



King's Research Portal

DOI:

[10.1530/ERC-18-0330](https://doi.org/10.1530/ERC-18-0330)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Xekouki, P., Lodge, E. J., Matschke, J., Santambrogio, A., Apps, J. R., Sharif, A., Jacques, T. S., Aylwin, S., Prevot, V., Li, R., Flitsch, J., Bornstein, S. R., Theodoropoulou, M., & Andoniadou, C. (2018). Non-secreting pituitary tumours characterised by enhanced expression of YAP/TAZ. *ENDOCRINE RELATED CANCER*. Advance online publication. <https://doi.org/10.1530/ERC-18-0330>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Non-secreting pituitary tumours characterised by enhanced expression of YAP/TAZ

Paraskevi Xekouki^{1,2}, Emily J. Lodge^{1,3}, Jakob Matschke⁴, Alice Santambrogio^{1,11}, John R. Apps^{5,6}, Ariane Sharif^{7,8}, Thomas S. Jacques^{5,6}, Simon Aylwin², Vincent Prevot^{7,8}, Ran Li⁹, Jörg Flitsch¹⁰, Stefan R. Bornstein^{3,11}, Marily Theodoropoulou^{12,*}, Cynthia L. Andoniadou^{1,11,*}

* Corresponding Authors

1. Centre for Craniofacial and Regenerative Biology, King's College London, London, UK
2. Department of Endocrinology, King's College Hospital NHS Foundation Trust, London, UK
3. Department of Endocrinology and Diabetes, King's College London, London, UK.
4. Institute of Neuropathology, University Hospital Hamburg-Eppendorf, Hamburg, Germany.
5. Birth Defects Research Centre, Developmental Biology and Cancer Programme, UCL Great Ormond Street Institute of Child Health, University College London, London, UK
6. Histopathology Department, Great Ormond Street Hospital NHS Trust, London, UK
7. INSERM, Laboratory of Development and Plasticity of the Neuroendocrine Brain, Jean-Pierre Aubert Research Centre, Lille, France
8. University of Lille, FHU 1000 days for Health, School of Medicine, Lille, France
9. Department of Neurosurgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, People's Republic of China
10. Department of Neurosurgery, Hamburg University Medical Center, Hamburg, Germany

11. Department of Internal Medicine III, Carl Gustav Carus Medical School, Technical University of Dresden, Dresden, Germany.
12. Medizinische Klinik und Poliklinik IV, Ludwig-Maximilians-Universität München, Munich, Germany

Cynthia L. Andoniadou (cynthia.andoniadou@kcl.ac.uk)

Marily Theodoropoulou (marily.theodoropoulou@med.uni-muenchen.de)

Word Count: 3,527

Abstract

Tumours of the anterior pituitary can manifest from all endocrine cell types but the mechanisms for determining their specification are not known. The Hippo kinase cascade is a crucial signalling pathway regulating growth and cell fate in numerous organs. There is mounting evidence implicating this in tumour formation, where it is emerging as an anti-cancer target. We previously demonstrated activity of the Hippo kinase cascade in the mouse pituitary and nuclear association of its effectors YAP/TAZ with SOX2-expressing pituitary stem cells. Here we sought to investigate whether these components are expressed in the human pituitary and if they are deregulated in human pituitary tumours. Analysis of pathway components by immunofluorescence reveals pathway activity during normal human pituitary development and in the adult gland. Poorly differentiated pituitary tumours (null cell adenomas, adamantinomatous craniopharyngiomas (ACPs) and papillary craniopharyngiomas (PCPs)), displayed enhanced expression of pathway effectors YAP/TAZ. In contrast, differentiated adenomas displayed lower or absent levels. Knock-down of the kinase-encoding *Lats1* in GH3 rat mammosomatotropinoma cells suppressed *Prl* and *Gh* promoter activity following an increase in YAP/TAZ levels. In conclusion, we have demonstrated activity of the Hippo kinase cascade in the human pituitary and association of high YAP/TAZ with repression of the differentiated state both *in vitro* and *in vivo*. Characterisation of this pathway in pituitary tumours is of potential prognostic value, opening up putative avenues for treatments.

Keywords: Hippo signalling, pituitary tumour, YAP, TAZ

Introduction

The Hippo kinase cascade is a crucial conserved signalling pathway regulating organ growth across diverse species, through the control of cell proliferation, apoptosis and differentiation during development (Zhao *et al.* 2011). This phosphorylation cascade in mammals includes core kinases MST1/2 (a.k.a. STK3/4, orthologues of *Drosophila* Hippo) that activate Large tumour suppressor homologs 1 and 2 (LATS1/2), which in turn phosphorylate and inhibit the activation of transcriptional co-activators Yes-associated protein (YAP), and WW domain containing transcription factor (TAZ/WWTR1), the major effectors of the cascade. YAP/TAZ act as co-activators to TEAD transcription factors (TEA domain family members 1-4) (Zhao *et al.* 2011). Nuclear YAP/TAZ are thus associated with low kinase activity and the promotion of growth, inhibition of apoptosis and the stem cell state, whilst cytoplasmic and phosphorylated YAP/TAZ are associated with active kinases and growth restriction. Deregulation of the mammalian Hippo signalling components has been implicated in the formation of tumours and cancers, with loss of MST1/2, LATS1/2, SAV, or MOB1 resulting in the development of different tumour types in mouse models (Mo *et al.* 2014). Furthermore, elevated levels and nuclear localisation of YAP and/or TAZ has been reported in a wide array of human cancers including hepatocellular carcinoma (HCC), prostate cancer, colorectal carcinoma (CRC), non-small cell lung cancer (NSCLC), ovarian cancer, clear cell renal carcinoma (ccRCC), pancreatic carcinoma, oesophageal squamous cell carcinoma, urothelial carcinoma of the bladder, and skin basal cell carcinoma (Zanconato *et al.* 2016).

Pituitary tumours account for 10% to 15% of intracranial neoplasms (Molitch 2017). Although in general they are considered benign tumours, an aggressive or invasive behaviour is not uncommon (Di Ieva *et al.* 2014). Hormonal secreting (functioning) pituitary tumours are commonly detected due to the clinical syndromes caused by hormonal hypersecretion (Chanson *et al.* 2015). Non-functioning subtypes are clinically challenging because they present at a later stage with local mass effects or hypopituitarism, as do craniopharyngiomas, both the adamantinomatous (ACP) and papillary (PCP) types. During the last years, progress has been made on the identification of mechanisms involved in anterior pituitary cell transformation and tumourigenesis; oncogene activation, tumour suppressor gene inactivation, epigenetic changes, and microRNA deregulation have all been shown to contribute to the initiation of pituitary tumours (Marques & Korbonits 2017). Recently, the isolation of cell subpopulations with stem-like characteristics was reported from human somatotropinomas and non-functioning pituitary adenomas (NFPAs), describing expression of stem cell markers (OCT4, SOX2, CD133, Nestin), sustained proliferation and a persistent undifferentiated compartment (Würth *et al.* 2016). There is evidence for a role of the Hippo signalling cascade in pituitary gland pathophysiology. Mice deficient for Lats1 (*Lats1^{-/-}*) present with hyperplasia of the anterior pituitary lobe, but reduced secretion of hormones such as LH, PRL and GH (St John *et al.* 1999). We recently mapped for the first time the activity of the Hippo-YAP/TAZ pathway in the murine pituitary during development and postnatal stages, and revealed an association of active Hippo effectors (i.e. nuclear YAP/TAZ localisation) with the uncommitted pituitary stem cells expressing SOX2 (Lodge *et al.* 2016).

