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YAP1/TAZ drives ependymoma-like tumour formation in mice

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YAP1 gene fusions have been observed in a subset of paediatric ependymomas. Here we show that, ectopic expression of active nuclear YAP1 (nlsYAP5SA) in ventricular zone neural progenitor cells using conditionally-induced NEX/NeuroD6-Cre is sufficient to drive brain tumour formation in mice. Neuronal differentiation is inhibited in the hippocampus. Deletion of YAP1's negative regulators LATS1 and LATS2 kinases in NEX-Cre lineage in double conditional knockout mice also generates similar tumours, which are rescued by deletion of YAP1 and its paralog TAZ. YAPI/TAZ-induced mouse tumours display molecular and ultrastructural characteristics of human ependymoma. RNA sequencing and quantitative proteomics of mouse tumours demonstrate similarities to YAP1-fusion induced supratentorial ependymoma. Finally, we find that transcriptional cofactor HOPX is upregulated in mouse models and in human YAP1-fusion induced ependymoma, supporting their similarity. Our results show that uncontrolled YAP1/TAZ activity in neuronal precursor cells leads to ependymoma-like tumours in mice.
YAP-associated protein 1 (YAP1) is a transcriptional regulator with oncogenic activity that is involved in cellular proliferation, maintenance of stem cell properties and tumorigenesis. YAP1 activity is the output of the conserved hippo signalling pathway, which is implicated in cancer. This canonical kinase signalling system/pathway transmits mechanical stimuli from the cell surface to regulate cell proliferation, survival and tissue size in multiple organs across species. The core kinase cascade consists of the Hippo kinase in *Drosophila* and its homologue Mammalian Sterile 20-like 1 (MST1) and MST2 in mammals, together with hippo’s downstream effector Warts in *Drosophila*, which is homologous to the Large tumour suppressor homologue 1 (LATS1) and LATS2 kinases in mammals. MST2/1 phosphorylates and activates LATS1/2, which in turn phosphorylates to inhibit YAP1 or its close homologue TAZ/WWTR1. YAP1 phosphorylation promotes its degradation via 14-3-3 binding and cytoplasmic sequestering, preventing its translocation to the nucleus. YAP1 activates TEAD1-4-dependent gene expression, which is required for its effects on cellular proliferation.

The role of hippo signalling in mammalian tumorigenesis has been demonstrated in vivo, for example, by conditional deletion of MST1/2 or LATS1/2 in liver, which deregulates progenitor cell proliferation and leads to tumorigenesis in mice. Moreover, activated YAP1 expression has been shown to drive embryonal rhabdomyosarcoma formation, whereas its expression in several cancer types makes YAP1 a considered target for novel treatments. However, its role in brain tumours, such as ependymoma, is not fully described.

Ependymoma is the third most common paediatric brain tumour. It accounts for 5–10% of all primary tumours of the central nervous system (CNS) in children and adolescents. Ependymomas have recently been classified into nine molecular subgroups based on genetic and epigenetic profiles, age of onset and location (supratentorial, posterior fossa and spinal). Surgery followed by radiotherapy are the main treatment modalities for most patients. Recurrence rates are however high and up to 40% of ependymomas are incurable. Approximately 70% of supratentorial ependymomas are characterised by a fusion between C11ORF95 and RELA, whereas ~4–10% carry the fusions of YAP1 with other genes encoding transcription factors: YAP1-MAML1, or YAP1-FAM118B. The group of ependymomas with YAP1 fusions occurs almost exclusively in children.

Ependymomas likely originate from a restricted population of radial glial-like stem cells that are found in the ventricles and spinal canal of embryos and adults. Radial glia and intermediate neural progenitors derived from them normally produce neurons, oligodendrocytes, astrocytes and ependymal cells. Signalling mechanisms that normally regulate cellular progenitor proliferation and orchestrate differentiation are therefore strong candidates to be involved in paediatric tumourigenesis. YAP1 is present in the embryonic ventricular zone and later in the ependyma of mice, where it has been implicated in ependymal cell differentiation.

We use conditional mouse models of active YAP1 expression and LATS1/2 deletion and show that suppression of YAP1 activity in NEX/NeuroD6 expressing neuronal precursor cells is essential for limiting proliferation and enabling neuronal differentiation in the brain, whereas activation of YAP1 is sufficient to produce tumours that display features similar to human ependymoma.

**Results**

**Active YAP1 expression in NEX-Cre lineage induces tumours.** Whether or not elevated YAP1 activity is tumourigenic in the developing mammalian brain is an open question. To target for cell types that normally express YAP1 in a developing brain, we first examined YAP1 localisation in mice brains using immunohistochemistry (IHC) with a specific anti-YAP1 antibody. YAP1 was highly expressed in the ventricular zone (VZ) at postnatal day 0 (P0) (Fig. 1a, Supplementary Fig. 1a), becoming localised in the ependymal layer at P20 (Supplementary Fig. 1a). A subset of YAP1-positive cells in the VZ at P0 was mitotically active and were of radial glia origin as shown by Ki67 and nestin co-expression, respectively (Fig. 1b, c). NEX-Cre positive neuronal precursor cells (NPCs) are known to be generated in the VZ starting at E11.5 and NEX/Math2/NeuroD6 lineage gives rise to pyramidal neurons of the cortex and hippocampus but does not contribute to astrocytes or oligodendrocytes. We crossed NEX-Cre-expressing mice with a Rosa26 tdTomato mouse reporter line. In addition to pyramidal neurons, we observed numerous tdTomato expressing cells in the VZ at P0 (Supplementary Fig. 1b–d). This finding is in agreement with previous reports showing NEX-Cre in dividing NPCs. TdTomato reporter positive cells also expressed YAP1, indicating that NEX-Cre driver can be used to manipulate a subpopulation of neural precursor cells (Supplementary Fig. 1b–d).

