The **HHIP-AS1** lncRNA promotes tumorigenicity through stabilization of dynein complex 1 in human SHH-driven tumors

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Most lncRNAs display species-specific expression patterns suggesting that animal models of cancer may only incompletely recapitulate the regulatory crosstalk between lncRNAs and oncogenic pathways in humans. Among these pathways, Sonic Hedgehog (SHH) signaling is aberrantly activated in several human cancer entities. We unravel that aberrant expression of the primate-specific lncRNA **HedgeHog Interacting Protein-AntiSense 1** (**HHIP-AS1**) is a hallmark of SHH-driven tumors including medulloblastoma and atypical teratoid/rhabdoid tumors. **HHIP-AS1** is actively transcribed from a bidirectional promoter shared with SHH regulator **HHIP**. Knockdown of **HHIP-AS1** induces mitotic spindle deregulation impairing tumorigenicity in vitro and in vivo. Mechanistically, **HHIP-AS1** binds directly to the mRNA of **cytoplasmic dynein 1 intermediate chain 2** (**DYNC1I2**) and attenuates its degradation by hsa-miR-425-5p. We uncover that neither **HHIP-AS1** nor the corresponding regulatory element in **DYNC1I2** are evolutionary conserved in mice. Taken together, we discover an lncRNA-mediated mechanism that enables the pro-mitotic effects of SHH pathway activation in human tumors.
Sonic hedgehog (SHH) signaling plays a pivotal role in promoting oncogenesis, tumor growth and progression. It is aberrantly activated in various common cancers in adults, including basal cell carcinoma (BCC), but also in pediatric neoplasms, including rhabdomyosarcoma and brain tumors such as medulloblastoma (MB) and atypical teratoid/rhabdoid tumors (ATRT). Pediatric brain tumors, like MB and ATRT, are the most common solid malignancies of childhood and the leading cause of cancer-related death in children. Both entities are highly heterogeneous and can be segregated into distinct subgroups by virtue of their divergent molecular characteristics. Notably, such classification, which is primarily based on inter-group differences detected at multi-omics level, is of clinical utility, as it correlates with specific clinicopathological and prognostic patterns. Specifically, MB comprises four main subgroups, designated as Wingless (WNT), SHH, Group 3 and Group 4. WNT and SHH MBs are named according to the signaling pathways that drive their formation and progression. Conversely, the other two MB subgroups are less defined in terms molecular etiology, although Group 3 MB acquires MYC amplification and/or overexpression, while Group 4 MB commonly show activation of the receptor tyrosine kinase signaling through aberrant expression of ERBB4 and the phosphorylated form of the tyrosine–protein kinase SRC. In the case of ATRT, three subgroups have been defined, namely tyrosine kinase (TYR), MYC and SHH, according to the distinctive overexpression of TYR or MYC genes, or the activation of SHH signaling.

Targeting the SHH signaling to extinguish its mitogenic effects in MB has shown efficacy in pre-clinical animal models and in humans. However, the clinical use of SHH inhibitors, such as the G protein–coupled receptor smoothened (SMO) antagonist Vismodegib (GDC-0449), is limited due to toxicity or emergence of resistance across species. Nevertheless, lncRNAs are known to show poor conservation across species and to deregulate their human cancer. In all, we discover a lncRNA circuitries in human cancer. In all, we discover a lncRNA-dependent blockage of endogenous RNA mechanism as an additional layer of epigenetic regulation mediated by HHIP-ASI as a human-specific target gene in SHH-dependent cell progression.

**Results**

**Overexpression of the long non-coding RNA HHIP-ASI is a hallmark of human SHH-driven tumors.** To discover global SHH-dependent gene expression patterns in cancer, we first determined differentially expressed transcripts in RNA sequencing data by comparing SHH MB (n = 58) to non SHH MB subgroup samples (n = 164) (Platform, R2). Our approach confirmed several known protein-coding SHH mediators including GLI1, GLI2, HHIP and Atonal BHLH Transcription Factor 1, and revealed lncRNAs deregulated in SHH MB (Fig. 1a, Supplementary Data file 1). Among these candidates, we identified HHIP-ASI as the most specifically overexpressed lncRNA in SHH MB (Fig. 1a, Supplementary Data file 1) without protein-coding potential (Fig. S1a). We next investigated whether HHIP-ASI was similarly overexpressed in other tumor entities with aberrant activation of the SHH signaling. Comparative expression analysis confirmed specific HHIP-ASI overexpression in SHH-driven entities including ATRT (Fig. 1b), cutaneous BCC (Fig. S1b) and rhabdomyosarcoma (Fig. S1c) compared to normal and cancerous control tissues. Consistent with its overexpression, we next revealed active transcription of HHIP-ASI from a hypomethylated promoter shared with HHIP, a well-established regulator of the SHH pathway and whose genomic localization shows a head-to-head orientation with the HHIP-ASI locus, exclusively in SHH MB using both H3K27ac ChIP-sequencing (Fig. 1c) and high-resolution DNA methylation data (Fig. 1d and S1d). Transcriptome-wide correlation analyses consistently revealed that HHIP and HHIP-ASI co-expressed in MB (n = 167, r = 0.990, p < 0.001, Fig. S1e) and in ATRT (n = 49, r = 0.836, p < 0.001, Fig. S1f). Furthermore, we could reveal that the two genes exhibited remarkably high and significant correlative co-expression scores in multiple expression datasets, including other cancer entities and control tissues (n = 39,990; p < 0.001, Fig. 1e). These observations prompted us to hypothesize that HHIP-ASI and HHIP may indeed share a unique bidirectional promoter. Therefore, we performed a luciferase reporter assay and revealed the existence of forward and reverse activity of the promoter region in vitro (Fig. 1f), suggesting that promoter hypomethylation may equally drive overexpression of both HHIP-ASI and HHIP transcripts in SHH-driven tumors.

Since HHIP-ASI was not previously described in murine models of SHH-driven tumors and lncRNAs are only partially conserved through species, we investigated to which extent HHIP-ASI was conserved across vertebrates. Remarkably, genome-scale comparisons revealed that HHIP-ASI was only conserved in primates and not in rodents (Fig. 1g and S1g), explaining why this lncRNA has not been identified in animal models so far.