Considering the involvement of the Hippo kinase cascade in tumorigenesis, our observations in the murine gland prompted us to examine the expression patterns of YAP and TAZ in the human normal pituitary and pituitary tumours. Herein we show for the first time that these proteins are expressed during human pituitary development as well as in the normal adult gland. YAP and TAZ were highly expressed in subsets of non-secreting pituitary tumours (null cell, ACP and PCP), but not in differentiated tumours. Activating this pathway *in vitro* by knocking down *Lats1*, decreased anterior pituitary hormone synthesis, further supporting a role for this cascade in repressing endocrine differentiation. Altogether, our data indicate a previously unappreciated involvement of the Hippo pathway in human pituitary differentiation, growth and tumour formation.

Materials and Methods

Tissue specimens

All procedures performed were in accordance with the ethical standards of the institutional research committee (King's College Research Ethics Committee (REC), approval number LRS-15/16-2126) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Anonymised archival FFPE specimens of five ACP (Andoniadou *et al.* 2012) and six PCP (Haston *et al.* 2017) were identified in the local pathology archive, through the Childhood's Cancer and Leukaemia Group Tissue Bank and BRAIN UK. The study also included ten null cell pituitary tumours, 16 prolactinomas, one PRL-secreting carcinoma (Winkelmann *et al.* 2002), 18 corticotropinomas and 10 somatotropinomas. Examples of confirmatory

testing for these cohorts are provided in Supplementary Figure 2. Fetal pituitary tissue (two samples at 17 weeks and one sample at 14 weeks corrected gestational age) was accessed through the Human Developmental Biology Resource (HDBR). Normal human pituitary glands were obtained from the School of Medicine, Lille, France from people who donated their body to science (two male, two female, ages 77-89 years old); permission to use human tissues was obtained from the French Agency for Biomedical Research (Agence de la Biomédecine, Saint-Denis la Plaine, France, protocol no. PFS16-002). Ischaemia time until fixation ranged between 8-39 hours. Pituitaries were fixed in 4% paraformaldehyde at 4°C for a minimum of 48 hours before processing for paraffin embedding.

Immunofluorescence

Samples were dewaxed in Histo-ClearTM (National Diagnostics) twice for 10 min, followed by rehydration through a descending ethanol series. Antigen retrieval was carried out in citrate-based DeclereTM unmasking solution (Cell Marque) in a Decloaking chamber NXGEN (Menarini Diagnostics) using the 110°C antigen retrieval protocol. Following blocking for one hour in TNB blocking buffer [0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.5% Blocking Reagent (FP1020, Perkin Elmer), samples were incubated overnight in primary antibodies at 4°C in TNB at the following dilutions: YAP (Cell Signaling Technology Cat. No. 4912, 1:1000), pYAP (S127) (Cell Signaling Technology Cat. No. 4911 1:1000), TAZ (Atlas Antibodies HPA007415, 1:2000), and SOX2 (Abcam ab97959, 1:2000). The following day, slides were washed in Tris-NaCl-Tween (TNT) buffer (0.1M Tris-HCl, pH7.5, 0.15M NaCl, 0.05% Tween-20) and incubated in species-specific biotinylated secondary antibodies (1:500, Abcam) diluted in TNB for 1 hour at room temperature. Following

washes in TNT, slides were incubated in ABC solution (Vector Laboratories PK-6100) for 30 min in the dark and in TSA-Cy3 diluted in TSA Stock Solution (Perkin Elmer NEL760001) for 10 min at room temperature. Subsequently, slides were washed and incubated in Hoechst labeling solution for 30 minutes at room temperature. After a final wash, they were mounted with soft-set mounting medium (Vector Laboratories, H1000). Immunofluorescence staining was assessed as follows: Type A: high levels of both YAP/TAZ, frequent nuclear staining; Type B: robust levels of TAZ with frequent nuclear staining and moderate levels of YAP with occasional nuclear staining; Type C: moderate levels of YAP/TAZ, predominantly cytoplasmic, in over 50% of the tumour; Type D: low levels of YAP/TAZ, predominantly cytoplasmic, between 20-50% of tumour; Type E: absent YAP/TAZ staining or low levels in under 20% of the tumour.

Cell culture, transfection and luciferase assays

GH3 cells (American Type Culture Collection) were cultured in 10% fetal calf serum Dulbecco's modified Eagle medium (DMEM) supplemented with 2.2 g/L NaHCO₃, 10 mM HEPES, 2 nM Glutamine and 10⁵ U/L penicillin-streptomycin. Cell culture materials were from Life Technologies (Karlsruhe, Germany), Nunc (Wiesbaden, Germany) and Sigma-Aldrich (St. Louis, MO, USA). Cells were transfected with SuperFect (Qiagen) following the manufacturer's instructions. siRNA were against rat *Lats1* (OriGene and Santa Cruz Biotechnology); a mix of scrambled non-specific siRNA was used as control. The GH and PRL promoter reporter vectors have the proximal (-593) rat *Gh* promoter and rat *Prl* promoter respectively upstream to the luciferase gene (both kind gifts of A. Gutierrez-Hartmann, University of Colorado, Denver, CO). After transfection, cells were left for 48 hours in low serum (2% FCS)

DMEM, before being treated and/or assayed. The transfection efficacy was determined by cotransfection with the RSV- β -gal construct and results are presented as luciferase: β -galactosidase activity ratio. Each experiment was done in triplicate.

Cell proliferation was determined 48 hours after transfection using the WST-1 colorimetric assay (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions.

Immunoblotting

GH3 cells were lysed in ice-cold RIPA lysis buffer supplemented with protease and phosphatase inhibitors cocktail (Roche). Proteins were separated by polyacrylamide gel electrophoresis and blotted using standard procedures (BioRad). Primary antibodies were against LATS1 (C66B5, #3477), YAP/TAZ (D24E4, #8418) and phosphorylated pYAP(S127) (D9W2I, #13008) (all rabbit mAb, Cell Signaling), and β -actin (mouse mAb, Chemicon). Anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies were used (Cell Signaling) and signal was developed with enhanced chemiluminescent solution (Roche). Each experiment was carried out in duplicate.

Results

YAP and TAZ are expressed in human fetal and adult pituitaries

To investigate the Hippo pathway activity in the human pituitary during development and adulthood we determined the expression of its downstream effectors YAP and TAZ. In the human fetal pituitary at 17 weeks, high expression of both was observed

in the posterior and anterior lobe within the epithelial remnants of Rathke's pouch (marginal zone epithelium, MZE) that highly express the stem cell marker SOX2; the main body of the anterior lobe presented with moderate YAP and TAZ immunoreactivity (Figure 1b). Both proteins mainly localised in the nucleus as well as the cytoplasm (arrowheads). The nuclear localisation was more prominent in the case of TAZ (arrowheads) throughout the MZE, similar to SOX2 expression. Nuclear immunoreactivity in the MZE was also observed for YAP, albeit to a lesser extent. The core Hippo kinases LATS1/2 phosphorylate YAP on serines S61, S109, S127, S164 and S381, leading to its inactivation through cytoplasmic retention (S127) and degradation (Zhao *et al.* 2007, 2010). To determine if the kinases are active, we performed immunostaining against pYAP(S127), and observed strong cytoplasmic as well as nuclear localisation in both the epithelium and parenchyma (arrowheads), suggesting that LATS kinases are active during embryonic development. These observations were confirmed in further fetal pituitary samples at 17 and 14 weeks of gestation (Supplementary Figure 1). To determine if expression of YAP and TAZ persists in the adult pituitary, we analysed immunoreactivities in adult pituitaries of advanced age (n=4) (Figure 1c). Strong nuclear TAZ staining persisted both in cells of the MZE and parenchyma, following the expression pattern of SOX2. YAP immunoreactivity was predominantly cytoplasmic in the MZE, while nuclear staining was occasionally observed in the parenchyma. Staining against pYAP(S127) persisted in the adult pituitary indicating the presence of an active Hippo cascade during adulthood. In summary, YAP and TAZ are expressed in the developing and adult human pituitary, where LATS kinases are active.