Based on this evidence, we assessed the effect of uncontrollable YAP1 activity in NEX-expressing NPCs, by using a Cre inducible mouse model, in which a hyperactive form of YAP1, nlsYAP5SA, is preceded by a floxed STOP codon. Upon Cre-mediated recombination these transgenic mice express phosphomutant YAP1 (S61A, S109A, S127A, S164A, S381A), which cannot be phosphorylated and inhibited by kinases, such as LATS1/2. In addition, nlsYAP5SA contains a nuclear localisation signal (nls) to direct YAP1 activity to the nucleus. nlsYAP5SA; NEX-Cre mice displayed multiple subependymal tumours at P14 along the lateral ventricles as well as in cerebellum. These results indicate that uncontrolled YAP1 in NEX-Cre lineage is sufficient for tumour formation.

**nlsYAP5SA suppresses hippocampal neuron differentiation.** We next determined the progression of the tumour formation at earlier developmental stages. In nlsYAP5SA; NEX-Cre mice, nuclear YAP1 was expressed throughout neocortex and hippocampus (Fig. 2a). In these animals, hippocampal neuronal cell bodies (CA1, CA3 and dentate gyrus) showed neuronal loss and disruption of normal layering as observed with the neuronal markers NeuN and CTIP2. We also identified multiple tumours adjacent to the ependymal layer. These tumours were positive for nestin, indicating that constitutive cells are of neuronal stem cell specification (Fig. 2c). Tumours did not express NeuN indicating that the tumour did not contain differentiated neurons, but several cells within the tumour mass expressed Ki67, a marker of proliferation, indicating that the tumours contain actively dividing cells. These data show that sustained nuclear YAP1 in NEX-Cre expressing NPCs lead to tumour formation by maintaining a neural stem cell-like fate and preventing hippocampal pyramidal neuronal differentiation.

**Deletion of LATS1/2 in NEX-Cre lineage causes brain tumours.** Despite several lines of evidence that supports an oncogenic role for YAP1, the impact of kinases regulating YAP1 activity in cancer is not well understood. In mammals, the canonical upstream phosphorylation and suppression of YAP1/TAZ activity is predominantly accomplished by two homologous kinases, LATS1 and LATS2. Constitutive knockout of LATS2 results in embryonic lethality, whereas constitutive LATS1 knockout leads to soft tissue sarcoma and ovarian tumour development, indicating essential roles of LATS2 during development and compensatory roles between the homologues. Conditional knockout of LATS1...
and LATS2 (LATS1/2) in mouse liver increases proliferation and represses the maturation of hepatocytes thereby causing pre-natal lethality. Whether or not LATS1/2 has a role in neuronal differentiation in mammals is not known. We tested if deficiency in YAP1 phosphoregulation by LATS1/2 is sufficient to cause tumour formation by conditionally knocking out Lats1 and Lats2, in NEX-Cre lineage (Fig. 3a). We found that dual LATS1/2 conditional knockouts (cKOs), but not individual LATS1 cKOs or LATS2 cKOs, have significantly reduced body weight by P19–P21 compared with control littermates, with an onset of difference at P15 (Supplementary Fig. 3a, b). Upon inspection of LATS1/2 cKO mice at P0–P21 age groups, we found that 67% at P5–P7 and 100% at P8–P21 developed tumours whereas no tumours were found before P5 (Supplementary Fig. 3c). Multiple clonal forebrain tumours, originating invariably at the ependymal layer were found in all LATS1/2 cKOs, but not in controls or individual LATS1 cKOs or LATS2 cKO at P20 (18/18 dual knockout, 0/3 LATS1 cKO, 0/3 LATS2 cKO, 0/6 controls), indicating redundancy between LATS1 and LATS2. Animals showed multifocal, discrete tumours indicating their synchronous origin (Fig. 3b).

Fig. 1 YAP1 is present in ependymal layer and its regulation is critical for brain development. a Coronal view of control P0 mouse brain sections immunofluorescence stained for YAP1. Arrowhead pointing at strong positive staining in the ependymal layer, scale bars = 1 mm. b–c Higher magnifications of ependymal layer/ventricular zone in control mice at P0. b, c Immunostainings with YAP1 and Nestin and c, Immunostainings with YAP1 and Ki67 show colocalization (arrows point to cells with colocalizations, overlap shown in white colour). Scale bars = 10 μm. d Breeding scheme to generate mice expressing nlsYAPSSA under the control of NEX-Cre (referred to as nlsYAPSSA). Mice with only one allele of NEX-Cre without the YAP1 transgene were used as controls. e Sagittal vibratome sections of the whole brain of Control and nlsYAPSSA mice are shown at P14. In nlsYAPSSA mice, ependymal layer is destructed, multiple tumours are formed across the brain as shown by DAPI stained nuclei and hippocampus is not developed. Arrowheads point at hippocampus. Scale bar = 1 mm.
LATS1/2 cKO brains were collected at predetermined time points (mostly at P20) as permitted by our project licence and were not assessed by survival (see methods). Based on the severity indications, we believe the majority of LATS1/2 cKO mice and all nlsYAP5SA mice were moribund before 2 months of age. YAP1 was expressed in all tumours including the smallest lesions observed in younger animals. This finding indicates that loss of LATS1/2 function increases YAP1 activity (Fig. 3c). In LATS1/2 deletion induced tumours, YAP1 was highly expressed in the cytoplasm of all cells and was enriched in the nucleus in a subset of cells in tumour, in a mosaic fashion (Fig. 3d, e). Although some cells expressed high levels of YAP1 in the nucleus, some had less-intense YAP1 levels in nucleus (Fig. 3d, e). These results indicate that in LATS1/2 cKO tumours YAP1 nuclear localisation can be regulated by additional factors, such as actomyosin and Src kinase, as previously shown. Furthermore, YAP1-positive tumours in this early stage of development showed increased nestin expression and nestin was present in cells expressing nuclear YAP1 as well as those that had increased YAP1 in cytoplasm (Fig. 3e). In addition to the periventricular tumours, we observed tumour formation in the cerebellum (Supplementary Fig. 3d), recapitulating the features of nlsYAP5SA; NEX-Cre mice (Fig. 1e). These data show that LATS1/2 loss in NEX-Cre expressing neural precursor cells cause YAP1-expressing tumours that are highly similar to tumours found in nlsYAP5SA; NEX-Cre mice. Our finding of much smaller clonal tumour formation in LATS1/2 cKO mice, when compared with nlsYAP5SA; NEX-Cre mice is likely owing to other regulators of YAP1/TAZ activity, such as Src and NDR kinases. Therefore, LATS1/2 activity in NPCs is necessary to control YAP1 activity during development.