**HHIP-ASI is functionally required in human SHH-driven brain tumors.** Since HHIP-ASI overexpression is a hallmark in SHH-driven tumors, we investigated whether HHIP-ASI expression was dependent on SHH signaling in tumor cells. Pharmacological activation of SMO receptor using SAG (SMO agonist) increased HHIP-ASI expression in two independent tumor cell lines, Daoy and CHLA-266, and one primary cell culture, HHU-ATRT1 (Fig. 2a) as well as in non-tumor cells with SHH activation,
**Fig. 1** The long non-coding RNA HHIP-AS1 is a hallmark of human SHH-driven tumors. **a** Long non-coding RNA (lncRNA) expression profiles in SHH-driven medulloblastoma (MB; right side) versus non SHH-driven MB (left side). The volcano plot illustrates the distribution of statistical significance (y-axis) and relative expression level (x-axis) for the lncRNAs profile. The red dot indicates HHIP-AS1 (Hedgehog Interacting Protein-Anti-Sense 1). Statistical analysis was performed using one-way ANOVA with post-hoc Tukey HSD; ***p < 0.001. **b** Violin plots display the expression level of HHIP-AS1 according to an integrative transcriptomic analysis of 3492 samples from neoplastic brain tissues with SHH activation (SHH MB and atypical teratoid/rhabdoid tumors (ATRT)) or without commonly reported SHH activation (brain tumors) and normal brain without tumor. Statistical analysis was performed using Kruskal-Wallis Test with Dunn’s Multiple Comparison Test; ***p < 0.001. Red dots = SHH-driven entities; black dots = non SHH-driven tumors and control tissue. **c** H3K27ac ChIP-sequencing profile on HHIP-AS1 and HHIP loci in the four MB subgroups. Bar graph indicates the expression level of HHIP-AS1 in the corresponding MB subgroups. Error bars represent ± SEM. **d** Scatter plot representing the degree of DNA methylation (B value) of the potential promoter region in relation to HHIP-AS1 expression levels in SHH MB (red dots) and other MB subgroups (black dots). Statistics were done by Pearson correlation. **e** Scatter plot displaying expression correlation of HHIP and HHIP-AS1 across datasets (n = 351). Mean expressions of both transcripts from 39,090 samples were analyzed in their respective datasets and plotted with error bars representing the SEM for both genes. Statistics were done by Pearson correlation. **f** The bar graph indicates the relative luciferase activity of empty luciferase vector (pLUC) or pLUC containing the cloned HHIP promoter sequence orientation (fw = forward, rv = reversed). The results are presented as the mean ± SD of three independent experiments. Student’s two-sided t-test; **p < 0.01, ***p < 0.001. **g** Identification of evolutionarily conserved regions corresponding to critical regulatory elements in large (>1 Mb), highly conserved gene desert regions flanking the human HHIP-AS1 gene located at chromosome 4q31.21 with two exons. Source data and exact p-values are provided as a "Source Data file".
Fig. 2 The long non-coding RNA HHIP-AS1 is functionally required in human SHH-driven brain tumors. a The relative gene expression levels of HHIP-AS1 and the SHH target gene GLI1 were tested in tumor cell lines (Daoy and CHLA-266) and in primary tumor cell cultures (HHU-ATRT1) upon pharmacological activation (SAG, Smoothened agonist) or inhibition (CYC, cyclopamine) of the SHH pathway. b Relative gene expression levels of indicated genes as measured by qRT-PCR upon transient knockdown of GLI1 and GLI2 in the depicted cell models normalized to control (si-negative-POOL; gene expression of target genes were normalized to housekeeping genes: HPRT, GUSB and PPIA). c Proliferation rate of Daoy, CHLA-266 and HHU-ATRT1 was measured by EdU incorporation upon transient (si-HHIP-AS1) or stable HHIP-AS1 knockdown normalized to control. d Self-renewal capacity of Daoy, CHLA-266 and HHU-ATRT1 was measured by colony formation assay upon transient (si-HHIP-AS1) or stable (sh-HHIP-AS1#1 and sh-HHIP-AS1#2) HHIP-AS1 knockdown normalized to control. In panel c + d corresponding controls (either with si-negative-POOL or sh-scr transfected Daoy, CHLA-266 and HHU-ATRT1 cells) were set to 100% and levels of knockdowns were calculated accordingly. e Proliferation rate of primary SHH MB cultures derived from freshly resected tumors (n = 2 patients) measured by EdU incorporation upon transient knockdown of HHIP-AS1 (si-HHIP-AS1) normalized to control (si-negative-POOL). f Cell viability of these primary SHH MB cultures derived from freshly resected tumors (n = 2 patients) measured by CellTiter-Glo upon transient knockdown of HHIP-AS1 (si-HHIP-AS1) normalized to control (si-negative-POOL). g Proliferation rate of SHH MB PDX cells (ICN-MB12) determined by BrdU incorporation and Ki67 immunostaining after transient knockdown of HHIP-AS1 (sh-HHIP-AS1#1) normalized to control. Bar graphs of panels a + b are presented as the mean ± SD, panels c–g are presented as the mean ± SEM of at least three independent experiments and corresponding controls were set to 100%. Student’s two-sided t-test; ***p < 0.001; **p < 0.01; *p < 0.05. Source data and exact p-values are provided as a “Source Data file”.
namely neuronal stem cells (NSC, Fig. S3a). Conversely, inhibition of SMO using cyclopamine (SMO antagonist) significantly reduced HHIP-AS1 expression in all cell models (Fig. 2a and S3a). Activation and inhibition of the SHH pathway were confirmed by quantification of GLI expression level through qRT-PCR (Fig. 2a and S3a) and immunoblotting (Fig. S2a–c). GLI proteins are known to function as regulators of the SHH transcriptional response, hence we next examined whether GLI could affect HHIP-AS1 expression. Transient knockdown of GLI1 and GLI2 reduced HHIP-AS1 and HHIP expression in all of the above-mentioned SHH-cancer cell models (Fig. 2b and S2), while on the other hand overexpression of GLI1 (Fig. S2d) resulted in increased expression of HHIP and HHIP-AS1 (Fig. S3b), confirming HHIP-AS1 as a target gene of SHH signaling. To evaluate the functional impact of HHIP-AS1, we silenced its expression in vitro using either siRNAs or shRNAs directed against this lncRNA. Strikingly, knockdown of HHIP-AS1 (Fig. S3c) resulted in reduced proliferation, monitored by EdU incorporation (Fig. 2c), as well as reduced clonogenicity (Fig. 2d) of Daoy, CHLA-266 and HHU-ATRT1. In addition, cell viability, assessed through metabolic assay (CellTiter-Glo), was diminished in the SHH MB cell line Daoy and in ATRT cell line CHLA-266 upon HHIP-AS1 silencing (Fig. S3d). We could verify these phenotypic changes after transient HHIP-AS1 knockdown in additional SHH-driven cell models, namely CHLA-04 (ATRT), RH30 (rhabdomyosarcoma), NSC and U1226 (BCC, Fig. S3e). Importantly, we also confirmed that HHIP-AS1 depletion reduced proliferation and viability in primary cultures from two SHH MB patients (Fig. 2e and f) and impaired proliferation of cultured cells of ICN-MB12 (SHH MB patient derived xenograft model; Fig. 2g).