Expression of YAP and TAZ in pituitary tumours

We next sought to determine the expression patterns of YAP and TAZ in human pituitary tumours. In human craniopharyngiomas, which are composed mainly of non-endocrine cells, the expression of SOX2/SOX9 (both progenitor/stem cell markers) has been well documented in both the papillary type (PCP), harbouring MAPK pathway mutations as well as the adamantinomatous type (ACP) that harbour *CTNNB1* mutations (encoding β -Catenin) (Hölsken *et al.* 2014; Thimsen *et al.* 2017; Haston *et al.* 2017). Strong YAP and TAZ stainings were observed in all PCPs tested and were predominantly nuclear in the basal cells and suprabasal squamous epithelium (asterisks in Figure 2a), described to robustly express SOX2 (Haston *et al.* 2017)(Figure 2a and Table 1). Strong nuclear staining for YAP and TAZ was observed in all five ACPs, both in whorl-like formations described to accumulate β -catenin (asterisks in Figure 2b) as well as in the palisading epithelium (arrows in Figure 2b). Null cell pituitary tumours do not show immunoreactivity for any of the pituitary hormones, although there is evidence that in their majority they express lineage-specific transcription factors (Mete & Lopes 2017; Nishioka H 2018). YAP and TAZ expression was variable and was subdivided into three different groups based on a semiquantitative scoring system as described in the methods (Table 1, Figure 2c). Four out of 10 null cell tumours displayed high immunoreactivity consistent with Type A, three were identified as Type B and three as Type C. There was no obvious correlation of the immunohistochemical data to age at presentation, rate of recurrence, Ki-67 index or p53 levels, although the study cohort is low for this to be accurately determined (Table 2).

Altogether our data from normal pituitaries and pituitary tumours suggest that high levels of nuclear YAP/TAZ are associated with a more uncommitted state in the

anterior pituitary, consistent with previous mouse data (Lodge *et al.* 2016). To explore this hypothesis further, we extended our study to include 16 prolactinomas, the most common type of secreting pituitary tumours, 10 somatotropinomas and 18 corticotropinomas. YAP and TAZ immunoreactivities were observed at much lower levels than in craniopharyngiomas and null cell pituitary adenomas. Based on our semiquantitative scoring system, two types of staining were detected in prolactinomas: Type D, characterised by cytoplasmic YAP/TAZ in 20-50% of tumour cells, and Type E, characterised by absence of staining or weak cytoplasmic in less than 20% of the tumour (Figure 3a). In the majority of prolactinomas (11 out of 16), YAP/TAZ staining was classified as Type E, where most of the tissue was negative for YAP and TAZ. In corticotropinomas staining ranged from Type B to Type D (Supplementary Figure 3a) and in somatotropinomas from Type B to Type E (Supplementary Figure 3b). Results are summarised in Table 1. To determine if YAP/TAZ become elevated in aggressive/invasive tumours, we analysed their expression in one PRL-secreting carcinoma. This tumour appeared entirely negative for both proteins (Figure 3b, Region 1) and only one area included cells with nuclear, but not cytoplasmic TAZ staining and cytoplasmic YAP immunoreactivity, similar to adult normal anterior pituitary (Figure 3b, Region 2). Therefore, YAP/TAZ levels are low in differentiated tumours, and in the single malignant prolactinoma sample analysed. In summary, robust expression levels and more abundant nuclear localization of YAP/TAZ are seen in non-secreting tumour types, harbouring a less differentiated cellular component, compared to tumours composed of differentiated cells.

Lats1* knockdown reduces *Gh* and *Prl* promoter activity in GH3 cells *in vitro

Our observations of a robust decrease or loss of YAP/TAZ in hormone-secreting pituitary tumours, compared to normal pituitary and non-secreting tumours, suggest a role for the Hippo kinases in promoting a hormone-secreting phenotype and/or repressing a progenitor/stem-like state. To this end, employing RNA interference, we inhibited LATS1, since it directly phosphorylates and marks YAP and TAZ for cytoplasmic retention and degradation. Knock-down of *Lats1* in rat mammosomatotrophinoma GH3 cells reduced YAP phosphorylation at S127 and increased YAP and TAZ protein levels (Figure 4a). This was accompanied by significant suppression of basal *Gh* and *Prl* promoter activity (Figure 4b). *Lats1* inhibition also reduced activation of the *Gh* promoter following stimulation with forskolin compared to scrambled siRNA control (Figure 4c). *Lats1* inhibition did not affect GH3 cell proliferation (Figure 4d). These *in vitro* findings indicate that deregulation of the Hippo pathway may repress pituitary hormone synthesis and compromise the pituitary cell response to physiological hormonal stimuli.

Discussion

Initially described in *Drosophila*, the Hippo pathway is now recognised as one of the most conserved molecular pathway in all metazoans, which is highly involved in fine-tuning of organ size through inhibition of proliferation and promotion of differentiation and cell death (Yu *et al.* 2015) with a contribution to tumourigenesis and cancer development (Mo *et al.* 2014). The present study demonstrates for the first time the expression patterns of two major downstream effectors of the Hippo pathway, YAP and TAZ, in the fetal and adult normal pituitary gland and provides

evidence of Hippo pathway activity during embryonic development that persists into adulthood. Interestingly the YAP/TAZ expression pattern is reminiscent of SOX2 in pituitary stem/progenitor cells recapitulating our previous findings in mice and indicating a potential link of the Hippo pathway to the stem/progenitor cell state (Lodge *et al.* 2016).

We investigated the expression of YAP/TAZ in three types of non-secreting/non-differentiated pituitary tumours: null-cell adenomas, ACPs and PCPs. YAP and TAZ were highly expressed in all ACP and PCP tumours. The expression pattern was nuclear and occasionally cytoplasmic in craniopharyngiomas that harbour known genetic mutations in *CTNNB1* or *BRAF*, and was located to well-characterised tumour compartments known to express progenitor markers. ACP tumourigenesis is mainly driven by mutations in the *CTNNB1* gene that encodes for β -catenin, the central regulator of the WNT pathway (Sekine *et al.* 2002). Gain-of-function alleles of BRAF activate the RAS-RAF-MEK-ERK pathway and may drive tumourigenesis in PCPs, which appear almost exclusively in adults (Brastianos *et al.* 2014; Larkin *et al.* 2014). In support of this, a mouse model overexpressing BRAF V600E in mouse pituitary precursors leads to increased proliferation of SOX2 stem cells and a block in differentiation (Haston *et al.* 2017). Interestingly both pathways were described to crosstalk with the Hippo signalling cascade. YAP forms a transcriptional complex with β -catenin that is required for tumour transformation and survival (Rosenbluh *et al.* 2012). When core Hippo kinases are active, YAP/TAZ are degraded or sequestered in the cytosol where they limit WNT- β -catenin signalling (Varelas *et al.* 2010). In contrast, WNT activation induces YAP/TAZ translocation to the nucleus and activation (Attisano & Wrana 2013; Azzolin *et al.* 2014). Similarly, RAS

signalling activates YAP and there is evidence for involvement of YAP in tumour resistance to pharmacological RAF-MEK inhibition (Reddy & Irvine 2013; Lin *et al.* 2015; You *et al.* 2015).