Tumours in LATS1/2 conditional knockout mice are YAP1/TAZ dependent. Although it is generally accepted that YAP1 is the main functional output of the hippo pathway and that its dysregulation leads to tumourigenesis, it remains to be tested whether YAP1 has an essential role in Lats1/2-dependent brain tumour generation. YAP1 exhibits largely redundant functions with its close homologue TAZ. Therefore, we used YAP1 f/f; TAZ f/+ mice to assess if YAP1/TAZ are required for tumorigenesis in LATS1/2 dual knockout mice. For this purpose, we crossed NEX-Cre; Lats1 f/f; Lats2 f/f mice with YAP1 f/f; TAZ f/+ mice (Fig. 4a). We found that all LATS1/2 cKO animals where one or both copies of YAP1 was deleted still exhibited tumours at P20 (n = 3 each), indicating that YAP1 is not the sole downstream factor regulated by LATS1/2. However, we observed that LATS1/2 cKO animals depleted of both copies of YAP1 exhibit a significant reduction (p < 0.001, Dunn’s multiplex comparisons test) in average tumour size compared with LATS1/2 cKO animals (Fig. 4b), indicating that level of YAP expression impacts tumour size. Further, using a random effects model to account for multiple tumours being recorded from each animal, we observe significant evidence of an additive dosage effect, specifically each YAP1 WT allele lost corresponds to a decrease in estimated tumour volume of ~0.03 mm$^3$ (p = 0.0068). In LATS1/2; YAP1 f/f mice, tumours were negative for YAP1 immunostaining supporting its specificity (Supplementary Fig. 4a). LATS1/2 cKO YAP1 f/+ TAZ f/+ mice have tumours (n = 3). However, tumour formation was suppressed at P20 in LATS1/2 cKO YAP1 f/+ TAZ f/+ mice (n = 3) (Fig. 4c). These results strongly support that activation of both YAP1 and TAZ mediate tumour formation in LATS 1/2 cKO mice (Fig. 4c). We conclude that YAP1 and TAZ are necessary for...
LATS1/2-dependent tumourigenesis arising from NEX expression NPCs.

LATS1/2 cKO tumours recapitulate features of human ependymoma. We next investigated the pathological features of the LATS1/2 cKO tumours. Serial sections of paraffin embedded P20 LATS1/2 cKO mice brains showed periventricular multifocal tumours in continuity with the ependymal layer (Fig. 5a). Histologically, the tumours appeared as moderately cellular lesions with a compressive margin. They were composed of uniform spindle or rounded cells with fibrillary or scanty cytoplasm and slightly hyperchromatic nucleus (Fig. 5b, c). We did not observe well-formed perivascular pseudo-rosettes. Some cells contained intracytoplasmic vacuoles (Fig. 5d). Mitoses were absent. The tissue surrounding these lesions showed reactive astrocytosis and microglial response. Tumour cells showed nuclear and lesser extent cytoplasmic YAP1 expression (Fig. 5e), they weakly expressed MUC1 (EMA) (Fig. 5f) and they were focally positive for GFAP (Fig. 5g) and cytokeratin 18 (Fig. 5h). Vimentin, nestin,
which are present in radial glia were also expressed in tumours, whereas neuronal marker NeuN was consistently absent (Supplementary Fig. 5a). Ki67 was expressed in tumours indicating cell division (Supplementary Fig. 5a). Notably, normal ependyma in mice also expressed cytokeratin 18 as previously shown (Supplementary Fig. 5b). Light microscopic features and immunoperoxidase of the tumours were consistent with ependymoma and similar to the human counterpart.

To further test the ependymal origin of LATS1/2 cKO tumours, we performed ultrastructural examination using transmission electron microscopy (TEM). After identifying the areas of the tumour mass using Micro-CT by comparing to nuclear 4′,6-diamidino-2-phenylindole (DAPI) fluorescent image of the brain (Supplementary Fig. 5c), we studied multiple regions of the tumour using serial blockface scanning electron microscopy (Supplementary Fig. 5d) and TEM (Fig. 5i–k, Supplementary Fig. 5e–f). Numerous microvilli and tight junctions (Fig. 5i–k, Supplementary Fig. 5f) were observed in different regions of the tumour. Presence of microvilli and tight junctions confirmed that YAP1 dysregulation and activation leads to the formation of ependymal tumours similar to the corresponding tumours seen in humans.

![Diagram](image_url)