Notably, overexpression of HHIP-AS1 rescued the reduced proliferation and viability (Fig. S3f), highlighting that HHIP-AS1 is not only aberrantly overexpressed in SHH-driven tumors, but also functionally relevant in these malignancies. Conversely, transfection of HHIP-AS1 siRNAs into non SHH MB cells (HD-MB03 and CHLA-01) with low expression of HHIP-AS1 (Fig. S3g) did not result in reduced proliferation and viability (Fig. S3h), ruling out potential off-target effects and confirming that the functional relevance of HHIP-AS1 is restricted to SHH-driven tumors. Remarkably, HHIP expression was not affected on mRNA or protein levels upon stable HHIP-AS1 depletion (Fig. S4a–c). Therefore, we rule out a cis-regulatory effect of HHIP-AS1 on HHIP in our MB and ATRT models.

Identification and functional validation of HHIP-AS1 downstream targets reveals that HHIP-AS1 binds to the mRNA of DYNCl12. In order to decipher the molecular mechanism controlled by HHIP-AS1, we investigated the impact of HHIP-AS1 knockdown on both transcriptome and proteome in two independent cell models (Daoy and CHLA-266). Interestingly, our integrative proteogenomic analysis identified two candidates that were highly and consistently perturbed upon HHIP-AS1 knockdown in both models, namely hydroxyprogesterone 17-beta dehydrogenase 10 (HSD17B10) and DYNCl12 (Fig. 3a, RNA sequencing data: NCBI GenBank GSE140741 and proteomic data: ProteomeXchange PRIDE database #PXD016550). We decided to focus on DYNCl12 based on its co-expression with HHIP-AS1 in primary MB samples (r = 0.336; p < 0.001, Fig. 3b), as well as its high expression level specifically in SHH MB subgroup (Fig. S5a) and its known functions in neurodevelopment and cell cycle progression. HSD17B10 did not show a significant correlation with HHIP-AS1 in primary MB samples (Fig. S5b).

We validated by qRT-PCR and immunoblots that HHIP-AS1 depletion reduced the mRNA and protein expression of DYNCl12 in Daoy, CHLA-266 and HHU-ATRT1 cells (Fig. S5c and d). To determine the underlying molecular mechanism, we investigated a potential interaction between HHIP-AS1 and DYNCl12 mRNA. First, we uncovered that both RNAs are co-localized in cells using RNA fluorescence in situ hybridization (Fig. 3c and S5e) and this co-localization can be enhanced or disrupted via SHH activation or inhibition, respectively (Fig. S5f). Second, we confirmed a direct interaction in each of our three cell models using an RNA-RNA-centric-pulldown probe set directed against HHIP-AS1 mRNA, which led to a specific enrichment for DYNCl12 mRNA compared to negative controls (Fig. 3d).

Finally, we computationally derived the sequence of HHIP-AS1 that is predicted to pair with DYNCl12 mRNA (Supplementary Table S1), identifying a 24 nucleotides long region (named HHIP-AS1 bind) able to bind the 5’UTR of DYNCl12. Furthermore, using bio-layer interferometry, we found that HHIP-AS1 bind could physically interact with DYNCl12 mRNA (Fig. 3e). In contrast, a negative control sequence of HHIP-AS1 (named HHIP-AS1 no), carrying the same GC content and number of nucleotides as HHIP-AS1 bind, but devoid of any in silico pairing potential to DYNCl12 mRNA, did not show any binding activity (Fig. S5g).

Remarkably, overexpression of HHIP-AS1 resulted in extended half-life of DYNCl12 mRNA (Fig. 3f and S5h), compared to a control condition where HHIP-AS1 was used. All these findings confirm the existence of a functional and direct physical interaction between these two RNAs in vivo and in vitro, which ultimately regulates DYNCl12 expression levels. In order to elucidate whether the functional interaction between HHIP-AS1 and DYNCl12 mediates the pro-proliferating phenotype controlled by HHIP-AS1, we evaluated the functional impact of DYNCl12 depletion in our cell models. Interestingly, transient DYNCl12 knockdown resulted in reduced proliferation and viability to a similar degree as observed upon HHIP-AS1 depletion (Fig. S6a and b). More importantly, when we overexpressed DYNCl12 using CRISPR-Cas9-based activation in HHIP-AS1-transiently depleted cells, both proliferation and viability were restored (Fig. 3g and h), indicating that HHIP-AS1 exerts its pro-proliferative effects by controlling DYNCl12 abundance in tumor cells.

**The interaction between HHIP-AS1 and DYNCl12 promotes mitosis.** Cytoplasmic dynein-complex 1 has been implicated in various phenotypes including cargo transport on cytoplasmic microtubules and it was recently reported that DYNCl12 loss disrupts mitotic spindle organization in zebrafish neural progenitor cells. Thus, we hypothesized that HHIP-AS1 loss may cause a similar effect in human tumor cells by reducing DYNCl12 availability. Indeed, we found that transient knockdown of HHIP-AS1 or DYNCl12 via siRNAs transfection significantly and consistently altered mitotic spindle organization in cells compared to siRNA control-transfected cells (Fig. 4a and b; Fig. S6c and d), leading to more DNA damage in the mitotic cells as well (Fig. S6e). Moreover, using two different shRNAs against HHIP-AS1, we could confirm the alteration in mitotic spindle organization in Daoy and CHLA-266 (Fig. 4c and d) upon stable HHIP-AS1 depletion. More importantly, induced overexpression of DYNCl12 as well as of HHIP-AS1 with CRISPR-Cas9-based activation, rescued the mitotic spindle organization (Fig. 4e and f) in the context of transient HHIP-AS1 knockdown, supporting that HHIP-AS1 promotes proliferation through mitotic spindle stabilization by controlling DYNCl12 abundance.