YAP and TAZ were also highly expressed in null cell pituitary tumours, but at variable levels. Similar to craniopharyngiomas, they also often displayed strong nuclear staining. There is mounting evidence that TAZ and YAP nuclear localisation correlates with metastatic potential, low response to treatment and worse patient outcome in several solid tumours (Zanconato *et al.* 2016). The relatively small number of cases did not allow us to correlate the pattern of staining with histological or clinical data but also recurrence may happen even after years of remission. Based on the recent WHO classification for pituitary adenomas, clinical aggressiveness is assessed by several clinical parameters such as tumour invasion (by MRI studies and/or intraoperative impression), in addition to mitotic count and Ki-67 index (Lopes MBS 2017). Whether YAP/TAZ may directly promote an oncogenic phenotype in the pituitary through activation of genes involved in proliferation/survival/invasion directly or secondary to other genetic defects like the ones found in ACP and PCP, is an intriguing possibility that remains to be explored.

In contrast to the majority of the non-differentiated tumours that often displayed strong nuclear staining, YAP/TAZ expression in the hormone secreting prolactinomas was very low or completely absent, suggesting that in the human pituitary YAP/TAZ expression may be associated with a shift from high to low secretory potential and therefore a less differentiated state. Indeed, our *in vitro* model showed that increasing YAP and TAZ expression in lactosomatotroph GH3 cells, by knocking down their

upstream regulator *Lats1*, dramatically reduces PRL as well as GH synthesis without affecting cell proliferation. Interestingly in the case of GH, the effect is prominent also after cAMP/PKA stimulation with forskolin. These data reflect the observations in the *Lats1*^{-/-} mice, which presented with low PRL and GH levels despite their hyperplastic pituitaries (St John *et al.* 1999). YAP/TAZ were shown to repress the differentiated state in other tissues (Lee *et al.* 2016; Yi *et al.* 2016; Cotton *et al.* 2017). Our data further support this notion and suggest that high levels of YAP/TAZ are associated with repression of hormone production and therefore a non-differentiated/progenitor state.

Treatment of pituitary tumours primarily relies on reducing hormone hypersecretion and its effects, decreasing the tumour mass and treating for any hormone deficiencies resulting from damage of normal pituitary tissue. Hippo signalling components and particularly YAP/TAZ have recently become attractive targets for new anti-cancer treatments (Johnson & Halder 2013; Nakatani *et al.* 2016). Our finding of increased expression of YAP/TAZ in non-secreting pituitary tumours reveals a previously unsuspected pathogenetic mechanism. Better understanding and targeting the Hippo signalling cascade could introduce novel improved treatments for these intriguing and hard to manage tumours.

Declaration of Interest

The authors declare no conflict of interest to disclose.

Funding

This work was supported by the Medical Research Council (MR/L016729/1) and a Lister Institute Research Prize to CLA. E.J.L., A.S., S.R.B., M.T. and CLA were supported by the Deutsche Forschungsgemeinschaft (DFG) within the CRC/Transregio 205/1 (Project A06 to C. Andoniadou and S.R. Bornstein and B17 to M. Theodoropoulou) and CRC/Transregio 127/2, as well as GRK 2251.

Author Contributions

Concept and design of experiments: P.X., M.T., C.L.A.

Provision of samples: J.M., J.R.A., A.S., T.S.J., V.P., R.L., J.F.

Acquisition of data: P.X., E.J.L., M.T., J.M., A.S.

Analysis and interpretation of data: P.X., E.J.L., J.M., M.T., C.L.A.

Writing and review of the manuscript: P.X., E.J.L., M.T., C.L.A.

Supervision of the work: S.A., S.R.B., M.T., C.L.A.

Acknowledgements

We thank the patients, their families and clinicians who have donated tissues to research. We thank the Childhood Cancer and Leukaemia Group (CCLG) Tissue Bank for access to samples, and contributing CCLG Centres, including members of the ECMC Paediatric network. The CCLG Tissue Bank is funded by Cancer Research UK and CCLG. The human embryonic and fetal material was provided by the Joint MRC/Wellcome Trust (099175/Z/12/Z) Human Developmental Biology Resource. Tissue samples were also obtained from Plymouth Hospitals NHS Trust as part of the UK Brain Archive Information Network (BRAIN UK), which is funded by the Medical Research Council and *braintrust*. Thank you to Prof. Juan Pedro Martinez-Barbera for critical reading of the manuscript.

References:

- Andoniadou CL, Gaston-Massuet C, Reddy R, Schneider RP, Blasco MA, Le Tissier P, Jacques TS, Pevny LH, Dattani MT & Martinez-Barbera JP 2012 Identification of novel pathways involved in the pathogenesis of human adamantinomatous craniopharyngioma. *Acta Neuropathologica* **124** 259–271. (doi:10.1007/s00401-012-0957-9)
- Attisano L & Wrana JL 2013 Signal integration in TGF- β , WNT, and Hippo pathways. *Fl1000Prime Reports* **5**. (doi:10.12703/P5-17)
- Azzolin L, Panciera T, Soligo S, Enzo E, Bicciato S, Dupont S, Bresolin S, Frasson C, Basso G, Guzzardo V *et al.* 2014 YAP/TAZ incorporation in the β -catenin destruction complex orchestrates the Wnt response. *Cell* **158** 157–170. (doi:10.1016/j.cell.2014.06.013)
- Brastianos PK, Taylor-Weiner A, Manley PE, Jones RT, Dias-Santagata D, Thorner AR, Lawrence MS, Rodriguez FJ, Bernardo LA, Schubert L *et al.* 2014 Exome sequencing identifies BRAF mutations in papillary craniopharyngiomas. *Nature Genetics* **46** 161–165. (doi:10.1038/ng.2868)
- Chanson P, Raverot G, Castinetti F, Cortet-Rudelli C, Galland F, Salenave S, Cazabat L, Foubert L, Bonneville JF, Gaillard S *et al.* 2015 Management of clinically non-functioning pituitary adenoma. *Annales d'Endocrinologie* **76** 239–247. (doi:10.1016/j.ando.2015.04.002)
- Cotton JL, Li Q, Ma L, Park JS, Wang J, Ou J, Zhu LJ, Ip YT, Johnson RL & Mao J 2017 YAP/TAZ and Hedgehog Coordinate Growth and Patterning in Gastrointestinal Mesenchyme. *Developmental Cell* **43** 35–47.e4. (doi:10.1016/j.devcel.2017.08.019)

