Non-canonical Hedgehog/AMPK-Mediated Control of Polyamine Metabolism Supports Neuronal and Medulloblastoma Cell Growth

Highlights
- Hh controls polyamine metabolism and medulloblastoma growth via ODC biosynthesis
- Sufu binds and stabilizes CNBP to regulate ODC translation
- AMPK phosphorylates and activates CNBP in response to Hh stimulation
- Targeting this non-canonical axis inhibits medulloblastoma growth

In Brief
D’Amico, Antonucci, and colleagues identify a non-canonical Hedgehog-dependent pathway that controls polyamine metabolism. The pathway involves the energy sensor AMPK and the translational regulator CNBP and is essential for Hh-dependent proliferation of medulloblastoma cells. The authors show that targeting this pathway efficiently restrains tumor growth.
Non-canonical Hedgehog/AMPK-Mediated Control of Polyamine Metabolism Supports Neuronal and Medulloblastoma Cell Growth

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SUMMARY

Developmental Hedgehog signaling controls proliferation of cerebellar granule cell precursors (GCPs), and its aberrant activation is a leading cause of medulloblastoma. We show here that Hedgehog promotes polyamine biosynthesis in GCPs by engaging a non-canonical axis leading to the translation of ornithine decarboxylase (ODC). This process is governed by AMPK, which phosphorylates threonine 173 of the zinc finger protein CNBP in response to Hedgehog activation. Phosphorylated CNBP increases its association with Sufu, followed by CNBP stabilization, ODC translation, and polyamine biosynthesis. Notably, CNBP, ODC, and polyamines are elevated in Hedgehog-dependent medulloblastoma, and genetic or pharmacological inhibition of this axis efficiently blocks Hedgehog-dependent proliferation of medulloblastoma cells in vitro and in vivo. Together, these data illustrate an auxiliary mechanism of metabolic control by a morphogenic pathway with relevant implications in development and cancer.

INTRODUCTION

The Sonic Hedgehog (Shh) pathway is a critical regulator of embryonic patterning and post-natal stem or progenitor cells (Northcott et al., 2012a). In the cerebellum, Shh promotes the post-natal mitotic expansion of cerebellar granule progenitors (GCPs) (Wechsler-Reya and Scott, 1999) and the aberrant activation of the signaling in these cells is a leading cause of medulloblastoma (MB) (Schüller et al., 2008; Yang et al., 2008), the most frequent brain malignancy of the childhood.

Shh ligand binds to the membrane inhibitory receptor Patched (Ptc), thus alleviating its inhibitory activity upon the transmembrane transducer Smoothened (Smoo). These events occur at the primary cilium and trigger a cascade of intracellular processes that involve the dynamic association between the Gli transcription factors (Gli1, Gli2, and Gli3) and Sufu, which in turn regulates their activity, processing, and cellular localization (Ryan and Chiang, 2012).

The aberrant activation of Shh pathway observed in MB can be attributed to mutations or amplifications of genes encoding pathway components, such as Ptc, Smo, Sufu, and Gli2 or other mechanisms (Schroeder and Gururangan, 2014).

Different small molecule inhibitors of the Shh signaling have been generated and tested, thus providing hope to MB patients. However, the majority of these compounds inhibits Smo activity and is thus inactive in case of mutations occurring at the downstream level. Additionally, trials in patients and animals with tumors driven by mutations of Ptc or Smo have shown that, despite a good initial response, they quickly develop resistance. For these reasons, it is now believed that alternative approaches, preferably targeting downstream components of the signaling, are required (Di Magno et al., 2015).

Polyamines are small intracellular polycations that control key aspects of cell biology, such as cell replication, translation, cell growth, differentiation, and survival and can be found upregulated in cancer (Casero and Marton, 2007). The metabolism of polyamines starts from the decarboxylation of ornithine to putrescine, a rate-limiting step catalyzed by ornithine decarboxylase (ODC), the crucial gatekeeper of polyamine metabolism. Putrescine is converted to spermine, which is in turn transformed to spermidine (Tavladoraki et al., 2012).

Given their critical role, the intracellular concentration of polyamines is kept under tight control by various mechanisms.
results

Hh-Dependent Proliferation Is Accompanied by an Increase of Polyamine Biosynthesis and Is Inhibited by DFMO

To determine whether Hh activation is associated with changes in polyamine metabolism, we studied cerebellar granule cell progenitors (GCPs), the cells of origin of Hh-dependent MB (Schüler et al., 2008). Exposure of GCPs to Shh induced a significant elevation of the three polyamines, putrescine, spermine, and spermidine, accompanied to a robust increase of cell proliferation (Figures 1A and 1B). The ODC inhibitor DFMO counteracted the increase of proliferation (Figure 1B), thus indicating that polyamine metabolism is necessary for Hh-dependent growth and that their inhibition can be exploited for treatment of Hh-dependent MB.

With the aim of identifying downstream inhibitors of Hh signaling, we have found a mechanism whereby this pathway controls polyamine metabolism via an AMP kinase (AMPK)-dependent control of ODC biosynthesis. This mechanism involves the small zinc finger protein CNBP, which promotes translation of ODC, in association with the cytoplasmic Hedgehog (Hh) transducer Sufu. Finally, we show that CNBP and polyamine metabolism are necessary for Hh-dependent growth and that their inhibition can be exploited for treatment of Hh-dependent MB.

