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Mutations in the sonic hedgehog pathway cause macrocephaly-associated conditions due to crosstalk to the PI3K/AKT/mTOR pathway

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Abstract

The hedgehog (Hh) pathway is highly conserved and required for embryonic patterning and determination. Mutations in the Hh pathway are observed in sporadic tumors as well as under syndromic conditions. Common to these syndromes are the findings of polydactyly/syndactyly and brain overgrowth. The latter is also a finding most commonly observed in the cases of mutations in the PI3K/AKT/mTOR pathway. We have identified novel Hh pathway mutations and structural copy number variations in individuals with somatic overgrowth, macrocephaly, dysmorphic facial features, and developmental delay, which phenotypically closely resemble patients with phosphatase and tensin homolog (PTEN) mutations. We hypothesized that brain overgrowth and phenotypic overlap with syndromic overgrowth syndromes in these cases may be due to crosstalk between the Hh and PI3K/AKT/mTOR pathways. To test this, we modeled disease-associated variants by generating PTCH1 and Suppressor of Fused (SUFU) heterozygote cell lines using the CRISPR/Cas9 system. These cells demonstrate activation of PI3K signaling and increased phosphorylation of its downstream target p4EBP1 as well as a distinct cellular phenotype. To further investigate the mechanism underlying this crosstalk, we treated human neural stem cells with sonic hedgehog (SHH) ligand and performed transcriptional analysis of components of the mTOR pathway. These studies identified decreased expression of a set of mTOR negative

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CONFLICT OF INTEREST

The authors have no potential conflict of interest.

DATA AVAILABILITY STATEMENT

Additional data are available to individual researchers by request to the corresponding author.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

regulators, leading to its activation. We conclude that there is a significant crosstalk between the SHH and PI3K/AKT/mTOR. We propose that this crosstalk is responsible for why mutations in PTCH1 and SUFU lead to macrocephaly phenotypes similar to those observed in PTEN hamartoma and other overgrowth syndromes associated with mutations in PI3K/AKT/mTOR pathway genes.

1 | INTRODUCTION

Hedgehog (Hh) signaling was first described in *Drosophila melanogaster* as a central regulator of “Wingless” (Wnt) orthologue signaling and patterning of the wing during development (Hartl & Scott, 2014; Ingham & Placzek, 2006). In vertebrates, a parallel role of Hh signaling in limb development was confirmed, as well as more complex involvement in neural cell type differentiation (Dessaud, McMahon, & Briscoe, 2008) and organ development (McMahon, Ingham, & Tabin, 2003). Hh signaling begins with the binding of Hh ligands to their receptors. In humans, there are three main Hh ligands such as sonic hedgehog (SHH), Indian hedgehog (IHH), and desert hedgehog (DHH) and two receptors such as Patched 1 (PTCH1) and Patched 2 (PTCH2). The effect of ligand binding is inhibition of PTCH, which normally inhibits smoothened (SMO) by sequestering it in the cytosol. The translocation to the membrane and subsequent activation of SMO leads to inhibition of Suppressor of Fused (SUFU) and Kinesin family member 7 (KIF7). With SUFU being inhibited, the main effectors of Hh signaling glioma-associated oncogenes 1–3 (GLI1–GLI3) can translocate to the nucleus where they act as transcription factors and modulate the expression of numerous target genes (Lee, Zhao, & Ingham, 2016; Liu et al., 2014; Matisse & Joyner, 1999). This translocation event is additionally regulated by the dual-specificity tyrosine phosphorylation-regulated kinase (DYRK1A, Figure 1) (Lee et al., 2016; Mao et al., 2002). The transcriptional targets of GLI1–GLI3 coordinate crosstalk with numerous other signaling cascades including Wnt, TGF β , and Notch (Borggreffe et al., 2016; Ma, Drannik, Jiang, Peterson, & Turnbull, 2017). These coordinated pathways play important roles in not only embryogenesis but also in stemness (Najafi et al., 2016), protein synthesis (D’Amico & Canettieri, 2016), and cell migration (Araujo, 2015). Given the widespread effects and roles of Hh signaling, it is not surprising that genetic disruptions in Hh pathway components result in multiple developmental abnormalities and neoplasms.

Mutations in the Hh signaling pathway that cause constitutive activation have been mostly described in basal cell carcinomas (Dahmane, Lee, Robins, Heller, & Ruiz i Altaba, 1997) and medulloblastoma (Taylor et al., 2002). Additionally, altered Hh signaling has also been reported in numerous other cancer types including breast (Koike et al., 2017), pancreas (Zou, Yang, Li, Liu, & Yuan, 2017), and prostate (Yang et al., 2017). Due to its widespread effects on cell proliferation, migration, and differentiation, Hh inhibitors have emerged as potential anticancer therapies (Figure 1). The goal of these therapies is to dampen Hh signaling and inhibit some of its proliferative and antiapoptotic functions (Gupta, Takebe, & Lorusso, 2010; Rimkus, Carpenter, Qasem, Chan, & Lo, 2016; Sonamoto et al., 2015). In addition to being associated with somatic mutations in numerous sporadic solid tumors, germline mutations of the Hh pathway lead to a plethora of human phenotypes and syndromic presentations.

Comparison of the brain, limb, neoplasm, and facial dysmorphic traits of each of the Hh pathways associated with syndromes reveals the trends in each tissue type that help to unify certain roles for Hh signaling in the developing human embryo. As initially described, Hh signaling plays an essential role in patterning and segmentation of the limbs. Nearly every Hh pathway-associated genetic syndrome presents with limb phenotypes, with polydactyly being the most common. The neoplasms observed in these syndromic conditions were similar to those observed in the Hh pathway-associated sporadic tumors including basal cell carcinomas and medulloblastoma, confirming the important role of Hh signaling in the skin and the brain. The brain phenotypes are varied and present in a range from widespread holoprosencephaly and megalencephaly to more focal and specific agenesis of the corpus callosum and anterior pituitary hypoplasia. The triad of brain–limb–dysmorphic phenotypes helps to define the Hh-associated syndromes, albeit with varying degrees and specific findings unique to each genetic etiology. Less well understood or reported are the behavioral phenotypes and general somatic growth trends for many of these conditions (Table 1; Figure 2).