- Haston S, Pozzi S, Carreno G, Manshaei S, Panousopoulos L, Gonzalez-Meljem JM, Apps JR, Virasami A, Thavaraj S, Gutteridge A *et al.* 2017 MAPK pathway control of stem cell proliferation and differentiation in the embryonic pituitary provides insights into the pathogenesis of papillary craniopharyngioma. *Development* **144** 2141–2152. (doi:10.1242/dev.150490)
- Hölsken A, Stache C, Schlaffer SM, Flitsch J, Fahlbusch R, Buchfelder M & Buslei R 2014 Adamantinomatous craniopharyngiomas express tumor stem cell markers in cells with activated Wnt signaling: further evidence for the existence of a tumor stem cell niche? *Pituitary* **17** 546–556. (doi:10.1007/s11102-013-0543-8)
- Di Ieva A, Rotondo F, Syro L V., Cusimano MD & Kovacs K 2014 Aggressive pituitary adenomas—diagnosis and emerging treatments. *Nature Reviews Endocrinology* **10** 423–435. (doi:10.1038/nrendo.2014.64)
- Johnson R & Halder G 2013 The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. *Nature Reviews Drug Discovery* **13** 63–79. (doi:10.1038/nrd4161)
- Larkin SJ, Preda V, Karavitaki N, Grossman A, & Ansorge O 2014 BRAF V600E mutations are characteristic for papillary craniopharyngioma and may coexist with CTNNB1-mutated adamantinomatous craniopharyngioma. *Acta Neuropathologica* **127**. (doi:10/1007/s00401-014-1270-6)
- Lee DH, Park JO, Kim TS, Kim SK, Kim TH, Kim MC, Park GS, Kim JH, Kuninaka S, Olson EN *et al.* 2016 LATS-YAP/TAZ controls lineage specification by regulating TGF β signaling and Hnf4 α expression during liver development. *Nature Communications* **7**. (doi:10.1038/ncomms11961)
- Lin L, Sabnis AJ, Chan E, Olivas V, Cade L, Pazarentzos E, Asthana S, Neel D, Yan JJ, Lu X *et al.* 2015 The Hippo effector YAP promotes resistance to RAF- and

- MEK-targeted cancer therapies. *Nature Genetics* **47** 250–256.
(doi:10.1038/ng.3218)
- Lodge EJ, Russell JP, Patist AL, Francis-West P & Andoniadou CL 2016 Expression analysis of the Hippo cascade indicates a role in pituitary stem cell development. *Frontiers in Physiology* **7**. (doi:10.3389/fphys.2016.00114)
- Lopes MBS 2017 The 2017 World Health Organization classification of tumors of the pituitary gland: a summary. *Acta Neuropathol.* (doi:doi: 10.1007/s00401-017-1769-8)
- Marques P & Korbonits M 2017 Genetic Aspects of Pituitary Adenomas. *Endocrinology and Metabolism Clinics of North America* **46** 335–374.
(doi:10.1016/j.ecl.2017.01.004)
- Mete O & Lopes MB 2017 Overview of the 2017 WHO Classification of Pituitary Tumors. *Endocrine Pathology* **28** 228–243. (doi:10.1007/s12022-017-9498-z)
- Mo J-S, Park HW & Guan K-L 2014 The Hippo signaling pathway in stem cell biology and cancer. *EMBO Reports* **15** 642–656.
(doi:10.15252/embr.201438638)
- Molitch ME 2017 Diagnosis and Treatment of Pituitary Adenomas. *JAMA* **317** 516.
(doi:10.1001/jama.2016.19699)
- Nakatani K, Maehama T, Nishio M, Goto H, Kato W, Omori H, Miyachi Y, Togashi H, Shimono Y & Suzuki A 2016 Targeting the Hippo signalling pathway for cancer treatment. *Journal of Biochemistry* **161** mvw074.
(doi:10.1093/jb/mvw074)
- Nishioka H IN 2018 New WHO classification of pituitary adenomas (4th edition): assessment of pituitary transcription factors and the prognostic histological factors. *Brain Tumor Pathol.* (doi:doi: 10.1007/s10014-017-0307-7.)

- Reddy BVVG & Irvine KD 2013 Regulation of Hippo Signaling by EGFR-MAPK Signaling through Ajuba Family Proteins. *Developmental Cell* **24** 451–471. (doi:10.1016/j.devcel.2013.01.020)
- Rosenbluh J, Nijhawan D, Cox AG, Li X, Neal JT, Schafer EJ, Zack TI, Wang X, Tsherniak A, Schinzel AC *et al.* 2012 β -Catenin-driven cancers require a YAP1 transcriptional complex for survival and tumorigenesis. *Cell* **151** 1457–1473. (doi:10.1016/j.cell.2012.11.026)
- Sekine S, Shibata T, Kokubu A, Morishita Y, Noguchi M, Nakanishi Y, Sakamoto M & Hirohashi S 2002 Craniopharyngiomas of adamantinomatous type harbor beta-catenin gene mutations. *The American Journal of Pathology* **161** 1997–2001. (doi:10.1016/S0002-9440(10)64477-X)
- St John MA, Tao W, Fei X, Fukumoto R, Carcangiu ML, Brownstein DG, Parlow a F, McGrath J & Xu T 1999 Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nature Genetics* **21** 182–186. (doi:10.1038/5965)
- Thimsen V, John N, Buchfelder M, Flitsch J, Fahlbusch R, Stefanits H, Knosp E, Losa M, Buslei R & Hölsken A 2017 Expression of SRY-related HMG Box Transcription Factors (Sox2) 2 and 9 in Craniopharyngioma Subtypes and Surrounding Brain Tissue. *Scientific Reports* **7**. (doi:10.1038/s41598-017-15977-3)
- Varelas X, Miller BW, Sopko R, Song S, Gregorieff A, Fellouse F a, Sakuma R, Pawson T, Hunziker W, McNeill H *et al.* 2010 The Hippo pathway regulates Wnt/beta-catenin signaling. *Developmental Cell* **18** 579–591. (doi:10.1016/j.devcel.2010.03.007)
- Winkelman J, Pagotto U, Theodoropoulou M, Tatsch K, Saeger W, Müller A,

- Arzberger T, Schaaf L, Schumann EM, Trenkwalder C *et al.* 2002 Retention of dopamine 2 receptor mRNA and absence of the protein in craniospinal and extracranial metastasis of a malignant prolactinoma: A case report. *European Journal of Endocrinology* **146** 81–88. (doi:10.1530/eje.0.1460081)
- Würth R, Barbieri F, Pattarozzi A, Gaudenzi G, Gatto F, Fiaschi P, Ravetti J-L, Zona G, Daga A, Persani L *et al.* 2016 Phenotypical and pharmacological characterization of stem-like cells in human pituitary adenomas. *Molecular Neurobiology* 1–17. (doi:10.1007/s12035-016-0025-x)
- Yi J, Lu L, Yanger K, Wang W, Sohn BH, Stanger BZ, Zhang M, Martin JF, Ajani JA, Chen J *et al.* 2016 Large tumor suppressor homologs 1 and 2 regulate mouse liver progenitor cell proliferation and maturation through antagonism of the coactivators YAP and TAZ. *Hepatology* **64** 1757–1772. (doi:10.1002/hep.28768)
- You B, Yang Y-L, Xu Z, Dai Y, Liu S, Mao J-H, Tetsu O, Li H, Jablons DM & You L 2015 Inhibition of ERK1/2 down-regulates the Hippo/YAP signaling pathway in human NSCLC cells. *Oncotarget* **6** 4357–4368. (doi:10.18632/oncotarget.2974)
- Yu FX, Zhao B & Guan KL 2015 Hippo Pathway in Organ Size Control, Tissue Homeostasis, and Cancer. *Cell* **163** 811–828. (doi:10.1016/j.cell.2015.10.044)
- Zanconato F, Cordenonsi M & Piccolo S 2016 YAP/TAZ at the Roots of Cancer. *Cancer Cell* **29** 783–803. (doi:10.1016/j.ccell.2016.05.005)
- Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L *et al.* 2007 Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes & Development* **21** 2747–2761. (doi:10.1101/gad.1602907)
- Zhao B, Li L, Lei Q & Guan KL 2010 The Hippo-YAP pathway in organ size control

and tumorigenesis: An updated version. *Genes and Development* **24** 862–874.

