SAPIENZA
UNIVERSITÀ DI ROMA

FACOLTÀ DI FARMACIA E MEDICINA

SCUOLA DI DOTTORATO IN MEDICINA MOLECOLARE

XXIX CICLO

A novel Granule Cell Progenitors culture method as a new tool for investigating Hedgehog and other pathways relevant to cerebellar development and tumorigenesis.

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a.a. 2015/2016

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SUMMARY

The Hedgehog (Hh) signaling is an essential pathway involved in development and cancer. Via the interaction with the 12-span transmembrane receptor Patch, Hh ligands (Sonic, Desert and Indian Hh) release the activity of the 7-span membrane protein Smoothened (Smo). Yet incompletely characterized molecular mechanisms, involving postranslational modifications of Smo and its shuttling within the primary cilium, translate the signal into the activation of the Gli family of transcription factors. The interplay between the three Gli members (Gil1, 2 and 3) regulates Hh-dependent transcription of a number of gene targets, many of which are master regulators of cell proliferation, such as N-Myc and Cyclin-D.

Hh pathway plays a pivotal role in cerebellar postnatal development, where it appears the main promoter of granule cell progenitors (GCPs) expansion, which in the mouse occurs between postnatal day (P)1 and P14, the time frame in which Hh signaling in the cerebellum raises up to its maximum (P7) and declines. By P14-P21 cerebellar maturation is completed. Silencing of Hh activity at the proper developmental time is molecularly regulated at multiple levels, some of which are still partially obscure, and appears to be essential to prevent transformation of GCPs into medulloblastoma.

Despite intense investigations, several aspects of Hh pathway activation and deactivation are not completely understood or conflicting interpretations exist. This is at least partially due to lack of appropriate models for the study of this pathway, *in vitro*. Indeed, Hh pathway gets quickly repressed in cell cultures, both in normal or cancer cells. Only transient cultures of freshly explanted primary granule cells (GC) allow Hh pathway investigation in a “naive” context, but only for few days.

Since this has long represented a big limitation in the field, we have recently developed a new procedure for GCPs culture, which overcome the limits of currently available models (transients cell cultures among other problems). Under the hypothesis that eliminating Hh-inhibiting growth factors and under the direct stimulation of Hh signal in the cell culture medium, GCs could be grown as neurospheres, we indeed generated a new primary model of GCPs with high and continuously active Hh signaling.

Under defined concentrations of the Smo agonist SAG, cells explanted from P7 cerebella can be indefinitely grown in culture as neurospheres. These neurospheres demonstrate complete activation overtime of the main effectors of Hh pathway Gli1 and N-Myc. They further show self-renewal capability *in vitro*, and constitutive expression of stemness genes, such as POU3f2, POU5f1, NANOG, and SOX2. These cultures do not get transformed during long periods in culture, as suggested by their continuous dependence on SAG.

SAG-dependent neurospheres express ZIC1, ATOH1 and NESTIN, which define their origin as GCPs. Consistently, these cells may be induced to differentiate into a homogeneous population of mature GC, expressing specifics markers such as TUBB3 and GABRA 6. Moreover, SAG-dependent cultures cannot be generated by the subventricular zone, but only from the mouse cerebellar explants taken at P1-P7.

Primary and continuously growing neurospheres of the same type can be generated by GC explanted from Patch KO animal, even without SAG, further suggesting that, in the absence of specific Hh inhibitory factors, constitutive Hh activation is the only element required for their growth and survival.

Overall, these data suggest that using culture conditions that mimic the appropriate biological signaling, allowed the isolation of transient amplifying GCP population in vitro, which is able to propagate in culture for undefined periods of time. This model represents a novel and important tool to study issues related to physiological and pathological aspects of Hh-dependent GCPs growth and differentiation in a cell autonomous environment.

By generating GC neurospheres from different mouse models we are now addressing important issues related to Hh signaling, but also to other pathways. In example, we have shown that the SmoM2 (Trp535Leu) mutation expressed in the transgenic ND2-SMOA1 medulloblastoma mouse model does not imply a full and constitutive activation of the SMO receptor, as commonly thought. Moreover, we have used these cultures to show that the knockout of the DNA repair gene NBS1 specifically impairs Hh-dependent GCPs growth.

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LIST OF ABBREVIATIONS

bFGF Basic Fibroblast Growth Factor

Ccdn1 CyclinD1

Ccdn2 Cyclin D2

Ccne Cyclin E

CK1 Casein Kinase 1

DDR DNA Damage Response

Dhh Desert Hedgehog

DSB Double Strand Break

E Embryonic Day (all timings are mouse unless stated otherwise)

EGF Epidermal Growth Factor

EGL External Granular Layer

En Engrailed Genes

GABRA2 GRABRA receptor alpha2

GC Granule Cell

GCP Cerebellar Granule Cell Progenitors

GEMMs Genetically Engineered Mouse Models

GF-cNS Growth Factor-dependent Cerebellar Neurospheres

GFAP Glial Fibrillary Acidic Protein

GL Granular Layer

Gli GLI Family Zinc Finger

GSk3β Glycogen Synthase Kinase 3 beta

Hh Hedgehog Signaling

IGL Inner Granule Layer

Ihh Indian Hedgehog

KIF7 Kinesin-like Protein KiF7

MB Medulloblastoma

MRN MRE11/RAD50 NBS1

ND2 Mouse neurogenic differentiation 2

ND2-SMOA1 Homozygous ND2:SMOA1 mouse model

NSC Neural Stem Cells

P Postnatal Day (all timings are mouse unless stated otherwise)

PC Purkinje Cell

PKA Protein Kinase A

Ptch-KO Math1-Cre/Ptc $^{C/C}$ mouse model

Ptch1 Patched1

ROS Reactive Oxygen Species

S-cNS SAG-dependent Cerebellar Neurospheres

SGZ Subgranular Zone

Shh Sonic Hedgehog

Smo Smoothened

SUFU Suppressor of Fused

SVZ Subventricular Zone

VZ Ventricular Zone

WNT Wingless

WT Wild Type mouse model

INTRODUCTION

Cerebellum structure and function

The cerebellum is a structure of the central nervous system, which is located at the back of the brain, underlying the occipital and temporal lobes of the cerebral cortex. It is responsible for coordinating movement and maintaining equilibrium, and it is also involved in cognitive and emotional functions. The cerebellum can be divided into two regions: the vermis and the hemisphere. Cerebellar function depends on well-organized neuronal connections and the integration of afferent and efferent fibers. The cerebellum is connected to the brainstem by three pairs of peduncles (superior, middle, and inferior), which are the pathways by which cerebellar afferents and efferents enter and exit. Cerebellar afferents can be categorized into two major types: mossy fibers and climbing fibers.[1] Mossy fibers are the majority of afferent fibers in the adult cerebellum and arise from multiple sources. They communicate with cerebellar nuclei neurons and with Purkinje (PC) cells through granule cells (GC)/parallel fibers. Climbing fibers directly synapse with the cerebellar nuclei and Purkinje cells (the sole output cells of the cerebellar cortex). Furthermore, a third set of afferents not categorized as mossy or climbing fibers exists, which terminate in all three layers of the cerebellar cortex and the cerebellar nuclei. Their functional role is neuromodulation, thus they are categorized as neuromodulatory cerebellar afferents.

The cerebellar cortex is a simple laminar structure, with two principal neurons, the granule cell (the most prevalent neuronal subclass within the cerebellum) and the Purkinje cell, plus a set of interneurons, which modulate the output of the Purkinje cell to the cerebellar nuclei.

The development of the cerebellum begins during the embryogenesis and continues after birth in both mice and humans [2]. Two distinct germinal zones, the primary and secondary, containing stem and/or progenitor cell populations, give rise to the various cell types of the mature cerebellum. The primary germinal zone, the ventricular zone (VZ), gives rise to GABAergic neurons, Purkinje cells and Golgi neurons. Progenitor cells of the secondary germinal zone originate in the rhombic lip and give rise to cerebellar granule cell progenitor (GCPs) that migrate rostrally across the cerebellum to form the external granule/germinal layer (EGL).