Sufu Binds and Stabilizes CNBP to Regulate ODC Translation

To shed light on this, we expressed FLAG-tagged Sufu in HEK293 cells and performed mass spectrometry analysis on FLAG immunoprecipitation purified Sufu-bound proteins. Among the hits with the highest coverage (Table S1), we found CCHC type Nucleic acid Binding Protein (CNBP), a zinc finger protein that binds single stranded RNA and DNA and functions as a nucleic acid chaperone (Calceterra et al., 2010). Notably, CNBP was shown to regulate ODC translation through an internal ribosome entry site (IRES)-mediated mechanism (Sammons et al., 2010). We generated and validated a CNBP antibody (Figure S2A) and confirmed the ability of CNBP to bind Sufu at an endogenous level and in vitro (Figures 2A and S2B). We also ruled out the involvement of CNBP in the Sufu-mediated regulation of Gli transcriptional activity, stability, and processing and any competition with the Gli/Sufu complex (Figures S2C–S2F).

Knock down of CNBP with different shRNAs caused a reduction of ODC protein levels (Figure 2B, middle). To confirm that this reduction was related to the ability of CNBP to regulate ODC IRES-dependent translation, we used the bicistronic ODC-Luc vector (Sammons et al., 2010) (Figure 2B, bottom). This construct contains: (1) the renilla CDS, (2) the luciferase CDS, and (3) the intercistronic ODC 5′ UTR. This cassette is expressed as a single transcript, under the control of the CMV promoter. Translation of the renilla gene (the most 5′ of the two open reading frames, ORFs) is cap-dependent and terminates with the TAA stop codon. Translation of the luciferase gene occurs through the ODC internal initiation site (IRES), located in the intercistronic region. Therefore, the luciferase values represent the ODC IRES activity of the intercistronic region and are normalized for the constitutive renilla activity (Sammons et al., 2010). Knockdown of CNBP reduced the luciferase, but not the renilla activity, confirming the ability of this protein to regulate ODC IRES-dependent translation (Figure 2B, top).

Northern blot analysis of RNA extracted from control or CNBP-deficient cells transfected with ODC-Luc did not show the presence of accessory bands, demonstrating that the effect of CNBP was not related to RNA splicing processes (Figure S2G) of the bicistronic transcript.

To rule out potential plasmid artifacts and to verify that this effect was a genuine IRES-dependent process, we transfected NIH 3T3 cells with in vitro transcribed, monocistronic IRESODC-Luciferase mRNA, conjugated to the non-physiological cap
an analog ApppG, and containing a stable stem loop structure at the 5' end to inhibit scanning (Gilbert et al., 2007) (Figure 2C, bottom). Compared to 7mGpppG-capped mRNA, the in vivo translational efficiency of the ApppG-capped Hairpin-containing mRNA lacking the IRES sequence (−IRES), was strongly reduced by 30-fold (Figure 2C, left). Insertion of the ODC
Figure 2. Identification of the CNBP-Sufu-ODC Axis
(A) CoIP of FLAG-tagged (top) or endogenous (bottom) Sufu with endogenous CNBP in MEF cells; Input 2.5%, In.
(B) The top image shows IRES translation assay on MEF cells transfected with ODC-Luc plasmid and either two distinct CNBP (shCNBP1 and shCNBP2) or scrambled shRNA vectors (shCtr). The results are expressed as fold change compared to the shCtr transfected cells. The luciferase values indicate the IRES-mediated translational activity of the ODC 5′UTR sequence and are normalized by the Renilla values, representing cap-dependent translation of the same transcript. The results are shown as the average ± SD of five independent experiments, each performed in triplicate (*p < 0.01). The middle image shows protein levels of ODC, CNBP, and actin (loading control). The bottom image shows a schematic representation of the ODC-Luc vector. See Results for details.

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5’ UTR IRES sequence (+IRES) significantly increased the translational efficiency of ApppG-Hairpin mRNA (Figure 2C, right). This confirmed the presence of genuine IRES activity in the ODC 5’ UTR (Pyronnet et al., 2000). CNBP depletion significantly reduced this IRES-dependent activity (Figure 2D, left), whereas exogenous CNBP induced translation of ApppG +IRES mRNA, but did not change the translational activity of control –IRES (Figure 2D, right). Thus, CNBP is a bona fide regulator of ODC IRES-dependent translation.

Ablation of CNBP decreased polyamines levels (Figure 2E, left) and the proliferation rate of MEF Ptc’h−/− cells, and this effect was rescued by addition of putrescine (Figure 2E, right), supporting that loss of CNBP impairs the same metabolic step of Sufu.

To determine whether Sufu and CNBP are both associated with translational complexes, we performed polysomal fractionation. As shown in Figures 2F and S2H, CNBP and Sufu copurified in the polyribosomes fractions and EDTA treatment, which did not change the translational activity of control –IRES (Figure 2D, right). Thus, CNBP is a bona fide regulator of ODC IRES-dependent translation.