(doi:10.1101/gad.1909210)

Zhao B, Tumaneng K & Guan K-L 2011 The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nature Cell Biology* **13** 877–883.

(doi:10.1038/ncb2303)

Figure Legends

Figure 1. YAP and TAZ are expressed in the human pituitary. (a) Haematoxylin and Eosin staining of sequential frontal sections of human fetal and adult pituitaries. Asterisks denote cysts between the anterior and posterior pituitary. (b) Frontal sections of fetal pituitaries at 17 weeks were stained for SOX2 (pituitary stem/progenitor marker), total TAZ, total YAP and pYAP(S127) marking inactive YAP. Arrowheads indicate examples of cells with nuclear protein. (c) Localisation of SOX2, TAZ, YAP and pYAP proteins in the adult anterior pituitary. Arrowheads indicate examples of cells with nuclear protein, and examples of cytoplasmic localisation for pYAP in (b). PL, posterior lobe; AL, anterior lobe; MZE, marginal zone epithelium; Par, parenchyma.

Figure 2. Expression of YAP and TAZ in non-secreting pituitary tumours. (a) Representative immunostaining against TAZ, YAP and pYAP(S127) in samples of papillary craniopharyngioma (PCP). Arrowheads denote examples of nuclear staining; asterisks, the suprabasal squamous epithelium. (b) Representative immunostaining against TAZ, YAP and pYAP(S127) in samples of adamantinomatous craniopharyngioma (ACP). Arrowheads indicate nuclear staining; arrows, the palisading epithelium; asterisks, characteristic whorl-like cluster cells. (c) Immunostaining against TAZ, YAP and pYAP(127) on null cell pituitary adenomas, showing representative staining from tumours classified as Type A, B and C. Arrowheads indicate nuclear staining. Scale bars 100µm.

Figure 3. Expression of YAP and TAZ in prolactin-secreting pituitary tumours. (a) Representative immunostaining against TAZ, YAP and pYAP(S127) in prolactinoma samples. Based on the staining patterns tumours were classified as Type D or Type E. (b)

Expression of TAZ and YAP in two regions of a prolactin-secreting carcinoma. Scale bars 100 μ m.

Figure 4. LATS1 inhibition with RNA interference suppresses the promoter activities of anterior pituitary hormones. (a) GH3 cells were transfected with a scrambled siRNA (control) or four different siRNA against rat *Lats1* for 48 hours. Immunoblot shows the knockdown efficacy and the impact of decreased LATS1 protein on YAP phosphorylation at Ser127 and total YAP and TAZ protein levels. (b) Effect of *Lats1* knockdown on basal *Prl* and *Gh* promoter activity. Data are luciferase to β -galactosidase ratio, means \pm standard deviation of three experiments (each in triplicate) presented as percentage of scrambled control. RLA, relative luciferase activity. * $P < 0.05$ to scrambled vehicle control. (c) Effect of *Lats1* knockdown on forskolin-induced (10 μ M, 6 hours treatment) *Gh* promoter activity. Data are means \pm standard deviation of three triplicates and presented as percentage of scrambled vehicle control. * $P < 0.05$ and *** $P < 0.001$ versus scrambled vehicle control, # $P < 0.05$ versus *siLats1* vehicle control. Veh, vehicle - the carrier in which forskolin was diluted. (d) *Lats1* knockdown does not affect cell proliferation (WST-1 colorimetric assay). Data are absorbance at OD450nm presented as percentage of scrambled siRNA control.

Supplementary Figure 1. YAP and TAZ are expressed in human fetal pituitaries. (a) Haematoxylin and eosin staining of sequential frontal sections of human fetal pituitaries at 14 and 17 weeks corrected gestational age. (b-c) Frontal sections of fetal pituitaries at 14 weeks (b) and 17 weeks (c) were stained for SOX2 (pituitary stem/progenitor marker), total TAZ, total YAP and pYAP(S127) marking inactive YAP. Arrowheads indicate examples of cells with nuclear protein. (c) Localisation of SOX2, TAZ, YAP and pYAP proteins in the adult

anterior pituitary. Arrowheads indicate examples of cells with nuclear protein. PL, posterior lobe; AL, anterior lobe; MZE, marginal zone epithelium; Par, parenchyma.

Supplementary Figure 2. Confirmatory testing for the diagnosis of tumours.

(a-b) Examples of confirmatory testing as part of the diagnosis of null cell adenoma, corticotropinoma, somatotropinoma and prolactinoma. (a) Haematoxylin and eosin staining, immunohistochemistry for FSH and SF1 on a null cell adenoma (top row) and a silent gonadotroph adenoma (bottom row) used as a positive control. (b) Examples of immunohistochemistry for specific hormones from two tumours of each cohort of corticotropinomas (ACTH), somatotropinomas (GH) and prolactinomas (PRL). All samples are counterstained with hematoxylin. Scale bars 50 μ m.

Supplementary Figure 3. Expression of YAP and TAZ in secreting pituitary tumours.

(a) Immunostaining against TAZ and YAP in samples of corticotropinoma showing representative staining from tumours classified as Types B, C and D. (b) Immunostaining against TAZ and YAP in samples of somatotropinoma showing representative staining from tumours classified as Types B, C, D and E. Arrowheads indicate nuclear staining; asterisk marks a blood vessel. Scale bars 100 μ m.

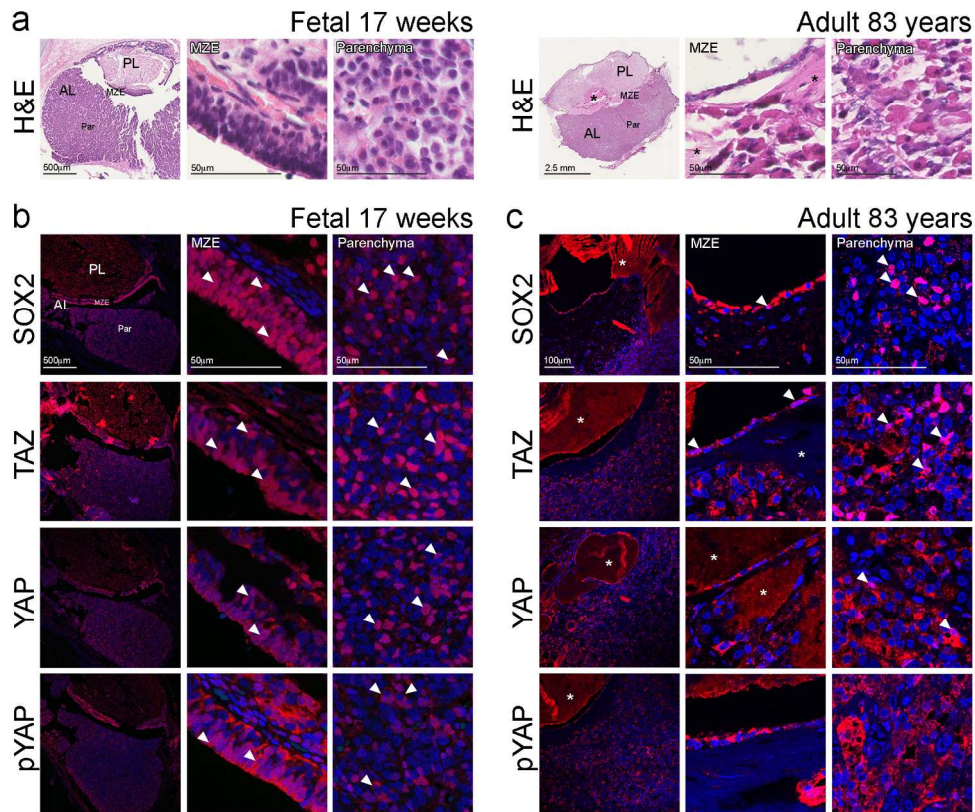
Table 1. Classification of YAP and TAZ staining in pituitary tumours