Concerning humans, the cerebellum undergoes its major growth in the third trimester and infant stage while, concerning mice it happens in the first two weeks after birth, primarily due to expansion of GCPs. The surface area of the cerebellum increases during the development period much more than its volume due to the formation of lobules. Such process named foliation begins at embryonic day 16.5 (E 16.5) in the mouse with the sequential formation of the base of each fissure, termed anchoring centers. In the mouse vermis, four initial anchoring centers defines the five initial lobes, which are further subdivided [3]. **(Fig.1)** The homeobox engrailed genes (En1/2) are fundamental to the patterning process as they determine when particular anchoring centers form.

The vermis of mice can have eight or nine lobules, as lobules I/II are not separated in some strains, IV/V are fused, and the hemispheres have four lobules that extend laterally from lobules VI and VII. **(Fig.1)** The basic pattern of vermis foliation is conserved throughout mammals, but foliation in the hemispheres is more variable than in the vermis and is very complex in primates.

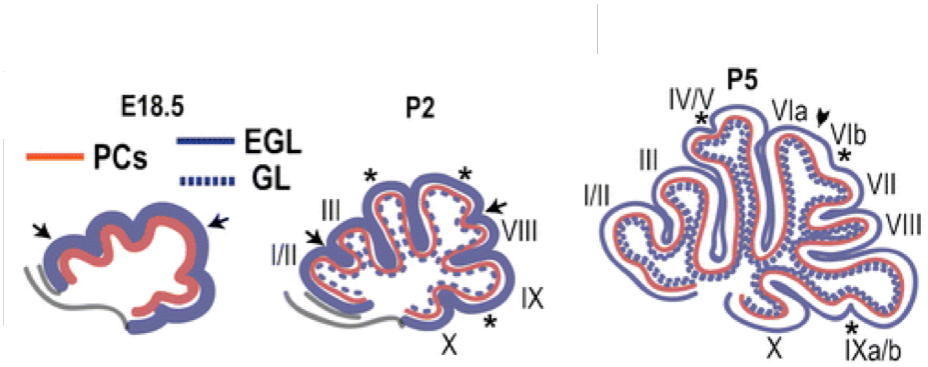

Fig. 1 *Mouse cerebellar foliation*
Anchoring centers and associated fissures aspect on particular days as the folia grow outward. Sagittal sections through the vermis, with lobules indicated in roman numerals. (Leto,K., et al 2016)

Cerebellar granule expansion

Between birth and the end of the second postnatal week, GCPs exit the cell cycle and move into the inner regions of the EGL where they extend parallel fibers and migrate along the radial fibers of Bergmann glial cells to a position beneath the Purkinje cells, where they form the inner granule layer (IGL). As GCPs exit the cell cycle, they decrease expression of the bHLH gene Atoh1/Math1 and upregulate expression of NeuroD1, Zic1,3 and the tumor suppressor cyclin-dependent kinase inhibitory protein p27Kip1. During their movement from the mantle of the EGL to the deeper layers of the EGL, GCPs extend long and parallel fiber axons that expresses the GPI-linked axonal glycoprotein TAG1, among others.

The process of expansion and migration of cerebellar granule cells is tightly regulated [4,5,6].

Mitogenic pathways that promote GCP proliferation

Mitogenic pathways maintain the rapid expansion of the pool of GCPs in the EGL. Purkinje neurons provide the mitogen sonic hedgehog (Shh) for GCP expansion in the EGL. Shh appears to regulate GCP proliferation through several mechanisms. First, Shh

signaling regulates the expression of the cell cycle regulators cyclin D1 (Ccnd1), cyclin D2 (Ccnd2), and cyclin E (Ccne)[7]. During cerebellar development, early post-natal GCPs express Ccnd1 while GCPs generated during the peak of GCP neurogenesis express both Ccnd1 and Ccnd2.

Notch2 signaling also stimulates granule cell proliferation and inhibits granule neuron differentiation. GCPs treated with Notch2 ligand, JAG1, or a constitutively activated form of the Notch2 receptor results in increased cell proliferation. In these cells, expression of the downstream transcription factor, HES1, is upregulated and HES1 overexpression has a similar ability to maintain proliferation in granule cell progenitor populations. HES1 expression is also induced by the SHH pathway, suggesting that it is a common downstream effector of these two pathways.

In addition, NMyc is also required for the rapid expansion of GCP proliferation in the postnatal cerebellum.

NMyc expression and down regulation of two cyclin dependent kinase inhibitory proteins p18Ink4c and p27Kip1, are both critical for GCP expansion during cerebellar development. [8]

Negative regulators of GCP proliferation

In addition to signaling pathways that promote growth, GCP cell cycle exit and differentiation depends on signaling pathways that provides negative growth regulation. Several signaling molecules antagonize the SHH- mediated proliferation of GCPs, including bFGF [9] and members of the BMP family [10]. Among the latter, BMP2 and BMP4 are expressed in granule cell progenitors, and in postmitotic, differentiating GCPs in the EGL. Studies have showed that Bmp4 antigonizes Shh signaling and induces differentiation of GCPs by rapid posttranscriptional turnover of Math1/Atoh1 [11]. Wnt3 has been identified as a novel negative regulator of GCP proliferation, which is provided by Bergmann glia in the developing cerebellum. [12]

Biological role of Hedgehog pathway

Hedgehog (Hh) pathway is essential for normal embryonic development and it plays critical roles in adult tissue maintenance, renewal and regeneration. Secreted Hh proteins act in a concentration- and time-dependent manner to initiate a series of cellular responses that range from survival and proliferation to cell fate specification and differentiation.

Proper levels of Hh signaling require the regulated production, processing, secretion and trafficking of Hh ligands– in mammals this includes Sonic (Shh), Indian (Ihh) and Desert (Dhh). All Hh ligands are synthesized as precursor proteins that undergo autocatalytic cleavage and concomitant cholesterol modification at the carboxy terminus and palmitoylation at the amino terminus, resulting in a secreted, dually-lipidated protein.

Shh is the most studied ligand of the hedgehog signaling pathway and it is present in cerebellar context [5,6].

Hh signalling [13,14] is initiated when the secreted ligand binds to the transmembrane receptor Patched 1 (Ptch1) and to the co-receptors GAS1, CDON and BOC. **(Fig.2)**

Unliganded Ptch1 inhibits the activity of GPCR-like protein Smoothened (Smo) which in turn controls the downstream modification of GLI Family Zinc Finger (Gli) transcriptional effectors. Gli proteins traffic through cilia and in the absence of Hh signaling are sequestered by suppressor of fused (SUFU) and kinesin-like protein KIF7 (KIF7), allowing for Gli phosphorylation by Protein kinase A (PKA), Glycogen synthase kinase 3 beta (GSK3β) and casein kinase 1 (CK1), and subsequent processing into transcriptional repressors (through cleavage of the carboxy-terminus) or targeting for degradation (mediated by the E3 ubiquitin ligase β-TrCP).

There are three Gli proteins in mammals; Gli2 acts predominantly as an activator and Gli3 as a repressor. Gli1 is not expressed in the absence of signalling but it is transcriptionally induced by Shh signalling and it acts as an activator. In the absence of

Shh, Smo is inactive and the full-length forms of Gli2 and Gli3 (GliFL) are proteolytically processed into repressive forms (GliR).

The binding of Shh to Ptch1 activates Smo that results in Smo accumulation in cilia and phosphorylation of its cytoplasmic tail. Smo mediates downstream signal transduction that includes dissociation of GLI proteins from kinesin-family protein, KIF7, and the key intracellular Hh pathway regulator SUFU, which in turn leads to the inhibition of GliFL processing and the production of the transcriptionally active forms of Gli (GliA). The net Gli activity that results from the balance between GliA and GliR regulates the expression of a number of target genes, including the receptor Ptch1, Gli1 and other transcription factors

The mechanism of Hh signalling is complex and it is not totally understood yet. Important questions are still obscure like how does Ptch1 regulate Smo?