**Fig. 4 YAP1 and TAZ are necessary for tumour development in LATS1/2 cKO mice.** a Breeding scheme to generate a rescue model. Available animals (Lats1f/f Lats2f/f, YAPf/+, and NEXCre/Cre) were crossed to generate mice with shown genotypes. b Measurements of size of tumours found in LATS1/2 cKO (n = 16 tumours), Mutant YAPf/+ (n = 15 tumours) and Mutant YAPf/f (n = 11 tumours) from n = 3 mice for each genotype. Colour shade indicates tumours from the same animal. LATS1/2 cKO tumour volumes compared with those measured in Mutant YAPf/f animals show a significant difference with padj = 0.0006 (two-sided Dunn’s multiple comparison test). Error bars = SEM. c Coronal vibratome sections of P20 brains stained for DAPI. No tumour formation identified in Rescue mice. Tumours (arrowheads) can be found in LATS1/2 cKO, Mutant YAPf/ and Mutant YAPf/+ animals. Scale bars = 1 mm.
RNA sequencing and proteomics in nlsYAP5SA brain. We performed total RNA sequencing of the right hemisphere of nlsYAP5SA mice (NEXCre+/−; YAP1nlsYAP5SA+/+) and unaffected littersmates (NEX+/++; YAP1nlsYAP5SA+/+) (n = 5 for each genotype) (Fig. 6a). Experiments included three additional non-littermate C57BL/6 controls, which clustered with the control animals, but were not included in later analysis (Supplementary Fig. 6a). First, we ranked all 30,280 identified genes based on their...
differential expression in nlsYAP5SA and control. We then applied gene enrichment analysis (GSEA) choosing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to identify gene sets that are overrepresented at both extremes of the ranked list. Among the top 10 upregulated gene sets in nlsYAP5SA we found pathways in cancer, cytokine receptor interaction and focal adhesion, in agreement with active YAP1’s capacity to induce cancer and a consequent immune response (Fig. 6b, c). On the other side, Oxidative Phosphorylation gene set among the most reduced, which may indicate changes in energy
Fig. 6 Transcriptome and proteome analysis of nlsYAP5SA mice exhibit further parallels to human ependymoma. a–e Gene expression analysis for nlsYAP5SA versus control. Five animals per genotype (control = NEX+/− YAPlnsYAP5SA/++; nlsYAP5SA = NEX−/− YAPlnsYAP5SA/+) were analysed utilising the Illumina HiSeq 4000. a Heat map of all significantly differentially expressed genes (2035 genes) between control and nlsYAP5SA animals. Rows represent genes and columns show individual animals. Legend indicates per gene median centred fold change. b Gene enrichment analysis (GSEA) of complete gene list (30,280 detected genes) from RNA sequencing (RNAseq) with gene sets derived from the KEGG pathway database. The top 10 annotated pathways with the highest normalised enrichment score (NES) are shown with number of genes in the data set linked to the indicated pathway (Size). c GSEA of KEGG annotation “Pathways in Cancer” with highest NES in analysis. d Gene list of YAP1 and RELA association (see methods) was used to assign specificity to detected genes in our expression data from nlsYAP5SA screen (350 genes of the 354 in reference list are present in our data set). Log2 fold change of control vs. nlsYAP5SA are ranked from highest to lowest. Teal and red colours are used to denote YAP-specific and RELA-specific genes, respectively. e The 10 highest ranked genes with assigned specificity all of these are associated with YAPI. f Venn diagrams illustrating numbers of identified genes in Transcriptome (RNAseq, blue) and proteome quantified by liquid chromatography mass spectrometry (LC-MS) (red). Left: all genes, right: significantly upregulated genes in nlsYAP5SA. g Density scatter plot of 6439 genes present in both proteome and transcriptome plotted by their expression difference between control and nlsYAP5SA (Welch difference is used for proteomics and stat value is used for transcriptomics). Data sets show a correlation coefficient of 0.5. Each point represents one gene. h Volcano plot of difference in protein levels between control and nlsYAP5SA cohort. Each point represents one protein. The x axis shows log2 transformed fold change and the y axis shows significance by −log10 transformed p value obtained by two-sided Welch t test. A gene is called significantly and differentially expressed if its FDR is <0.05 and s0 > 0.1, which is indicated by the black line. Proteins associated with astrocytes (magenta) and microglia (green) are displayed. Cell type association was generated by integrating previously published data (see methods). i Area shown in h, enlarged to display and name significantly upregulated proteins in nlsYAP5SA brains.

metabolism tumours (Supplementary Fig. 6b). In a complementary analysis, we analysed the 2035 significantly differentially expressed genes between the two genotypes utilising the online tool DAVID with KEGG defined gene sets. The significantly differentially expressed gene list was generated using the DESeq2 R package applying the Wald significance test with a threshold of 0.05 for significance. As expected, one of the most-enriched gene sets was the hippo signalling pathway in addition to terms including immune and infection-related genes (Supplementary Fig. 6c). Specifically, in nlsYAP5SA mice numerous known YAP1 downstream effectors were significantly upregulated at mRNA level, including AMOTL2, CTGF, CYR61, ANKRDI and AXL (Supplementary Data 1).

Next, we inspected how gene expression signatures in our nlsYAP5SA mouse models compare with published gene expression data from human ependymoma subtypes. For this we used a list of genes that was previously established to be differentially expressed between YAPI-MAMLD1 fusion and RELA fusion-positive human ependymoma subtypes, as well as between YAPI-MAMLD1 and C11orf95-RELA-driven mouse ependymoma models. Of these 354 published unique genes (151 YAP1 associated and 203 RELA-associated), 350 were present in our transcriptomic data, which we ranked with respect to fold change in nlsYAP5SA compared with control mice (Fig. 6d). We found that although RELA fusion-associated genes were equally represented among increased and reduced genes in nlsYAP5SA model (Fig. 6d, red), genes that are specific for YAPI-MAMLD1 fusion-positive ependymoma were clearly overrepresented among upregulated genes in the nlsYAP5SA model (Fig. 6d; teal, Supplementary Data 2). When we restricted the analysis to mRNAs that are significantly changed between control and nlsYAP5SA mice (89/354 genes were found in our significantly differentially expressed gene list, matched by gene name) we found that this pattern was even more striking. In all, 45 out of the 46 (98%) YAPI-MAMLD1 fusion-associated mRNAs were significantly increased in our mouse model and only one mRNA was reduced, in contrast 20 out of 43 (46%) RELA fusion-specific genes were increased and 23 were reduced in nlsYAP5SA mice (Supplementary Fig. 6d, Supplementary Data 2). These data show that the proportion of YAP-associated genes that are increased in nlsYAP5SA are much higher than RELA-associated genes (p < 0.0001, Chi-squared test), indicating that YAP-MAMLD1 fusion-associated gene expression signatures are present in nlsYAP5SA model. The 18 genes with the highest expression changes in nlsYAP5SA included known YAPI downstream target genes such as Ankrdi1, Cyr61 and Ctgf (Fig. 6e). Interestingly, cytokeratin 18, a marker expressed in the ependymal layer and in our LATS1/2 cKO tumours was among the top 10 increased genes in nlsYAP5SA mice (Fig. 6e). These data support that nlsYAP5SA expression in NPCs lead to a gene expression profile that resembles human ependymomas with YAPI-MAMLD1 fusion. We attempted to perform a principle component analysis between multiple types of paediatric brain tumour gene expression and our nlsYAP5SA mice-sequencing results. We did not observe a strong clustering of the individual patient tumour types (Supplementary Fig. 6e), thus we were not able to compare nlsYAP5SA tumours either, leading this analysis to be inconclusive. Substantial technical caveats were associated with comparing microarray data set with our next-generation sequencing data set (see methods).