In a cohort of macrocephaly cases, we have identified germline mutations affecting negative regulators of Hh signaling. These cases are associated with mutations in *PTCH1* and *SUFU*, both as part of structural copy number variants (CNVs) diagnosed via chromosomal microarray and single nucleotide variants (SNVs) diagnosed via clinical exome sequencing (CES). We present phenotypic data for each of the cases and show that there is striking somatic overgrowth, macrocephaly, and behavioral phenotypes. This constellation of phenotypes was most similar to the syndromes associated with germline mutations in phosphatase and tensin homolog (*PTEN*) and other genes within the PI3K/AKT/mTOR pathway. We therefore hypothesized that there might be crosstalk between the activated Hh pathway and the PI3K/AKT/mTOR signaling cascade. To explore this hypothesis, we generated *PTCH1* and *SUFU* heterozygote cell populations via Crispr/Cas9; furthermore, we treated human neural stem cells with SHH ligand and assayed the transcriptional consequences of Hh activity. Our findings demonstrate that the phenotypic overlap between Hh and PI3K/AKT/mTOR-associated syndromes is due to underlying molecular crosstalk.

2 | MATERIALS AND METHODS

This study was approved by the University of California, Los Angeles (UCLA) Institutional Review Board, and informed consent was obtained.

2.1 | Crispr/Cas9 cloning

PTCH1 and *SUFU* were deleted from HEK293T cells utilizing the Crispr/Cas9 plasmid (pSpCas9(BB)-2A-Puro (PX459) V2.0, #62988 [Ran et al., 2013]) from Addgene. Guide RNAs (gRNAs) were designed using the Benchling software. gRNAs were selected that showed the highest “on-target” score (Doench et al., 2016) and “off-target” score (Hsu et al., 2013). The 20-bp gRNAs were flanked with CACC- on the 5′ end of their forward oligos and AAAC- on the 5′ end of their reverse oligo. These overhangs facilitate ligation into the parental plasmid once it is digested with BBSI. Briefly, the oligos were annealed and phosphorylated using T4 PNK enzyme (NEB, M0201S), and then annealed oligos were then

diluted and added to a digestion–ligation reaction containing parental plasmid, tango buffer (Life Tech By5), DTT, ATP, BBSI (Thermo, FD1014), and T7 Ligase (NEB, M03185). After six cycles of 37°C for 5 min and 23°C for 5 min, the reaction was treated with plasmid safe exonuclease (Epicentre, E3101K). Finally, plasmids were transformed in Stbl3 *Escherichia coli* and grown under ampicillin selection. Plasmid mini-preps were performed on three colonies and sanger sequencing confirmed the cloning event using the human U6 primer sequence: 5′-CGATACAAGGCTGTTA-3′ (gRNA sequences in Data S1).

2.2 | Cell culture and transfection

HEK293T cells were grown in basal media including Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin-neomycin (PSN), and 10% fetal bovine serum (FBS). Twenty-four hours before transfection, the cells were released from T25 flask with TrypLE Express Enzyme (1×). Cell concentration and viability were determined with the Invitrogen Countess I. After plating 2×10^5 viable cells onto a 12-well culture dish, volume was brought to 1 ml of basal media. On the day of transfection, the medium was aspirated, and 500 µl of basal media was added to each well. GeneIn Transfection reagent was combined with 2 µg of plasmid, brought to a final volume of 200 µl, vortexed, and then incubated at room temperature for 15 min and added to each well. The cells were incubated for 24 hr, followed by a 12-hr nutrient starvation in DMEM lacking glutamine, glucose, and FBS. To assay signaling changes as a result of PTCH1, SUFU, and LAMTOR3 loss, we chose to analyze populations of cells that had been edited (Figure 3). gRNAs were designed using the benchling software, which maximizes the efficiency and minimizes off-target effects. These gRNAs are then cloned into parental CRISPR plasmids that also carry puromycin resistance. Following transfection and puromycin selection, we analyzed populations of the cells. This is advantageous because of the few passages required, which gives the cells less time to compensate and allows biological differences to be observed. We further isolated clonally expanded cell lines for the purpose of observing morphological changes and to confirm the presence of homozygote and heterozygote genotypes. This was achieved by serial dilution to a concentration of 1 cell per 10 µl and then plated in a 96-well format. Following expansion, replica plating was performed and genotyping was pursued using the Zymo Research Quick-DNA 96 kit (Cat D3010).

2.3 | Western blot analysis

Cells were lysed on 24-well or 12-well plates with 200 µl or 400 µl of passive lysis buffer, prepared with phosphatase and protease inhibitors, and incubated by shaking at 4°C for 1 hr. After lysis, the tubes were spun at 7500 Rcf for 5 min to collect debris. The supernatant was transferred to a new tube and then protein concentration was determined with the Coomassie Plus Bradford reagent (Life Technologies) following manufacturer's instructions. Protein samples were diluted to a final concentration of 10 µg/20 µl into western loading buffer with beta-mercaptoethanol and then boiled for 5 min. Western blots were run on 12% acrylamide gels followed by transfer onto nitrocellulose membranes using the Transblot Turbo apparatus from BioRad. The membrane was blocked in 5% bovine serum albumin in Tris-buffered saline plus Tween (TBST) for 30 min, then incubated in primary antibodies for 72 hr against STAG2 (Cell Signaling #5882, Dilution 1/500) and beta-actin (Cell Signaling #4970, Dilution 1/5000). Blots were then rinsed with Tris-buffered saline (TBS) and washed three

times with TBST. Blots were then incubated in rabbit secondary antibodies conjugated to horseradish peroxidase at a dilution of 1/3750. Then secondary incubation membranes were rinsed with TBS and then washed twice with TBST. Blots were exposed using Western Clarity reagents from BioRad and imaged on the BioRad ChemiDoc and viewed in the ImageLab and ImageJ Software (The full list of antibodies is provided in Data S1).

