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 adrenocorticotropic hormone (Acth) production and proliferation of Sox2+ 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 productionsecretion.
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/adrenocorticotropic 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\(^+\) (stem) cells and a meshwork of non-endocrine Sox2\(^+\) 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 homoeostasis 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 Smoothened (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. \textit{Gli1, Gli2} or \textit{Ptch}) (reviewed in (Bangs and 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 (Franca et al., 2010, Flemming et al., 2013) or hyperplasia of the pituitary (Treier et al., 2001), respectively. Several lines of evidence additionally point towards a regulative function of the pathway in stem cell maintenance and regenerative processes in the adult pituitary. Thus, our group described enhanced proliferation of Sox2\(^+\) 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, Vila et al., 2005b, 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 (Bale et al., 1991, Bale et al., 1994, Wicking et al., 1997, Lo Muzio et al., 1999, Hahn et al., 1998, Cramer and Niederdellmann, 1983, Kahn and Gordon, 1967, Codish et al., 1973, Marcos et al., 1982, Kimonis et al., 1997). 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⁺ cells. However, it never has been analysed 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 substantiate 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).
Material 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: \textit{Ptch}\textsubscript{1}\textsuperscript{tm1Hahn} (\textit{Ptch}\textsuperscript{flox/flox} (Uhmann et al., 2007), JAX stock \# 012457), \textit{Smo}\textsuperscript{tm2Amc} (\textit{Smo}\textsuperscript{flox/flox} (Long et al., 2001), JAX stock \# 004526), \textit{Tg(Pomc-cre/ERT2)}\textsuperscript{#Jke} (\textit{PomcCreERT2}, (Berglund et al., 2013) a kind Gift from J.K. Elmquist), \textit{Gli1}\textsuperscript{tm3(cre/ERT2)Alj} (\textit{Gli1CreERT2} (Ahn and Joyner, 2004), JAX stock \#007913), \textit{Gt(ROSA)26Sor}\textsuperscript{tm9(CAG-tdTomato)Hze} (\textit{tdT}, (Madisen et al., 2010), JAX stock \#007905) and \textit{Tg(S100b-EGFP)}\textsuperscript{11Lgrv} (\textit{S100b-EGFP}, (Vives et al., 2003) a kind Gift from C. Legraverend and P. Mollard).

\textit{Ptch}\textsuperscript{flox/flox}, \textit{PomcCreERT2}, \textit{tdT} and \textit{S100b-EGFP} strains were maintained on C57BL/6 and \textit{Smo}\textsuperscript{flox/flox} and \textit{Gli1CreERT2} mice on a 129/Sv background. Both genders of transgenic mice were used. No sex-specific differences were observed. Genotyping of the mice were conducted by PCR on genomic DNA isolated from tail or ear biopsies using primer pairs recommended by the donating investigators (Berglund et al., 2013, Vives et al., 2003) or by The Jackson Laboratory (https://www.jax.org/jax-mice-and-services). For CreERT2-mediated homozygous deletion of \textit{Ptch} or \textit{Smo} \textit{Ptch}\textsuperscript{flox/flox} or \textit{Smo}\textsuperscript{flox/flox} mice, respectively, were bred to the respective CreERT2-deleter mouse strain. For lineage tracing experiments the CreERT2-deleter strains were crossed to \textit{tdT} mice that in some experiments additionally carry the \textit{S100b-EGFP} transgene. The CreERT2-activity of the transgenic mice was induced by five single intraperitoneal injection (i.p.) of 1 mg tamoxifen on five consecutive days at an animal age of 8 weeks (Uhmann et al., 2007). Untreated mice without the respective CreERT2-recombinase gene and solvent-treated mice carrying the...
floxed allele and the respective CreERT2-recombinase genes served as controls. For lineage tracing experiments mice were analysed after the first tamoxifen application as indicated in the respective figure legends. Body weight and blood samples of PomcCreERT2 Ptch\textsuperscript{fl/o}, PomcCreERT2 Smo\textsuperscript{fl/o} 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 (see Fig. 1B). Measurements of blood glucose and serum hormone levels are described in the Supplemental methods section. The number of analysed animals is given in Table S1 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 (β-Ala-Lys-N(ε)-AMCA) was obtained from Carbosynth (Berkshire, UK) and Smoothened Agonist (SAG) from Cayman Chemical (Ann Arbour, USA). β-Ala-Lys-N(ε)-AMCA was dissolved in HBSS (0.952 mM CaCl\textsubscript{2}·2H\textsubscript{2}O, 5.36 mM KCl, 0.411 mM KH\textsubscript{2}PO\textsubscript{4}, 0.812 mM MgSO\textsubscript{4}·7H\textsubscript{2}O, 136.7 mM NaCl, 0.385 mM Na\textsubscript{2}HPO\textsubscript{4}, 25 mM D-Glucose·H\textsubscript{2}O, 10 mM HEPES). SAG was dissolved in dimethyl sulfoxide (DMSO). The preparation of tamoxifen/ethanol/sunflower oil for \textit{in vivo} application has been previously described (Uhmann 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, Carlsbad, USA).
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 containing 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 analysed by marker gene expression analyses and immunofluorescent stainings against marker proteins as shown in Figure S3. 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 Supplemental methods section.

**Detection of recombination of the $Ptch^{\text{flox}}$ and the $Smo^{\text{flox}}$ 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 $Ptch^{\text{flox}}$ or $Smo^{\text{flox}}$ locus the primer pairs indicated in Fig. 1H and G were used. The sequences of the primers are given in Table S2.

**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 Table S3. 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 above) were analysed. RNA quality control (Fragment Analyzer, Agilent Technologies, USA), 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 Supplemental methods section. 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 β-Ala-Lys-N(ε)-AMCA incubation see Supplemental
methods. Used antibodies, antibody dilutions and antigen retrieval procedures are summarized in Table S4.

