe. Scale bars: 33  $\mu$ m (A), 10  $\mu$ m (C top, D top), 3.3  $\mu$ m (B, C bottom, D bottom, E, F). Each open circle indicates one pituitary. Mean  $\pm$  s.e.m.

cells, potentially by secreted factors (reviewed in Morris & Christian 2011). To test this hypothesis, we treated GH3 or AtT-20 cells with conditioned medium from SAG-stimulated (CM-TtT/GF<sub>SAG</sub>) or solvent-treated TtT/GF cells (CoM-TtT/GF) (for confirmation of Hh signaling activity in TtT/GF cells after SAG treatment see Fig. 5A and Supplementary Fig. 6A), and analyzed the expression levels of *Gli1*, *Gli2*, *Ptch* and *Gh* or *Pomc*. CM-TtT/GF<sub>SAG</sub>-treatment neither alters Hh signaling activity in GH3 (Fig. 5B) and AtT-20 cells (Supplementary Fig. 5B), the proliferative activity of GH3 cells (Fig. 5C)

nor the *Pomc* expression (Supplementary Fig. 5B) or Acth secretion level of AtT-20 cells (Supplementary Fig. 5C). However, CM-TtT/GF<sub>SAG</sub>-incubation significantly increases *Gh* expression levels (Fig. 5B) and *Gh* secretion of GH3 cells compared to the respective CoM-TtT/GF-treated controls (Fig. 5D).

These data demonstrate that Hh activation in the FSC cell line TtT/GF apparently induces the release of paracrine factors that initiate *Gh* production/release from GH3 cells. The factors, however, do not initiate Acth production/release from AtT-20 cells.