Based on previous data illustrating the ability of Sufu to bind and stabilize Gli proteins (Chen et al., 2009; Humke et al., 2010; Wang et al., 2010), we hypothesized that Sufu could play a similar role in this context, by preventing CNBP degradation. Toward this end, we incubated wild-type (WT) and Sufu−/− cells with the protein synthesis inhibitor cycloheximide (CHX) for different time points and observed that CNBP half-life was significantly shorter in Sufu-deficient cells (Figure 3A). The specific involvement of Sufu was demonstrated by its retroviral-mediated reconstitution in Sufu−/− cells, which restored CNBP levels (Figure 3B). Incubation of Sufu−/− cells with the proteasome inhibitor MG132 rescued the expression of CNBP and of Gli3, used as control (Chen et al., 2009), indicating the involvement of proteasomal degradation in this process (Figure 3C). In agreement with this observation, we found that both ectopic and endogenous CNBP were efficiently polyubiquitinated, a process that was increased by Sufu knockdown and decreased by its overexpression (Figures 3D, 3E, S3A, and S3B). Thus, Sufu binds CNBP and prevents its ubiquitination and proteasomal degradation, thereby increasing its stability.

**Hh Activation Increases ODC Biosynthesis via AMPK/CNBP**

Since we observed increased expression of ODC protein and polyamine biosynthesis in Hh-activated cells (Figures 1A, 1C, and S1B–S1D), we next wondered whether and how activation of Hh pathway perturbs this Sufu/CNBP-mediated mechanism.

In co-immunoprecipitation studies with equal amounts of CNBP, we observed that upon exposure of cells to SAG, the binding to endogenous Sufu was promptly increased (Figures 4A and S4A), leading to accumulation of CNBP protein (Figures 4B and S4B). Similarly, CNBP levels were higher in Ptc’h−/− cells, compared to control cells and in GCPs treated with Shh peptide (Figures 4C and S4C).

To determine whether this upregulation is associated with an increased translational activity of CNBP, we performed polysomal fractionation analysis in control and Hh-activated cells.

Following SAG activation, CNBP was loaded on polysomal fractions and EDTA treatment disrupted this association (Figures 4D, S4D, and S4E). Consistently, treatment with SAG increased the recruitment of CNBP to ODC 5’UTR in RNA-IP experiments (Figure 4E). Paralleling CNBP, ODC protein levels were upregulated in Ptc’h−/− MEF cells, compared to WT MEF cells, and knock down of CNBP abolished this effect (Figure 4F). The increase of ODC protein was not dependent on differences in mRNA synthesis and/or stabilization, since ODC mRNA levels

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(C) In vivo translation of monocistronic 7mGpppG-capped (7mGpppG-Luc) and ApppG-capped polyA+ Luciferase mRNA either without (ApppG-Hairpin-Luc, -IRES) or with human ODC IRES (ApppG-Hairpin-ODC-Luc, +IRES). After in vitro translation and modifications (see Experimental Procedures), mRNA were transfected in NIH 3T3. The luciferase values were normalized by qPCR analysis of luciferase mRNA levels (p < 0.05). The results represent the average ± SD of four independent experiments, each performed in triplicate. The bottom image shows a schematic representation of the monocistronic vector.

(D) In vivo translation of ApppG-capped polyA+ monocistronic Hairpin-Empty-Luc (–IRES) or Hairpin-ODC-Luc (+IRES) vectors in NIH 3T3 cells transfected with siCNBP, siCtrl (left), and with either CNBP or empty expression plasmids (right). The values indicate luciferase activity normalized to luciferase mRNA levels as assessed by qPCR (p < 0.05 siCtrl, −IRES versus siCtrl, −IRES; p < 0.05 siCNBP, +IRES versus siCtrl, +IRES; and *p < 0.01 CNBP, +IRES versus Empty, +IRES). The results are shown as the average ± SD of three independent experiments, each performed in triplicate.

(E) Polyamine levels (left) in MEF Ptc’h−/− cells transfected with either CNBP (shCNBP) or scrambled (shCtrl) shRNAs (p < 0.05 shCNBP versus shCtrl). The image on the right shows a cell proliferation assay from cells transfected as above (p < 0.05 shCNBP versus shCtrl 48 hr and *p < 0.01 shCNBP+Put versus shCNBP 48 hr). The results are shown as the average ± SD of three independent experiments, each performed in triplicate (putrescine, Put).

(F) Analysis of CNBP and Sufu association with polysomes in subconfluent WT MEF cells. The lysates were separated on a 15%–50% sucrose gradient (+EDTA, right). The presence of CNBP and Sufu protein in each fraction was analyzed by western blotting. Distribution of ribosomal proteins and purity of the fractions along the gradient was controlled with rpS19 and vinculin staining, respectively.

(G) RNA-IP of ODC mRNA. The NIH 3T3 cells were crosslinked and lysed. The lysates were immunoprecipitated with CNBP, Sufu, or control IgG antisera. The eluted mRNAs were reverse transcribed and quantified by qPCR with primers encompassing actin and ODC 5’UTR. The results are indicated as fold difference, relative to IgG control (p < 0.05). The results are shown as the average ± SD of four independent experiments, each performed in triplicate.

(H) The top image shows IRES-translation assay on MEF cells transfected with ODC-Luc and either Sufu (siSufu1 and siSufu2) or scrambled siRNAs (siCtrl) (p < 0.01). The results are shown as the average ± SD of three independent experiments, each performed in triplicate. The bottom image shows Sufu and actin protein levels.