Statistics

Statistical analyses were performed using the GraphPadPrism 6 software (GraphPad Software Inc.). The used statistical tests are given in the respective Figure legends.
Results

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

Constitutive activation of Hh signaling by a Rosa26-CreERT2-driven homozygous deletion of *Ptch* in *ex vivo* cultured adult pituitaries leads to an increase in *Pomc, Gh* and *Prl* expression and enhanced BrdU-incorporation of Sox2$^+$ pituitary cells (Pyczek et al., 2016). Since the Rosa26-CreERT2-deleter recombines the *Ptch*$_{\text{flax}}$ locus in virtually every pituitary cell, these experiments did not allow for 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*$_{\text{flax/flax}}$ or *Smo*$_{\text{flax/flax}}$ 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$^+$ cells isolated from adult pituitaries of solvent-treated *Pomc/tdT* mice (experimental setup see Fig. S1A), the *Pomc/tdT* reporter was highly or mildly leaky in the intermediate lobe or the AL, respectively (Fig. S1B). However, tamoxifen-application strongly increased the number of tdT$^+$ 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 (Fig. S1B). 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 (Fig. S1B) 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\textsuperscript{flox/flox} or Smo\textsuperscript{flox/flox} mice. Irrespective of the leakiness of the deleter, PomcCreERT2 Ptch\textsuperscript{flox/flox} (Pomc/Ptch\textsuperscript{f/f}) and PomcCreERT2 Smo\textsuperscript{flox/flox} (Pomc/Smo\textsuperscript{f/f}) 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\textsuperscript{f/f} and Pomc/Smo\textsuperscript{f/f} mice showed signs of a deregulated hormone status [e.g. alopecia, weight loss/gain (Fig. 1C,D), abnormal blood glucose levels (Fig. 1C,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\textsuperscript{flox} or Smo\textsuperscript{flox} loci were efficiently recombined in Pomc/Ptch\textsuperscript{f/f} and Pomc/Smo\textsuperscript{f/f} mice (Fig. 1H). In addition, no histological abnormalities were observed in Pomc/Ptch\textsuperscript{f/f} and Pomc/Smo\textsuperscript{f/f} pituitaries (Fig. 2A and 2B, respectively). Thus, the distribution of hormone releasing cells was normal and the Pomc/Ptch\textsuperscript{f/f} or Pomc/Smo\textsuperscript{f/f} pituitaries did not show any signs of hyperplasia (Fig. 2A) or hypoplasia (Fig. 2B), respectively, compared to the controls. Moreover, the percentage of Acth\textsuperscript{+} cells was not altered in tamoxifen-treated Pomc/Ptch\textsuperscript{f/f} (Fig. 2C) or Pomc/Smo\textsuperscript{f/f} 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\textsuperscript{f/f} mice nor in the controls (Fig. 2E).

Together, Ptch or Smo depletion in Pomc/Ptch\textsuperscript{f/f} and Pomc/Smo\textsuperscript{f/f} 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 \textit{in vivo}. These results are surprising, because \textit{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 \textit{Gli1}

Because the above-mentioned \textit{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. To shed light on this we performed \textit{Gli1} lineage tracing experiments by generating \textit{Gli1CreERT2 R26-tdTomato (Gli1/tdT)} mice and visualized the pituitary progeny of Gli1\textsuperscript{+} cells under normal physiological conditions (same experimental setup as for \textit{Pomc/tdT} mice, Fig. S1A). Leakiness of the \textit{Gli1CreERT2}-deleter strain was excluded by simultaneously investigated pituitary glands of solvent-treated \textit{Gli1/tdT} mice (Fig. 3A). Remarkably, analyses of tamoxifen-treated \textit{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 (β-Ala-Lys-N(ε)-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 \textit{Gli1/tdT/S100b-EGFP} mice, in which the progeny of Gli1\textsuperscript{+} cells and cells that express the FSC marker S100b are marked simultaneously. Indeed, this approach revealed that tdT\textsuperscript{+} stellate-shaped pituitary cells express EGFP (Fig. 3C) indicating that FSC represent progenies of Gli1\textsuperscript{+} pituitary cells. Moreover, combined
RNAscope/immunofluorescent analyses (Fig. 4A-F) and subsequent quantification of Gli1+ and Gli2+ cells (Fig. 4G-J) verified that 33% (SEM 1.5%) or 38% (SEM 3.9%) of all somatotrophs (Fig. 4G,H) and 31% (SEM 3.2%) or 34% (SEM 7.2%) of S100b-EGFP+ FSC (Fig. 4I,J) express Gli1 or Gli2 transcripts, respectively, and thus show active Hh signaling. The distribution and number of Gli1+ somatotrophs and Gli1+ 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 above-mentioned *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 (Fig. S2A) and express high levels of the FSC markers Sox2 (Fig. S2A), S100b, Vegfa, Mif and Fst (Fig. S2B), whereas GH3 or AtT-20 cells express Gh and Ghhr (Fig. S2C,D,E) or Pomc and Atch, respectively (Fig. S2F,G). In contrast to GH3 and AtT-20 cells, TtT/GF cells furthermore express robust Gli1 levels (Fig. S3A) and show unambiguous Smo localization to primary cilia (Fig. S3B-D), indicating basal Hh signaling activity. In addition, Smoothened Agonist (SAG)-treatment elevates the basal Gli1 and
Gli2 transcription in TtT/GF (Fig. S4A) but not in GH3 (Fig. S4B) or AtT-20 cells (Fig. S4C). 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 cells, potentially by secreted factors (reviewed in (Morris and Christian, 2011)). To test this hypothesis, we treated GH3 or AtT-20 cells with conditioned medium from SAG-stimulated (CM-TtT/GF\textsubscript{SAG}) 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 S6A), and analysed the expression levels of Gli1, Gli2, Ptch and Gh or Pomp. CM-TtT/GF\textsubscript{SAG}-treatment neither alters Hh signaling activity in GH3 (Fig. 5B) and AtT-20 cells (Fig. S5B), the proliferative activity of GH3 cells (Fig. 5C) nor the Pomp expression (Fig. S5B) or Acth secretion level of AtT-20 cells (Fig. S5C). However, CM-TtT/GF\textsubscript{SAG}-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.