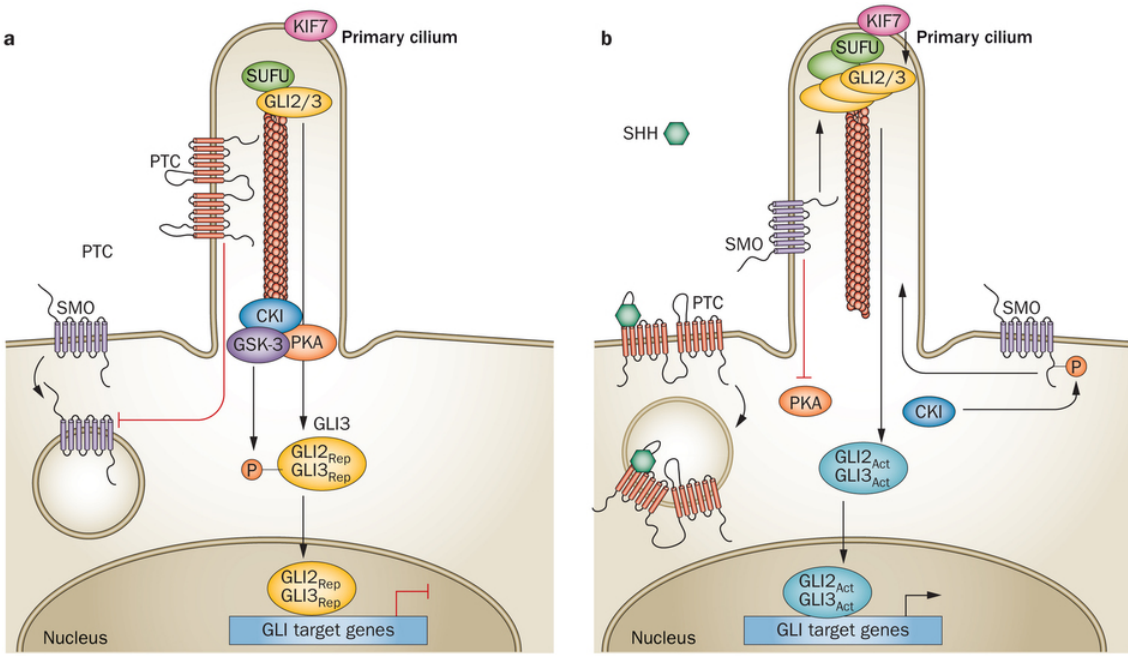

Fig.2 *In vertebrates, primary cilia acts as an organizing centre for processing GLI proteins a) In the absence of Hedgehog ligand binding, Patch1 is localized in the cilium and inhibits SMO through an undetermined mechanism. SUFU directly interacts with GLI2 and GLI3, enabling their phosphorylation by PKA, CKI and GSK-3, which promotes their*

proteolytic processing to GLI2Rep and GLI3Rep. b) Shh binding to Patch1 promotes removal of Patch1 from the cilium. Enzymes (including CKI) phosphorylate Smo, which is activated thereby and accumulates in the primary cilium, resulting in translocation of SUFU–GLI protein complexes to ciliary tips. KIF7 acts through both SUFU-dependent and SUFU-independent mechanisms to control the ciliary localization and dissociation of SUFU–GLI protein complexes, which results in translocation of GLI2Act and GLI3Act into the nucleus, promoting GLI target gene transcription. (B.A. Alman,2015)

Shh and Medulloblastoma

The role of dysregulated Hh signaling in cancer was first characterized by studies of basal cell nevus syndrome. Basal cell nevus syndrome, also known as Gorlin syndrome, is an autosomal dominant disorder that presents itself with craniofacial and skeletal abnormalities and a notably increased risk of advanced basal cell carcinoma and medulloblastoma. The major breakthrough in the understanding of Hh signaling in cancer came from the discovery that mutations in Ptch1 were the cause of Gorlin syndrome suggesting that aberrant Hh pathway activity was responsible for the development of these cancers. These findings were reinforced by the discovery of mutations of Ptch1, Smo, and SUFU in a large percentage of spontaneous basal cell carcinomas and medulloblastomas [15,16].

Hh-driven medulloblastomas represent an intermediate prognosis subgroup. This kind of medulloblastoma has an incidence in childhood and in adulthood.
Germline mutations affecting either Ptch1 or SUFU, which encode negative regulators of the Shh signaling pathway, as well as activating mutations of Smo, result in the development of Shh-type medulloblastoma. Somatic mutations of Ptch1 and occasionally additional pathway components, as MYCN and Gli2 , are common in this subgroup [17].

Medulloblastoma mouse models

In the last 20 years, conventional and advanced conditional genetically engineered mouse models (GEMMs) as well as xenografts have been an indispensable tool in basic cancer biology and translational cancer research. As mammals, mice are closely related to humans at a physiological, biochemical, and genetic level. During the last decade, enhanced knowledge of human and mouse genomes have facilitated genetic manipulation, allowing a better understanding of gene function and making possible a more accurate modelling of human diseases. Ideally, a faithful recapitulation of human disease in genotype and phenotype is an essential element of mouse modelling.

Wingless (WNT) and the Hh pathways have been the primary area of focus in studies related to Medulloblastoma (MB) biology **(Table.1)**. The majority of published MB mouse models thus were associated with these developmental signaling pathways [18].

Table. 1 Summary of many of the GEMMs MB mouse models currently published in the literature. *(Wu X, et al 2011)*

Medulloblastoma genetically engineered mouse models (GEMMs)			
Genotype	**MB profile**	**MB incidence**	**Tumor latency (weeks)**
Ptc+/−	Shh/desmoplastic	14%	5–25
Ptc+/− P53−/−	Shh/desmoplastic	95%	4–12
Ptc+/− Ink4c−/− or +/−	Shh/desmoplastic	30%	12–36
Ptc +/− Kip1−/− or +/−	Shh/desmoplastic	60%–70%	16–18
Ptc+/− Hic1−/−	Shh/desmoplastic	~40%	by 25
Math1-Cre/PtcC/C	Shh/desmoplastic	100%	8–12
Gfap-Cre/PtcC/C	Shh/desmoplastic	100%	3–4
Sufu+/−/P53−/−	Shh/desmoplastic	58%	by 28
Hemizygous ND2-SmoA1	Shh/desmoplastic	48%	25
Homozygous ND2-SmoA1	Shh/desmoplastic	94%	by 24
Gfap-Cre/Rbloxp/loxp/Tp53−/− or loxp/loxp	Shh/desmoplastic	>84%	12
Lig4−/−/p53−/−	Shh/desmoplastic	100%	3–9
Nestin-Cre/Xrcc4loxp/loxp/p53−/−	Shh/desmoplastic	87%	12–14
Nestin-Cre/Xrcc2loxp/loxp/p53−/−	Shh/desmoplastic	>90%	14–16
Nestin-Cre/Lig4loxp/loxp/p53−/−	Shh/desmoplastic	>90%	14–16
Nestin-Cre/Brca2loxp/loxp/p53−/−	Shh/desmoplastic	>90%	14–16
Parp1−/−/p53−/−	Shh/desmoplastic	49%	8–24
GTML	Classic or LCA	75%	by 29
Blbp-Cre/Ctnnb1+/lox(ex3)/Tp53flx/flx	Wnt-subgroup	15%	~41

We focused on describing two models of medulloblastoma that were used in this work: homozygous ND2:SmoA1 and Math1-Cre/Ptc $^{C/C}$.