**Figure 5**

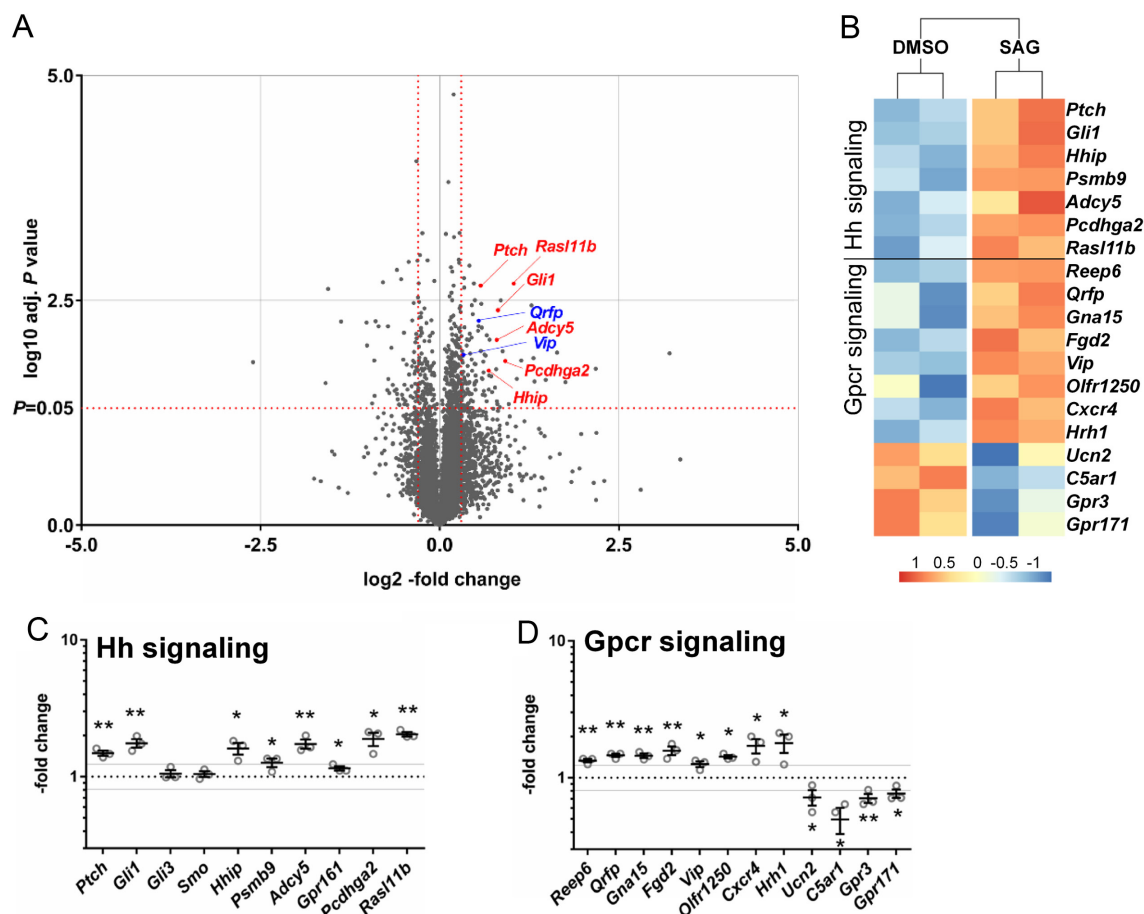
Upregulated Gh production of somatotrophs via paracrine signal transduction of Hh-activated FSC. (A and B) Gene expression levels of *Gli1*, *Gli2*, *Ptch* (A and B) and *Gh* (B) of (A) TtT/GF cells after serum starvation followed by 48 h, 100 nM Smoothened Agonist (SAG) or solvent treatment (dotted line) in GH3 serum starvation medium and (B) of GH3 cells after serum starvation followed by 48 h, 100 nM SAG (gray circles, same data as shown in Supplementary Fig. 5B) or solvent treatment (dotted line) or by 48 h incubation with conditioned media from TtT/GF cells (shown in A) treated with SAG (CM-TtT/GF<sub>SAG</sub>, red circles) or solvent (dotted line). Please note: *Gli2* expression most likely is not induced by general Hh signaling activation by remaining SAG in the CM-TtT/GF<sub>SAG</sub> medium since SAG-treatment of GH3 cells rather leads to reduced *Gli2* expression levels (Supplementary Fig. 5). (C) Relative BrdU incorporation of GH3 cells after serum starvation followed by 48 h incubation with GH3 starvation medium (dotted line), conditioned media from TtT/GF cells treated with SAG (CM-TtT/GF<sub>SAG</sub>, red circles) or solvent (CoM-TtT/GF). (D) Gh protein concentration in supernatant of GH3 cells after serum starvation followed by 48 h incubation with conditioned media from TtT/GF cells (shown in A) treated with SAG (CM-TtT/GF<sub>SAG</sub>, red circles) or solvent (dotted line). Gene expression levels were normalized to *18S* rRNA expression and to the respective gene expression levels of solvent-treated control cells (dotted lines). Gh concentration was normalized to the Gh concentration of solvent-treated control cells (dotted lines). Each open circle indicates one biological replicate measured in technical triplicates (A, B and D) or sextuplets (C). Mean  $\pm$  s.e.m. Significant differences were tested using the non-parametric Holm-Sidak method. Significant differences to the respective base line (dotted lines) are indicated by asterisks above the data. \*\* $P = 0.01$ ; \*\*\* $P = 0.001$ ; \*\*\*\* $P = 0.0001$ ; n.s., not significant.

### Vasoactive intestinal peptide is a candidate molecule for mediating Gh production/secretion upon Hh signaling activation in folliculo-stellate cells

The current knowledge about the functional regulation of endocrine cells by FSC and the involved signal transduction molecules is sparse. However, growth factors and peptides may play a role in this process (Allaerts & Vankelecom 2005, Morris & Christian 2011). To identify potential candidate molecules that are upregulated upon Hh signaling activation and potentially mediate Gh production in GH3 cells in a paracrine manner, we conducted comparative transcriptome analyses of SAG- vs solvent-treated TtT/GF cells. This approach revealed that SAG-treatment leads to an up- and downregulation of 108 or 63 genes, respectively (Fig. 6A). Significantly upregulated genes included 8 genes associated with Hh signaling activation (*Ptch*, *Gli1*, *Hhip*, *Psmb9*, *Adcy5*, *Pcdhga2*, *Gpr161*, *Rasl11b*) (Fig. 6A, B and C) and 8 genes associated with G protein-coupled receptors (Gpcr) signaling (*Reep6*, *Qrffp*, *Gna15*, *Fgd2*, *Vip*, *Olf1250*, *Cxcr4*, *Hrh1*) (Fig. 6A, B and D) whereas the expression of four genes associated with Gpcr signaling were downregulated (*Ucn2*, *CSar1*, *Gpr3*, *Gpr171*) (Fig. 6B and D). Additionally, SAG-treatment increased the expression levels of *insulin-like growth factor-binding protein 2* (*Igfbp2*),