**HHIP-AS1 blocks endogenous hsa-miR-425-5p function to maintain DYNCl12 level.** To further mechanistically elucidate how HHIP-AS1 stabilizes DYNCl12 mRNA, we evaluated the genetic sequence of RNA-RNA interaction. Our analysis revealed six potential miRNA binding sites (Supplementary Table S2)
Fig. 3 Identification and functional validation of HHIP-AS1 downstream targets reveals that HHIP-AS1 binds to mRNA of DYNC1I2. 

a. Scatter plot indicates the correlation analysis of RNA sequencing (x-axis) and protein mass spectrometry (y-axis) data in two different cell models (Daoy and CHLA-266) upon HHIP-AS1 knockdown (using sh-HHIP-AS1#1 and sh-HHIP-AS1#2) versus control cells (sh-scr, n = 3 independent samples per condition and cell model). b Scatter plot displaying expression correlation of DYNC1I2 and HHIP-AS1 sequencing data comparing FPKM expression values in 167 MB patient samples. Samples are color coded for MB subgroups. Statistics were done by Pearson correlation. c Representative image of co-localization of HHIP-AS1 and DYNC1I2 mRNA in Daoy obtained through two-color fluorescence in situ hybridization (FISH). White frame indicates the location of the zoom out picture at the right side. Green: HHIP-AS1 lncRNA, red: DYNC1I2 mRNA, blue: DAPI, Nucleus. Scale bar: 5 µm. This experiment was repeated twice with similar results. d Enrichment of DYNC1I2 mRNA upon HHIP-AS1 raPOOL pulldown in Daoy and CHLA-266 cell lines and HHU-ATRT1 primary cells. Bar graphs are presented as the mean ± SD of three independent experiments. e Bio-Layer interferometry was used for detecting direct interaction between DYNC1I2 mRNA and HHIP-AS1. f DYNC1I2 mRNA stability upon transfection of a control (HHIP-AS1neg) or the HHIP-AS1 interacting sequence (HHIP-AS1bind). Calculation was done in comparison to the mRNA level at time point “0 h” in each condition. Data are presented as the mean ± SEM of five independent experiments; Student’s two-sided t-test; ***p < 0.001. g The immunoblot shows a representative blot of DYNC1I2 protein expression in control (Ctrl) or DYNC1I2-overexpressing (DYNC1I2 OE) cells. ACTB immunoblotting was used as loading control. This experiment was done twice with similar result. Source data and exact p-values are provided as a “Source Data file".

h Bar graph indicating the proliferation rate or viability of Daoy cells in control condition (Ctrl), upon overexpression of DYNC1I2 (DYNC1I2 OE) and upon transient HHIP-AS1-knockdown (si-HHIP-AS1) in DYNC1I2 overexpression (DYNC1I2 OE + si-HHIP-AS1). Data are represented as the mean ± SD of at least six independent experiments normalized to the control condition. Student’s two-sided t-test; ***p < 0.001; n.s. not significant.
HHIP-AS1 transient
n.s. not significant
experiments (with a total condition or upon rescue of HHIP-AS1 by endogenous gene transcriptional activation through CRISPR-Cas9 technology in the context of transient independent shRNAs (sh-)
expression of these miRNA candidates with control (Ctrl
DYNC1I2 mRNA may interfere with miRNA-dependent regula-
within the predicted interaction region between HHIP-AS1 and DYNC1I2 mRNA (Fig. 5a), suggesting that HHIP-AS1 binding to DYNC1I2 mRNA may interfere with miRNA-dependent regulation of DYNC1I2 expression. Therefore, we correlated the expression of these miRNA candidates with DYNC1I2 using RNA sequencing data of 167 primary MB samples. Out of these six miRNAs, four were not detected in MB patient samples, while two miRNAs showed expression in MB patient samples, namely hsa-miR-425-5p (see binding site in Fig. 5a) and hsa-miR-1915. Notably, hsa-miR-425-5p expression level showed no differences across molecular subgroups of MB patients and cell models (Fig. S7a and b), but demonstrated anti-correlation with DYNC1I2 expression in patient samples \( r = -0.312; p < 0.001, \) Fig. S7c) compared to hsa-miR-1915 (Fig. S7d). After dividing the dataset into SHH and non SHH MB, the anti-correlation was only maintained in non SHH MB samples \( r = -0.303; p < 0.001, \) Fig. S7e). We therefore tested whether a functional relationship existed between hsa-miR-425-5p and DYNC1I2. First, we cloned the 5’UTR of DYNC1I2 in front of a luciferase reporter and found that hsa-miR-425-5p functionally binds this sequence causing a reduction of luciferase expression (Fig. 5b), hence confirming our previous in silico prediction (Fig. 5a). Importantly, this effect was abrogated upon mutation of the miRNA binding sequence on DYNC1I2 5’UTR (Fig. 5b). Second, we demonstrated that hsa-miR-425-5p inhibition significantly increased DYNC1I2 mRNA levels upon stable HHIP-AS1

Ac-tubulin = Microtubuli
DAPI = Chromosomes
Pericentrin = Spindle pole

Fig. 4 The interaction between HHIP-AS1 and DYNC1I2 promotes mitosis. a Representative images of immunofluorescence analysis show spindle assembly in mitotic cells by immunostaining for acetylated tubulin (Ac-tubulin, red) and pericentrin (green). Chromosomes are visualized with DAPI (blue). White scale bar: 5 µm. b-d Bar graphs display the percentage of dividing cells displaying normal, disrupted or multipolar spindle mitosis under control (Ctrl = si-negative-POOL or sh-sc) condition and DYNC1I2- or HHIP-AS1-knockdown using siRNAs for transient knockdown in Daoy (b) or two independent shRNAs (sh-HHIP-AS1#1 and sh-HHIP-AS1#2) for stable HHIP-AS1 knockdown in Daoy (c) and in CHLA-266 (d). e Bar graphs showing the percentage of dividing cells displaying normal, disrupted or multipolar spindle mitosis under control (Ctrl) condition or upon rescue of DYNC1I2 expression by endogenous gene transcriptional activation through CRISPR-Cas9 technology in the context of transient HHIP-AS1 knockdown (DYNC1I2 OE + si-HHIP-AS1) in Daoy cells. f Bar graphs showing the percentage of dividing cells displaying normal, disrupted or multipolar spindle mitosis under control (Ctrl) condition or upon rescue of HHIP-AS1 expression by endogenous gene transcriptional activation through CRISPR-Cas9 technology in the context of transient HHIP-AS1 knockdown (HHIP-AS1 OE + si-HHIP-AS1) in Daoy cells. All bar graph values are representative of n at least ten independent experiments (with a total n > 50 counted mitotic cells) and data are shown as mean ± SEM. Student’s two-sided t-test; ***p < 0.001; **p < 0.01; *p < 0.05; n.s. not significant. Source data and exact p-values are provided as a "Source Data file".
knockdown in three SHH-driven cell models, while transfection of a negative control miRNA inhibitor did not restore DYNC1I2 expression (Fig. 5c). This effect could be also observed after in vitro transfection of stable sh-HHIP-AS1 Daoy with only HHIP-AS1\textsuperscript{bind} sequence resulting in higher expression of DYNC1I2 mRNA independent of hsa-miR-425-5p inhibition (Fig. S8a), compared to a control condition where HHIP-AS1\textsuperscript{neg} was used. Interestingly, the corresponding regulatory element of DYNC1I2, where hsa-miRNA-425-5p is binding, is not evolutionary conserved in mice (Fig. S9). Third, inhibition of hsa-miR-425-5p rescued the decreased proliferation phenotype in Daoy and CHLA-266 obtained with HHIP-AS1 knockdown (Fig. 5d).