- *Homozygous ND2:SmoA1 Mouse Model*

Smo/Smo transgenic mice express the constitutively active human point mutation SmoM2 (Trp535Leu) in the mouse protein (corresponding aminoacid: Trp539Leu) which is referred to as SmoA1. This is under the control of the mouse neurogenic differentiation 2 (ND2) promoter. Transgene expression is specific to cerebellar granule cells. The Smo/Smo model is the first mouse medulloblastoma model that show leptomeningeal spread [19].

The ND2:SmoA1 transgene containing the entire mouse smoothened homolog (Smo) gene, with the constitutively active point mutation SmoA1, is also tagged with both His6 and Myc3. The hemizygotes animals can be inter-crossed to produce homozygotes.

- *Math1-Cre/Ptc$^{C/C}$ Mouse Model*

Mice heterozygous for mutations in Ptch1 develop cerebellar tumours that resemble human medulloblastoma. In these mice, Ptch1 is inactivated in all cells (including GCPs and Neural Stem Cells (NSCs)), so definitive conclusions about the cell of origin were not possible. To determine whether defeat of Ptch1 in GCPs can lead to medulloblastoma, a Ptch1 knockout specific for GCP was created. To this end, conditional Ptc$^{C/C}$ mice were generated by breeding mice homozygous for the floxed Ptch1 allele to Math1-Cre mice: Math-Cre/Ptc $^{C/C}$ (Ptch KO) [20].

Math1-Cre mice were generated using a construct containing the bacteriophage P1 cre recombinase coding sequence downstream of a 1.4 kb Math1 enhancer element that has been previously used to express transgenes in GNPs.
Conditional Ptch1 knockout (Ptc $^{C/C}$) mice have loxP recombinase recognition sites flanking a portion of the Ptch1 gene.

Neurodegenerative disorders associated with DDR defects

Neurons display high rates of transcription and translation associated with high rates of metabolism and mitochondrial activity. The amount of oxygen consumed by the brain relative to its size far exceeds that of other organs. This high activity coupled with high oxygen consumption creates a stressful environment for neurons. It produces primarily reactive oxygen species (ROS) that constantly attacks neuronal genomic and mitochondrial DNA.

If DNA damage occurs, an elaborate signaling network, a sort of cellular defence system known as the DNA damage response (DDR), is then activated [21-23].
One of the most powerful activator of the DDR is the double strand break (DSB) in the DNA.

DSB response is a multi-tiered process that begins with rapid recruitment to the break sites of early damage response proteins (commonly named “sensors”), which create expanding nuclear foci at these sites. Among them are the MRE11/RAD50/NBS1 (MRN) complex, the tumor suppressor protein BRCA1, the p53-binding protein 53BP1, MDC1, RNF8, RAP80 and Abraxas. These proteins participate in the initial process of the damage and gradually activate a signaling cascade that leads to the activation of the transducers. The primary transducer of the DSB alarm is the nuclear serine-threonine kinase ATM. In response to DSB induction, inert ATM is rapidly activated while undergoing several post-translational modifications, and a portion of activated ATM is recruited to the DSB sites. ATM then phosphorylates several effectors, which are players in a variety of damage response pathways. ATM belongs to a conserved family of “PI3K-like protein kinases” (PIKKs), which includes among others two other major DDR transducers, the DNA-dependent protein kinase (DNA-PK), and ATR (ataxia-telangiectasia and Rad3-related).

Evidence of the connection between DNA damage and neurodegeneration is found in genetic disorders affecting the DDR, chronic neurodegenerative disorders, and studies of neurodevelopment and aging processes [24]. **(Fig.3)**

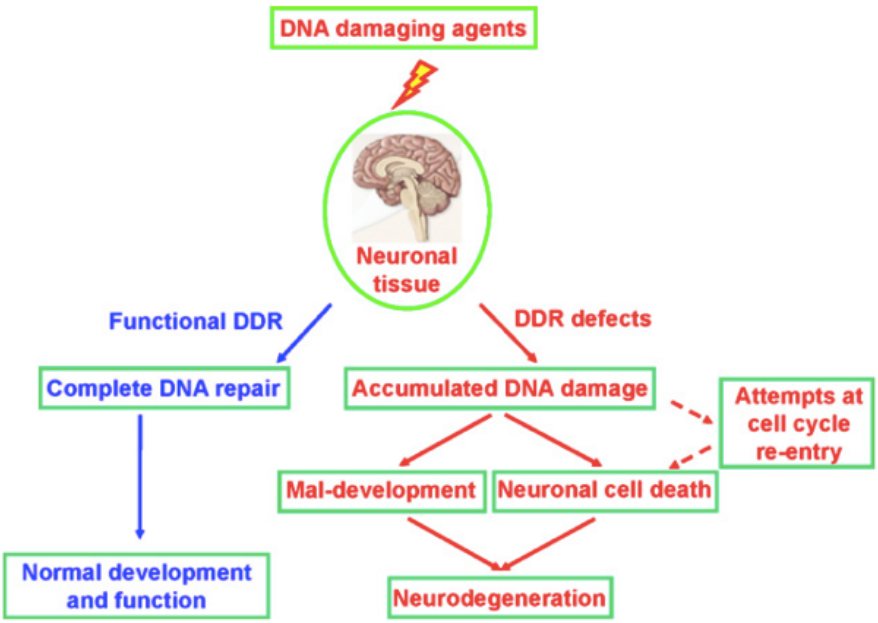

Fig.3 *Effects of defective DDR pathways on the CNS (A.Barzilai, et al 2008)*

Several human monogenic defects affecting DDR pathways are characterized by neurological deficits **(Table.2)**

DSB response				
Syndrome	Affected protein and pathway	Affected brain area	Main neuronal implications	Non-neuronal symptoms
Ataxia telangiectasia (A-T)	ATM (activation of the DSB response)	Cerebellum; extrapyramidal movement system	Early onset of cerebellar ataxia degeneration of Purkinje and granule neurons; progressive ataxia; oculomotor deficits; dysarthria	Immunodeficiency; telangiectasia; radiosensitivity; lymphoid malignancy; sterility
A-T like disorder (A-TLD)	MRE11 (sensing and initial processing of DSBs; ATM activation)	Cerebellum	Late onset of cerebellar ataxia; degeneration of Purkinje and granule neurons; dysarthria; abnormal eye movement;	Radiosensitivity; chromosome fragility
Nijmegen breakage Syndrome (NBS)	NBS1 (MRN complex function; ATM recruitment; other, unknown pathways)	Cerebral cortex	Microcephaly; mental deficiency	Immunodeficiency; radiosensitivity; chromosome fragility; lymphoid malignancy
ATR Seckel syndrome (ATR-SS)	ATR; PCNT (ATR-mediated signaling)	Cerebral cortex	Microcephaly; mental deficiency	Growth retardation; skeletal abnormalities; dysmorphic facies
Primary microcephaly (PM)	MCPH1/BRIT	Cerebral cortex	Defective neurogenesis; mental retardation	Breast, ovarian and prostate cancer
Immunodeficiency with microcephaly	Cernunnos/XLF	Cerebral cortex	Microcephaly	Growth retardation; lymphopenia; cellular radiosensitivity
Ligase 4 syndrome	LIG4 (NHEJ pathway of DSB repair)	Cerebral cortex	Microcephaly; cognitive delay	Developmental and growth delay; immune deficiency; pancytopenia; dysmorphic facies; lymphoid tumors; cellular radiosensitivity
SSB response				
Ataxia with oculomotor apraxia (AOA1)	Aprataxin	Cerebellum; nigro-striatal pathway	Early onset ataxia; neurodegeneration (Purkinje neurons); oculomotor apraxia; dysarthria; limb dysmetria	Hypoalbuminemia; hypocholesterolemia
Ataxia-ocular apraxia 2 (AOA2)	Senataxin	Cerebellum spinal cord	Cerebellar atrophy; axonal sensorimotor neuropathy; oculomotor apraxia; dysarthria; distal amyotrophy	Elevated α-fetoprotein
Spinocerebellar ataxia with axonal neuropathy (SCAN1)	Tyrosyl-DNA phosphor-diesterase (TDP1)	Spinocerebellar pathway	Cerebellar ataxia; axonal sensorimotor neuropathy	Hypoalbuminemia; hypercholesterolemia
Nucleotide excision repair				
Xeroderma pigmentosum (XP) with neurological disease	XPA, XPD, XPG	Cerebrum	Sensorineural deafness; progressive neurodegeneration; cerebral and cerebellar atrophy; dysmyelination; calcification of basal ganglia (30% of patients)	Sunlight sensitivity; increased skin pigmentation; multiple skin lesions; skin cancer
Cockayne's syndrome (CS) and XP/CS	CSA, CSB, XPB, XPD, XPG	Cerebral cortex	Microcephaly; progressive neurodegeneration; cerebral and cerebellar atrophy; sensorineural deafness; dysmyelination	Growth retardation; sunlight sensitivity; congenital cataracts; progeric symptoms; immature sexual development
Trichothiodystrophy (TTD)	XPB, XPD TFB5/TTDA	Cerebral cortex	Microcephaly; hypomyelination; psychomotoric abnormalities	Brittle hair; sunlight sensitivity; short stature; developmental delay; congenital cataracts;