Tumour Type	Classification	Number	TAZ	YAP
Normal Pituitary	Type B	4	+++ n c	++ n c
PCP	Type A	6	++++ n c	++++ or +++ n c
ACP	Type A	5	++++ n c	++++ or +++ n c
Null cell adenoma	Type A	4	++++ n c	++++ or +++ n c
	Type B	4	+++ n c	++ n c
	Type C	2	++ or + c	++ or + c
Corticotropinoma	Type B	5	+++ n c	++ n c
	Type C	7	++ or + c	++ or + c
	Type D	6	+ c	+ c
Somatotropinoma	Type B	2	+++ n c	++ n c
	Type C	2	++ or + c	++ or + c
	Type D	5	+ c	+ c
	Type E	1	-	-
Prolactinoma	Type D	11	+ c	+ c
	Type E	5	-	-

ACP, adamantinomatous craniopharyngioma; PCP, papillary craniopharyngioma; +, positive staining; -, negative staining; n, nuclear; c, cytoplasmic.

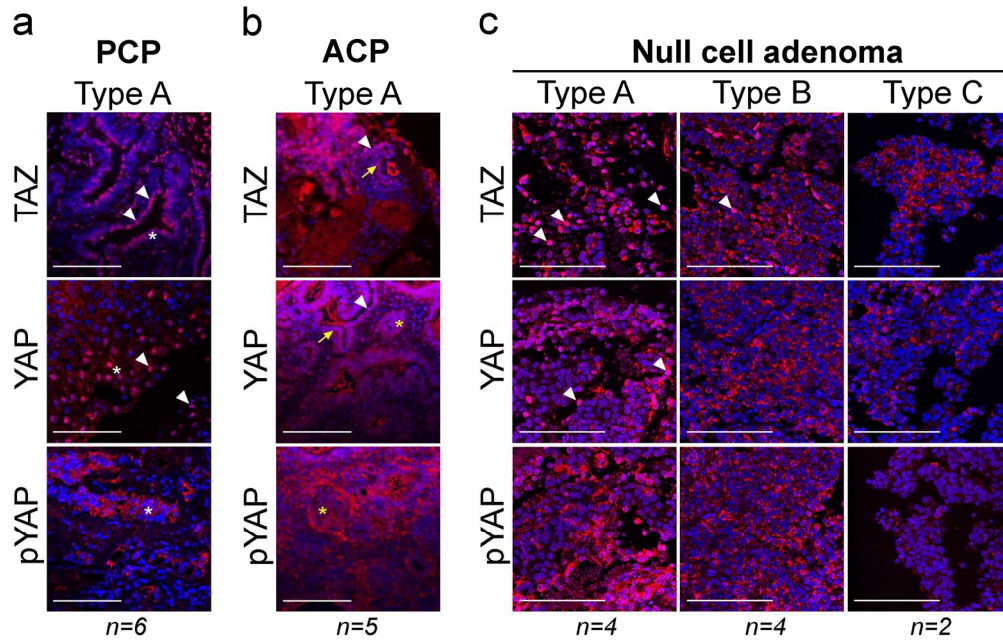
Table 2. Related data for null cell pituitary adenomas

Sample	Type	Histology	Ki-67	Notable Characteristics	p53 Status	Age at Presentation	Sex
Null1	A	null cell adenoma	2-3%		-	46	F
Null2	A	null cell adenoma	3-5%		2-3%	27	F
Null3	A	null cell adenoma	3-5%		>1%	48	F
Null4	A	null cell adenoma	< 3%		-	36	F
Null5	B	null cell adenoma	5-7%	recurrence	< 1%	41	F
Null6	B	null cell adenoma	2-3%		-	73	F
Null7	B	null cell adenoma	2-3%		-	31	F
Null8	B	null cell adenoma	1-2%		-	49	F
Null9	C	null cell adenoma	< 2%	necrotic areas	-	48	M
Null10	C	null cell adenoma	2.5%		-	33	F



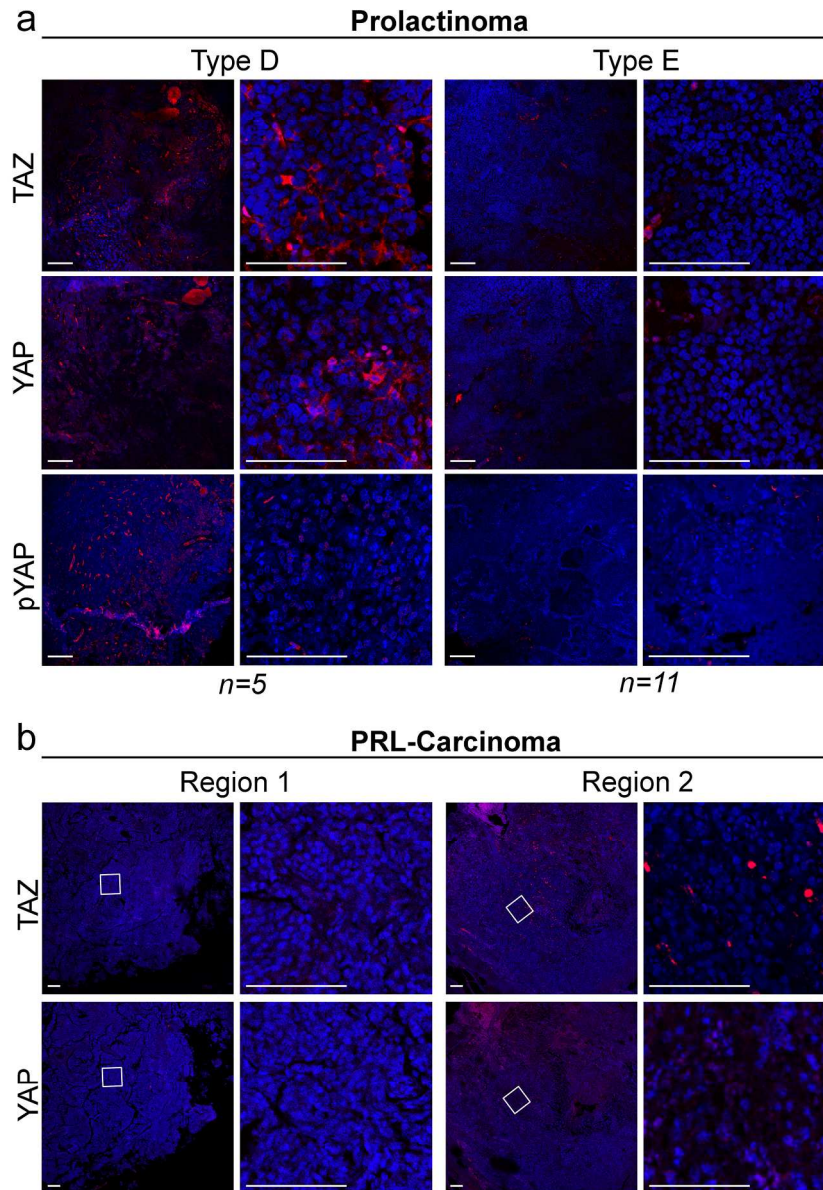
YAP and TAZ are expressed in the human pituitary. (a) Haematoxylin and Eosin staining of sequential frontal sections of human fetal and adult pituitaries. Asterisks denote cysts between the anterior and posterior pituitary. (b) Frontal sections of fetal pituitaries at 17 weeks were stained for SOX2 (pituitary stem/progenitor marker), total TAZ, total YAP and pYAP(S127) marking inactive YAP. Arrowheads indicate examples of cells with nuclear protein. (c) Localisation of SOX2, TAZ, YAP and pYAP proteins in the adult anterior pituitary. Arrowheads indicate examples of cells with nuclear protein, and examples of cytoplasmic localisation for pYAP in (b). PL, posterior lobe; AL, anterior lobe; MZE, marginal zone epithelium; Par, parenchyma.