(i) CNBP and Sufu protein (top) and mRNA levels (bottom) normalized by HSP70 and GAPDH levels, respectively, in WT and Sufu−/− MEF cells. The results are shown as the average ± SD of three independent experiments, each performed in triplicate. See also Figure S2.
were unchanged (Figure S4F), neither could be attributed to variations of protein stability, as there were no differences in ODC degradation rate between WT and Ptch1−/− cells (Figure S4G).

The addition of the protein synthesis inhibitor CHX prevented the Hh-induced upregulation of ODC (Figure S4H), thus indicating that the increase was dependent on translation. To directly demonstrate an increase in ODC protein synthesis, we performed in vivo metabolic [35S]-methionine labeling followed by ODC immunoprecipitation (IP). Compared to WT cells, Ptch1−/− cells showed a significant increase of metabolically labeled, newly synthesized ODC protein in the presence of the proteasome inhibitor MG132 (Figures 4G and S4I). Consistently, treatment of cells with SAG strongly increased the ODC-Luc IRES-dependent activity, and this effect was disrupted by CNBP knockdown (Figure 4H). Therefore, Hh induces ODC translation through CNBP.

We next sought to understand the signaling pathway connecting Smo to CNBP. Toward this end, we analyzed the CNBP sequence to identify putative residues that could be subjected to post-translational modifications. We noticed a potential AMPK-consensus site (LARECT), evolutionary conserved (Figure 5A), located in the C-terminal region that diverged from the optimal AMPK site for having the T in position +4 instead of a hydrophobic residue. A similar variation was previously identified in one of the AMPK sites of FOXO3 (Greer et al., 2007).

![Figure 3. Sufu Prevents CNBP Degradation](image)

(A) Left, CNBP and actin levels in WT or Sufu−/− MEF cells incubated with CHX (100 μg/mL) for the indicated times. The image on the right shows a densitometric analysis. The value 1 was assigned to time 0. The results represent the average ± SD of three independent experiments (p < 0.05).

(B) CNBP protein levels in Sufu−/− MEF cells transduced with empty (Ctr) or Sufu (rSufu) retroviruses (actin, loading control).

(C) Effect of 6 hr treatment with 50 μM MG132 on CNBP levels in Sufu−/− MEF cells. The full length Gli3 (Gli3FL) levels are shown as control of MG132 efficacy.

(D) Ubiquitination assay in MEF cells transfected with FLAG-tagged CNBP, HA-Ub, and siSufu (left) or Myc-Sufu (right). The filters were probed with HA antibody to detect ubiquitination and with FLAG, MYC, and actin antibodies for the other proteins.

(E) Endogenous ubiquitination assay in MEF cells transfected with HA-taggedUb and Myc-tagged Sufu vectors. The filters were probed as indicated. See also Figure S3.

Since it was recently shown that Hh activation directly activates AMPK via non-canonical Ca2+–dependent activation of CAMKK2 (Teperino et al., 2012), we tested if AMPK is involved in the Hh-regulated CNBP activation.

SAG-dependent increase of CNBP, ODC protein levels, and the activity of ODC-Luc reporter were disrupted in AMPK−/− compared to WT cells (Figures 5B and 5C). Similarly, the AMPK inhibitor Compound C (CC) prevented the increase of ODC and polyamines in SAG-treated MEF cells, in Ptch1−/− MEF cells, and Shh-treated GCPs (Figures S5A, S5B, and S5D). Elevation of CNBP and ODC proteins, polyamines, and ODC-Luc activity could also be observed upon exposure of cells to the direct AMPK activator A769662 (Figures 5E and S5D) and to KAAD cyclopamine (Figures 5F, S5E, and S5F), which binds Smo and acts as an inhibitor of the canonical pathway, but as a partial agonist of the non-canonical branch (Teperino et al., 2012). To determine if cyclopamine activates the non-canonical branch also in tumor cells driven by constitutive Hh activation, we tested its effect in primary MB cells generated in conditional Math1-Cre/Ptc1−/− mice (Yang et al., 2008). We will refer to these cells as “primary MB cells” within text and figures. Exposure of these cells to cyclopamine did not induce AMPK activation and polyamine production, while it robustly inhibited the expression of the Hh target gene Gli1 (Figure S5G).

Consistent with previous observations (Teperino et al., 2012), activation of this non-canonical mechanism is dependent on the integrity of the primary cilium, since SAG failed to induce CNBP, AMPK phosphorylation, and putrescine content in cilia-deficient Itf88−/− MEF cells (Figure S5H).

Thus, activation of the non-canonical Hh-AMPK pathway promotes upregulation of ODC and polyamines in normal cells, but not in tumors sustained by loss of Ptch1.

To determine if CNBP is phosphorylated by AMPK at the putative threonine 173, we performed in vitro kinase assay with recombinant CNBP or its T173A mutant. WT CNBP efficiently incorporated 32P in the presence of AMPK, whereas the T173A mutant did not (Figure 5G), thus demonstrating that AMPK phosphorylates CNBP only at this residue. We then immunoprecipitated ectopically expressed CNBP from WT and AMPK/C0 cells and performed western blotting with an antibody recognizing AMPK-phosphorylated substrates. CNBP was phosphorylated in WT, but not AMPK/C0 cells (Figure 5H). Activation of Hh with SAG increased the AMPK-mediated phosphorylation of both endogenous and ectopic CNBP (Figures 5I and 5J), and this modification was completely abrogated by mutation of the T173 residue to alanine (Figure 5J). To study the activity of the T173A mutant and to rule out the possibility of overexpression artifacts, we knocked down endogenous CNBP with shRNA.
Figure 5. AMPK Promotes ODC Translation in Response to Hh Agonists by Phosphorylating CNBP at the Conserved Threonine 173

(A) Protein alignment of the amino acid sequence of CNBP surrounding T173. The optimal AMPK consensus motif is shown.