***Table 2**. DDR defects associated with neurodegenerative disorders. (A.Barzilai, et al 2008)*

In this study we used the NBN-CNS-del animal model to approach our study on DDR defects in development. The protein encoded by NBS1 (called Nbn in mice), was eliminated in the developing nervous system. NBN-CNS-del mice show extensive genome damage in neural precursor cells, which is thought to lead to apoptosis, and extensive cell loss and abnormal development of the cerebellum [25].

- *NBN-CNS-del Mouse Model*

Frappat et al. previously showed that the disruption of exon 6 results in Nbn null mutation, leading to early embryonic lethality. Thus, they first used gene targeting to engineer a mouse strain in which exon 6 was deleted (Nbn+/ Δ) . Nbn Δ/ Δ embryos did

not survive, suggesting that the deletion of exon 6 caused a null mutation of Nbn. They next generated mice carrying the Nbn allele modified by flanking exon 6 with two loxP sites (Nbn+/F6). NbnF6/F6 homozygous mice were healthy and phenotypically normal. To disrupt the Nbn gene in the CNS, these mice were crossed with Nbn+/- mice and with nestin-Cre transgenic mice generating mouse 'Nbn-CNS-del'.

Neural stem cells

Neural stem cells (NSCs) in the adult mammalian brain has fostered several translational and preclinical studies to investigate future therapeutic approaches for the cure of neurodegenerative diseases [26,27].

The isolation and characterization of multipotent NSCs from multiple locations within the mammalian brain has represented one of the most significant advancements in neuroscience and has provided accruing evidence of endogenous NSC potential to respond to neurological injuries. Thus far, germinative zones have been identified in the subgranular zone (SGZ) of the hippocampus, in the olfactory bulb, in the subventricular zone (SVZ) surrounding the ventricles, and in the subcallosal zone underlying the corpus callosum. Some studies have reported the presence of active neurogenesis even in the adult cerebellum.

However, the precise mechanism by which NSCs and progenitor cells self-renew and generate neurons at the same time is unclear. Because adult NSCs maintain a glial identity resembling that embryonic radial glia, many studies suggest that the principles elucidated during the development can be considered valid also for adult neurogenesis. Endothelial cells secrete factors that enhance self-renewal and neuron generation from progenitor cells, suggesting that the vascular niche harbors mostly activated progenitors and supporting the concept of a niche-dependent self-renewal. Increasing distance from the niche or under restrictive conditions, NSCs spontaneously arrest to proliferate and differentiate, they even die. This behavior is likely due to the absence of factors promoting self-renewal and also to the presence of signals fostering NSCs toward differentiation.

Following the acceptance of the existence of multipotent NSCs in the adult mammalian brain able to self-renew and differentiate in vitro, questions arose about

specific selection criteria to establish stable NSC lines for either basic research or therapeutic purposes. NSCs are characterized by the expression of markers such as Sox2, glial fibrillary acidic protein (GFAP), nestin, and Musashi 1 and 2, but no specific combination of markers has so far been identified to definitely distinguish an NSC from a transiently amplifying progenitors (neural precursor cells [NPCs]). Alternatively, the neurosphere assay is based on the self-renewal capacity of NSCs, which is dependent on the presence of the mitogenic factors basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), and it was developed as a procedure for the isolation and expansion of NSCs in vitro. Under these culture conditions, NSCs grow in suspension as floating cell clusters, called neurospheres, which are heterogeneously composed of stem, progenitor, and differentiating cells. This method highlights that actively proliferating progenitors also generate neurospheres in vitro.

NCSs demonstrate differentiation capability to the three neural lineages: neuronal, astrocytic and oligodendrocytic [28].

As mentioned above, the cerebellum is mainly composed by cerebellar granules that nevertheless can not be obtained by neurospheres culture from P7 murine cerebellum (using serum free culture conditions supplemented with EGF and bFGF), isolated cells positive for ATOH1-GFP are not responsive to SHH stimulation in these culture conditions [29].

Current culture conditions of the neurospheres limit the study to only some lineage of the central nervous system.

A novel, Hedgehog pathway driven, culture of GCPs.

In a previous and yet unpublished work, our group described novel and specific culture conditions to indefinitely grow primary untransformed GCPs as neurospheres. This was obtained by modifying the classical protocol of neurospheres generation aiming to maintain the constitutive activation of the Hedgehog (Hh) pathway in GCPs, *in vitro*.

Cells explanted from postnatal day 7 (P7) murine cerebellum can be grown in a serum-free neurosphere culture condition in the presence of defined concentration of SAG, a Smoothened agonist. Addition of other growth factors typically used in neurospheres generation, such as bFGF, need to be totally excluded, since they are potent inhibitors of the Hh pathway [9] **(Fig. 4a)**. Under these conditions, GCPs neurospheres can be cultured for extended periods always maintaining Hh pathway activation **(Fig. 4b)**. We have named these cultures SAG-dependent cerebellar neurospheres (S-cNS) as compared to growth factor-dependent cerebellar neurospheres (GF-cNS)

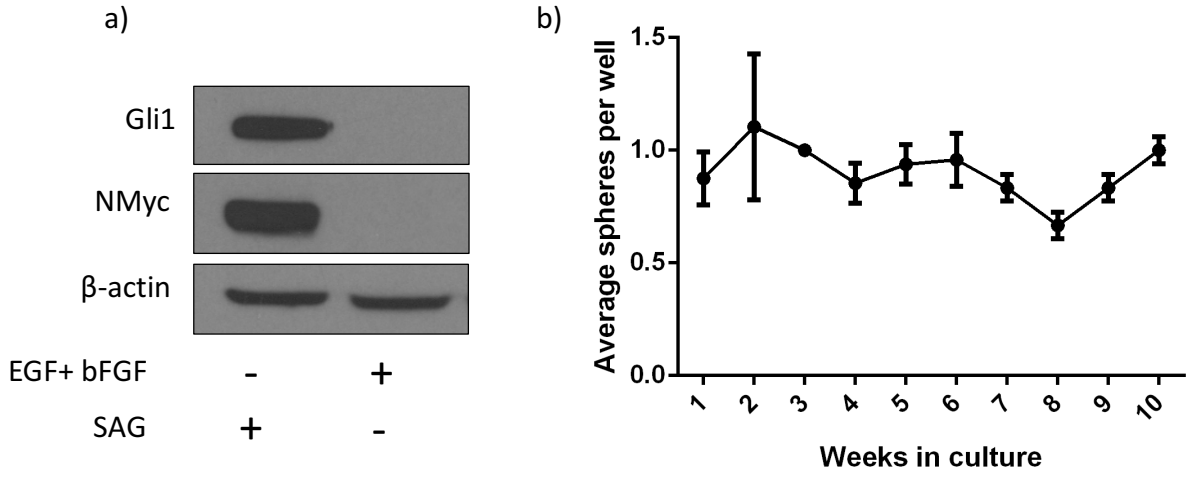

Fig.4 *GCPs grow as neurospheres by activating the HH pathway a) Western blot illustrated divergence in HH activation pathway between GF-cNS and S-cNS (1 week cultures). S-cNS maintains high levels of Gli1 and NMYC. B) Clonogenic assay was performed each week for a total of 10. S-cNS has self-renewal capability and constant rate of proliferation. Figures from C. Heil (PhD thesis)*