Thus, our data unravel a regulatory network requiring HHIP-AS1 to bind to DYNC1I2 mRNA, to prevent DYNC1I2 depletion...
mediated by binding of hsa-miRNA-425-5p to its 5'UTR. Together, our correlative data analysis approach in primary MB samples combined with in silico predictions and mechanistic validation, uniformly point to a previously poorly explored regulatory function of lncRNAs which consists in binding to a specific miRNA and thereby blocking endogenous miRNA binding sites.

Loss of HHIP-AS1 extends survival in SHH-driven brain tumors in vivo. We next evaluated whether targeting HHIP-AS1 affected tumor growth in vivo. To this end, we initially utilized two well-established orthotopic brain tumor models with aberrant SHH signaling activation. Remarkably, stable knockdown of HHIP-AS1 in both orthotropically engrafted Daoy and CHLA-266 cells significantly extended the survival of recipient mice compared to corresponding isogenic controls (Daoy, p = 0.0045; CHLA-266, p = 0.0011; Fig. 6a and b). Repeated luminescence measurement indicated that the loss of HHIP-AS1 affected tumor formation in vivo consistently (Fig. S10a–c). We next aimed at validating our finding using a well-characterized patient-derived xenograft model of SHH MB. We transduced cells from the SHH-Med-1712-FH PDX model in vitro with sh-HHIP-AS1 or control shRNA prior to transplantation into the cerebella of recipient mice. Knockdown of HHIP-AS1 and corresponding DYNC1I2 reduction was confirmed in this model via qRT-PCR (Fig. S10d).

Interestingly, silencing of HHIP-AS1 in injected PDX cells delayed the appearance of signs of morbidity in animals, whose mean survival was significantly extended compared to control mice (p = 0.0011; Fig. 6c). Lastly, HHIP-AS1 depletion markedly reduced cell proliferation index in Med-1712-FH PDX tumors compared to control tumors, when these samples were tested for Ki67-immunoreactivity (Fig. 6d). Accordingly, we observed more differentiated cells in tumor tissue compared to control (Fig. S11a). In line with our in vitro data, we detected no change in caspase activity (cleaved caspase) in Med-1712-FH PDX tumors (Fig. S10e and S11b). However, we observed more DNA damage in tumor tissue compared to control (Fig. 6e) and in NSC (Fig. S9f) after HHIP-AS1 depletion. Overall, these results provide compelling evidence that HHIP-AS1 promotes tumorigenicity of SHH-driven human tumors in vivo.

Discussion

Until now the complexity of the human SHH signaling pathway and regulatory feedback loops is not fully understood. Specifically, the precise knowledge of the molecular mechanisms underlying SHH pathway pro-oncogenic activity in human cells is urgently required. To this aim, the widespread use of genetic animal models of SHH-driven malignancies has been successful at identifying and describing a large number of protein-coding genes and protein post-translational modifications implicated in regulation and downstream functions of SHH signaling in tumors43–46. Nevertheless, despite accounting for ~70% of the transcribed genome in humans47, mechanistic insights into the role of lncRNAs in SHH-driven cancer biology have been gained only for a very few species. A reason for this poor consideration relies on the general low level of inter-species conservation of lncRNAs, which has inevitably hindered their discovery and functional characterization in the commonly used genetic rodent models. In our study, we employed a large comparative transcriptome analysis approach across several thousands of human normal and cancerous tissues, and identified HHIP-AS1 as a lncRNA that is specifically upregulated in SHH-driven tumors and functionally required for mediating the pro-proliferative effects of oncogenic SHH signaling. Interestingly, we found that HHIP-AS1 constitutes a downstream transcriptional target of SHH signaling, providing an explanation to its restricted and elevated expression in SHH-driven entities. Specifically, we uncovered that HHIP-AS1 transcription is initiated from a bidirectional promoter shared with the known SHH-pathway regulator HHIP. However, unlike HHIP or other key protein-coding gene of the SHH pathway, HHIP-AS1 is poorly conserved across vertebrates, showing high sequence similarity between humans and other primates, but substantially no conservation between humans and rodents. Therefore, our findings pinpoint the importance of species-specific evaluation of oncogenic signaling pathways, and emphasize how some key regulatory networks or pro-oncogenic programs may not be faithfully recapitulated by rodent tumor models, despite their generally well-accepted value to study human pathogenic processes. Furthermore, by using a combined proteomic and RNA sequencing approach, we uncovered that HHIP-AS1 acts in trans by regulating the expression of DYNC1I2, which encodes for an intermediate chain of the cytoplasmic dynein-complex 1, and not in cis by regulating HHIP48. We, among others, could already demonstrate the power of integrated proteogenomic approaches, showing that proteomic combined with transcriptomic profiles provides a profound insight into active oncogenic gene expression regulation in pediatric brain tumors and other cancers46,25.

The involvement of the cytoplasmic dynein-complex 1 provides an intriguing aspect of SHH pathway regulation and function. Indeed, dynein motor complexes have been long implicated in the activity of SHH signaling. However, this association was only restricted to cytoplasmic dynein-complex 2, due to its requirement at the level of primary cilia in mediating ciliary retrograde transport49. By characterizing the functional interaction between HHIP-AS1 and DYNC1I2 in cancer cells and in NSC, we unraveled the existence of an additional layer whereby SHH signaling sustains cell growth and progression. SHH signaling is already known to directly promote cell cycle progression by primarily activating the expression of MYCN and Cyclin D genes, which drive cells toward the G1/S transition50. By sustaining the transcription of HHIP-AS1, the SHH signaling guarantees effective spindle assembly and chromosome segregation through maintenance of appropriate levels of DYNC1I2. Interestingly, we could observe in cells with HHIP-AS1 depletion that the dysregulation of mitosis led to more DNA damage in mitotic cells. Although the DNA damage response and the mitotic cell division pathways were thought to be distinct and unrelated, it was shown in several studies that mitotic cells can experience DNA damage either endogenously due to unrepaird premiotic damage or exogenously from cancer therapies amongst other causes51–53. As we demonstrate that SHH exerts a control on cell division during the time of mitosis in cancer cells and in NSC, therapeutic limitations may exist. Targeting the SHH signaling pathway seems to provide a highly innovative therapeutic option for a broad variety of cancers. Nevertheless, as SHH pathway inhibitors seem to be safe in adults, this still remains to be shown in children treated for MB and taking into consideration our findings in NSCs. While the introduction of targeted therapies represents an exciting era in personalized medicine, compounds that target developmental signaling pathways (including SHH signaling) should be carefully evaluated for specific toxicities and their potential benefits.