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 transducing molecules is sparse. However, growth factors and peptides may play a role in this process (Morris and Christian, 2011, Allaerts and Vankelecom, 2005). 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- versus 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-C) and 8 genes associated with G protein-coupled receptors (Gpcr) signaling (Reep6, Qrfp, Gna15, Fgd2, Vip, Olfr1250, Cxcr4, Hrh1) (Fig. 6A,B,D) whereas the expression of 4 genes associated with Gpcr signaling were downregulated (Ucn2, C5ar1, Gpr3, Gpr171) (Fig. 6B,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 coxsackie virus and adenovirus receptor (Cxadr) (Fig. S6A). In contrast, SAG-treatment merely altered FSC marker gene expression (e.g. Sox2, S100b, Mif, Anxa1) (Fig. S6B) albeit it significantly increased the transcript levels of Cxcr4 (Fig. 6D) and Cxadr (Fig. S6A) 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 (Qrfp) and vasoactive intestinal peptide (Vip), which are known to regulate pituitary hormone release (Leprince et al., 2017, Bluet-Pajot et al., 1987, Chihara et al., 1982, Matsushita et al., 1981, Denef et al., 1985, Fazekas et al., 2000, Abe et al., 1985, Vleck and Patrick, 1999, Christian et al., 2007, Bjoro et al., 1990, Alexander and Sander, 1994, Mazzocchi et al., 1998) and...
whose expression levels were significantly elevated in SAG-stimulated TtT/GF cells compared to
the controls (Fig. 6A,B,D). qRT-PCR-based expression analyses verified the significant increase
of Vip expression in SAG-treated TtT/GF cells (Fig. 7A), whereas the absolute Qrfp reads remained
under qRT-PCR detection level. Importantly, measurement of Vip protein concentration revealed
a significant increase of Vip protein in CM-TtT/GF_{SAG} compared to CoM-TtT/GF (Fig. 7B; Suppl.
Fig. S7). 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
analysed whether GH3 cells respond to Vip. Strikingly, treatment of GH3 cells with the hybrid Vip
antagonist KPRPYTDNYTRLRKQMAVKKYLNSILN-NH$_2$ efficiently inhibited the CM-
TtT/GF$_{SAG}$-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$^+$ 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 (Roessler et al., 2003, Franca et al., 2010, Flemming et al., 2013, Treier et al., 2001). However, its function in the adult gland is far from clear. Recently we demonstrated that Hh signaling activation in the adult pituitary gland leads to Acth, Gh and Prl production and proliferation of Sox2+ 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 Pome-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 Pome-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 f/f 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 Pome/Ptch f/f and Pome/Smo f/f mice Hh signaling is activated/inactivated cell-specifically in Pome-expressing cells. Moreover, the fact that murine Pome-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 Pomp/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 towards 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 suggests that Hh signaling influences the functionality of FSC, which activate hormone production in somatotrophs in a paracrine way. FSC represent 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 and Vankelecom, 2005)). However, the exact mechanisms how FSC regulate 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 SAG-induced Gh production from GH3 cells, this paracrine mechanism most likely encompass 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 (Murakami et al., 1995, Bluet-Pajot et al., 1987, Chihara et al., 1982, Matsushita et al., 1981, Denef et al., 1985, Fazekas et al., 2000). Apart from that Vip also induces Prl (Fazekas et al., 2000, Abe et al., 1985, Vleck and Patrick, 1999, Christian et al., 2007, Bjoro et al., 1990) and Acth release (Alexander and 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/GFSAG 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* (see Fig. 7H). This may explain the unresponsiveness of AtT-20 cells towards Vip-enriched CM-TtT/GFSAG 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 towards 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, Ptc1 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, Cimpean et al., 2017, Voit et al., 1999, Horvath and Kovacs, 2002) 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.
Conflict of interest statement

The authors have declared that no conflict of interest exists.

Author contributions

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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Figures legends

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,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\textsuperscript{f/f} and Ptch\textsuperscript{f/f} or Pomc/Smo\textsuperscript{f/f} and Smo\textsuperscript{f/f} mice. Analysed animal numbers are given in Table S1, 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 +/- SEM), circles in C-E represent mean +/- SEM. No significant differences were detected by using non-parametric Holm-Sidak method or Mann-Whitney tests.

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,B) Representative (immune) histological analyses and (C,D) percentage of Acth\textsuperscript{+} cells of pituitaries of tamoxifen- or solvent-treated \textit{(A,C)} Pomc/Ptch\textsuperscript{f/f} and Ptch\textsuperscript{f/f} and \textit{(B,D)} Pomc/Smo\textsuperscript{f/f} and Smo\textsuperscript{f/f} mice 265 days post tamoxifen or solvent treatment. \textit{(E)} Representative Gli1 RNAscope/anti-Acth antibody stainings of pituitaries of tamoxifen- or solvent-treated of Pomc/Ptch\textsuperscript{f/f} and Ptch\textsuperscript{f/f} mice 265 days post tamoxifen or solvent treatment (for RNAscope control stainings please see Fig. 4A). Analysed animal numbers are given in Table S1. Boxes: zoomed areas. Black arrows: Ki67\textsuperscript{+} cells. White arrows: \textit{Gli}1\textsuperscript{+} cells. Scale bars: 50 \textmu m (A,B), 10 \textmu m (A insets, B insets, E). Circles in C,D
represent mean +/- SEM. No significant differences were detected by using non-parametric Holm-Sidak method or Mann-Whitney tests.

**Figure 3: Somatotrophs and FSC of the adult pituitary gland descent from Gli1+ cells.**
Representative fluorescence analyses of adult (A,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 Figure 1. Analyses were conducted on pituitaries of at least 3 animals per cohort. Arrow heads: somatotrophs; double arrows: FSC. Scale bars: 10 μm.

**Figure 4: Somatotrophs and FSC of the adult pituitary gland express Gli1 and Gli2. (A-F)**
Representative fluorescence analyses and (G-J) quantification of Gli1+ (G) and Gli2+ somatotrophs (H) and of Gli1+ (I) and Gli2+ 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 3 animals per cohort. Arrow heads: somatotrops; double arrows: FSC. secAB, secondary antibody only; negative, negative control probe. Scale bars: 33 μm (A), 10 μm (C top, D top), 3.3 μm (B, C bottom, D bottom, E, F). Each open circle indicates one pituitary. Mean +/- SEM.
Figure 5: Upregulated Gh production of somatotrophs via paracrine signal transduction of Hh-activated FSC. (A,B) Gene expression levels of Gli1, Gli2, PtcH (A,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 GH cells after serum starvation followed by 48 h 100 nM SAG (gray circles, same data as shown in Fig. S5B) 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 (see Suppl. Fig. S5). (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,D) or sextuplets (C). Mean +/- SEM. 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.
Figure 6: Comparative transcriptome analysis of Hh signaling activated TtT/GF cells. (A-D)

Comparative transcriptome analysis of Smoothened Agonist (SAG)- versus solvent-treated (DMSO) TtT/GF cells. (A) Volcano plot of all expressed gene transcripts (cut off $\log^2 -0.8$- or $\log^2 1.2$-fold change, red vertical lines), (B) heat map and (C,D) expression profile of significant differential 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,D). Differential expression with adjusted $P$ values (non-parametric Holm-Sidak method) below 0.05 were consider to be significant (red horizontal line in A, asterisks in C,D). Significant differences to the respective base line (dotted lines) are indicated by asterisks above/below the data.

*, $P=0.05$; **, $P=0.01$; ***, $P=0.001$; ****, $P=0.0001$.