S-cNS express markers of neural progenitors/stem cells such as SOX2, Nanog, POU3f2 and POU5f1, whose function is known to contribute to maintenance of stem-like features [30,31]. At variance from GF-cNS, S-cNS could only be grown from cerebellar explants, but not from sub-ventricular zone explants. Moreover they specifically express ATOH1, ZIC1 and NESTIN, which are known markers of proliferating GCPs. These data strongly support that S-cNS take origin from proliferating GCPs

Conventional neurospheres are said to be multipotent since in a defined differentiation medium they have the capability to differentiate into cells of three lineages of the NSC, neuronal, astrocytic and oligodendrocytic [29]. In the same differentiation medium, S-cNS adhere to the substrate, down-regulate ATOH1 expression and more than 90% of them differentiate into Granule Cells , expressing specific markers, such as beta3-tubulin and GABRA receptor alpha 2 (GABRA2). At variance from differentiated GF-cNS, S-cNS do not express the glial cell marker GFAP **(Fig. 5a)**.

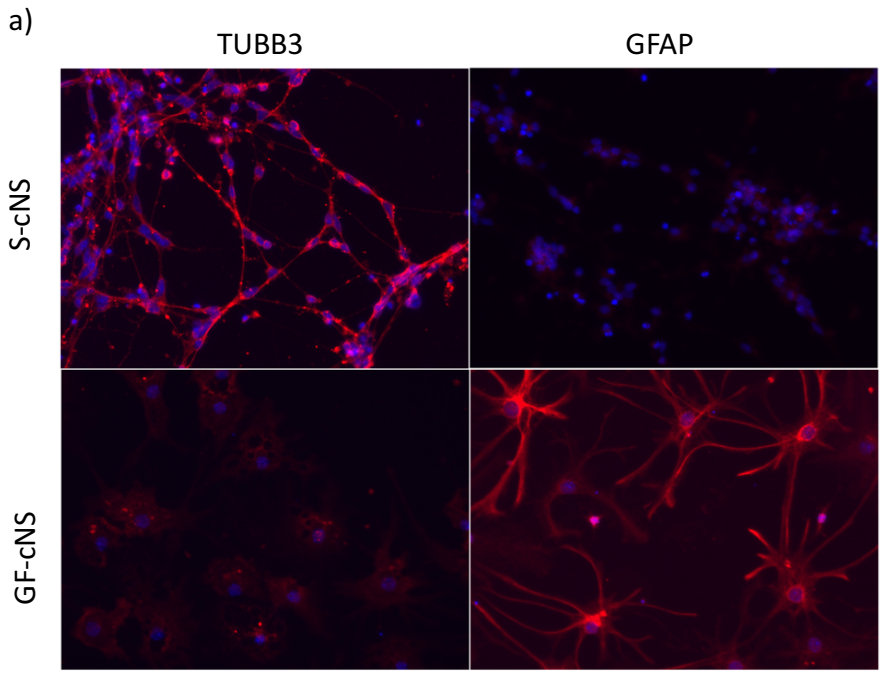

Fig.5 *S-cNS and GF-cNS differentiated towards different lineages a) Inmunostaining after 5 days in differentiation medium; S-cNS cells were highly positive for TUBB3 while GF-cNS cells were mostly positive for GFAP. Figures from C. Heil (PhD thesis)*

AIM OF THE WORK

Our group established for the first time a novel culture protocol to grow cerebellar granule cell progenitors (GCPs) as neurospheres by maintaining a constitutive activation of the Hedgehog pathway, by means of the Smoothened agonist, SAG.

Following up this work, we have now addressed different aspects of SAG-dependent cerebellar neurospheres (S-cNS) in order to improve and complete their characterization and to experience possible applications.

We have appropriately defined the SAG concentration dependence during the "genesis" of the culture, and at the steady state on well-established cultures. Moreover, we defined the cerebellar mouse developmental time frame in which S-cNS can be generated from cerebellar explants.

Several aspects of the Hh pathway remain poorly understood, largely due to the lack of appropriate models for in vitro studies. Therefore, we tested whether S-cNS can be used as a new tool to answer specific questions regarding the regulation of Hh signaling. To this end we selected murine models of Hh-dependent medulloblastoma, the Math1-Cre/Ptc $^{C/C}$ (Ptch KO) model and the Homozygous ND2:SMOA1 (ND2-SmoA1) transgenic model, in order to assess whether and how these mutations affect Hh activity in a cell-autonomous condition.

While, as expected, Patch KO allowed generation of cNS even independent of SAG, confirming that Hh pathway is constitutively on in GC from this model, surprisingly we demonstrated for the first time that the SmoM2 (Trp535Leu) mutation expressed in the transgenic ND2-SMOA1 mouse model is not constitutively active in a cell autonomous environment.

To further show the potentiality of the newly developed neurospheres model, we also generated cNS from the conditional NBN-CNS-del model. This approach allowed us to show that while NBN KO is detrimental for cell proliferation in multiple environments, it is absolutely essential for GCPs.

Thus, our work proves that S-cNS represents a novel and important *in vitro* tool to study GCPs pathophysiology

RESULTS

1- GCPs neurospheres are continuously dependent on SAG.

To further characterize the *in vitro* properties of the newly described S-cNS model, we generated cultures using different SAG concentrations. In order to do this P7 cerebellum explants from wild type (WT) mouse were disaggregated and the cells counted and plated with SAG concentrations ranging from 0 to 1 uM. SAG concentrations from 0 to 0.2 uM clearly provided a dose-response effect on the clonogenic assay, while a plateau was reached at higher concentrations **(Fig. 6a**). This clonogenic effect was associated to the expression of typical Hh targets such as Gli1 and N-Myc **(Fig. 6b)**. Thus, we established 0.2 uM SAG as the minimal and optimal concentration that allows a stable GCPs proliferation over time.

S-cNS can be maintained in culture for long times. Nonetheless, these cells remain strictly dependent on SAG for survival and proliferation. Indeed, SAG washout quickly caused cell death, as indicated by the conversion of the bright and translucent appearance of S-cNS into dark and opaque spheres containing picnotic cells **(Fig. 6c)**. Consistently, 24 hours of SAG deprivation was sufficient to induce PARP1 cleavage, supporting the occurrence of cell death, possibly by apoptosis. At the same time we observed disappearance of Gli1 and Ciclin-D1 expression, supporting the complete shut-off of Hh pathway in this culture condition **(Fig. 6d).** These data indicate that SAG is not only essential for proliferation but also for survival of primary GCPs, in neurospheres culture conditions.

2- Generation of SAG-dependent neurospheres from explanted murine cerebella is possible from P1 to P7

H&E staining of cerebellar section form WT (C57BL/6J) mice taken at different time points clearly illustrate the different phases of foliation, GC expansion and migration **(Fig. 7a)**. In fact it is very well characterize that, after an initial expansion time (between P1 and P7) GC start to migrate to the IGL and to differentiate into mature GC (by P15). The initial expansion phase is largely dependent on Hh activity, which indeed declines between P7 and P21 (see Gli1 and N-Myc expression levels, **Fig. 7b**) when maturation of the mouse cerebellum is complete.