Depleting cells of HHIP-AS1 or blocking its interaction with DYNC1I2 mRNA exposes the latter to hsa-miRNA-425-5p-mediated degradation, thereby compromising mitotic fidelity and consequently blocking cell progression in vitro and in vivo. As the power of miRNA in regulation of mRNA has been explored elaborately, we are beginning to recognize also the complex interactions of miRNAs and lncRNAs and ensuing RNA regulatory networks. Our work provides a previously unmatched molecular understanding of lncRNA function by actively competing with a miRNA binding site on a target mRNA. We show
that the IncRNA HHIP-ASI constitutes a so far unexplored target gene of SHH signaling, which enables the pro-mitotic effects of this pathway by blocking hsa-miR-425-5p-dependent inhibition of DYNC1I2 expression. This mechanism is of fundamental relevance in SHH-driven neurogenesis and tumorigenesis. Further experiments are required to elucidate the role of the regulatory HHIP-ASI/DYNC1I2/miR425 axis and its potential pro-mitotic effects, further experiments are required to elucidate the relevance in SHH-driven neurogenesis and tumorigenesis.

**Methods**

**Animal models.** For the orthotopic brain tumor models we used 8 week old female NMRI-Foxn1nu/nu that were purchased from Janvier Labs, Le Genest-Saint-Ise, France. We followed both European and national regulations for animal housing, care and experimentation (Directive 86/609). The use of animals was approved by the reporting ethical committee and the ministry under the agreement #03130.20. Ethics committee: CCEA-IC, Instances: Higher Education of the Ministry for Education and Research (France).

**Cell culture.** The cell line Daoy was obtained from American Type Culture Collection (ATCC, Manassas, VA) and was grown in Dulbecco’s Modified Eagle’s medium (DMEM)- GlutaMAX 1 high glucose medium (Thermo Fisher Scientific, Waltham, MA, #11965092) supplemented with 10% fetal bovine serum (FBS) (Merck, #41105021) and 1% penicillin/streptomycin (P/S) (Merck, #P4333). Cell line CHLA-266 was purchased from CCcells (Childhood Cancer Repository) and was grown in 1x Iscove’s modified Dulbecco’s medium (IMDM; Thermo Fisher Scientific, #2155-0542) supplemented with 20% FBS, 2 mM L-glutamine (Thermo Fisher Scientific, #25300022) and 1% insulin transferrin selenium (Thermo Fisher Scientific, #4140045). HHU-ATRT1 cells were generated at the University Hospital Düsseldorf (ethical permission #2018-102) and were grown in Neurobasal-A medium (Thermo Fisher Scientific, #12440053) supplemented with 2% FBS, 4 mM L-glutamine (Thermo Fisher Scientific, #25300032) and 1% insulin transferrin selenium (Thermo Fisher Scientific, #4140045). HUH-ATRT1 cells were generated at the University Hospital Düsseldorf (ethical permission #2018-102) and were grown in Neurobasal-A medium (Thermo Fisher Scientific, #10088022) complemented with 2 mM L-glutamine, 1% P/S, 0.0075% bovine serum albumin (BSA), B-27 supplement 1x (Thermo Fisher Scientific, #25030022) and recombinant epidermal growth factor (EGF, 10 ng/ml, Thermo Fisher Scientific, #RP-10914) and basic
fibrillar growth factor (FGF, 10 ng/mL, Biozol, #HZ-1285). The cell line HD-MB03 was a generous gift of Dr. Till M ü lde, KITZ, HOpf Children’s Cancer Centre Heidelberg, Germany (#HPA040619, 1:1000), PIP1D1-HIP1D1 (Thermo Fischer Scientific, #Hs.PT.58.4562781; GLI1 (#Hs.PT.58.42648279), GLI2 (#Hs.PT.58.45624781); GUSB (#Hs.PT.58.27737538); HHIP-AS1 (#Hs.PT.58.40948331); and HHIP-AS2 (#Hs.PT.58.613732); HPRT1 (#Hs.PT.58.45621572); MALAT1 (#Hs.PT.58.42645167.g); PIA (#Hs.PT.39a.22418515); p16 (Mm.PT.39a.2.g2); b2m (#Mm.PT.39a.22418535), gusb (#Mm.PT.39a.22414864), hibg (#Mm.PT.58.29296649); and gusb (#Mm.PT.58.11938234); siRNA pools, consisting of 30 different siRNAs, were purchased from siTOOLs Biotech (Martinsried, Germany); HHIP-AS1 (Hs#1464576- 10 nmol); DYNC12 (#18718- 10 nmol); pPUS, consisting of 30 biotinylated mRNA probes, were also from siTOOLs Biotech: HHIP-AS1 (RPA#L66576-5- 5 nmol).

**Immunoblot experiments.** Immunoblots were performed using the following primary antibodies: anti-DYNC12 (Atlas Antibodies, Bromma, Sweden; #PA040619, 1:1000), mouse anti-β-Actin (CA) (Santa Cruz Biotechnology, Heidelberg, Germany, #sc-7778, 1:1000) or mouse anti-β-Actin (Cell Signaling Technology, Danvers, MA, #3700, 1:1000), mouse anti-HHIP (Afnova Germany; #H0006399-M01; 1:1000); mouse anti-GLI2 (Santa Cruz Biotechnology, Heidelberg, Germany, #sc-271786, 1:500); rabbit anti-GLI1 (Cell Signaling Technology, Danvers, MA, #81812, 1:1000).

**Luciferase reporter assay.** Promoter insert (Chromosome 4, 144,645,400-144,647,801; hg19 coordinates) was subcloned into pGL4.22 [luc2CP/Puro] Vector (Promega, #DQ188841) in two orientations. The forward sequence (“HHIP-fw”) was flipped around for the “HHIP-re” sequence. The correctness of insert orientations was confirmed by sequencing. The reporter activity was assayed by using “Dual-Luciferase Reporter Assay System” (Promega, #E1910) according to the manufacturer’s instructions. The 3' and 5' Luciferase reporter gene assays were performed as described previously57 except for transfecting cells with 50 nM of pre-miR miRNA precursor hsa-miR-425-5p (Thermo Fischer Scientific, #171000) and hsa-miR-425-5p with 50 nM of pre-miRNA negative control #1 (Thermo Fischer Scientific, #AM17110).