Figure 7: Paracrine signal transduction of Hh-activated FSC to somatotrophs is mediated by Vip/Vipr2 signaling. (A,B) qRT-PCR- (E, same samples as shown in Fig. S5)) and EIA-based (F) verification of increased Vip/Vip expression/secretion of SAG-treated TtT/GF cells (E) or supernatant (F, for total Vip concentration see Suppl. Fig. S8). (C-E) Vip mRNA (C), Vip protein (D) and Vipr2 mRNA expression of TtT/GF (C-E), GH3 and AtT-20 cells compared to NIH/3T3 cells (C,E). (F) qRT-PCR-based analysis of Gh expression level of GH3 cells treated with CM-TtT/GFSAG or CoM-TtT/GF supplemented with 1 µM 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 +/- SEM. 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\(^+\) TtT/GF cells. White double arrows: \( Gli^+ \) Vip\(^+\) stellate-shaped pituitary cells. Scale bars: 10 \( \mu m \) (D), 3.3 \( \mu m \) (G).
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,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/f and Ptch/f or Pomc/Smof/f and Smof/f mice. Analysed animal numbers are given in Table S1, 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 +/- SEM), circles in C-E represent mean +/- SEM. No significant differences were detected by using non-parametric Holm-Sidak method or Mann-Whitney tests.
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,B) Representative (immune) histological analyses and (C,D) percentage of Acth+ cells of pituitaries of tamoxifen- or solvent-treated (A,C) Pomc/Ptchf/f and Ptchf/f and (B,D) Pomc/Smof/f and Smof/f mice 265 days post tamoxifen or solvent treatment. (E) Representative Gli1 RNAScope/anti-Acth antibody stainings of pituitaries of tamoxifen- or solvent-treated of Pomc/Ptchf/f and Ptchf/f mice 265 days post tamoxifen or solvent treatment (for RNAScope control stainings please see Fig. 4A). Analysed animal numbers are given in Table S1. Boxes: zoomed areas. Black arrows: Ki67+ cells. White arrows: Gli1+ cells. Scale bars: 50 µm (A,B), 10 µm (A insets, B insets, E). Circles in C,D represent mean +/- SEM. No significant differences were detected by using non-parametric Holm-Sidak method or Mann-Whitney tests.
Figure 3: Somatotrophs and FSC of the adult pituitary gland descend from Gli1+ cells. Representative fluorescence analyses of adult (A,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 Figure 1. Analyses were conducted on pituitaries of at least 3 animals per cohort. Arrow heads: somatotrophs; double arrows: FSC. Scale bars: 10 µm.

61x34mm (600 x 600 DPI)
Figure 4: Somatotrophs and FSC of the adult pituitary gland express Gli1 and Gli2. (A-F) Representative fluorescence analyses and (G-J) quantification of Gli1+ (G) and Gli2+ somatotrophs (H) and of Gli1+ (I) and Gli2+ 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 3 animals per cohort. Arrow heads: somatotrops; double arrows: FSC. secAB, secondary antibody only; negative, negative control probe. Scale bars: 33 µm (A), 10 µm (C top, D top), 3.3 µm (B, C bottom, D bottom, E, F). Each open circle indicates one pituitary. Mean +/- SEM.

69x65mm (600 x 600 DPI)
Figure 5: Upregulated Gh production of somatotrophs via paracrine signal transduction of Hh-activated FSC.

(A,B) Gene expression levels of Gli1, Gli2, Ptch (A,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 GH cells after serum starvation followed by 48 h 100 nM SAG (gray circles, same data as shown in Fig. S5B) 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/GFSAG, 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/GFSAG medium since SAG-treatment of GH3 cells rather leads to reduced Gli2 expression levels (see Suppl. Fig. S5). (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/GFSAG, 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/GFSAG, 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,D) or sextuplets (C). Mean +/- SEM. 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.

128x103mm (600 x 600 DPI)
Figure 6: Comparative transcriptome analysis of Hh signaling activated TtT/GF cells. (A-D) Comparative transcriptome analysis of Smoothened Agonist (SAG)- versus solvent-treated (DMSO) TtT/GF cells. (A) Volcano plot of all expressed gene transcripts (cut off log2 -0.8- or log2 1.2-fold change, red vertical lines), (B) heat map and (C,D) expression profile of significant differential 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,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,D). Significant differences to the respective base line (dotted lines) are indicated by asterisks above/below the data. *, P=0.05; **, P=0.01; ***, P=0.001; ****, P=0.0001.
Figure 7: Paracrine signal transduction of Hh-activated FSC to somatotrophs is mediated by Vip/Vipr2 signaling. (A, B) qRT-PCR- (E, same samples as shown in Fig. S5)) and EIA-based (F) verification of increased Vip/Vip expression/secretion of SAG-treated TtT/GF cells (E) or supernatant (F, for total Vip concentration see Suppl. Fig. S8). (C-E) Vip mRNA (C), Vip protein (D) and Vipr2 mRNA expression of TtT/GF (C-E), GH3 and AtT-20 cells compared to NIH/3T3 cells (C,E). (F) qRT-PCR-based analysis of Gh expression level of GH3 cells treated with CM-TtT/GFSAG or CoM-TtT/GF supplemented with 1 µM 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 +/- SEM. 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+ TtT/GF cells. White double arrows: Gli1+ Vip+ stellate-shaped pituitary cells. Scale bars: 10 µm (D), 3,3 µm (G).

54x36mm (600 x 600 DPI)
Table S1: Animal numbers used for the experiments shown in Figure 2, 3 and S2.

|                                | Pomp/Ptch<sup>f/f</sup> | Ptch<sup>f/f</sup> | Pomp/Smo<sup>f/f</sup> | Smo<sup>f/f</sup> |
|--------------------------------|--------------------------|-------------------|------------------------|------------------|
|                                | tamoxifen               | solvent          | tamoxifen             | solvent          |
| birth rate                     | Fig. 1A left <br>88     | 108               | Fig. 1A right <br>126  | 111              |
| body weight                    | Fig. 1C top <br> &gt; 4* | &gt; 6*          | &gt; 3*                | Fig. 1D top      | &gt; 9*          | &gt; 6*          | &gt; 8*          |
| blood glucose level            | Fig. 1C bottom <br> &gt; 3* | &gt; 3*          | &gt; 4*                | Fig. 1D bottom   | &gt; 17*         | &gt; 22*         | &gt; 13*         |
| A&horbar;h serum level         | Fig. 1E                  | 2<sup>-20 d</sup> | 2<sup>-4</sup>         | Fig. 1E          | 2<sup>-20 d</sup> | 5<sup>-5</sup>  | 4<sup>-4</sup>  |
|                               | 85-130 d                | 5<sup>-3</sup>   | 4<sup>-4</sup>         | 85-130 d         | 5<sup>-5</sup>   | 4<sup>-4</sup>  |
|                               | 250-265 d               | 5<sup>-3</sup>   | 4<sup>-4</sup>         | 250-265 d        | 5<sup>-5</sup>   | 4<sup>-4</sup>  |
| pituitary weight               | Fig. 1F                  | 39                | 17<sup>-44</sup>       | Fig. 1F          | 39                | 36                | 47              |
| gene expression                | Fig. 1G                  | Gli1 expression  | 7<sup>-6</sup>         | Fig. 2G          | Gli1 expression  | 17<sup>-17</sup>  | 17<sup>-17</sup>  |
|                               | Gli2 expression         | 7<sup>-6</sup>   | 6<sup>-6</sup>         | Gli2 expression  | 17<sup>-17</sup>  | 17<sup>-17</sup>  |
|                               | Pomp expression          | 7<sup>-6</sup>   | 6<sup>-6</sup>         | Pomp expression  | 17<sup>-17</sup>  | 17<sup>-17</sup>  |
|                               | Gh expression           | 7<sup>-6</sup>   | 6<sup>-6</sup>         | Gh expression    | 17<sup>-17</sup>  | 17<sup>-17</sup>  |
|                               | Prl expression          | 7<sup>-6</sup>   | 6<sup>-6</sup>         | Prl expression   | 16<sup>-17</sup>  | 17<sup>-17</sup>  |
| immune-<br>(histological)<br>stainings | Fig. 2A                  | 23                | 11<sup>-18</sup>       | Fig. 2B          | 13                | 11                | 18              |
| % A&horbar;h cells             | Fig. 2C                  | 5<sup>-3</sup>   | 4<sup>-4</sup>         | Fig. 2D          | 5<sup>-4</sup>   | 4<sup>-4</sup>  |
| Gli1/A&horbar;h staining       | Fig. 2E                  | 5<sup>-2</sup>   | 3<sup>-3</sup>         |                   |                   |                   |