(B) ODC, CNBP, total, and phosphorylated AMPK protein levels in WT and AMPK−/− MEF cells treated with DMSO or SAG for 6 hr (actin, loading control).

(C) Activity of the bicistronic ODC-Luc vector transfected for 48 hr in WT and AMPK−/− MEF cells and treated with DMSO or SAG for 6 hr (*p < 0.01 WT SAG versus WT DMSO and **p < 0.01 AMPK−/− SAG versus WT SAG). The results are shown as the average ± SD of three independent experiments, each performed in triplicate.

(D–F) CNBP, ODC, total, and phosphorylated AMPK protein levels in GCPs stimulated with Shh for 6 hr in the absence or presence of CC (20 μM) (D), or with the AMPK agonist A769662 (25 μM) (E), or treated with KAAD-cyclopamine (KAAD, 0.1 μM) for the indicated times (F) (tubulin, loading control).

(G) In vitro AMPK-phosphorylation assay of recombinant GST-CNBP WT or T173A mutant. The incorporation of 32P was determined by autoradiography and the protein levels were detected by Coomassie blue staining.

(H) WT and AMPK−/− MEF cells were transfected with a plasmid encoding FLAG-CNBP. The cell extracts were immunoprecipitated with anti-FLAG antibody and phosphorylation was revealed with an anti-phospho AMPK substrate (pAMPKsub) antibody. Equivalent amounts of FLAG-CNBP are shown after FLAG immunoblot.

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targeting the 3'UTR region and reintroduced WT and T173A CNBP (Figure S5I). Reconstitution of CNBP-deficient cells with CNBP WT rescued ODC-Luc activity and putrescine levels, whereas the T173A mutant had a significantly reduced activity (Figures 5K and S5J). The CNBP T173A mutant was no longer upregulated by SAG (Figure S5J) and showed a significantly reduced half-life compared to the WT protein (Figure S5K).

Since the stability of CNBP depends on its association with Sufu, we tested the binding of Sufu to CNBP WT, T173A, and the phosphomimetic T173D mutant. Compared to WT CNBP, the T173A mutant bound Sufu with reduced affinity, while the T173D showed an increased affinity (Figures 5L and S5L), indicating that phosphorylation of T173 favors the formation of the complex.

In keeping with this finding, the phosphomimetic T173D mutant promoted significantly higher ODC-Luc activity and polyamines production (Figure S5M).

Collectively, these data indicate that T173 phosphorylation represents the critical regulated checkpoint of this Hh/AMPK-mediated regulation of CNBP.

**ODC and CNBP Are Elevated in MB, and their Targeting Prevents MB Growth**

To explore the pathophysiological relevance of this mechanism, we analyzed CNBP and ODC levels in Hh-dependent MB developed in conditional Math1-Cre/PtcC/C0 mice, where Hh signaling is constitutively hyperactive (Figure S6A). Compared to a normal adult cerebellum, CNBP, ODC, Sufu, and phosphorylated AMPK were elevated in MB samples (Figures 6A and S6B), and this was accompanied by a strong upregulation of the three polyamines (Figure 6B).

We next analyzed CNBP and ODC expression in a cohort of 42 human MBs, divided according to the molecular subgroup (five wingless, WNT; seventeen SHH; ten group 3; and ten group 4) as previously described (Mastronuzzi et al., 2014; Miele et al., 2015).

CNBP and ODC staining were strongly increased in the SHH molecular subgroup compared to a normal adult cerebellum, which showed a negative staining for ODC and a weak positivity for CNBP. A positive staining of CNBP and ODC was also observed in the group 3, while the WNT and group 4 molecular subgroups displayed a weaker staining (Figure 6C). Negative controls were also performed (Figure S6C).

We next analyzed the cohort of seventeen human SHH MBs belonging to the three histological subgroups: desmoplastic, classic, and large cell anaplastic (LCA) (Kool et al., 2012; Northcott et al., 2012b). The staining for both CNBP and ODC was strongly positive in all the MB analyzed independently of their histological classification (Figure 6D).

The strong increase of CNBP, ODC, and polyamine levels in Hh-dependent tumors prompted us to evaluate the consequence of turning off this mechanism in tumor cells.

We first tested different shRNA expressing lentiviruses targeting CNBP for their efficacy in knocking down CNBP and consequent ODC downregulation in primary SHH MB (Figure 7A). We selected the shRNA #58 and used it in all the subsequent experiments.

Ablation of CNBP caused a significant reduction of polyamine content (Figure 7B) and cell proliferation in primary SHH MB cells and in Shh-treated GCPs, as assessed by BrdU incorporation assays (Figures 7B, 7C, and S7A).