As already mentioned above, initially we focused on P7, the time when Hh-dependent GC expansion peaks, as the right developmental time to generate S-cNS. After that, we asked ourselves if S-cNS could be generated in other cerebellar developmental stages. Thus, we attempted the generation of S-CNS at different time points representative of specific cerebellar developmental stages: P1, P7, P14, P21. These experiments showed that S-cNS culture can be generated from P1 to P7, but not from P14 and P21 cerebellar explants. **(Fig. 7c)**.

Therefore, these data indicate that only proliferating GCPs populating the cerebellum between P1 and P7 can be grown in culture in the presence of SAG. GC explanted from the cerebellum at later time points; on the contrary, seem to have unreversably lost the possibility to respond with proliferation and/or survival to Hh stimulation.

3- Constitutive activation of the Hedgehog pathway allows genesis of GCPs neurospheres in the absence of SAG.

Activation of the Hh pathway is common in about 30% of human medulloblastoma. In many instances this is dependent on known gain- or loss-of-function mutations of genes encoding components of the Hh signaling (Smo or PTCH1/2 and SUFU, respectively). Consistently, numerous murine models of medulloblastoma with a hyper activation of the Hh pathway could be generated by introducing mutations in the same or other genes **(Table 1).** These models strongly improved our understanding of Hh-related developmental issues and tumorigenesis. Nonetheless many aspects of Hh-signaling which might also have significant impact on Hh-tumorigenesis are still obscure.

Even the simple comparison of the phenotypes of these mouse models may highlight some major differences. In example, ND2-SmoA1 transgenic mice [19] significantly differ from Patch KO [20]. In principle, both mutations should drive a similar constitutive activation of the Hh pathway due to either Ptch1 loss or direct Smoothened activation. Nonetheless the two models significantly differ in the timing of medulloblastoma development, which occurs much earlier in the Patch KO than in the ND2-SmoA1 model. Much more they differ in their cerebellar developmental appearance. Light images of hematoxylin-eosin stained cerebellar tissue from P1 to P21 illustrate large homology between ND2-SmoA1 and WT animals in the early development of the cerebellum **(Fig. 8a).** However, there are important events that should be mentioned. Indeed ND2-SmoA1 prolongs GC expansion to later times, as described by [19]. A greater EGL thickness can be observed in the ND2-SmoA1 cerebellum compared to WT, at P14 **(Fig. 8b).** Moreover, migration from EGL to IGL, that largely occurs in the correct developmental time frame, remains incomplete in the ND2-SmoA1 model, since some cells never migrate and remain in EGL at p21 **(Fig. 8b).**

In sharp contrast, the Patch KO shows a completely subverted postnatal development, with an astonishingly enlarged EGL, which persists from P7 to P21, to become a tumor shortly after **(Fig. 8a, 8b)**

These differences at the morphological observation of the structural modifications of the cerebellum during postnatal development suggest important differences in the timing and perhaps strength of the Hh activity. These differences are indeed paralleled by differences in the expression of Hh markers from P1 to P21. Indeed, examination of Gli1 and N-Myc expression highlights that Hh activity is still flourishing at P14 in the ND2-SmoA1, but not WT cerebella, but is substantially silenced in both models at P21 **(Fig. 8c)** Expression levels of Gli1 and N-Myc are very high in the Patch KO cerebella through all developmental stages and remain very high also at P21.

To gain more hints on the features of Hh activity in the Patch KO and ND2-SmoA1 models we established S-cNS, under the idea that both mutations (Patch KO and ND2-SmoA1) should be associated with constitutive Smo (and thus Hh) activity, such that cNS could grow also without SAG when explanted from these models. Therefore, we explanted cells front the cerebellum of the different models at relevant times of cerebellar development P1, P7, P14 to P21.

First, we analyzed the number of cells isolated. Confirming a much stronger activation of the Hh pathway in the Patch KO model, the cells obtained from it were always largely superior compared with WT and ND2-SmoA1 cerebella **(Fig. 9a).**

Then, we plated an equal number of cells explanted from P7 cerebellum of each model in the absence or presence of SAG.

As expected WT explants only generated S-cNS, while no spheres could be obtained without SAG. In sharp contrast, explants from the Patch KO could be generated either in the presence or absence of SAG **(Fig. 9b)**, confirming that release from Patch inhibition is sufficient for Smo activity and that a constitutively active Hh pathway is all is needed for maintenance and propagation of GCPs in vitro.

4- The SmoM2 (Trp535Leu) mutation expressed in the transgenic ND2-SMOA1 medulloblastoma mouse model is not sufficient for a constitutive activation of the Hedgehog pathway.

Interestingly, we obtained only modestly increased number of GC from ND2-SmoA1 compared to WT explants **(Fig. 9a)**, and definitely much lower numbers compared with Patch KO explants, suggesting once again a huge difference in the activity of the two mutations.

Surprisingly, explants from the ND2-SmoA1 model were not competent for growth and survival in the absence of SAG. These data indicate that, in a cell autonomous context, the SmoM2 (Trp535Leu) mutation expressed in the transgenic ND2-SmoA1 mouse model is not constitutively active.

Nonetheless, ND2-SmoA1 explants appeared more efficient than WT explants for the generation of S-cNS **(Fig. 9b)**. To further characterize the differences in the two mouse models, we performed GCPs cultures at different developmental times. These experiments again confirmed that WT explants could only give rise to S-cNS between P1 and P7, while ND2-SmoA1 explants were still capable of generating S-cNS up until P14. Only Patch KO explants could originate cNS at any developmental stage, even without SAG **(Fig. 10b)**. This data is consistent with the presence of Ki-67 positive GC in the ND2-SmoA1 but not WT model, at P14 and at any time point in the Patch KO model **(Fig. 10a)**.

Thus, although the mutant SmoA1 is clearly different than WT SMO, it clearly can still be inhibited by Patch in our cell autonomous GCPs culture.

To further address this issue, we performed SAG dose-response experiments on WT and ND2-SmoA1 explants. For this purpose we performed clonogenic assays using SAG concentration from 0.005 uM to 0.200 uM. **(Fig 11a)**. Once again, we obtained no neurospheres growing in the absence of SAG from both WT and ND2-SmoA1 explants, while 0.050 uM SAG was already sufficient to support an average of 1 neurospheres/well in the ND2-SmoA1, but not WT explants. Consistently, at the 0.200uM SAG concentration on average ND2-SmoA1 explants gave rise to 3 neurospheres/well

compared to 1 neurospheres/well obtained from WT explants.

As previously shown, in WT S-cNS SAG deprivation leads to early Gli1 and later N-Myc decline, indicating a drop in Hh signaling, followed by cell death **(see Fig.6d)**. Interestingly ND2-SmoA1 S-cNS undergoing SAG deprivation experienced cell death and Hh pathways shut off at much later times, compared to WT S-cNS **(Fig. 11b)**. Once again, these data confirm that the SmoM2 mutation is not constitutively active in a cell autonomous context and requires SAG for transducing Hh-signaling. However, they also clearly indicate major differences in activity between WT and mutant Smo.

An alternative possibility that would explain lack of SAG independence in the SmoA1 S-cNS would be loss or reduction of the transgene expression at culture conditions. To exclude this possibility we took advantage of the presence of a Myc-tag in the ND2-SmoA1 construct that was employed for the generation of the ND2-SmoA1 model [19] **(Fig. 12a)**. Indeed, we first showed that a Myc-tag specific RbAb could detect transgenic Smo in cerebellar extracts from the ND2-SmoA1 but not from WT mouse **(Fig. 12b)**. Then we showed that the transgene is expressed also in S-cNS cultures, eventually at higher levels compared to P7 cerebellar extracts **(Fig. 12c**). Further experiments confirmed the high transgene expression at different times of S-cNS cell culture.

5- NBS1 depletion abolish proliferation of SAG-dependent, but not GF-dependent, cerebellar neurospheres

Here we addressed the hypothesis that S-cNS might be used to asses different issues concerning GCPs physiopathology.