To investigate whether the changes we observed in mRNA expression are translated to protein level changes, we took a mass spectrometry approach. The left hemispheres of the nlsYAP5SA and control animals (n = 5, each) that were used in the transcriptomic screen were labelled with a TMT (Tandem Mass Tag) 10-plex reagent to enable relative proteome quantification. We identified a total of 6643 proteins, 21.3% of the identified mRNAs were represented at the protein level and we find an overlap of 85 genes and proteins that were significantly increased in both data sets in nlsYAP5SA compared with control when matched by gene name (Fig. 6f). The principal component analysis of the proteomics data shows two distinct clusters clearly separating the different genotypes (Supplementary Fig. 6f). We observed a correlation between the transcript (Stat value) and protein (Welch difference) levels with a Pearson’s correlation coefficient of 0.5 (Fig. 6g, Supplementary Data 3). Next, we calculated categorical enrichment in two dimensions as described previously. Gene ontology (Biological Processes, Molecular Function and Cellular Compartment) and KEGG were annotated both on the transcript and the protein level. These data revealed a good correlation between the transcriptome and proteome data on the categorical level (Supplementary Fig. 6g, Pearson’s r = 0.844 correlating 2D enrichment scores). GFAP, vimentin and ANXA1 were specific examples of genes that were increased at mRNA as well as protein levels in nlsYAP5SA brains (Fig. 6g) and these were previously associated with ependymoma.

To investigate if increased proteins can be associated with a particular cell type we analysed our proteomics data separately by overlaying it with an existing data set of full proteomics performed on isolated CNS cells. We show that in tumour bearing mice there is an increase in microglia and astrocyte associated proteins (Fig. 6h, i, Supplementary Fig. 6h, i; Supplementary Data 4),
indicating a microglial response to the tumour. In addition, complement cascade proteins such as C1qa, C1qb, C1qc and C4b are highly upregulated in agreement with the GSEA pointing towards immune response components (Fig. 6b, Supplementary Fig. 6c). Interestingly, the known YAP1 target CTGF was upregulated in mRNA level but was unchanged at protein level (Fig. 6g), which was further confirmed with western blot (Fig. 7a), suggesting additional posttranscriptional control. Overall, we observed good correlation of our proteomics and transcriptomics analysis, which together strongly support that nlsYAP5S brain tumours resemble YAP1-fusion-positive ependymoma and activate known YAP target genes.

**YAP1 effectors are highly expressed in LATS1/2 cKO tumours.** First, we confirmed that Vimentin, GFAP and HOPX were highly
increased in nlsYAP5SA brains, where FLAG-tagged YAP1 overexpression can be observed (Fig. 7a). Next, we wanted to know if genes and proteins upregulated in nlsYAP5SA mice were also upregulated in LATS1/2 knockout mouse tumours. Using immunofluorescence, we found that YAP1 downstream effectors identified in mRNA analysis, ANKR1D, AMOTL2 and AXL were increased in LATS1/2 cKO tumours (Fig. 7b). ANKR1D was abundant in the tumour centre, whereas AMOTL2 and AXL were higher at periphery, where C3-positive microglia were also observed (Supplementary Fig. 7a). These results suggest that canonical YAP1 signalling effectors are relevant to tumorigenesis of ependymoma-like tumours in both of our mouse models.

In addition to YAP1, Homeodomain-only protein (HOPX) was the only transcription co-factor that was significantly increased in mRNA sequencing and proteomics analysis (Fig. 6g-1). HOPX is a transcriptional regulator expressed in many tissues including pseudo-rosettes in neuronal stem cells with astrocytic fate62,63. HOPX was present in a transcriptional regulator expressed in many tissues including pseudo-rosettes in a neuronal stem cell62,63. HOPX has been reported to be absent in various models, including human YAP1-fusion ependymoma32. For example, isolated cerebral embryonic neural stem cells from Ink4a/Arf-deleted mice, a genetic aberration previously observed in human ependymoma33 were transformed by an Ephb2-expressing virus72. This mouse model, mEPephb2ze, was used in high-throughput screens, which identified 5-fluorouracil as an effective treatment74, a hypothesis that is being tested clinically75. More recently, a mouse model, RELAFC11 was generated by transducing nestin expressing mouse stem cells with a c11ORF95-RELA fusion protein72. In both mouse models, upon engrafting, tumours with profound similarities to ependymoma were formed72,73. Similar to other ependymoma mouse models57,72,73, in LATS1/2 cKOs we did not observe rosettes or well-formed pseudo-rosettes, which are commonly observed in human ependymomas and are also present in the mEPephb2ze model72. We speculate that formation of these structures may depend on tumour size and species.