*angiotensin-converting enzyme* (*Ace*), *glutamate ionotropic receptor kainate type subunit 4* (*Grik4*) and the putative pituitary stem/progenitor marker *cox sackie virus and adenovirus receptor* (*Cxadr*) (Supplementary Fig. 6A). In contrast, SAG-treatment merely altered FSC marker gene expression (e.g. *Sox2*, *S100b*, *Mif*, *Anxa1*) (Supplementary Fig. 6B) albeit it significantly increased the transcript levels of *Cxcr4* (Fig. 6D) and *Cxadr* (Supplementary Fig. 6A) that are also known to be expressed in FSC (Horiguchi *et al.* 2012, Chen *et al.* 2013).

To this end, we focused on the two neuropeptides RF(Arg-Phe)amide family 26 amino acid peptide (Qrffp) and vasoactive intestinal peptide (Vip), which are known to regulate pituitary hormone release (Matsushita *et al.* 1981, Chihara *et al.* 1982, Abe *et al.* 1985, Denef *et al.* 1985, Bluett-Pajot *et al.* 1987, Bjoro *et al.* 1990, Alexander & Sander 1994, Mazzocchi *et al.* 1998, Vleck & Patrick 1999, Fazekas *et al.* 2000, Christian *et al.* 2007, Leprince *et al.* 2017) and whose expression levels were significantly elevated in SAG-stimulated TtT/GF cells compared to the controls (Fig. 6A, B and D). qRT-PCR-based expression analyses verified the significant increase of *Vip* expression in SAG-treated TtT/GF cells (Fig. 7A), whereas the absolute *Qrffp* reads remained under qRT-PCR detection level. Importantly, measurement of *Vip* protein concentration revealed a significant increase of

**Figure 6**

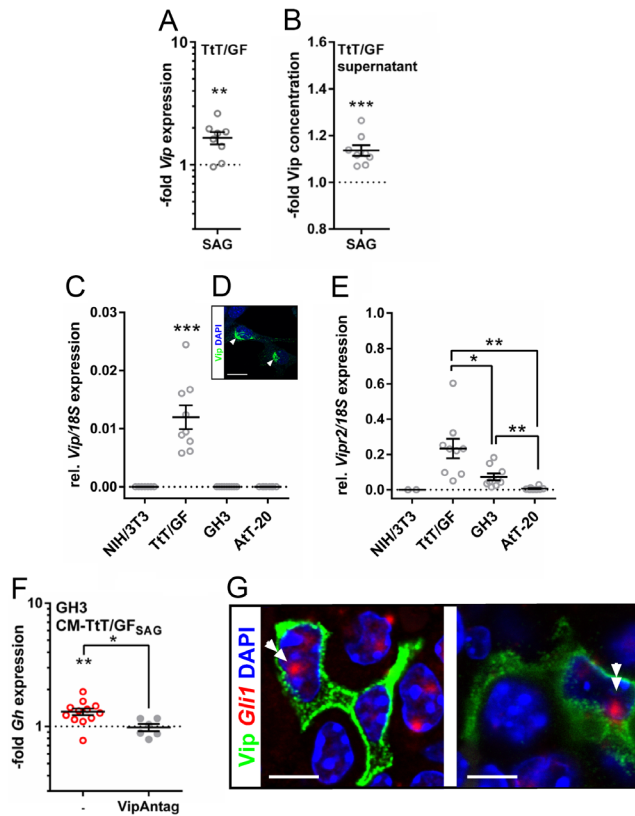
Comparative transcriptome analysis of Hh signaling activated TtT/GF cells. (A, B, C and D) Comparative transcriptome analysis of Smoothed Agonist (SAG)- vs solvent-treated (DMSO) TtT/GF cells. (A) Volcano plot of all expressed gene transcripts (cut off log<sub>2</sub> -0.8- or log<sub>2</sub> 1.2-fold change, red vertical lines), (B) heat map and (C and D) expression profile of significant differentially expressed genes (cut off 0.8- or 1.2-fold change, grey lines) associated with Hh and Gpcr signaling. Transcriptome analyses were conducted in biological triplicates (open circles). Gene expression of SAG-treated cells were normalized to solvent-treated controls (dotted lines in C and D). Differential expression with adjusted *P* values (non-parametric Holm–Sidak method) below 0.05 were considered to be significant (red horizontal line in A, asterisks in C and D). Significant differences to the respective baseline (dotted lines) are indicated by asterisks above/below the data. \**P* = 0.05; \*\**P* = 0.01; \*\*\**P* = 0.001; \*\*\*\**P* = 0.0001.