Exposure of primary SHH MB cells to DFMO resulted in a robust inhibition of tumor cell proliferation (Figure 7D), without affecting Gli1 mRNA levels (Figure S7B). Accordingly, the levels of the three polyamines putrescine, spermine, and spermidine in MB cells were downregulated by this drug (Figure 7E).

To determine whether DFMO could inhibit proliferation of tumors sustained by upregulation of Gli, independently of upstream activation of Smo, we used the TC-71 tumor cells. Gli1 drives the proliferation of these cells as a consequence of downstream events causing its aberrant transcription (Beauchamp et al., 2009, 2011). Indeed, the direct Gli inhibitor ATO strongly inhibits proliferation of these cells, while they do not respond to the treatment with cyclopiamine (Beauchamp et al., 2011).

Compared to the Ptch–/– MEF cells, TC-71 cells showed comparable levels of Gli1 (Figure 7F). Treatment of these cells with DFMO did not affect their proliferation, while ATO had a strong inhibitory effect (Figure 7G). Knock down of Gli1 caused a significant decrease of their proliferation, without affecting polyamine levels (Figure 7H). Thus, Gli1-driven proliferation does not require changes of polyamine content.

To study the effect of targeting the CNBP/ODC axis in vivo, we allografted tumor cells explanted from Ptch–/– MB and stably transduced with shCNBP or control lentiviruses into nude mice. When the tumor reached the volume of 100 mm³, half of the mice from each group were treated with 0.5% DFMO in the drinking water until the end of the experiment, monitoring the growth of the tumor every other day. As shown in Figures 7I, 7J, and 7K, MB growth and polyamine content were strongly reduced in CNBP-deficient tumors, compared to controls. Inhibition of ODC with DFMO also had a similar inhibitory effect on tumor growth and polyamine content. Notably, DFMO did not cause a further decrease of the growth and polyamine levels, neither a reduction of Gli1 mRNA (Figure S7C), in tumors.
signals. We show here that following Hh activation, CNBP is an attractive therapeutic target to control the proliferation of cerebellar GCPs and their tumor counterpart by regulating ODC, the key gatekeeper of polyamines metabolism.

In this work, we have demonstrated the ability of Hh signaling to control a druggable metabolic process, which is required for normal and MB growth.

The non-canonical Smo/AMPK-dependent signaling route was originally identified in adipocytes, muscle cells, and fibroblasts, where it promotes a rapid metabolic rewiring toward glycolysis and increase of glucose uptake (Teperino et al., 2012). Here, we provide evidence that the same transduction axis is essential to control the proliferation of cerebellar GCPs and their tumor counterpart by regulating ODC, the key gatekeeper of polyamines metabolism.

The levels of ODC are controlled by CNBP, a small conserved protein known to regulate neuronal cell proliferation and forebrain development (Chen et al., 2003), in response to unknown signals. We show here that following Hh activation, CNBP is phosphorylated at T173 by AMPK and promotes translation of ODC, thereby controlling cell proliferation. Thus, in contrast to the classical mode of action as a tumor suppressing kinase, here AMPK is engaged in a process that supports, rather than counteracts, tumor cell growth. These observations are in line with the emerging view that AMPK may act as a tumor suppressor or a contextual oncogene, depending on degree or duration of its activation (Liang and Mills, 2013). It is likely that Hh signaling activates AMPK to a level where it prevails its tumor promoting effect. Interestingly, we also show a role for AMPK in promoting, rather than inhibiting, a translational process. Indeed, our findings illustrate that, by activating CNBP, AMPK promotes IRES-dependent translation, an emergent process. Indeed, our findings illustrate that, by activating CNBP, AMPK promotes IRES-dependent translation, an emergent process. Indeed, our findings illustrate that, by activating CNBP, AMPK promotes IRES-dependent translation, an emergent process.

Figure 6. CNBP and ODC Proteins Are Highly Expressed in Mouse and Human MB
(A) Western blot analysis of WT cerebella and Ptch-/- MB samples. The staining for CNBP, ODC, pAMPK, AMPK, Sufu, and actin as loading control are shown.
(B) Polyamine levels in normal cerebellum and Ptch-/- MB tissue samples. The results are the average ± SD of three different samples for each condition (*p < 0.05 MB versus Cerebellum).
(C) Representative images of immunohistochemistry (IHC) staining for CNBP and ODC in human adult normal cerebellum and human MB, subdivided according to their molecular subgroup (wingless, WNT; SHH; group 3; and group 4). The nuclei were counterstained with hematoxylin (40x original magnification at light microscopy, and the scale bars represent 100 μM).
(D) Immunohistochemistry of human adult normal cerebellum and human LCA, Classic, and Desmoplastic MB of the SHH subgroup, stained with anti-CNPB and anti-ODC antibodies. The nuclei were counterstained with hematoxylin, and the scale bars represent 100 μM. See also Figure S6.

DISCUSSION

In this work, we have demonstrated the ability of Hh signaling to control a druggable metabolic process, which is required for normal and MB growth.