The tumours generated by increased YAP/TAZ activity in our nlsYAP5SA and LATS1/2 cKO mouse models are located in the periventricular region, similarly to ependymoma with YAP1-MAML1 fusion in humans, which consistently shows a periventricular location, with or without an intraventricular component32, in contrast with RELA-fused ependymoma, which tends to occur more often in cortical-centred locations64. A recent publication showed that YAPI-MAML1 fusion in nestin-positive neural stem cells induce ependymoma-like tumours in mice57. In this model, MAML1 was necessary for the nuclear localisation of YAP1 and confers specific properties to YAP1-dependent gene regulation57. Our nlsYAP5SA and LATS1/2 cKO mouse models indicate the notion that YAP/TAZ activity alone is sufficient for ependymoma-like tumour formation. This result supports that the main function of fusion proteins such as MAML1 may be to induce human ependymoma, while largely absent in three ependymoma models57. We were not able to demonstrate a clustering of our

Discussion
Our study demonstrates that control of YAP1/TAZ activity in neuronal precursor cells at VZ by LATS1/2 kinases is critical for limiting their proliferation. Upon nlsYAP5SA expression, hippocampal neuron differentiation is severely affected. These results are in line with the findings that NF2, a positive regulator of hippo signalling, also alters hippocampal formation by limiting progenitor numbers and enabling hippocampal neuron differentiation; effects mediated by YAP168,69. Our LATS1/2 cKO mouse model data indicate that LATS kinases play a role in suppressing YAP1 activity in progenitors, thus establishing a requirement for the canonical LATS-YAP1 signalling pathway in limiting progenitor proliferation.

Mouse models of ependymoma are important for understanding the pathogenesis of this tumour and its subtypes and developing therapeutic interventions. Alongside patient-derived xenografts70,71, mouse models of ependymoma that mimic genetic causes in cells of origin are invaluable for studying tumour development57,72,73. For example, isolated cerebral embryonic neural stem cells from Ink4a/Arf-deleted mice, a genetic aberration previously observed in human ependymoma33 were transformed by an Ephb2-expressing virus72. This mouse model, mEPephb2ze, was used in high-throughput screens, which identified 5-fluorouracil as an effective treatment74, a hypothesis that is being tested clinically75. More recently, a mouse model, RELAFC11 was generated by transducing nestin expressing mouse stem cells with a c11ORF95-RELA fusion protein72. In both mouse models, upon engrafting, tumours with profound similarities to ependymoma were formed72,73. Similar to other ependymoma mouse models57,72,73, in LATS1/2 cKOs we did not observe rosettes or well-formed pseudo-rosettes, which are commonly observed in human ependymomas and are also present in the mEPephb2ze model72. We speculate that formation of these structures may depend on tumour size and species.
Methods
to reach a more comprehensive understanding of ependymoma. Experimental mice were immune compromised. Both male and female mice were on a 12 hr light/dark cycle, with food and water provided ad libitum. None of the mice were approved by institutional ethical reviews. Mice were group housed and maintained in large cohorts of ependymoma subtypes would be needed to assess subtype-specificity of this marker. Nevertheless, these data support the use of nlsYAP5SA and LATS1/2 cKO mice as ependymoma models.

Our proteomics screen showed a striking increase in microglia-associated proteins, such as the ANXA1 and complement factors. Genomics signatures from whole tissues would inevitably contain signatures of all cellular types. High-throughput tissue staining would be needed to clarify cellular subtypes in tumours. In our LATS1/2 cKO mouse model, although some of the tumour markers were highly present in the centre of the tumour mass, e.g., ANKRD1, Vimentin, CK18, nestin and Ki67, some were localised towards the edges of the tumour, e.g., HOPX, AMOTL2, AXL and microragal marker C3. For the latter category, further studies would be needed to distinguish between a cell autonomous effects of YAP1 expression in Cre recombinant cells from an effect of YAP1-induced tumours on the surrounding wild-type cells. Our results show that nlsYAP5SA and LATS1/2 cKO mouse models are similar to ependymoma in general, in terms of histology, immunoprofle and ultrastructural features. Specifically, nlsYAP5SA mouse tumours highly resemble YAP1-fusion ependymoma in gene expression. Our study suggests that nlsYAP5SA and LATS1/2 cKO mice are valuable models of YAP1-fusion ependymoma for biological and preclinical research. Small tumour sizes, early lethality and lack of pseudo-rosettes may have limitations. Complementary models, such as xenografts, could be used in conjunction with genetically defined mouse ependymoma models, to reach a more comprehensive understanding of ependymoma.

Methods
Human tissue and staining. Tissue of six patients were used in this study. Written consent was given by the patient (one adult RELA tumour) or by legal representatives (five children, three YAP and two RELA ependymoma). All tumours at diagnosis without any previous treatments such as chemotherapy or radiotherapy. For IHC tissue analysis of YAP1, HOPX and RELA expression, the patient tissues were deparaffinised and subject to Heat-Mediated Antigen Retrieval (pH 6.0 citric Buffer, microwave 23 min). The slides were incubated with YAP1 (1:400, Cell Signalling #14074), HOPX (1:250, Proteintech #11419-1-AP), NF-κB p65 (1:800, Cell Signalling #8242) in signal stain diluent overnight at 4 °C and 45 min in secondary antibody. Diaminobenzidine was utilised as the chromogen and the sections were counterstained with haematoxylin. Images were acquired with a Leica DM2500 LED.

Animals. Maintenance and handling of animals were performed under regulations of the Animal (Scientific procedures) Act 1986 of the United Kingdom by institutional ethical reviews. Mice were group housed and maintained on a 12 hr light/dark cycle, with food and water provided ad libitum. None of the experimental mice were immune compromised. Both male and female mice were used and randomly allocated to experimental groups according to genotypes.
a cylindrical specimen holder with Devcon S-210 epoxy glue. The mounting was done with the longitudinal axis of the brain section oriented vertically. Tomo- 
graphical images were acquired in an Xradia Versa 510 (Carl Zeiss SMT, San Jose, CA, USA). A low resolution scan was captured at sx/5kV, with x projections and a pixel size of x. The region of interest was identified using 3DViewXviewer software (Zeiss) and co-ordinates located for positioning in the Xradia Versa 510. A high-resolution scan was captured at sx/kV, with x projections and a pixel size of x. The data were exported as tiff, to be visualised in 3D in ClearVolume, a plug-in of the Fiji framework. The samples were then trimmed to a small trapezoid, excised from the resin block, and attached to a SBF SEM specimen holder using conductive epoxy resin (Circuitworks CW2400). Prior to commencement of a SBF SEM imaging session, the samples were coated with a 2 nm layer of platinum to further enhance conductivity.