Vip protein in CM-TtT/GF<sub>SAG</sub> compared to CoM-TtT/GF (Fig. 7B and Supplementary Fig. 7). Expression analyses of *Vip* and its receptors *Vipr1* (*vasoactive intestinal peptide receptor*), *Vipr2* and *pituitary adenylate cyclase-activating peptide (Pacap) type 1 receptor (Adcyap1r1)* in TtT/GF, GH3, AtT-20 and NIH/3T3 (used as negative control) cells revealed that *Vip* transcripts were only detectable in TtT/GF cells (Fig. 7C) that also showed *Vip* protein expression (Fig. 7D). In addition, both TtT/GF and GH3 cells showed robust *Vipr2* mRNA levels (Fig. 7E). None of the cell lines expressed *Vipr1* or *Adcyap1r1* (data not shown). Finally, we analyzed whether GH3 cells respond to *Vip*. Strikingly, treatment of GH3 cells with the hybrid *Vip* antagonist KPRRPYTDNYTRLRKQMAVKKYLNSILN-NH<sub>2</sub> efficiently inhibited the CM-TtT/GF<sub>SAG</sub>-mediated Gh production (Fig. 7F).

These data show that Hh signaling activation in the FSC cell line TtT/GF stimulates the production and release of the neuropeptide *Vip*, which induces Gh production/secretion in the GH3 cells most likely via *Vipr2* signaling. Moreover, the fact that *Gli1*<sup>+</sup> stellate-shaped pituitary cells of the adult pituitary gland also express *Vip* (Fig. 7G) strongly points to a similar circuit in the pituitary *in vivo*.

## Discussion

The Hh signaling pathway plays a prominent role in the development of the pituitary (Treier *et al.* 2001, Roessler *et al.* 2003, França *et al.* 2010, Flemming *et al.* 2013). However, its function in the adult gland is far from clear. Recently we demonstrated that Hh signaling activation

**Figure 7**

Paracrine signal transduction of Hh-activated FSC to somatotrophs is mediated by Vip/Vipr2 signaling. (A and B) qRT-PCR- (E, same samples as shown in Supplementary Fig. 5) and ELISA-based (F) verification of increased Vip/Vip expression/secretion of SAG-treated TtT/GF cells (E) or supernatant (F, for total Vip concentration see Supplementary Fig. 8). (C, D and E) Vip mRNA (C), Vip protein (D) and Vipr2 mRNA expression of TtT/GF (C, D and E), GH3 and AtT-20 cells compared to NIH/3T3 cells (C and E). (F) qRT-PCR-based analysis of Gh expression level of GH3 cells treated with CM-TtT/GF<sub>SAG</sub> or CoM-TtT/GF supplemented with 1  $\mu$ M Vip antagonist (VipAntag) or solvent, respectively. (G) Combined visualization of Gli1 transcripts and Vip protein expression in an adult murine C57Bl6/N wildtype pituitary gland. Gene expression levels in A, C, E and F were normalized to 18S rRNA expression and in A and F additionally to the Vip or Gh expression level of solvent-treated control cells, respectively (dotted lines in A and F). Vip protein concentration was normalized to the Vip protein concentration of solvent-treated control cells (dotted line in B). Each open circle indicates one biological replicate measured in technical triplicates. Mean  $\pm$  s.e.m. Significant differences were tested using the non-parametric Holm–Sidak method. Significant differences to the respective base line (dotted lines) are indicated by asterisks above the data. \* $P = 0.05$ ; \*\* $P = 0.01$ ; \*\*\* $P = 0.001$ . White arrows: Vip<sup>+</sup> TtT/GF cells. White double arrows: Gli1<sup>+</sup> Vip<sup>+</sup> stellate-shaped pituitary cells. Scale bars: 10  $\mu$ m (D), 3.3  $\mu$ m (G).