2.4 | Immunofluorescence

A total of 100,000 cells were plated on Geltrex (Invitrogen)-coated coverslips. After 24 hr of growth, the cells were fixed using paraformaldehyde for 20 min and then blocked with 10% normal goat serum in TBST overnight. The following day, coverslips were washed three times in TBST. Coverslips were incubated in Phalloidin 647 (Thermo, A22287) in 3% BSA in TBST for 1 hr. Coverslips were then washed three times in TBST and incubated in secondary antibody (Jackson Laboratories, AffiniPure Donkey Anti-Rabbit, 711-165-152, dilution 1/1000, and invitrogen, Alexa Fluor 647 Phalloidin, A22287, dilution 1/100) in 3% BSA in TBST for 1 hr. Coverslips were then washed three times in TBST and then three times in water. Coverslips were mounted on glass slides using ProLong Gold Antifade Mountant with DAPI (4',6-diamidino-2-phenylindole, Invitrogen, P36931). Slides were imaged using a Zeiss LSM-800.

2.5 | Treatment of human neural stem cells with SHH ligand

Human neural progenitor cells (hNPCs) were added to a single cell suspension using Accumax. The concentration of the cells was determined using an Invitrogen Countess and then was plated at a density of 750×10^5 cells per well in a 6-well plate. The cells were suspended in 3 µg/ml SHH ligand for the “treated” wells or standard growth media for the “untreated” wells. The cells were allowed to grow for 24 hours before they were harvested for RNA extraction.

2.6 | Transcriptional analysis

Cells were collected after 24 hr with or without SHH treatment and pelleted by brief centrifugation and 800 µl of Invitrogen TRIzol reagent was added to each cell pellet. Tubes were vortexed and frozen at -80°C . RNA extraction was performed using the Zymo Direct-Zol as per manufacturer's instructions, and DNase was performed off the column. RNA was quantified and checked for quality before performing real-time polymerase chain reaction (RT-PCR) analysis. For RT-PCR, we used 400 ng of RNA and created a cDNA library using the Qiagen RE3 mix. Following generation of cDNA, a SYBR Green (BioRad) Master Mix was created for each sample and aliquots were loaded onto the Qiagen RT2 Profiler PCR array, which is manufactured using RT-PCR primers in every well that are validated to quantitate the expression of the components of the mTOR pathway. The 96-well plates were run on the 7900 HT at a program of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

2.7 | Transcription factor-binding analysis

After mTOR transcriptional targets were identified, they were screened for common transcription factor-binding sites. This was achieved by manually entering the 5 kb upstream

region for each identified gene into the transcription factor search engine TFSearch. The results from this analysis were manually curated and entered to a spreadsheet to identify common shared transcription factors.

3 | RESULTS

We recruit patients to an ongoing study when they meet the inclusion criteria of having overgrowth defined as height, weight, and/head circumference greater than or equal to the 95th percentile. Macrocephaly is the most common reason for patients being referred to and enrolled in the study. If the patients have developmental delay or autism with macrocephaly, they are tested for PTEN mutations as this is the most common cause of the autism/macrocephaly phenotype. We are systematically subjecting a cohort of PTEN-negative macrocephaly cases with developmental delay to chromosomal microarray and whole exome sequencing testing. Through this effort, we identified a subset of four cases with mutations or copy number variants affecting negative regulators of SHH signaling. Mutations and summary of physical findings are summarized in Tables 2 and 3.

3.1 | Clinical presentations

Case 1—Limited clinical information is available in this case, as the patient was referred to UCLA from an outside physician and subsequently lost to follow up. Briefly the patient presented, at age 5, with somatic overgrowth (height 95th percentile and weight >97th percentile), as well as macrocephaly (OFC > 97th percentile). Additionally, the patient had a diagnosis of autism, the combination of autism and macrocephaly led to the suspicion and testing for PTEN mutations. When PTEN testing was negative, clinical exome sequencing identified a heterozygous c.4014_4034del21, p.Trp1339_Arg1345del in-frame variant in the *PTCH1* gene. The heterozygous deletion variant was of unknown inheritance as parental samples were not available for testing. This patient also underwent microarray, chromosomal analysis, Fragile X, and deletion/duplication for 15q11 testing, all of which was normal.

Case 2—Baby boy was born at 37 weeks' gestation to a 35-year-old mother after an uncomplicated pregnancy. He required a 2-week hospital stay due to respiratory distress syndrome. He had difficulty in breathing and was admitted for congestion, trouble breathing, and hypoxia. Birth weight was 3,500 g (50th percentile) and length 54.5 cm (95th–97th percentile). He also had bilateral inguinal hernias with repair at 6 weeks of age. At 3 years of age, he displayed overgrowth with weight (22.8 kg), height (104 cm), and head circumference (58 cm), all above the 97th percentile. Additionally, dysmorphic features and developmental delay were observed. He had a flat face with downslanting palpebral fissures, a short nose with anterior nares, frontal bossing, and a wide forehead. His ears were low set, and he had very small ear canals. He had clinodactyly of the 5th digits bilaterally and doughy hands. He had a sacral dimple on the back, as well as hypermobility of the ankles. History of narrow ear canals and chronic serous otitis media requiring tympanostomy tube placement was reported. The parents also reported gastroesophageal reflux disease, constipation, and torticollis, which were resolved. The patient had significant developmental delay: he said very few words at 18 months and walked after the age of 2 years. He was receiving speech therapy twice per week and physical therapy once per week. Brain

magnetic resonance imaging (MRI) showed dilated ventricles, with no tumors or any other abnormalities. Comparative genomic hybridization testing identified a 3.9 Mb heterozygous copy number loss of chromosomal material at region 9q22.31q22.33, including *PTCH1*. Clinical exome sequencing (trio) reveals no other convincing disease causing variants.