*at each time point
Table S3: Oligonucleotide primers used for qRT-PCR analyses.

| Name   | sequence                                            | location | amplicon size | species |
|--------|-----------------------------------------------------|----------|---------------|---------|
| 18S-F  | 5’-CGC AAA TTA CCC ACT CCC G-3’                   | exon 1   | 81 bp         | m/r     |
| 18S-R  | 5’-TTC CAA TTA CAG GGC CTC GAA-3’                 | exon 1   |               |         |
| Fn1-F  | 5’-TTC AAG TGT GAT CCC CAT GAA G-3’                | exon 43  | 154 bp        | m/r     |
| Fn1-R  | 5’-CAG GTC TAC GGC AGT TGT CA-3’                  | exon 45  |               |         |
| Fst-F  | 5’-TGC TGC TAC TCT GGC AGT TC-3’                  | exon 1   | 130 bp        | m/r     |
| Fst-R  | 5’-GTG CTG CAA CAC TCT TCC TTG-3’                 | exon 2   |               |         |
| Gh-F   | 5’-AAG AGG GCA TCC AGG CTC T-3’                   | exon 4   | 111 bp        | m/r     |
| Gh-R   | 5’-CGT CGT CGC TGC GCA TGT T-3’                   | exon 5   |               |         |
| Gli1-F | 5’-TAC ATG CTG GTG GTG CAC ATG-3’                  | exon 9   | 115 bp        | m/r     |
| Gli1-R | 5’-ACC GAA GGT GGG TCT TGA GG-3’                  | exon 10  |               |         |
| Gli2-F | 5’-GTT CAT CTA CGA GAC CAA CTG C-3’                | exon 8   | 272 bp        | m/r     |
| Gli2-R | 5’-GTG TCT CTA GCT TCT CCA GGC-3’                 | exon 9   |               |         |
| Hhip-F | 5’-GGA GCC TTA CTT GGA CAT TCA CAA-3’              | exon 4   | 143 bp        | m/r     |
| Hhip-R | 5’-ACC GTT CCT GGT TGG TGG TAT AA-3’               | exon 5   |               |         |
| Mif-F  | 5’-GCC AGA GGG GTT GGT TGT CTC G-3’                | exon 1   | 118 bp        | m       |
| Mif-R  | 5’-GTT CGT GCC GCT AAA AGT CA-3’                  | exon 2   |               |         |
| Pomp-F | 5’-GAC ACC TTA GTG TGG ACC GTG-3’                  | exon 3   | 111 bp        | m/r     |
| Pomp-R | 5’-GCG GAA GTG ACC CAT GAC GTA C-3’                | exon 4   |               |         |
| Prl-F  | 5’-GAG AGC TGT TGT ACC GTG TGG-3’                  | exon 2   | 196 bp        | m/r     |
| Prl-R  | 5’-GAT GAC CTT GAC CAT AAA CTC AC-3’               | exon 3   |               |         |
| Pech-F | 5’-TAC AGT CCG GGA CAG CAT ACC-3’                  | exon 5   | 152 bp        | m/r     |
| Pech-R | 5’-GTA CCC ATG CCC ACC CAC GTC TAC C-3’            | exon 6   |               |         |
| S100b-F| 5’-AAC AAG GAC ATG CAC TCT ACC ACC-3’              | exon 2   | 105 bp        | m/r     |
| S100b-R| 5’-CTG GAA GTG ACA ACC CCC ATC-3’                  | exon 3   |               |         |
| Vip-F  | 5’-AGC AGA AAA TGG CAC ACC CTA-3’                  | exon 2   | 85 bp         | m/r     |
| Vip-R  | 5’-AAA GAG TCT GTC GTA ATC GCC GGT-3’               | exon 3   |               |         |
| Vegfa-F| 5’-CCC AGC ACA GAA AGG GAG CAG AAG-3’              | exon 2   | 159 bp        | m       |
| Vegfa-R| 5’-CAT CAG CGG CAC ACA GGA CGG-3’                  | exon 3   |               |         |
| Vipr2-F| 5’-TAC AAC GAC CCC GAG GAT GA-3’                   | exon 4   |               |         |
| Vipr2-R| 5’-TCA GAG AAA CAC TGT AGC CCA-3’                  | exon 5   | 85 bp         | r       |
**Table S2: Oligonucleotide primers used for analyses of the recombination of the Ptch\textsuperscript{flax} and the Smo\textsuperscript{flax} locus.**

| Name   | sequence                                      | amplicon size |
|--------|-----------------------------------------------|---------------|
| Exon7-F | 5'-AGG AAG TAT ATG CAT TGG CAG GAG-3'         |               |
| neoR    | 5'-GCA TCA GAG CAG CCG ATT GTC TG-3'          |               |
| Smo-F   | 5'-GGG TTC CCA GGG TTG AAG ACA GCT TCG ATC TCC AG-3' |    |
| Smo\textsuperscript{d}-R | 5'-GTA GCG CAA AGG CTC GCA GTG G-3' | Smo\textsuperscript{d}: 590 bp |
| Smo-F   | 5'-GGG TTC CCA GGG TTG AAG ACA GCT TCG ATC TCC AG-3' | Smo\textsuperscript{d}: 1700 bp |
| Smo\textsuperscript{d}-R | 5'-TGC CAG TTT GAG GGG ACG ACA GTA TCG GCC TC-3' |               |
Table S4: Primary and secondary antibodies used for Western blot, immunohistochemical or immunofluorescent stainings of paraffine or cryotome-sections or of in vitro cultured cells.