in the adult pituitary gland leads to Acth, Gh and Prl production and proliferation of Sox2<sup>+</sup> cells. Unfortunately, these experiments were not conclusive with respect to the Hh-responsive pituitary cell type in the normal gland (Pyczek *et al.* 2016). However, this information is of great importance because GLI1 and SHH are highly expressed

by GH-, PRL- and ACTH-expressing human pituitary adenoma, which suggests that Hh signaling has an impact on pituitary tumor formation (Pyczek *et al.* 2016).

Here we demonstrate that a cell-specific deregulation of Hh signaling in Pomc-expressing cells does not affect the homeostasis of corticotrophs *in vivo*. This conclusion is based on our findings that homozygous depletion of Ptch or Smo in Pomc-expressing cells neither leads to defective development of the gland nor to disturbed Hh signaling activity or defective homeostasis of the adult pituitary. At the first glance, these results are contrary to our previous *ex vivo* studies on Rosa26-CreERT2/Ptch<sup>fl/fl</sup> pituitaries that revealed a higher Acth release upon Hh signaling activation (Pyczek *et al.* 2016). However, Rosa26-CreERT2-driven recombination targets every pituitary cell, whereas in Pomc/Ptch<sup>fl/fl</sup> and Pomc/Smo<sup>fl/fl</sup> mice Hh signaling is activated/inactivated cell-specifically in Pomc-expressing cells. Moreover, the fact that murine Pomc-expressing cells never stained positive for tdT in Gli1 lineage tracing experiments or for Gli1 transcripts in RNAScope stainings supports the conclusion that cell-intrinsic Hh signaling is not important for corticotrophs. Currently, we cannot be completely sure whether this also applies to the human pituitary since some ACTH-expressing cells of the human pituitary are immunopositive for SHH (Vila *et al.* 2005a, Pyczek *et al.* 2016). Nevertheless, our new data demonstrate that Pomc/Acth production in corticotrophs must also involve an indirect (e.g. paracrine) effect of Hh signaling.

Beyond that, our RNAScope and Gli1 lineage tracing approaches revealed that subpopulations of somatotrophs and FSC show active Hh signaling *in vivo*. These findings are remarkable because they suggest that Hh signaling is important for homeostasis of both pituitary cell types. However, our analyses of the Hh signaling status and responsiveness toward SAG-treatment in well-accepted pituitary cell lines revealed that GH3 cells express extremely low Gli1 levels, show very rarely ciliary Smo localization and are unresponsive to Hh signaling activation upon SAG-treatment. These facts impaired further *in vitro* analyses using GH3 cells to investigate the cell-intrinsic impact of Hh signaling in somatotrophs and the most elegant way to do so would be *in vivo* approaches. Unfortunately, until now no somatotroph-specific CreERT2-deleter mouse strains exist.

In addition, our data strongly suggest that Hh signaling influences the functionality of FSC, which activates hormone production in somatotrophs in a paracrine way. FSC represents a small (5–10%)