Case 3—Baby girl was born as a 34-week monoamniotic twin via C-section to a previously healthy 26-year-old G2P3 mother and a 36-year-old father. She did well postpartum and met normal developmental milestones in infancy, but afterwards language and learning delays were observed. She had increasing head circumference and congenital right microtia. By the age of 2 years, headaches without neurologic deficits and clumsiness were reported, which prompted brain imaging studies that identified a large cerebellar mass with resultant obstructive hydrocephalus and no other signs of gross or microscopic metastases. The patient underwent gross total resection of the mass via craniotomy and ventriculoperitoneal shunt placement. Pathology was found to be consistent with a desmoplastic medulloblastoma. She underwent treatment with high-dose chemotherapy and autologous hematopoietic stem cell transplant as per Head Start 3, without complications. At 3 years 8 months old, her height was 94 cm (placing at the 15.71 percentile), weight was 16.8 kg (placing at the 79.54 percentile), and head circumference was 53 cm. Her body mass index was 10.01 kg/m² (placing at the 97.96 percentile). Dysmorphic features included right-sided microtia, hypertelorism, downslanting palpebral fissures, and fifth finger clinodactyly. A combination of developmental delay and macrocephaly led to the suspicion and testing for PTEN mutations. When PTEN testing was negative, clinical exome sequencing identified a heterozygous c.111delC frameshift variant in the *SUFU* gene. The heterozygous c.111delC frameshift variant was confirmed in this patient using Sanger sequencing as an alternate methodology. It was also confirmed to be absent (wildtype) in the mother. The twin sister (identical, monoamniotic) was tested and she carried the same heterozygous *SUFU* frameshift variant c.111delC (p.Tyr38Thrfs*58). She has multiple benign melanocytic nevi, but no medulloblastoma. Surveillance brain MRIs have all been normal, and she is developmentally appropriate for age. Additional genetic testing in the proband included microarray and MYC/MYCN sequencing, which were both negative. Finally, fluorescent in situ hybridization (FISH) and karyotype of the tumor sample was normal.

Case 4—Baby boy was born full-term to a 39-year-old mother by cesarean due to breech position. The perinatal period was uncomplicated. At the time of evaluation, he was 18 months old with overgrowth as demonstrated by weight (13.1 kg) in the 75–90th percentile, height (87.5 cm) in the 75–90th percentile, and head circumference (52.5 cm) above the 98th percentile. Dysmorphic facial features included frontal bossing, bilateral posterior, and superior plagiocephaly, and depressed nasal bridge. He had clinodactyly of the 4th and 5th fingers and 5th toe bilaterally. At the time of evaluation, his mother noted that he did not look at her when she called his name until about 12 months. His milestones were delayed, especially in speech. He was reported to be “clumsy” and experienced frequent falls. Brain MRI showed multiple areas of ectopic gray matter in the ventricular lining, but they did not appear to be tumors or subependymal nodules, suggestive of tuberous sclerosis. Array comparative genomic hybridization (CGH) was performed and showed a 325 kb deletion at chromosome 10q24.32, which included *SUFU*. Fragile X testing was negative.

We further expanded upon our cohort by searching the Decipher database for the cases with mutations and copy number variations in *PTCH1* and *SUFU*. We identified 14 cases with mutations/deletions (2 SNVs and 12 CNVs) in *PTCH1* (Table 4) and one case with a deletion of *SUFU* (Table 5). From the cases with *PTCH1* deletions, we searched the available phenotypic data from 14 cases to determine whether there was phenotypic overlap, which would support our claim that these mutations are associated with a core triad phenotype of macrocephaly/overgrowth, dysmorphia, and developmental delays. A total of 10 of the 14 cases reported overgrowth (71%), 11 of the 14 had developmental delays (79%), and 10 of the 14 cases reported dysmorphic facial features (71%). A total of 5 of the 14 cases (36%) reported neoplasms. We were able to identify one additional *SUFU* deletion: this case presented with developmental delays and dysmorphia; however, overgrowth was not a reported phenotype. We were also able to identify duplications involving *PTCH1* (n = 5) and *SUFU* (n = 4). From these duplications, we observe that many cases display undergrowth: three of the five *PTCH1* cases (60%) and two of the four *SUFU* cases (50%). Additionally, developmental delays were observed in 60% of *PTCH1* cases and 25% of *SUFU* cases, and finally, dysmorphia was observed in 60% of *PTCH1* cases and 100% of *SUFU* cases. In support of this reciprocal relationship, there are additional reports of familial microcephaly associated with *PTCH1* duplications (Derwinska et al., 2009). Overall, these phenotypes help to establish the role of *PTCH1* and *SUFU* haploinsufficiency in the etiology of overgrowth, developmental delay, and a characteristic dysmorphic phenotype and additionally demonstrate mirror phenotypes associated with undergrowth, potentially due to dosage sensitivity of *PTCH1* and *SUFU* deletions versus duplications.

3.2 | Deletion of *PTCH1* and *SUFU* alters PI3K/AKT/mTOR signaling and cell size

A number of genetic syndromes have been recently described associated with germline or somatic mosaic mutations in genes that encode components of the PI3K/AKT/mTOR signaling pathway. These syndromes present with macrocephaly, somatic overgrowth, developmental delay, intellectual disability, and dysmorphic features, including polydactyly. These features are similar to those present in our four cases, prompting the hypothesis that activation of Hh signaling may in turn activate mTOR and promote brain growth. The PI3K pathway plays an important role in brain neural progenitor proliferation and regulates brain size. Having identified deletions of *PTCH1* and *SUFU* in macrocephaly cases, we were thus interested in determining the effects of either heterozygous gene deletion on PI3K/AKT/mTOR signaling. We generated heterozygous deletions of *PTCH1* or *SUFU* using Crispr-Cas9 gene editing. Following transfection of *PTCH1* and *SUFU* gRNA plasmids and 48-hour puromycin selection, we confirmed *PTCH1/SUFU* deletions via PCR analysis utilizing a primer set, which flanks the cut sites and observed the presence of an edited band. This band is of the expected size, which confirms that the CRISPR-Cas9 system edited efficiently and correctly (Figure 4a,b). This was further confirmed via RT-PCR analysis of RNA extracted from clonally expanded homozygous cell populations, which shows no wild-type *PTCH1* transcript in the homozygotes, intermediate *PTCH1* levels in the heterozygotes, and normal *PTCH1* levels in the wild-type controls (Figure S1).