| antigen | antibody | host | reactivity | clone/catalogue# | manufacturer | application | antigen retrieval | dilution | fluorochrome-labeled secondary antibody (dilution, manufacturer, catalogue#) |
|---------|----------|------|------------|------------------|--------------|-------------|------------------|----------|-----------------------------------------------------------------------|
| Acth    | rb anti-Acth | rb   | rat        | AFP-15610278     | NHPP         | DIF (P), IHC (P) | citric acid, pH6 | 1:1000   | d anti-rb-Alexa488 (1:200; Jackson ImmunoResearch; #712-545-150)  |
|         |          |      |            |                  |              |              |                  |          | d anti-rb-Cy3 (1:200; Jackson ImmunoResearch; #712-165-150)          |
| α tubulin | ms anti α tubulin | ms | ms/h/r | DLN-0999         | Dianova      | WB          | -                | 1:10,000 | rb anti-ms-HRP (1:10,000; Jackson ImmunoResearch #15-035-003)       |
| acetylated tubulin | ms anti su | ms | su | #T6793; clone 6-11B-1 | Sigma-Aldrich | DIF (P), IHC (P), ICC | citric acid, pH6 | 1:100   | d anti-ms-TRITC (1:200; Jackson ImmunoResearch; #715-025-150)       |
| GFP     | gt anti GFP   | gt   | -         | NB100-1770       | Novus Biologicals | DIF (P), IHC (P), | citric acid, pH6 | 1:1000   | bov anti-gt-Alexa488 (1:200; Jackson ImmunoResearch; #805-545-150) |
| GFP     | rb anti GFP   | rb   | -         | 600-401-215S     | Rockland     | DIF (P), IHC (P), | citric acid, pH6 | 1:500    | d anti-rb-Alexa488 (1:200; Jackson ImmunoResearch; #712-545-150)  |
|         |            |      |            |                  |              |              |                  |          | d anti-rb-Cy3 (1:200; Jackson ImmunoResearch; #712-165-150)          |
| Gh      | rb anti-Gh   | rb   | rat        | AFP-5641801      | NHPP         | DIF (P), IHC (P) | citric acid, pH6 | 1:500    | d anti-rb-Alexa488 (1:200; Jackson ImmunoResearch; #712-545-150)  |
|         |            |      |            |                  |              |              |                  |          | d anti-rb-Cy3 (1:200; Jackson ImmunoResearch; #712-165-150)          |
| Ghrhr   | rb anti Ghrhr | rb | ms/h/r | ABIN1386028     | antibodies-online | ICC, WB | - | 1:500 (ICC) 1:5,000 (WB) | d anti-rb-Cy3 (1:200; Jackson ImmunoResearch; #712-165-150)  |
| K14     | ms anti-K14  | ms   | h          | ab7800           | Abcam        | IHC (P)      | citric acid, pH6 | 1:1000   | d anti-ms-TRITC (1:200; Jackson ImmunoResearch; #715-545-150)       |
| K5      | rb anti-K5   | rb   | ms/h       | Poly19055        | BioLegend    | IHC (P)      | citric acid, pH6 | 1:1000   | d anti-rb-Alexa488 (1:200; Jackson ImmunoResearch; #712-545-150)  |
| Ki67    | ms anti-Ki67 | ms | ms/h | B56           | BD Pharmingen | IHC (P) | citric acid, pH6 | 1:50     | gt anti-ms-Alexa488 (1:200; Invitrogen; #A11029)                     |
| Pdgfra  | gt anti-Pdgfra | gt | ms | AF1062       | R&D Systems  | IF (C)       | - | 1:20     | bov anti-gt-Alexa488 (1:200; Jackson ImmunoResearch; #805-545-150) |
| Pome    | rb anti Pome | rb | ms/h | NBP2-57719    | Novus Biologicals | DIF (P), IHC (P) | citric acid, pH6 | 1:1000   | d anti-rb-Alexa488 (1:200; Jackson ImmunoResearch; #712-545-150)  |
|         |            |      |            |                  |              |              |                  |          | d anti-rb-Cy3 (1:200; Jackson ImmunoResearch; #712-165-150)          |
| Prl     | rb anti Prl  | rb   | ms         | AFP-425-10-91    | NHPP         | DIF (P), IHC (P) | citric acid, pH6 | 1:1000   | d anti-rb-Alexa488 (1:200; Jackson ImmunoResearch; #712-545-150)  |
|         |            |      |            |                  |              |              |                  |          | d anti-rb-Cy3 (1:200; Jackson ImmunoResearch; #712-165-150)          |
| RFP* | gt anti-RFP | gt | ms | MBS448122 | MyBioSource | DIF (P) | citric acid, pH6 | 1:200 | bov anti-gt-Alexa488 (1:200; Jackson ImmunoResearch; #805-545-150) |
|------|-------------|----|----|----------|-------------|--------|----------------|------|-------------------------------------------------------------|
| RFP* | rb anti-RFP | gt | ms | 600-401-379S | Rockland | DIF (P) | citric acid, pH6 | 1:500 | d anti-rb-Alexa488 (1:200; Jackson ImmunoResearch; #712-545-150) |
| Smoothened | rb anti Smo | rb | ms/rat/h | ab38686 | Abcam | DIF (P), ICC | citric acid, pH6 | 1:1000 | d anti-rb-Alexa488 (1:200; Jackson ImmunoResearch; #712-545-150) |
| Sox2 | rat anti-Sox2 | rat | ms/h | 14-9811 | eBioscience | DIF (P), ICC | citric acid, pH6 | 1:100 | d anti-rat-Cy3 (1:200; Jackson ImmunoResearch; #712-165-150) |
| Vip | rb anti Vip | rb | ms/r/h | ab272726 | Abcam | DIF (P), ICC | citric acid, pH6 | 1:500 | d anti-rb-Alexa488 (1:200; Jackson ImmunoResearch; #712-545-150) |

* detects tdTomato; m: mouse; r: rat; rb: rabbit; g: goat; d: donkey; IF: immunofluorescence; DIF: double immunofluorescence; IHC: immunohistochemistry; WB: Western blot; P: paraffine sections; C: cryosection
Supplemental methods

**SAG treatment, preparation of conditioned medium and medium transfer experiments,**

**BrdU incorporation analysis**

For SAG treatment, 5x10^4 TtT/GF, 4x10^5 GH3 or 1x10^5 AtT-20 cells per well of 6-well-plates were seeded in the respective normal growth medium. 24 h after seeding the cells were starved for 24 h in the respective starvation medium followed by 48 h incubation in starvation medium supplemented with 100 nM SAG or solvent (DMSO). Cells and supernatant were collected for RNA isolation or neuropeptide level measurement, respectively.