SBF SEM data were collected using a View3DP (Gatan Inc., Pleasanton, CA) attached to a Sigma VP SEM (Carl Zeiss Ltd.). Inverted back-scattered electron images were acquired through the entire extent of the region of interest. For each 30 nm slice, a low resolution overview image (horizontal frame width 800 µm for 0.2/384 µm for 0.3; pixel size of 78 nm for 0.2/48 µm for 0.3; using a 2 µm dwell time) were acquired. The SEM was operated in variable pressure mode at 5 Pa. The 30 µm aperture was used, at an accelerating voltage of 2.5 kV for 0.3, 2 kV for 0.2. In total, 130 (for 0.2) and 400 (for 0.3) slices were necessary to get enough 3D information for an entire tumour. As data were collected in variable pressure mode, only minor adjustments in image alignment were needed. All the images were converted as tiff in Digital Micrograph (Gatan Inc.), and the tiff stacks were automatically aligned using TrakEM2, a plug-in of the Fiji framework (see supplementary). After SBF SEM, the samples were serial sectioned using a UC7 ultracutmicromtome (Leica Microsystems, Vienna, Austria) and 70 nm sections were picked up on Formvar-coated 2 mm slot copper grids (Gilder Grids Ltd., Grantham, UK). The first sections were viewed using a 120 kV Tecnai G2 Spirit transmission electron microscope (FEI, Eindhoven, Netherlands) and images were captured using an Orics CCD camera (Gatan Inc.).

Tissue collection from mice. For RNA sequencing and mass spectrometry control and nlsYAP5SA littermates from two matings were collected at the age of P11 (five animals per genotype). In addition, three age-matched C57BL/6 animals where used. The mice were killed by cervical dislocation, decapitated and the brain dissected out of the skull. Olfactory bulbs and cerebellum were split off and the hemispheres were separated. Although the right hemisphere was put into RNAlater (Qiagen, #76104), the left hemisphere was snap frozen in liquid nitrogen.

For western blotting, control and nlsYAP5SA were collected at the age of P11 (three animals per genotype). The mice were killed by cervical dislocation, decapitated and the brain dissected out of the skull. As before the olfactory bulbs and cerebellum were split off and the hemispheres were separated, flash freezing the right hemisphere in liquid nitrogen.

RNA extraction and sequencing. RNA of tissue stabilised in RNAlater was extracted using the RNeasy Mini Kit (Qiagen #74104) according to manufacturer protocol. The samples were quantified via a NanoDrop (ThermoFisher #84868). All samples were dried and resolubilised in 0.1% TFA prior to TMT labelling efficiencies were performed prior to quenching the labelling reaction. The samples were combined, pooled, dried and then cleaned on a C18 SepPak and aliquoted. Subsequently, one-tenth of the sample was resolubilised and fractionated into eight fractions using the Pierce High Purity Reversed-Phase Peptide Fractionation Kit (ThermoFisher #84868). All samples were dried and resolubilised in 0.1% TFA prior to LC-MS/MS analysis. Peptides were separated on a 50 cm, 75 µm I.D. Pepmap column and eluted directly into a hybrid quadrupole-linear ion trap mass spectrometer (Orbitrap Fusion Lumos ETD) with HCD MS2 fragmentation and in a second injection (7 µl each) analysed in MS3. Xcalibur software was used to control the data acquisition. The instrument was run in data-dependent acquisition mode with the most abundant peptides selected for MS/MS by HCD fragmentation. MaxQuant v1.6.6 was used to process the raw data acquired with a reporter ion quantification method. Adjusted reporter ion isotopic distributions according to the TMT Lot number. Protein database searching was done by Andromeda search engine using the Uniprot KB database of mammalian proteins (updated 08 Jan 2016). The protein group table was uploaded into Perseus 1.4.0.2 for subsequent statistical data analysis and data visualisation. A two-sided t-test was run with threshold set to FDR = 0.05 and s0 = 0.1 was used to display significance.

To investigate association of proteins to particular cell types the data were merged with existing data sets from57. From this publication, Supplementary Data 6 were used to identify protein association to isolated cell types and Supplementary Data 15 were used to identify protein association to cultured cell types. The data sets were partial merged (by matching gene name, integrating the log2 fold expression columns per cell type of the other data set into ours) and association to particular isolated CNS cell type was defined by expression value being a minimum of three in one of the four isolated cell types (astrocytes, microglia, neurons and oligodendrocytes) and with a value of at least double in one compared with any of the other cell types.

To investigate the resemblance of nlsYAPSSA mouse model to human embryonic subtypes, we utilised previously reported microarray data on differential gene expression at embryonic day 11.5 (E11.5) (Carl Zeiss SMT, San Jose, CA, USA) and nlsYAP5SA induced RELA mouse models of embryodroma15,16. The summarised expression of these subtype-associated, differentially expressed genes listed in Supplementary Data 6 from ref. 57 was compared with our complete gene list as well as our significantly differentially expressed gene list. Log2 fold change of our data set was plotted and the identified of specification was taken from the other data set using R 3.5.1.

For comparison of the nlsYAPSSA gene expression profile with a previously published human tumour data sets we downloaded the gene lists from either Glioblastoma, medulloblastoma and pilocytic astrocytoma15,57. Adjustments made prior to the comparative analysis: the human Affymetrix microarray report 21,050 probes of which 10,347 (49.2%) have a corresponding Ensemble identifier using the latest version of the Bioconductor annotation (hgu133aENSEMBL2PROBE). Of these, 5771 (27.4%) are assigned a gene symbol with an annotated human homologue with a well-defined Ensemble identifier. Almost all of these 5750 are included in the annotation that we use for the RNA-sequencing analysis. In all, 68 of these had duplicates leaving a total of 5682 RNA-sequencing probes from the original 21,050 gene probes of the Affymetrix microarray. In total, 5682 gene expression data (27% of initial Affymetrix probes) were compared with the sequencing results from Illumina. Normalisation was performed gene-wise; each data set was individually normalised by median centering and scaling by the standard deviation for each gene. These scaled data sets then were merged and the same median centering and standard deviation scaling applied, per gene, to this merged data set. The heat map was generated with the R package pheatmap in R 3.1.2. (details on correction for the detrimental reduction of the data set and normalisation protocols described above constitute considerable caveats for this experiment."