We then assayed whether *PTCH1* or *SUFU* deletion alters the activation of the PI3K/AKT/mTOR pathway. Cells with *PTCH1* deletions show strongly increased levels of the

mTORC1 target p4EBP1, an activated translation initiation factor that increases cell proliferation. We also observed increases in pmTOR2448, a marker of mTORC1 activity. There were no changes in pAKT473 or pAKT308 leading us to conclude that this mTORC1 activation was AKT independent (Figure 4c,e). Cells with *SUFU* deletions show stronger increases in mTORC1 activation marked by elevated pmTOR 2448 and p4EBP1 levels. In addition, there are increases in pAKT473 and pAKT308 levels, which show that there is distinct activation of both mTORC1 and AKT (Figure 4d,f). We imaged the *PTCH1* deleted cells and show that they form large multinucleated, circular, and irregular clusters in cell culture. These abnormal cells are not only larger, but also more prevalent in homozygous *PTCH1* deletions than in heterozygous *PTCH1* cells (Figure 4g). These findings demonstrate that haploinsufficiency for *SUFU* or *PTCH1* is sufficient to activate mTOR signaling, albeit through AKT-dependent (*SUFU*) and AKT-independent (*PTCH1*) pathways.

3.3 | Activation of PI3K/AKT/mTOR signaling resulting from *SUFU* haploinsufficiency is mediated through protein–protein interactions with regulators of mTOR

Our observation that *SUFU* haploinsufficiency increases AKT phosphorylation and *PTCH1* deletion does not prompted us to query the mechanistic etiology for this difference. Using GeneMania to access publicly available protein–protein interaction datasets, we searched proteins that have direct physical interaction with *PTCH1* and *SUFU*. We further compared differential interactors between the two proteins that might mediate AKT activation. From this analysis, we pulled out a potential candidate, Late Endosomal/Lysosomal Adaptor, MAPK And mTOR Activator (LAMTOR3, Figure 5). As its name suggests, LAMTOR3 is a direct activator of mTOR, and we hypothesized that *SUFU* exerts an inhibitory effect on this protein via direct protein–protein interactions. Activation of Hh signaling decreases *SUFU* levels, which leads to mTOR and Akt activation, perhaps through LAMTOR3 activity. To test the hypothesis that LAMTOR3 is modulating mTOR signaling, we engineered cells with LAMTOR3 heterozygous deletions and show that they have decreased pmTOR, pAKT308, and p4EBP1. We propose that loss of *SUFU*-mediated negative regulation of LAMTOR3 upon Hh pathway activation is responsible for the observed differences between *PTCH1* and *SUFU* syndromes, including the susceptibility to medulloblastomas (Figure 6a).

3.4 | SHH signaling in neural stem cells transcriptionally modulates regulators of PI3K/AKT/mTOR signaling

We next explored whether Hh-dependent changes in PI3K/AKT/mTOR signaling are mediated by transcriptional changes in the expression of genes that regulate the latter pathway. We treated human neural stem cells with recombinant SHH ligand and searched for SHH-mediated gene expression changes that may explain the observed increases in PI3K/AKT/mTOR signaling. We identified a group of genes with significantly decreased expression following treatment (Figure 7a,b). The strongest candidates due to significance and magnitude are *PIK3C3*, *PRKAG2*, *RHOA*, *STRADB*, and *RRAGB*. Interestingly, all these genes with the exception of *PIK3C3* are negative regulators of mTOR, suggesting that PI3K/AKT/mTOR pathway activation is facilitated by Hh-dependent downregulation of its known inhibitors. We next performed transcription factor-binding analysis of the 5 kb upstream region for each of the five genes identified. We found an overrepresentation of binding sites for two transcription factors, Lyf1-1 and Nrk2.2. Lyf1-1 binding sites were

found in all five candidate genes while the Nkx2.2 binding sites were found in three out of five candidate genes: PRKAG2, RHOA, and RRAGB. The presence of these common binding sites suggests that these genes are controlled in a coordinated fashion (Figure 7c). Our findings demonstrate that SHH mediates transcriptional downregulation of mTOR pathway negative regulators to increase mTOR activity mediated by the coordinated function of transcription factors present in neural progenitors.

4 | DISCUSSION

4.1 | Mutations/deletions in PTCH1 and SUFU are associated with overgrowth, macrocephaly, developmental delays, and dysmorphism

We have presented a series of four cases, which help to establish somatic overgrowth, macrocephaly, developmental delays, and dysmorphism as part of the phenotypic spectrum of PTCH1 and SUFU mutations/deletions. Interestingly, this phenotype is similar but distinct from that of the basal cell carcinoma syndrome (BCNS, MIM#: 109400). This is mainly due to the fact that the reported cases, with the exception of Case 3, do not have neoplastic disease (Table 2), which may be due to our cases all being children, and this finding develops later in life. Most recently, the brain morphology of BCNS cases was studied and reported to display overgrowth of the cerebrum, thinning of the corpus callosum and ventricular enlargement (Shiohama et al., 2017). These studies suggested that the brain phenotypes were similar to those observed in the PI3K/AKT-related overgrowth syndromes and proposed that the pathways may be interacting; however, experimental evidence to support this claim has been lacking. Additionally, gain of function mutations in smoothened and activation of Hh signaling causes the Curry-Jones syndromes, which present with hemimegalencephaly (Twigg et al., 2016). In aggregate, these syndromes are very similar to those associated with mutations in the PI3K/AKT pathway, and we have provided evidence for the molecular basis of this crosstalk. Finally, the dysmorphic facial features of our cohort also show similarities to those observed in PI3K/AKT overgrowth, primarily downslanting palpebral fissures, ear phenotypes, and fifth finger clinodactyly being present in two cases (50%, Table 3).