**RNA fluorescence in situ hybridization (FISH).** FISH was carried out based on the Stellaris RNA FISH protocol according to the manufacturer. Daoy and CHLA-266 cells were fixed in 4% formaldehyde (Merck, #100496.8350) at room temperature for 10 min. Cells were permeabilized in 70% ethanol at 4°C for 1 h followed by hybridization with FITC-labeled HHIP-AS and ATTO655-labeled DYN122 probes in hybridization buffer (10% formamide, 10% dextran sulfate, 2x saline sodium citrate) at 37 °C overnight. Nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI). Images were acquired using a wide field fluorescence microscope Axio Observer.Z1 (Carl Zeiss Microscopy, Jena, Germany) with an ApoTome 2 (Zeiss) attachment.

**Bio-Layer interferometry.** Bio-Layer interferometry (BLI) has been a promising technique to study the RNA-RNA binding interaction58. We used the “Fortebio Blitz System” (Fortebio, Fremont, CA) to study the interaction between the proposed RNA sequences along with a negative control sequence. We used 1x RNA binding buffer (RBB) containing 10 mM Tris-HCl pH 8.0, 125 mM NaCl, 125 mM KCl & 25 mM MgCl2 to perform all binding studies. We used a modified protocol from58 to study the binding interaction in our system. Basically, SSA biosensor tips were hydrated in RBB for at least 10 min. Next, 200 nM of biotinylated DYNC12 sequence (ordered from IDTdna, see section “In silico analysis of RNA binding”) was loaded for 15 min after performing an initial baseline of the unloaded sensors for 30 s in RBB. Subsequently, the loaded RNAs were displaced in RBB for 1 min. The loaded RNAs were incubated with 200 nM of HHIP-AS1 pos/neg sequence (ordered from IDTdna, see section “In silico analysis of RNA binding”) for 5 min in the association phase, and dissociation of the bound sequences was studied for 5 min again in RBB. All the RNAse free buffer components were purchased from “Thermo Fisher Scientific”. The biotinylated and non-biotinylated RNA sequences were ordered from IDTdna. The Super-Steptavidin (SSA) coated biosensors were purchased from Fortebio.
Merck, #PEG550816) pre-coated with poly-D-lysine (Merck, #A-003-E) and Matrigel (Dexter, Brumath, France #354230). Lentiviral transduction with siRNA constructs was performed 2 h after seeding. Cells were maintained in culture for 10 days, passing them when confluence was reached. A pulse of 10 μM BrdU (BD Biosciences, #550891) was provided before fixation with 4% PFA for 20 min. For orthotropic SHH MB tumor tissues of Med-1712-FH PDX line, whole mouse brains bearing the tumor were fixed for 10 h in 4% PFA. The tissue was then embedded in paraffin and cut into 5 μm sections for immunostaining (Leica, Wetzlar, Germany). Proliferation analysis in Doxa, CHLA-266, HHU-ATRT1 and patient-derived MB cells was performed 72 h after transfection, siRNA-mediated knockdown of HHIP-AS1 using the “Click-iT EdU Alexa Fluor 488 Imaging Kit” (Thermo Fisher Scientific, #C10337) according to the manufacturer’s instructions. PDX tissues in vitro and orthotopic PDX tissue sections were instead stained with 1:400 rabbit anti-Ki67 (Merck Millipore, Darmstadt, Germany, #AB9260), 1:1000 rabbit anti-NeuN (Abcam, #ab177487), 1:100 Rabbit anti-Cleaved Caspase 3 (D175, Cell Signaling, #9661S), 1:100 mouse anti-phospho-H2A.X (BWB301.Merck Millipore, Darmstadt, Germany, #05-636-1) for in vivo tissue slides or 1:400 rabbit anti-phospho-histone H2A.X (Cell Signaling, Frankfurt, Germany #9718) for in vitro staining and 1:500 anti-BrdU (Bio-Rad Abbott Serotec, Oxford, UK, #OB00300G) primary antibodies. For DNA damage in vitro studies, neuronal stem cells or Doxa were seeded on 8-well glass slides (Nunc® Lab-Tek® II Chamber Slide® System, Thermo Fisher Scientific, #154534) fixed in paraformaldehyde, rinsed in phosphate-buffered saline, and incubated with γH2AX antibodies and then Alexa 568-conjugated goat anti-rabbit IgG as secondary antibody. Slides mounted with Slow-Fade antifade reagent were imaged on an Zeiss inverted Apotome microscope with a ×63 oil immersion objective (Zeiss). Before counting foci, digital images were processed with ImageJ (LOCI, University of Wisconsin) to adjust brightness and contrast. Cells were evaluated as “positive” for γH2AX foci if they displayed >10 discrete dots of brightness. For tumor sections, only distinct and bright foci for γH2AX were counted as positive cells. Mitotic spindle staining was achieved by staining cells with 1:500 rabbit anti-tubulin (Merck, #T6793) primary antibodies, detecting the centrosomes and the γ-microtubules of the spindle, respectively. In all cases, secondary antibodies were species-specific: chicken anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor-488 labeled (Thermo Fisher Scientific, #A-21441) and 1:500 mouse anti-acetylated tubulin (Merck, #4494) primary antibodies, detecting the centrosomes and the microtubules of the spindle, respectively.

In all cases, secondary antibodies were species-specific: chicken anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor-488 labeled (Thermo Fisher Scientific, #A-21441) and goat anti-mouse IgG2b cross-adsorbed secondary antibody, Alexa Fluor-594 labeled (Thermo Fisher Scientific, #A-21145).