180x148mm (300 x 300 DPI)



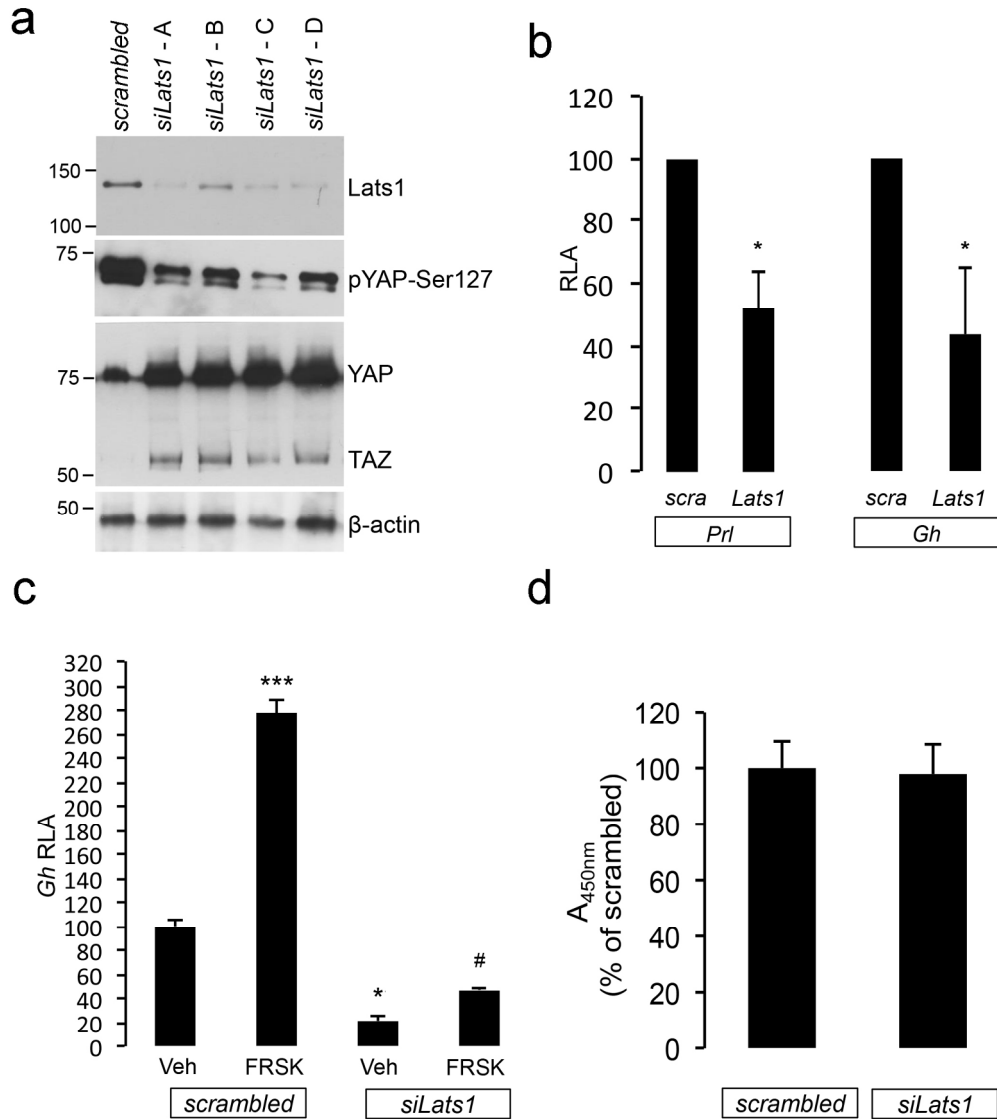
Expression of YAP and TAZ in non-secreting pituitary tumours. (a) Representative immunostaining against TAZ, YAP and pYAP(S127) in samples of papillary craniopharyngioma (PCP). Arrowheads denote examples of nuclear staining; asterisks, the suprabasal squamous epithelium. (b) Representative immunostaining against TAZ, YAP and pYAP(S127) in samples of adamantinomatous craniopharyngioma (ACP). Arrowheads indicate nuclear staining; arrows, the palisading epithelium; asterisks, characteristic whorl-like cluster cells. (c) Immunostaining against TAZ, YAP and pYAP(127) on null cell pituitary adenomas, showing representative staining from tumours classified as Type A, B and C. Arrowheads indicate nuclear staining. Scale bars 100 μ m.

180x114mm (300 x 300 DPI)



Expression of YAP and TAZ in prolactin-secreting pituitary tumours. (a) Representative immunostaining against TAZ, YAP and pYAP(S127) in prolactinoma samples. Based on the staining patterns tumours were classified as Type D or Type E. (b) Expression of TAZ and YAP in two regions of a prolactin-secreting carcinoma. Scale bars 100 μ m.

180x259mm (300 x 300 DPI)



LATS1 inhibition with RNA interference suppresses the promoter activities of anterior pituitary hormones. (a) GH3 cells were transfected with a scrambled siRNA (control) or four different siRNA against rat Lats1 for 48 hours. Immunoblot shows the knockdown efficacy and the impact of decreased LATS1 protein on YAP phosphorylation at Ser127 and total YAP and TAZ protein levels. (b) Effect of Lats1 knockdown on basal Prl and Gh promoter activity. Data are luciferase to β -galactosidase ratio, means \pm standard deviation of three experiments (each in triplicate) presented as percentage of scrambled control. RLA, relative luciferase activity. * $P < 0.05$ to scrambled vehicle control. (c) Effect of Lats1 knockdown on forskolin-induced (10 μ M, 6 hours treatment) Gh promoter activity. Data are means \pm standard deviation of three triplicates and presented as percentage of scrambled vehicle control. * $P < 0.05$ and *** $P < 0.001$ versus scrambled vehicle control, # $P < 0.05$ versus siLats1 vehicle control. Veh, vehicle - the carrier in which forskolin was diluted. (d) Lats1 knockdown does not affect cell proliferation (WST-1 colorimetric assay). Data are absorbance at OD450nm presented as percentage of scrambled siRNA control.