4.2 | Deletion of PTCH1 and SUFU cause activation of PI3K/AKT/mTOR signaling

It has been previously reported that SHH overexpression activates PI3K/mTOR signaling via the DYRK1B kinase (Singh, Dhanyamraju, & Lauth, 2017). Here, we demonstrate that loss of negative regulators of Hh pathway signaling is sufficient to activate PI3K/mTOR signaling. Heterozygous *PTCH1* loss results in moderate activation of MTOR, demonstrated by pmTOR2448 levels and increased phosphorylation of a major mTORC1 target p4EBP1 (Thr37/46). This signaling is AKT independent as we observed no activation of AKT at the mTORC1-associated residue 308 or the mTORC2-associated residue 450 or 473. This led us to suspect that the crosstalk of the two pathways mediated by PTCH1 may occur downstream of AKT (Figure 6b). Examples have been described of direct activation of the PI3K/AKT/mTOR pathway through SHH activation in esophageal adenocarcinoma (Wang et al., 2012) and pancreatic tumors (Sharma et al., 2015). In contrast, loss of SUFU is distinct and demonstrates stronger activation of pmTOR, similar increases in its target p4EBP1 (Thr37/46), and also shows increases in pAKT levels at both residues 308 and 473

but not 450. We propose that not only the increased pmTOR levels are due to the combinatorial effect of the same mechanism demonstrated by PTCH1-mutated cells, but we also suggest that cytoplasmic SUFU is acting as a negative regulator of AKT or mTOR. This additional activation of mTOR may be necessary for the more extreme phenotype, which can include medulloblastoma (Mohan et al., 2012), as observed in Case 3. We demonstrate that the constitutive, ligand-independent activation of Hh signaling can directly modulate PI3K/AKT/mTOR signaling in both AKT-independent and AKT-dependent mechanisms. We believe that this activation of mTOR and subsequent increased phosphorylation of its targets cause the phenotypic overlap between SHH- and PI3K-related overgrowth disorders.

4.3 | SHH-dependent activation of PI3K/AKT/mTOR signaling is mediated through transcriptional downregulation of negative regulators of mTOR

We hypothesized that activation of PI3K/AKT/mTOR signaling upon loss of Hh-pathway-negative regulators was due to GLI-mediated expression changes resulting from activation of SHH signaling, similar to what has been recently described with DYRK1B expression following SHH activation (Singh et al., 2017). We confirmed this via transcriptional analysis of human neural stem cells treated with SHH ligand. We were able to identify negative regulators of mTOR, which show decreased expression including RHOA (Gordon et al., 2014), PRKAG2 (Reiter, Bolster, Crozier, Kimball, & Jefferson, 2005), and STRADB. Most interestingly, the 5 kb upstream region to these negative regulators were all shown to contain transcription factor-binding sites for Lyf1-1, and all except STRADB were shown to have a binding site for Nkx2.2. The Nkx2.2 homeobox gene is known to not only be SHH responsive but is additionally integral to determine neuronal identity (Briscoe et al., 1999). We believe that this supports a model by which GLI-mediated alterations in Nkx2.2 decrease the expression and therefore translation of negative regulators of mTOR and lead to its AKT-independent activation, as seen in the PTCH1 mutants (Figure 6b).

4.4 | Individuals with germline mutations in SHH pathway components may benefit from combination therapies that incorporate PI3K/mTOR inhibitors

Our data show that individuals with mutations that cause constitutive activation of the SHH pathway have phenotypic similarities to those with overactive PI3K/AKT/mTOR signaling. We therefore propose that these patients may be candidates for combination therapies that are composed of not only SHH but also PI3K/AKT/mTOR inhibitors. These therapies have already been empirically shown to combat certain tumor types including rhabdomyosarcoma (Graab, Hahn, & Fulda, 2015) and pancreatic cancer (Miyazaki et al., 2016). Interestingly in *PTEN* mutant glioblastoma cell lines, Hh inhibition has demonstrated additive therapeutic effects (Filbin et al., 2013). We expand upon the link between these two pathways, which suggests a potential benefit of a combined PI3K/SHH inhibition approach. In particular, we propose that a precision therapeutic approach to these cases would be different depending on the mutated gene: patients harboring mutations in SUFU may require a higher dose of mTOR inhibition or may require an AKT inhibitor, while patients with PTCH1 mutations may potentially benefit from an mTOR inhibitor. Additionally, there may be a role for the use of DYRK1B inhibitors in these patients (Singh et al., 2017). It is possible that other macrocephaly-associated conditions are acting via crosstalk to either of the PI3K and SHH

pathways, and future studies will shed light on additional combinatorial therapies to potentially treat these conditions.

5 | CONCLUSIONS

We expand the phenotypic spectrum of patients carrying mutations in *PTCH1* and *SUFU* to include overgrowth, macrocephaly, developmental delays, and dysmorphism. We demonstrate that the strong phenotypic overlap between these conditions and those associated with PI3K/AKT/mTOR-related overgrowth syndromes is due to crosstalk between the two pathways, which is mediated by GLI-associated transcriptional changes and alterations in regulators of mTOR activity in addition to the already described increases in the DRYK1B kinase (Singh et al., 2017). Surprisingly, mutations in *SUFU* not only overlap with the transcriptional changes associated with active Hh signaling but also demonstrate additional PI3K activation, which may be due to the loss of suspected inhibition of LAMTOR3. We propose that these cases as well as those affected by the more classical basal cell carcinoma nevroid syndrome may benefit from dual inhibition of PI3K/AKT/mTOR and SHH signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Hedgehog signaling pathway. Initial ligand binding activates hedgehog signaling. Sonic hedgehog binds to the receptor PTCH1, which disrupts the inhibition of cytoplasmic smoothed, allowing it to translocate to the membrane where it is active. Membrane-bound smoothed then activates SUFU, which facilitates the translocation of GLI1–3 into the nucleus. Once in the nucleus, the GLI1–3 transcription factors coordinate the expression of numerous genes. Due to its involvement in numerous sporadic tumor types, inhibitors of the Hh pathway activity have been developed. The two main targets of inhibition are smoothed and GLI1. The main inhibitors are labeled in red

FIGURE 2.