non-hormone secreting cell population in the adult AL and are implicated in the regulation and maintenance of hormone-secreting cells by delivering paracrine factors (e.g. interleukin-6, vascular endothelial growth factor, annexin-1) (reviewed in [Allaerts & Vankelecom 2005](#)). However, the exact mechanisms of how FSC regulates endocrine cells are not well understood. Our *in vitro* approaches now demonstrate for the first time that activation of Hh signaling in the FSC cell line TtT/GF induces Gh production/secretion in GH3 cells via a paracrine mechanism. Since Vip expression and concentration are significantly increased in TtT/GF cells and in the respective supernatant after SAG-treatment, and since Vip antagonist treatment can block CM-TtT/GF<sub>SAG</sub>-induced Gh production from GH3 cells, this paracrine mechanism most likely encompasses the neuropeptide Vip. In addition, this peptide is well known for its specific capacity to stimulate Gh production/secretion in GH3 and adenoma cells and in *in vivo* approaches ([Matsushita \*et al.\* 1981](#), [Chihara \*et al.\* 1982](#), [Denef \*et al.\* 1985](#), [Bluet-Pajot \*et al.\* 1987](#), [Murakami \*et al.\* 1995](#), [Fazekas \*et al.\* 2000](#)). Apart from that Vip also induces Prl ([Abe \*et al.\* 1985](#), [Bjoro \*et al.\* 1990](#), [Vleck & Patrick 1999](#), [Fazekas \*et al.\* 2000](#), [Christian \*et al.\* 2007](#)) and Acth release ([Alexander & Sander 1994](#), [Mazzocchi \*et al.\* 1998](#)) from the respective cell lines and endocrine and/or pituitary adenoma cells. In the normal pituitary gland Vip is expressed throughout the organ ([Arnaout \*et al.\* 1986](#), [Hsu \*et al.\* 1989](#)) including in a so far unidentified pituitary cell type with FSC-like morphology ([Hagen \*et al.\* 1986](#)). Vip signal transmission into the target cells is mediated by binding to the G protein-coupled membrane-bound receptors Vipr1 or Vipr2 (type 2 receptors), but not via the Pacap-specific Pac1 receptor (type 1 receptor, encoded by *Adcyap1r1* gene) (reviewed in [Hirabayashi \*et al.\* 2018](#)). Interestingly, TtT/GF cells express neither *Pacap*, *Adcyap1r1* nor *Vipr1*. However, they express *Vip* and *Virp2* and the expression and concentration of Vip increases upon Hh signaling activation in TtT/GF cells. Most strikingly, GH3 cells express *Vipr2* but not *Vip*, *Adcyap1r1* or *Vipr1*. Thus, the increased Gh production/release of GH3 after incubation with CM-TtT/GF<sub>SAG</sub> is most likely transmitted via Vip/Vipr2 signaling. Similar findings have been reported for the AtT-20 substrains AtT-20/D16-16 (Cellosaurus CVCL\_GZ35) and AtT20/D16v (Cellosaurus CVCL\_4W08), in which Vip-binding to the Vipr2 receptor induces Acth-release ([Reisine \*et al.\* 1982](#), [Aoki \*et al.\* 1997](#)). Paternal AtT-20 cells (Cellosaurus CVCL\_2300) used in our study do not express *Vipr2* ([Fig. 7H](#)). This may explain the

unresponsiveness of AtT-20 cells toward Vip-enriched CM-TtT/GF<sub>SAG</sub> in our setting.

Together, our data demonstrate for the first time that Hh signaling is involved in FSC-mediated regulation of Gh production/release at least *in vitro*. Moreover, our results strongly hint toward a similar role of Hh signaling *in vivo*. Nevertheless, additional studies are needed to show whether this concept is indeed transferrable to the *in vivo* situation and potentially also to Acth-expressing cells. For this purpose, *in vivo* depletion of *Gli1*, *Ptch* or *Smo* in FSC would be advantageous which is so far hampered by missing availability of an FSC-specific Cre- or CreERT2-deleter mouse strain. However, our findings could be of importance for several pituitary adenoma subtypes, in which HH signaling is activated ([Pyczek \*et al.\* 2016](#)). It is possible that Hh signaling activation in tumor-associated FSC, which are found in large numbers at the periphery of adenomas and other pituitary lesions ([Nishioka \*et al.\* 1991](#), [Voit \*et al.\* 1999](#), [Horvath & Kovacs 2002](#), [Cimpean \*et al.\* 2017](#)) support hormone production from tumor cells. This opens the intriguing possibility that hormone production of tumor cells depends on Hh signaling activity in adjacent FSC, which thus might represent a target for future therapeutic intervention.

#### Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0388>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Author contribution statement

D S B designed and performed research, collected and analyzed data, prepared the figures and wrote the manuscript. N B, A F and I H performed research and collected data. A W analyzed data, A Z analyzed data, H H contributed vital reagents and analytical tools and reviewed the paper. R B contributed vital reagents and analytical tools and reviewed the paper. A U designed research, collected and analyzed data, prepared the figures and wrote the manuscript. All authors reviewed the manuscript.

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