Taken together, these data show that CNBP and ODC are elevated in Hh-dependent MB and that genetic or pharmacological targeting of the Hh-ODC axis is effective in counteracting the growth of MB both in vitro and in vivo.
This observation is seemingly paradoxical, since Sufu is generally described as a tumor suppressor, and its monoallelic deletion appears to be sufficient to confer a Hh gain-of-function phenotype in mice (Cooper et al., 2005; Svárd et al., 2006). However, MB does not develop in Sufu+/− mice, unless they carry a p53+/− background (Lee et al., 2007) and conditional cerebellar Sufu−/− mice do not display signs of early MB or pre-tumoral lesions, such as thickening of the external granule layer (Kim et al., 2011; Yang et al., 2008), typically observed in other Hh gain of function models, such as the cerebellar ptch deletion or smo activating mutations (Wu et al., 2011). Thus, it is possible that the lack of MB development in Sufu deficient animals may reflect the ability of this protein to mediate both tumor suppressing and promoting functions of Hh signaling, like polyamine biosynthesis.

Mono and biallelic mutations of Sufu have been also found in human MB (Brugières et al., 2012; Pugh et al., 2012; Robinson et al., 2012) and are believed to cause activation of the canonical pathway. Whether these mutations also affect the AMPK-dependent route remains to be determined.

In addition to SHH subgroup, CNBP and ODC protein levels are elevated in group 3 MBs. While the mechanism underlying the increase of CNBP has to be established, it is possible that the elevation of ODC could be attributed, at least in part, to overexpression of cMy, a typical alteration found in this molecular subgroup (DeSouza et al., 2014). Indeed, ODC is a transcriptional target of cMy and is typically overexpressed in cMy-driven malignancies (Bello-Fernandez et al., 1993; Nilsson et al., 2009).

A key finding of this work is that targeting of this Hh/CNBP/ODC metabolic axis efficiently prevents the growth of MB cells in vitro and in vivo by reducing intracellular polyamine content. Increased polyamine levels had been observed in MB in an old study (Scalabrino et al., 1982), but their role and the underlying mechanisms were not known, as well as the existence of different molecular MB subgroups. Here, we demonstrate that a non-canonical, Smo-dependent, and Gli-independent mechanism is responsible for the elevations of the polyamine content observed in SHH MB.

This evidence appears to be relevant since a growing body of evidence is documenting the occurrence of resistance to Smo antagonists in the treatment of MB and other Hh driven tumors. Explanations to this phenomenon have been provided with the occurrence of novel Smo mutations and/or with the identification of oncogenic signaling pathways involving ERK, AKT, and mTOR/S6K1, which promote Smo-independent Gli activation or non-canonical Gli-independent cascades (Di Magno et al., 2015). These observations all indicate that full inhibition of the Hh pathway requires alternative approaches, inhibiting the signaling at multiple levels rather than targeting the receptor apparatus alone. Interestingly, the canonical Smo antagonists cyclopamine and GDC-0449 appear to function as selective partial agonists of this non-canonical Hh/AMPK branch in metabolic cells (Teperino et al., 2012) and in GCPs. Cyclopamine inhibits canonical Hh signaling and counteracts cell growth only in tumors with constitutive activation of the receptor apparatus (i.e., in Ptc1 loss of function). In these tumors, the activity of Smo is constitutively increased on both canonical and non-canonical branches. In addition, cyclopamine efficiently represses Gli-dependent transcription, but does not result in any further activation of the AMPK-polyamine axis in cultured Ptc1−/− SHH-MB cells. Therefore, the effect of cyclopamine differs between normal and tumor cells: in normal cells cyclopamine acts as a partial agonist of the non-canonical Hh signaling, but inhibits the canonical route. In contrast, in Hh-dependent tumors, cyclopamine inhibits the canonical pathway, but is unable to induce any further activation of the non-canonical branch.

To address the relevance of the non-canonical AMPK-ODC pathway in SHH MB, we have targeted both CNBP (shRNA) and/or ODC (DFMO) and observed a significant inhibition of the growth of MB cells from conditional Ptc1 KO mice, where Smo is constitutively activated. Thus, turning off the non-canonical branch limits MB growth, even if the canonical signaling is still hyperactive, indicating that both pathways are required for growth.

Further supporting our findings, a previous report showed that treatment of Ptc1−/− mice with DFMO prevented the formation of UV-induced basal cell carcinoma, although the underlying mechanism was not clarified (Tang et al., 2004).

Compared to other chemotherapeutic compounds, DFMO is a well-tolerated drug with a modest toxicity, consisting in thrombocytopenia, gastrointestinal effects, and reversible hearing loss. Recent clinical trials have demonstrated its significant effect as a chemopreventive agent in tumors like neuroblastoma, skin, and colon cancers (Jeter and Alberts, 2012). Our preclinical data support the use of DFMO also as a pharmacological agent for MB. Whether this approach, alone, or in combination with other strategies, will be successful in human MB patients, thus, represents a relevant open question raised in this work.

**EXPERIMENTAL PROCEDURES**

**Drug Treatments**

The ODC inhibitor DFMO (Sigma, 5 mM) and putrescine (Sigma, 10 μM) were added to the medium for the indicated times.

For SAG treatment, NIH 3T3 cells were incubated in low serum (0.5% BS) overnight. MEF WT and Itch−/− were incubated in 1% BSA overnight and then exposed to SAG (200 mM, Enzo Life Sciences) or KAAD (0.1 μM, Calbiochem) for the indicated times. MEF Sufu−/− and HEK293T cells were treated with MG132 (50 μM, Sigma) for 6 hr, MEFs cells were treated for the indicated times with 100 μg/ml protein-synthesis inhibitor CHX (Sigma). GCPs were treated with recombinant Shh-N-terminal peptide (R&D Systems, 3 μg/ml in BSA), with KAAD (0.1 μM, Calbiochem), A769662 (25 μM, Santa Cruz Biotechnology), for the indicated times.