Clonogenic, caspase 3/7 activity and cell viability assays. For the analysis of self-renewal capacity, clonogenicity was analyzed. Doxa, CHLA-266 and HHU-ATRT1 were seeded on 10 cm dishes at the appropriate density and cultured for 1–3 weeks. For transient knockdown approach, transfected cells were co-transfected with siPOOL against HHIP-AS1 as described above after 24 h of seeding. Stable shRNA models were analyzed 2 h after seeding. Cells were maintained in culture for 10 days, passing them when confluence was reached. A pulse of 10 μM BrdU (BD Biosciences, #550891) was provided before fixation with 4% PFA for 20 min. For orthotopic SHH MB tumors of Med-1712-FH PDX line, whole mouse brains bearing the tumor were fixed for 10 h in 4% PFA. The tissue was then embedded in paraffin and cut into 5 μm sections for immunostaining (Leica, Wetzlar, Germany). Proliferation analysis in Doxa, CHLA-266, HHU-ATRT1 and patient-derived MB cells was performed 72 h after transfection, siRNA-mediated knockdown of HHIP-AS1 using the “Click-iT EdU Alexa Fluor 488 Imaging Kit” (Thermo Fisher Scientific, #C10337) according to the manufacturer’s instructions. PDX tissues in vitro and orthotopic PDX tissue sections were instead stained with 1:400 rabbit anti-Ki67 (Merck Millipore, Darmstadt, Germany, #AB9260), 1:1000 rabbit anti-NeuN (Abcam, #ab177487), 1:100 Rabbit anti-Cleaved Caspase 3 (D175, Cell Signaling, #9661S), 1:100 mouse anti-phospho-H2A.X (BWB301.Merck Millipore, Darmstadt, Germany, #05-636-1) for in vivo tissue slides or 1:400 rabbit anti-phospho-histone H2A.X (Cell Signaling, Frankfurt, Germany #9718) for in vitro staining and 1:500 anti-BrdU (Bio-Rad Abbott Serotec, Oxford, UK, #OB00300G) primary antibodies. For DNA damage in vitro studies, neuronal stem cells or Doxa were seeded on 8-well glass slides (Nunc® Lab-Tek® II Chamber Slide® System, Thermo Fisher Scientific, #154534) fixed in paraformaldehyde, rinsed in phosphate-buffered saline, and incubated with γH2AX antibodies and then Alexa 568-conjugated goat anti-rabbit IgG as secondary antibody. Slides mounted with Slow-Fade antifade reagent were imaged on an Zeiss inverted Apotome microscope with a ×63 oil immersion objective (Zeiss). Before counting foci, digital images were processed with ImageJ (LOCI, University of Wisconsin) to adjust brightness and contrast. Cells were evaluated as “positive” for γH2AX foci if they displayed >10 discrete dots of brightness. For tumor sections, only distinct and bright foci for γH2AX were counted as positive cells. Mitotic spindle staining was achieved by staining cells with 1:500 rabbit anti-tubulin (Merck, #T6793) primary antibodies, detecting the centrosomes and the microtubules of the spindle, respectively. In all cases, secondary antibodies were species-specific: chicken anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor-488 labeled (Thermo Fisher Scientific, #A-21441) and goat anti-mouse IgG2b cross-adsorbed secondary antibody, Alexa Fluor-594 labeled (Thermo Fisher Scientific, #A-21145).

In silico analysis of RNA binding. The potential binding between HHIP-AS1 and DYNC1I2 mRNA was analyzed in silico using IntARNA™ and http://tools.cbr.ijp/ bioinformatics web servers. We detected a 24 nt long sequence (5’CCCTTCCTGTTCAACCCAGACTGACAGA 3’) in HHIP-AS1, which binds to the 5’ UTR region of DYNC1I2. This sequence was used as a “positive binding” probe for mRNA stability assay and we designed another sequence with a similar G/C content from HHIP-AS1 (5’ AAAGACGTCTGCTCCTTATT 3’) that was predicted not to bind DYNC1I2, as a negative control (TTCAGGCCTCAAGGGGCCTTATT). Secondary structures of two RNAs forming dimers were predicted with RNAcofold™, which takes into account intra- as well as intermolecular base pairs of both sequences. Secondary structures of single sequences were predicted with RNAfold. All calculations were performed at T = 37 °C and all used programs were from the ‘ViennaRNA package’ v. 2.4.9.6.

mRNA stability assay. Cells were pretreated either in the presence or absence of positive binding probe HHIP-AS1 (5’ AAAGACGTCTGCTCCTTATT 3’), so called “ribofootprint” experiment or “not binding” sequence HHIP-AS1 (5’ AAAGACGTCTGCTCCTTATT 3’) before the addition of actinomycin D (Hyckatek; 10 μg/ml final concentration), a potent inhibitor of mRNA synthesis. Afterwards, total mRNA was extracted at 0-9 h and DYNC1I2 abundance was measured by qRT-PCR.

RNA sequencing and conservation analysis. For RNA sequencing analysis, reads generated from Doxa scr (control), Doxa sh-HHIP-AS1#1, Doxa sh-HHIP-AS1#2, CHLA-266 scr (control), CHLA-266 sh-HHIP-AS1#1 and CHLA-266 sh-HHIP-AS1#2 were filtered, normalized and aligned to the human genome hg38 using STAR (v2.4.1d), unaligned reads were further aligned using BOWTIE2 (v2.2.5) and combined reads were quantified using the partek expectation-maximization algorithm (version 2.14.1.14, Thermo Fisher Scientific) with 0.01% FDR (false discovery rate) to identify significantly deregulated genes for each genotype or sample, and the used statistical test is indicated in each figure legend. Graphs were generated by using GraphPad Prism * Version 9 Graphpad Software.

Computational mass spectrometric data analysis. Proteome discoverer (version 1.4.1.14, Thermo Fisher Scientific) was applied for peptide/protein identification with mascot (version 2.4, Matrix Science) as search engine employing the UniProt database (human; including isoforms; date 2016-11-01). A false discovery rate of 1% (p ≤ 0.01) on peptide level was set as the identification threshold. Proteins were quantified with Progenesis QI for Proteomics (Version 2.0, Nonlinear Dynamics, Waters Corporation).

Statistics. Unless otherwise indicated in the figure legends, error bars represent mean ± SD or SEM of at least three independent experiments for each genotype or sample, and the used statistical test is indicated in each figure legend. Graphs were generated by using GraphPad Prism * Version 9 Graphpad Software.

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**Author contributions**

M.R., J.B., O.A. and G.R. planned and directed the study. J.B. designed and performed in vitro experiments supported by F.B., J.T., G.C., I.M.K., M.W., L.B., D.Pa., V.M., M.K., C.Mö., D.B., F.K., T.B., C.Mu and S.G. F.B., M.Z., and A.F. conducted in vivo experiments with support from G.C. and H.Y. D.Pi. analyzed RNA sequencing data and provided bio-informatics analysis and F-D.M. carried out RNA sequencing sample preparation. N.Q. designed overexpression strategies. Q.G. and P.A.N. provided H3K27ac ChIP–sequencing profile data and visualized data. S.N. carried out Bio-Layer interferometry experiments and design, and analyses were done by A.B., D.W. and G.S. M.L. performed proteomic sample preparation and A.S., K.S. analyzed proteomic data. B.S., B.R., J.T., J.S., S.D., V.R., A.O., U.F., G.L., M.Z., and M.D.T. provided intellectual contributions to the project and to the interpretation of the results. G.S. performed in silico experiments. M.K., H.S., P.L., and S.M.P. provided methylation and RNA sequencing data and supported the analysis of data. J.B., M.Z., M.R., O.A., A.B. and G.R. wrote the manuscript with support of S.M.P., M.K., J.S., J.T., P.L., S.D. and M.D.T. Figures were designed and drafted by J.B., F.B., M.R., G.R., M.Z., and O.A. All authors edited and contributed to the final manuscript.

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**Competing interests**

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**Additional information**

**Supplementary information**

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