Summary of Hh-associated phenotypes. As presented in Table 1, we have summarized the key findings of Hh-related syndromes. The main manifestations are those affecting the development of the brain, limbs, and face. This reproducible triad marks the specific events and processes in development that are altered by abnormal Hh signaling

FIGURE 3.
Experimental design for generation of PTCH1, SUFU, and LAMTOR3 deletion populations in HEKS293Ts

FIGURE 4.

Modeling PTCH1 and SUFU loss in HEK293T with CRISPR/Cas9: (a) PCR showing deletion of critical exon 2 in PTCH1; (b) PCR showing deletion of critical exon 1 in SUFU; (c) western blot analysis of PTCH1(+/-) cells that demonstrates increased mTORC1 targets including p4EBP1 (Ser65) and pmTOR2448; however, there were no increases in AKT473 and AKT308; (d) western blot analysis of SUFU(+/-) cells that demonstrate increased mTORC1 targets including p4EBP1 (Ser65) and pmTOR2448 and increases in AKT473 and AKT308; (e) quantified expression levels of mTOR-phosphorylated substrates (relative to loading control B-actin) in PTCH1 heterogeneous populations. *denotes statistically significant $p < .05$. (f) Quantified expression levels of mTOR-phosphorylated substrates (relative to loading control B-actin) in SUFU heterogeneous populations. *denotes statistically significant $p < .05$. (g) Morphological changes observed in clonal PTCH1(+/-)

and PTCH1(−/−) cells, which demonstrate increased cell size and multinucleated cells (shown by yellow arrows). Ph. phalloidin

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FIGURE 5.

LAMTOR3 physically interacts with SUFU and activates mTOR. To explain the differences in mTOR activation, we searched for proteins that physically interact with (a) PTCH1 and (b) SUFU. We identified an activator of mTOR, LAMTOR3. To determine if LAMTOR3 regulates mTOR signaling, we (C) engineered LAMTOR knockout cell lines and show that (d) loss of LAMTOR3 decreases the activity of mTORC1 and additionally pAKT308, which supports the model by which increased LAMTOR3 would have the opposite effect

FIGURE 6.

Loss of SUFU and PTCH1 has different effects on PI3K/AKT activation. (a) Loss of SUFU causes transcription as well as LAMTOR3-mediated AKT activation of mTOR: Loss of SUFU lessens the inhibition on GLI translocation. When GLI translocates to the nucleus, it activates the expression of *Lyf* and *Nkx2.2*, which inhibits the expression of *PRKAG2*, *RHOA*, and *STRADB*; these are all negative regulators of mTOR. The loss of negative inhibition leads to activation of mTOR, which we have demonstrated via increased phosphorylation of p4EBP1 and mTOR itself. Additionally, loss of SUFU liberates LAMTOR3 from inhibition and allows it to activate AKT, which we have demonstrated via increased phosphorylation of pAKT473 and pAKT308. (b) Loss of PTCH1 causes transcriptional, AKT-independent activation of mTOR: Loss of PTCH1 lessens the inhibition of smoothened, which allows it to activate SUFU and remove the negative inhibition of SUFU on GLI translocation. When GLI translocates to the nucleus, it activates the expression of *Lyf* and *Nkx2.2*, which inhibits the expression of *PRKAG2*, *RHOA*, and *STRADB*; these are all negative regulators of mTOR. The loss of negative inhibition leads to activation of mTOR, which we have demonstrated via increased phosphorylation of p4EBP1 and mTOR itself

FIGURE 7. Expression changes in neural progenitors following SHH treatment. (a) Scatterplot showing differentially regulated gene expression in SHH treated versus untreated neural progenitor cells. (b) Volcano plot identifying the most significant and greatly affected expression of mTOR-related genes in response to SHH treatment. (c) Transcription factor-binding analysis that demonstrates that the five strongest candidates identified share a Lyf1-binding site and Lyf2 share a Nkx2.2 binding site. These transcription factors are known to be SHH responsive

TABLE 1
Syndromes associated with mutations in the Hh signaling pathway and associated phenotypes

Hedgehog ligands								
Gene symbol	Gene name	Associated syndrome (S)	Type of mutation/effect on pathway	Reference	Brain phenotype	Limb phenotype	Neoplasms	Dysmorphic
SHH	Sonic hedgehog	Holoprosencephaly 3	Inactivating/ downregulation	Wong et al. 2015	Holoprosencephaly Single brain ventricle	n/a	n/a	Proboscis Midface hypoplasia
IHH	Indian hedgehog	Acrocallosal syndrome	Inactivating/ downregulation	Yuksel-Apak et al. 2012	Disproportionate macrocephaly	Short-limbed dwarfism Brachydactyly	n/a	n/a
Membrane bound receptors								
PTCH1	Patched 1	Basal cell nevus syndrome	Inactivating/ upregulation	Evans and Farnon 1993	Megalencephaly	Brachydactyly Short 4th metacarpal Short thumb terminal phalanx	Medulloblastoma Basal cell carcinoma	Broad facies Frontal and biparietal bossing Mild mandibular prognathism Odontogenic keratocysts of jaws
PTCH2	Patched 2	Basal cell nevus syndrome	Inactivating/ upregulation	Fujii et al. 2013	Megalencephaly	Brachydactyly Short fourth metacarpal Short thumb terminal phalanx	Medulloblastoma Basal cell carcinoma	Frontal bossing Mild mandibular prognathism Odontogenic keratocysts of jaws
SMO	Smoothened	Curry-Jones syndrome	Activating/ upregulation	Twigg et al., 2016	Hemimegalencephaly Megalencephaly Polymicrogyria	Broad thumbs Duplicated thumbs Preaxial polydactyly	Desmoplastic medulloblastoma of cerebellum	Facial asymmetry
Cytoplasmic/nuclear signaling								
SUFU	SUFU negative regulator of hedgehog signaling	Basal cell nevus syndrome	Inactivating/ upregulation	Evans and Farnon 1993	Megalencephaly	Brachydactyly Short 4th metacarpal Short thumb terminal phalanx	Medulloblastoma Basal cell carcinoma	Broad facies Frontal bossing Prognathism Odontogenic keratocysts of jaws
GLI2	GLI family zinc finger 2	Culler-Jones syndrome, Holoprosencephaly 9	Inactivating/ downregulation	Ribeiro et al. 2005	Anterior pituitary hypoplasia Ectopic posterior pituitary Thin pituitary stalk	Postaxial polydactyly	n/a	Midface hypoplasia Hypotelorism Cleft lip Cleft palate
GLI3	GLI family zinc finger 3	Greig cephalopolysyndactyly syndrome	Inactivating/ downregulation	Sobetzko et al. 2000	Macrocephaly Hydrocephaly Agenesis of corpus callosum	Postaxial polydactyly Broad thumbs Syndactyly Camptodactyly	n/a	High forehead Frontal bossing Hypertelorism Downslanting palpebral fissures Broad nasal root