Mass spectrometry and data processing. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to analyse differences in protein expression in P11 nlsYAPSSA compared with control animals (n = 5 each). The left hemisphere of each animal was taken and lysed in lysis buffer containing 8 M Urea, 50 mM HEPES pH8.2, 10 mM Glycerol 2-phosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM sodium vanadate, 1 mM dithiothreitol (DTT), 1× Protease Inhibitor Cocktail (Roche), 1× Phosphatase inhibitor Cocktail, 1 µM okadaic acid and incubated for 30 minutes on ice. Protein concentration was determined with BCA protein assay according to manufacturer instructions. Protein concentration was reduced with DTT, IAA and CHAPS and the samples were quenched with DTT and digested with lysisC (Trypsin Endopeptidase, Mass Spectrometry Grade (Lys-C), 01-10506, Lot#CAR2347) and Trypsin (Pierce Trypsin Protease, MS, Grade, 90058, Lot#TK276718). After acidification with trifluoroacetic acid (TFA) the samples were desalted on C18 MacroSpin columns (Next Generation) followed by freeze drying them in liquid nitrogen prior to drying them on the speed vac. Subsequently, the samples were labelled with a TMT 10-plex kit (ThermoFisher, 90110, Lot#TF268160) according to manufacturer protocol. TMT labelling efficiency was confirmed (>99%) and mixing check was performed prior to quenching the labelling reaction. The samples were combined, pooled, dried and then cleaned on a C18 SepPak and aliquoted. Subsequently, one-tenth of the sample was resolubilised and fractionated into eight fractions utilising the Pierce High Purity Reversed-Phase Peptide Fractionation Kit (ThermoFisher #84868). All samples were dried and resolubilised in 0.1% TFA prior to LC-MS/MS analysis. Peptides were separated on a 50 cm, 75 µm I.D. Pepmap column and eluted directly into a hybrid quadrupole-linear ion trap mass spectrometer (Orbitrap Fusion Lumos ETD) with HCD MS2 fragmentation and in a second injection (7 µl each) analysed in MS3. Xcalibur software was used to control the data acquisition. The instrument was run in data-dependent acquisition mode with the most abundant peptides selected for MS/MS by HCD fragmentation. MaxQuant v1.6.6 was used to process the raw data acquired with a reporter ion quantification method. Adjusted reporter ion isotopic distributions according to the TMT Lot number. Protein database searching was done by Andromeda search engine using the Uniprot KB database of mammalian muscles sequences (dated 08 Jan 2016). The protein group table was uploaded into Perseus 1.4.0.2 for subsequent statistical data analysis and data visualisation. A two-sided t-test was run with threshold set to FDR = 0.05 and s0 = 0.1 was used to display significance.
Western blots. Flash frozen Mouse brains were homogenised by sonication in sample buffer containing 0.2 M DTT. Lysates were centrifuged at 13,000 g for 15 minutes and supernatants denatured at 95 C for 10 minutes. Equal amount of protein from the brain lysates were ran on a NuPAGE 4–12% Bis-Tris poly-acrylamide gels (Invitrogen) and then transferred to a polyvinylidene difluoride membrane (Millipore). After blocking in 5% non-fat milk in TBST for 60 minutes, the membrane was incubated with the primary antibodies overnight at 4 °C. The following antibodies were used: CTGF (1:1000, Abcam, #ab69922); CYR61 (1:1000, Abcam, #ab24448); GAPDH (1:5000, Abcam, #ab8245); GAP (1:1000, Sigma-Aldrich, #G6171); HOXA (1:10000, ProteinTech, #11419-1-AP); TAZ (1:1000, Abcam, #ab84927); Vimentin (1:5000, Abcam, #ab52457) and YAPI (1:2000, Cell Signalling, #14074). Subsequently, membranes were incubated with the horseradish peroxidase-conjugated (HRP) secondary antibody for 60 minutes—HRP-conjugated mouse (1:10,000; Jackson #711-035-152). Finally, the membrane was developed with ECL system (Pierce) and visualised by a chemiluminescence detection system (Amer sham WB System). Western blot analysis was performed using ImageJ software (version 1.52r).

Tumour measurements. Three animals of LATS1/2 cKO, Mutantf/+ , Mutantf/f and rescue were subject to detailed tumour size analysis. The brains were sectioned coronal using the vibratome with 50 nm thickness. Every second section was stained with a tumour marker (Vimentin, YAPI or GAPI) to aid tumour identification and DAPI. All sections were evaluated and imaged using the Olympus IX83. The area of each identified tumours was measured in all sections using Fiji and volume was calculated by section thickness and taking gap sections into account. Kruskal–Wallis test was performed to evaluate if the tumour size is significantly different from any group to another (p = 0.0009). Post hoc the Dunn’s multiple comparisons test was performed to identify which groups are significant from each other.

In addition, the effect of change of genotype on estimates volume was determined by encoding genotype as an ordered factor (Mutantf/f < Mutantf/+ < LATS1/2 cKO) and a threshold value of 0.02. Finally, the membrane was developed with ECL system (Pierce) and visualised by a chemiluminescence detection system (Amer sham WB System). Western blot analysis was performed using ImageJ software (version 1.52r).

Statistical analysis and reproducibility. Statistical analysis was performed using GraphPad Prism 7 software. R 3.3.1 or R 3.5.1 using statistical tests as indicated in the text. Statistical significance was visualised as: ns = not significant (p > 0.05), * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

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Author contributions
N.E. and S.K.U. conceived the project idea. N.E., F.R., M.-C.D., L.C., B.T., H.R.F, A.P.S. performed electron microscopy experiments and A.T.L. performed western blots. S.C. and M. & A.B. performed targeted sequencing analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 4–29 (2009).

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Competing interests
The authors declare no competing interests.

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