MEF cells and GCPs were pre-treated with CC (20 μM, Millipore) for 20 min and then stimulated with SAG or Shh, respectively, for the indicated times. For polyamine quantitation, MEF Ptc1−/− and GCPs were treated with 10 μM CC for 24 hr (Teperino et al., 2012). Primary MB cells were treated with KAAD (0.5 μM) for the indicated times.

TC-71 cells were treated with 5 mM DFMO (Sigma) or 5 μM arsenic trioxide (ATO, Sigma) for the indicated times.

**Polyamine Analysis**

Polyamine content was determined by gas chromatography-mass spectrometry (GC-MS). Cells or tissues were resuspended in 0.2 M HClO4 and processed. Values were normalized by the protein concentration.

**In Vivo Translation Assay**

m7GpppG- and AppG-capped polyA+ transcripts were obtained by in vitro transcription of the monocistronic luciferase pSP64-Hairpin-ODC-Luc (+IRES) or of pSP64-Hairpin-Empty-Luc (−IRES) vector, using the...
MEGAscript SP6 kit (Ambion), in reactions containing physiological m7GpppG or nonphysiological ApppG caps. mRNA transfections were performed with Lipofectamine 2000 reagent (Invitrogen). After 6 hr of transfection, luciferase assays were performed and the values were normalized by quantitative qPCR analysis of luciferase mRNA levels.

RNA IP
Cells were treated, formaldehyde crosslinked, lysed, and sonicated. Lysates were immunoprecipitated with the specified antibodies and the immunocomplexes washed extensively.

Eluted mRNAs were analyzed by real-time qRT-PCR.

MB Allograft Models
A parental murine MB model was derived from Math1-Cre/PtchC/C mice (Yang et al., 2008). Nude female mice were housed under standard conditions. Briefly, freshly isolated MB were mechanically dissociated into single-cell suspensions in HBSS (GIBCO) supplemented with Pen/Strep. The 5 x 10^6 viable cells were harvested in duplicate and, after 24 hr, infected with control shRNA or shCNBP58 lentiviral particles. After 96 hr, 2 x 10^6 viable cells were suspended in equal volumes of PBS and Matrigel (BD PharMingen) and implanted subcutaneously in both flanks in adult athymic nude mice (Charles River Laboratories). Animals from the two subgroups were randomized and divided in two further subgroups. Treatment was initiated when tumor volume reached 100 mm^3. Mice (control: n = 5 and shCNBP: n = 5) were treated with 0.5% DFMO in the drinking water or only water (control: n = 5 and shCNBP: n = 5) and tumors sizes were measured every day using a caliper. Volume was calculated as V = (L x W^2)/ 2 (Kim et al., 2013). Growth patterns were summarized graphically by plotting the mean and SD for each treatment group over time. Study events were recorded and analyzed using GraphPad Prism software (v6.05).

CNBP knockdown was confirmed by qPCR in all tumor samples at the end of the experiment.

Statistical Analysis
Statistical analysis was performed using StatView 4.1 software (Abacus Concepts). Results are expressed as mean ± SD from at least three independent experiments, each performed in triplicate. Statistical differences were analyzed with the Mann-Whitney U test for non-parametric values and a p < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.09.008.

AUTHOR CONTRIBUTIONS
G.C. conceived and coordinated the project, designed experiments, analyzed the data, and wrote the paper; D.D.A., L.A., L.D.M., S.C., G.S., E.M., and P.I. designed and performed experiments and analyzed the data; A.M. and E.A. performed measurements and analysis of the polyamine data; B.C. performed experiments and analyzed the data of polysome fractionation, in vitro binding assays, and translational analyses; E.D.S., E.F., L.D.M., L.C., F.G., J.R.Y., I.S., and A.G. designed and analyzed the data. All authors critically revised and edited the manuscript.

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Figure 7. Targeting CNBP/ODC Axis Impairs MB Growth In Vitro and In Vivo
(A) Primary MB cells infected with different shCNBP-containing lentiviral particles (clones 58, 59, and 66) or a control lentivirus (shCtr) for 96 hr. The top image shows qPCR analysis of CNBP, ODC, and Gli1 mRNA levels, normalized by GAPDH (*p < 0.01). The results represent the average ± SD of three independent experiments, each performed in triplicate.

(B) Polyamine levels in primary MB cells infected with shCNBP clone 58 or control (shCtr) lentivirus for 96 hr (*p < 0.05). The results represent the average ± SD of three independent experiments, each performed in triplicate.

(C) BrdU incorporation assay in primary MB cells infected with shCNBP clone 58 or control lentivirus (shCtr) for 96 hr and stained by BrdU (red) and nuclear fluorescent stain Hoechst 3342 (blue). The BrdU incorporation was carried out for the last 24 hr. The % of BrdU positive cells was measured and the results expressed as fold change, relative to cells infected with scrambled (*p < 0.05). The results are expressed as fold change relative to untreated mice (H2O) and are shown as the average ± SD (*p < 0.01). The results are analyzed with the Mann-Whitney U test for non-parametric values and a p < 0.05 was considered significant.

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