Hedgehog ligands								
Gene symbol	Gene name	Associated syndrome (S)	Type of mutation/effect on pathway	Reference	Brain phenotype	Limb phenotype	Neoplasms	Dysmorphic
Kif7	Kinesin family member 7	Acrocallosal syndrome, Joubert syndrome 12	Inactivating/upregulation	Ali et al. 2012	Macrocephaly Hypoplastic or absent corpus callosum	Tapered fingers Fifth finger clinodactyly Brachydactyly Polydactyly	n/a	Prominent forehead Short philtrum Hypoplastic midface

Notes: We presented a list of the congenital genetic syndromes associated with mutations to the Hh pathway. We stress the occurrence of brain, limb neoplastic, and dysmorphic phenotypes within this group.

TABLE 2

Cases with mutations in PTCHI and SUFU with macrocephaly

Case	Method	Growth parameters		Genetic change	Genes of interest	Macrocephaly	Dysmorphic	Neoplasm	Developmental delay
1	CES	Wt >97%	Ht 95% OFC >97%	Het del c.4014–4034	<i>PTCHI</i>	+	+	–	+
2	CMA	Wt >97%	Ht 95% OFC >97%	Del 9q22.31–33 (96109699–973826)	<i>PTCHI</i>		+	–	+
3	CES	Wt >97%	Ht 95% OFC >97%	Het c.111 delC	<i>SUFU</i>	+	+	+	+
4	CMA	Wt 75–90%	Ht 90–95% OFC >95%	Del 10q24.32 (104345460–104670581)	<i>SUFU</i>	+	+	–	+

Notes: Summary of each of the four cases presented with their growth parameters, genetic changes, and presence or absence of specific phenotypes; +, present; –, absent.
Abbreviations: CMA, chromosomal microarray, WES, whole exome sequencing.

TABLE 3

Specific clinical findings in cases

Case	Flat face	Downslanting palpebral fissures	Ear phenotype	Clinodactyly
1	?	?	?	?
2	+	+	+	+
3	-	+	+	+
4	-	-	-	+

Notes: We focused on the most common phenotypes in this cohort and report on the occurrence of downslanting palpebral fissures, ear phenotypes, and fifth finger/toe clinodactyly. +, present; -, absent; ?, unknown.

PTCH1 cases from decipher

TABLE 4

Decipher ID	Genomic change	Overgrowth	Developmental delay	Dysmorphic	Neoplasm
Single nucleotide variations					
276843	9-98231437-98231437 T>C	+	+	+	NR
258983	9-98241382-98241382 A>C	+	+	+	NR
Deletions					
321763	9-94358815-99785745 Deletion	+	+	+	+
268610	9-91877380-99121811 Deletion	+	+	+	+
248719	9-94599654-98659874 Deletion	+	+	+	+
268609	9-95461101-104307286 Deletion	+	+	+	NR
282572	9-95441416-104307086 Deletion	+	+	+	NR
290013	9-98267081-98272376 Deletion	+	+	NR	NR
761	9-97336045-102370397 Deletion	+	+	NR	NR
249750	9-96208745-104600969 Deletion	+	NR	+	NR
1002	9-97336045-102019119 Deletion	NR	+	+	+
251468	9-96742321-104692852 Deletion	NR	+	+	NR
322740	9-98181648-98333827 Deletion	NR	NR	NR	+
270439	9-98218538-110294149 Deletion	NR	NR	NR	NR
Duplications					
256837	9-71950016-99525487 Duplication	+	+	+	NR
295745	9-220253-141102518 Duplication	+	+	+	NR

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Decipher ID	Genomic change	Overgrowth	Developmental delay	Dysmorphic	Neoplasia
288238	9:204090–141087916 Duplication	+	NR	+	NR
285789	9:220253–141102518 Duplication	NR	+	NR	NR
286236	9:220253–141102518 Duplication	NR	NR	NR	NR

Notes: We collected all cases from the DECIPHER database that had genetic mutations in the PTCH1 gene. We were able to find numerous PTCH1 deletions that were associated with overgrowth, developmental delays, and dysmorphia, and some with neoplasms. Most interestingly from this collection, we also identified cases with duplications, and some present with undergrowth, developmental delay and dysmorphia. +, present; NR, not reported.

TABLE 5

SUFU cases from decipher

Decipher ID	Genomic change	Overgrowth	Developmental delay	Dysmorphic	Neoplasia
Deletions					
264272	10:103852285–104696098 Deletion	NR	+	+	NR
Duplications					
2578	10:94214092–111401818 Duplication	+	+	+	NR
249450	10:101711724–104823513 Duplication	+	NR	+	NR
249514	10:101711724–104823513 Duplication	NR	NR	+	NR
328230	10:104021180–105357898 Duplication	NR	NR	+	NR

Notes: We collected a case from the DECIPHER database that had a genetic mutation in the SUFU gene. We were able to find numerous SUFU deletions, which were associated with developmental delays and dysmorphia. Most interestingly from this collection, we also identified cases with duplications, and some present with overgrowth, developmental delay, and dysmorphia. +, present; NR, not reported.