For preparation of conditioned medium 5x10^4 TtT/GF cells per well of a 6-well plate were seeded in normal growth medium. 24 h after seeding the cells were starved for 24 h and subsequently cultured for 48 h in the respective medium of the target cell line (GH3 or AtT-20 starvation medium) supplemented with 100 nM SAG (CM-TtT/GF\textsubscript{SAG}) or solvent (CoM-TtT-GF). Afterwards the cells were harvested for RNA isolation and the supernatant was filtered through a 0.22 µm filter and stored at 4 °C until further use (maximum 14 days). For expression and supernatant analysis 4x10^5 GH3 and 1x10^5 AtT-20 cells per well of a 6-well-plate and for BrdU incorporation 5,000 GH3 cells per well of a 96-well-plate were seeded in their respective normal growth medium. 24 h after seeding the growth medium was discarded, cells were starved for 24 h in their respective starvation medium followed by incubation with 1 ml or 100 µl CM-TtT/GF\textsubscript{SAG} or CoM-TtT-GF per well of a 6-well-plate or a 96-well-plate, respectively, for 48 h. Afterwards the cells and the supernatants were collected for RNA isolation or hormone level measurement, respectively. For Vip antagonist (KPRRPYTDNYTRLRKQMAVKKYLNSILN-NH\textsubscript{2}, Bachem, Switzerland) treatment, GH3 cells were starved as described above and then pretreated with 1 µM Vip antagonist in GH3 starvation medium for 1h. Afterwards the cells were incubated for 48h with
CM-TtT/GF <sub>SAG</sub> or CoM-TtT/GF supplemented with 1 µM Vip antagonist or the respective volume of solvent (water). BrdU pulsing (24 h prior analysis) and BrdU incorporation analysis was performed as recently described (Pyczek et al., 2016).

**Paraffin, cryotome and vibratome sections**

For histological analyses tissue samples were fixed in 4% paraformaldehyde, embedded in Cryo Embedding Medium (Medite Inc., Burgdorf, Germany) or dehydrated and embedded in paraffin and sectioned on a cryotome (Leica Microsysteme, Germany) or a sliding microtome (Reichert-Jung, Germany), respectively. For vibratome sections of freshly isolated murine pituitary glands, the glands were embedded in 2% low melting agarose (Carl Roth, Karlsruhe, Germany) and placed in HBSS. 200 µm sections were made on a LEICA VT1000S vibratome (Leica, Wetzlar, Germany). The sections were directly incubated with 50 µM β-Ala-Lys-N(ε)-AMCA dissolved in HBSS at 37°C, 5% CO<sub>2</sub> for 3 hrs. Afterwards fixed in 4% paraformaldehyde for 10 min and mounted in Mowiol (4,3 mM Mowiol 4-88, 3016,24 mM Glycerol, 18502,811 mM H<sub>2</sub>O, 133 mM Tris pH 8.5, 34,05 mM 1,4-diazabicyclo[2.2.2]octane).

**Western blot analysis**

Protein isolation and Western blot analysis were conducted as previously described (Becker et al., 2020).

**Measurements of blood glucose, serum and supernatant hormone/neuropeptide levels**
Blood glucose concentration was measured every other week between 9 am and 10 am with a blood glucose meter (Contour XT, Bayer, Leverkusen, Germany) using 10 µl blood from the murine retroorbital plexus. For Acth measurements 150 µl of blood were collected from the retroorbital plexus at specific time points during the observation period (see Fig. S2C). The blood was coagulated for two hours at 4 °C and centrifuged for 10 min at 3,000 rpm. The serum was removed and stored at -80 °C until use. Acth concentration in serum or supernatants was measured in duplicates or triplicates with an ELISA for Acth (Biomatik Corporation, Cambridge, Canada) according to manufacturer’s instructions using a microplate reader (BioTek, Vermont, USA). Gh and Vip levels in the supernatant of cultured cells were measured in triplicates using a Gh ELISA (Biomatik Corporation) or Vip EIA (Phoenix Pharmaceuticals, Inc., Burlingame, USA) according to manufacturer’s instructions by using a microplate reader (BioTek, Vermont, USA).

**RNAscope, immunohistological and immunofluorescent antibody stainings**

Immunohistological and immunofluorescent antibody stainings of paraffin and cryosections have been described previously (Pyczek et al., 2016). For immunofluorescent stainings of adherent or non-adherent cells they were cultured on poly-L-lysine (Sigma-Aldrich) coated coverslips or on SHI-FIX™ (Everest Biotech, Oxfordshire, UK) coverslips, respectively. Afterwards the cells were fixed with 4 % paraformaldehyde for 10 min, incubated with PBS containing 0.5 % Triton X-100 for 30 min. Blocking was performed with 0.2 % (w/v) I-Block (Applied Biosystems, Waltham, USA) in TBS for 30 min. The cells were incubated for 1 hr with the primary antibody and 45 min with the secondary antibody at RT diluted in PBS. Specimens were mounted with ProLong™ Gold Antifade Mountant with DAPI (Invitrogen, Carlsbad, USA). Used antibodies, antibody dilutions and antigen retrieval procedures are summarized in Table S4.
For quantification of corticotrophs, Acth⁺ cells of 1,600-3,300 nucleated cells per murine
AL mouse pituitary (the numbers of analysed animals are given in Suppl. Table S1) were counted.

RNAscope® analyses of paraffin-embedded murine samples were performed using
RNAscope® Probe Mm-Gli1 (311001), Mm-Gli2 (405771), Mm-Ppib (313911), Negative Control
Probe-DapB (310043) and the RNAscope® 2.5 HD Reagent Kit-RED (322350; Advanced Cell
Diagnostics, Newark, USA) according to the manufacturer’s recommendations. In brief, paraffin
sections were cut one day before staining, dried over night at 37 °C, incubated for 1 hr at 60 °C
and afterwards de-paraffinized/rehydrated in xylene and ascending RNA-pure EtOH series. After
air drying, the slides were incubated for 20 min with RNAscope H₂O₂ at room temperature, washed
in autoclaved double destilled H₂O and heated in RNAscope Target Retrieval Reagent for 10 min
at 135 W and for 6 min for 180 W in a microwave. The slides were washed with autoclaved double
destilled H₂O and 99 % RNA-pure EtOH and dried over night at room temperature in a wet
chamber. Next, the sections were incubated for 30 min with RNAscope Protease Plus at 40 °C in a
wet chamber followed by washing in autoclaved double destilled H₂O. Hybridization was
conducted by applying two drops of the respective probe on the section for 2 hrs at 40 °C in a wet
chamber. Afterwards the slides were washed for 4 min in 1 x RNAscope Wash Buffer and
incubated with two drops of AMP1 (30 min), AMP2 (15 min), AMP3 (30 min), AMP4 (15 min),
AMP5 (1 hr) and AMP6 (15 min), respectively at 40 °C in a wet chamber, including respective
washing steps between the AMP probes each for 4 min in 1 x RNAscope Wash Buffer. After
AMP6-incubation the slides were washed two times with 1 x RNAscope Wash Buffer for 4 min.
Signal detection was performed with the Fast-RED reagent for 10 min at room temperature in a
wet chamber. Subsequent antibody staining was performed directly after the signal detection with
Fast RED. For combined RNAscope/immunofluorescent stainings RNAscope staining was
conducted as described above followed incubation with primary and secondary antibodies for
immunofluorescent stainings. For quantification of Gli1+ and Gli2+ somatotrophs or FSC, all Gh+, Gli1+ Gh+ and Gli2+ Gh+ cells out of 182-300 nucleated cells/pituitary of 4 wildtype C57BL/6N pituitary glands and all EGFP+, Gli1+ EGFP+ and Gli2+ EGFP+ out of 123-241 nucleated cells/pituitary of 5 or 4 S100b-EGFP pituitary glands, respectively, were counted at 1,800-fold magnification.

Fluorescent or immunohistological stainings were examined with an Olympus confocal laser scanning microscope equipped with Fluoview FV100 software or an Olympus BX60 equipped with CellSense software (Olympus corporation, Shinjuku, Japan), respectively.

Transcriptome analyses

Three biological replicates of RNA from TtT/GF cells treated with either 100 nM SAG or solvent (see above) were analysed. RNA quality control (Fragment Analyzer, Agilent Technologies, USA), 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. In detail, all fastq files are single end reads with 51 basepair readlength. They were aligned against the reference of mus musculus (Mus_musculus.GRCh38 Ensembl 96) genome with the RNA-Seq splice aware aligner STAR (Dobin et al., 2013) version 2.7.0f. Afterwards, aligned reads were counted towards their features (genes) with rsem calculate expression (Li and Dewey, 2011) (RSEM v1.1.1), not only utilizing unique matched reads, but multi matched reads as well, allowing for the most comprehensive usage of the input read data. The resulting expected counts were the baseline input for further analysis in the programming language R (3.6.1) using the limma + voom (Law et al., 2014) (limma version 3.40.6) package for
differential gene expression analysis. Following GSEA utilizing the clusterProfiler v3.12 (Yu et al., 2012) package in R. For quality control during the analysis pipeline multiQC (Ewels et al., 2016) version 1.8, evaluating the output of fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the log files of STAR, was used. Final data visualization was performed with Rstudio (Rstudio, Inc., USA) and GraphPadPrism 6 (GraphPad Software, Inc., San Diego, USA).
Supplemental Figure legends

Figure S1: Long-term characterization of the specificity and inducibility of the PomcCreERT2-deleter in adult pituitary glands. (A) Experimental setup and (B) representative immunofluorescence analyses of adult Pomc/tdT pituitaries 7, 14, 50, 100, 150, 200 and 250 days post-tamoxifen. Analyses were conducted on pituitaries of at least 3 animals per cohort. AL: anterior lobe; IL: intermediate lobe. Arrows: double positive cells. Scale bars: 500 µm (left panels), 10 µm (right panels).

Figure S2: Characterization of the murine FSC cell line TtT/GF, the rat somatotroph cell line GH3 and the murine corticotroph cell line AtT-20. (A,B) TtT/GF cells grow with a stellate-shaped morphology (A left), express the FSC- and stem cell marker Sox2 (A right) and show high expression of the FSC marker genes S100b, Vegfa, Mif and Fst (B). (C-E) GH3 cells express Gh (C, left) and Ghrhr protein (C right, E) as well as high levels of Gh transcripts (D). (F,G) AtT-20 cells express Pomc (F left) and Acth (F right) protein as well as high levels of Pomc transcripts (G). Gene expression levels were normalized to 18S rRNA expression and to the respective gene expression levels of NIH/3T3 cells (dotted lines in B and D). Pomc transcript levels of NIH/3T3, TtT/GF and GH3 remained below detection level. Each open circle indicates one biological replicate measured in technical triplicates. Mean +/- SEM. 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; ****, P=0.0001. glycosyl. Ghrhr: glycosylated Ghrhr variants (Chu et al., 2016). Scale bars: 200 µm (A left), 10 µm (A right, C, F)

Figure S3: Characterization of Hh signaling activity of the murine FSC cell line TtT/GF, the rat somatotroph cell line GH3 and the murine corticotroph cell line AtT-20. (A) Gli1 expression analysis of TtT/GF, GH3 and AtT-20 cells compared to the fibroblast cell line NIH/3T3. Gene expression levels were
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**Figure S5:** Medium of Smoothened Agonist-stimulated TtT/GF cells does not impact on Hh signaling activity or Pomc expression levels of AtT-20 cells. Gene expression analyses of Hh signaling target genes (A,B) and Pomc (B) of (A) TtT/GF cells after serum starvation followed by 48 h 100 nM Smoothened Agonist (SAG) or solvent treatment (dotted line) in AtT-20 serum starvation medium and (B) of AtT-20 cells after serum starvation followed by 48 h 100 nM SAG (gray circles, same data as shown in Fig. S5C) 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$_{SAG}$, red circles) or solvent (dotted line). (C) Acth protein concentration in supernatant of AtT-20 cells after serum starvation followed by 48 h incubation with conditioned media from TtT/GF cells (shown in A)
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**Figure S6:** Graphical representation of gene expression levels of the murine FSC cell line TtT/GF after Smoothened Agonist treatment determined by comparative transcriptome analyses. Expression profile of (A) differential expressed and (B) FSC marker genes of Smoothened Agonist (SAG)- versus solvent-treated (DMSO) TtT/GF cells based on transcriptome analyses (see Fig. 7). Gene expression of SAG-treated cells were normalized to solvent-treated controls (dotted lines). Differential expression (cut off 0.8- or 1.2-fold change, grey lines) with adjusted P values (non-parametric Holm-Sidak method) below 0.05 were consider to be significant. *, P=0.05; **, P=0.01.

**Figure S7:** EIA-based Vip protein measurements. (A) Standard curve of the EIA-based measurement of Vip protein concentrations and (B) absolute and (C) relative Vip protein levels in the supernatant of SAG- (CM-TtT/GF
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