The MRE11/RAD50/NBN (MRN) complex is a major sensor of DNA double strand breaks, which also plays a pivotal role in controlling faithful DNA replication and preventing replication stress. Our group recently published that N-Myc promotes MRN complex expression in Hh-induced mouse cerebellar GCPs and also demonstrated that the MRN complex is required for N-Myc-dependent proliferation. [32]

Enhancing the understanding of MRN complex functions in different neuronal cell compartment is important to improve our comprehension of MRN-related pathologies such as Nijmegen Breakage Syndrome or AT-like Syndrome, due to inherited defects in the NBN and MRE11 genes, respectively [33,34,35]

The NBN-CNS-del mouse, characterized by the nestin-dependent and CRE-mediated NBN KO in neuronal cells, is characterized by typical Nijmegen Breakage Syndrome-like features including microcephaly and strongly impaired cerebellar development due to impaired proliferation/survival of GCPs.

In fact, the NBN-CNS-del mouse shows a large decrease in the number of GCPs in the EGL compared to WT animals, at multiple stages in cerebellar postnatal development **(Fig. 13a)**, a difference already remarkable at P5. This can be further documented by counting the cells obtained by cerebellar explants in NBN-CNS-del compared to WT mice, at P5 **(Fig. 13b)**.

By performing primary GC and S-cNS cultures from cerebellar explants from the NBN-CNS-del mouse we observed several features. Firstly, three-to five times more NBN-CNS-del GC should be put in culture to obtain a similar number of adhering on polylisine and surviving cells compared to WT samples **(Fig. 14a)**. Moreover, these cells show a much lower proliferative response to 48 hours of exposure to maximal doses of SAG **(Fig. 14a)**. Importantly, no S-cNS could be grown by NBN-CNS-del cerebellar explants, while GF-cNS could be obtained, although inefficiently **(Fig. 14b)**. After establishment of GF-cNS from WT and NBN-CNS-del mice, spheres were dissociated and plated clonally. Both genotypes gave rise to spheres, although GF-cNS from NBN-CNS-del showed always lower clonogenic capability than GF-cNS from WT mouse. **(Fig. 14c)** Interestingly, GF-cNS can be re-seeded in SAG-dependent medium and they may regenerate S-cNS, while the converse is not possible (not shown). Therefore, we switched to SAG-containing medium the GF-cNS from WT and NBN-CNS-del explants. Again, NBN-CNS-del S-cNS were unable to form.

Overall, these data indicate that NBN function plays a 'special' role in GCPs, which appear to be unable to survive in the absence of NBN. In contrast NBN depletion is detrimental but not totally intolerable for GF-dependent spheres, suggesting that NBN is important, but not essential for GF-cNS survival. These data are consistent with a much stronger defect of cerebellar GC compared to other cell types that is observed in the NBN-CNS-del mouse. The S-cNS and GF-cNF cultures will be of primary relevance to further analyze this issue.

DISCUSSION

A number of issues related to Hh signalling and tumorigenesis are still largely not understood. In example, how Patch inhibitory signal is transduced to Smoothened in order to prevent downstream signaling is not completely clarified. As a consequence, how exactly hedgehog ligands remove the inhibitory function of Patch on Smoothened is also poorly characterized, at the molecular level. At least in part, this is due to lack of long-term cell models that may allow biological/biochemical characterization of those issues, in a physiological context.

Indeed most studies in the field are performed either on transient primary GC cultures, or medulloblastoma cell lines, or Patch-/- MEF or in cell models genetically engineered for overexpressing one or more Hh-signaling components. Although each of these models provided valuable help in understanding Hh pathway, each of them has is own limitations. Transient primary GC cultures promote differentiation and cannot be passaged in vitro beyond few cycles of division, even in activating conditions of Hh signaling, which is shown to promote proliferation [7]. Thus, the limited time frame and the relatively small amount of cells that can be explanted from individual animals represent the major inconvenient of this model. In contrast, continuous medulloblastoma cell lines can be grown in culture in big amounts and for unlimited time intervals. However, the expression and activation of Hh components and signaling decline over time, reaching levels very often approaching detection limits in different biological and biochemical assays. Patch-/- MEF, and their derivatives, are important models for performing biochemical studies. But the results obtained in fibroblasts may not be directly extended to Hh activity in neurons or skin cells. Finally, overexpression systems are very versatile, but overexpression per se may lead to phenotypes, which not always compare with the physiological level of expression of specific signal transduction components.

Our lab defined novel culture conditions to grow freshly explanted cerebellar GCPs in the form of neurospheres, which we have named SAG-dependent cerebellar neurospheres (S-cNS). Interestingly, these cells: i) can be generated from cerebellum,

but not SVZ; ii) show unlimited replication in vitro; iii) express GCPs and stemness markers, as well as high Hh-activity; iv) can be induced to differentiate into mature GC; v) maybe generated from Patch-KO mice, without addition of mitogens; all of which indeed supports their identity as transit-amplifying granule cell progenitors. Importantly, these cells, generated by removing Hh-inhibitory GF and by adding SAG as a main driver of Hh-activity, maintain SAG-dependence even after long time in culture, and immediately shut off Hh pathway and die on SAG removal.

Interestingly, these cells may be generated from P1 to P7 cerebella, but not from P14 to P21 developmental stages, suggesting not only that transit-amplifying GCP have a limited existence in vivo, but also that GC existing in the cerebellum at p14 and later developmental stages cannot be recruited any longer into an Hh-dependent survival and proliferation program.

Such a model may be surely useful in a number of studies not only concerning Hh pathway, but more in general addressing the physiopathology of cerebellar GC. To provide initial evidence of this, we have used the S-cNS cultures to address whether loss and gain of function mutations in the Hh pathway may indeed lead to its constitutive activation in a "cell autonomous" context.

The possibility to indefinitely grow cNS from Patch-KO cerebellar explants preserving an high Hh activity in cell autonomous culture conditions, from all postnatal developmental stages tested so far, without supplementation with any mitogen allows a number of conclusions. Firstly, cNS indeed represent Hh-dependent proliferating GCPs. Second, in the absence of Patch, Smoothened is a constitutively and fully active signal transducer, whose activity is sufficient to drive Hh-dependent survival and proliferation signals, in GCPs. Third, this activity is sufficient to drive continuous proliferation of GCPs in vitro and in vivo, despite local conditions may allow GC growth arrest, migration into the IGL and differentiation, in the Patch-KO cerebellum, suggesting that exogenous signals may suppress Hh-activity independent of Patch. Forth, the continuous presence of big numbers of proliferating GCPs in the cerebellum of Patch-KO animals even up to p21 indicate that this continuous proliferation is the basis for the early transformation into medulloblastoma observed in this model.

At variance from Patch-KO mice, ND2-SmoA1 mice develop medulloblastoma much later. Moreover, while postnatal cerebellar development is diverted in Patch-KO mice, ND2-SmoA1 mice show and increased and prolonged GC expansion phase (up to P14), but by P21 the cerebellum is substantially identical to a WT. A minor, but significant difference is provided by the persistence of few and isolated nests of proliferating GC defining hyperplastic regions, which are though to be precursors of medulloblastoma.

Interestingly the SmoM2 mutation expressed in the ND2-SmoA1 transgenic model was reported to be "constitutively active" (that is unrepressed by Patch) in multiple reports, although partially contrasting evidenced have also been reported [36]. Our attempt to generate SAG-independent cNS from the ND2-SmoA1 model repeatedly failed, indicating that SmoM2 mutant can be efficiently repressed by Patch in a cell autonomous context. Moreover SmoA1 S-cNS continuously require SAG for proliferation and survival, similar to WT counterparts. Nonetheless, our experiments also support that the SmoM2 mutation is affecting Smo function making it more prone to activation by SAG. Indeed, SmoA1 S-cNS could be generated in lower SAG concentration compared to WT. Also, SAG deprivation resulted in a longer interval before Hh-pathway shut off and cell death in SmoA1 S-cNS, compared to WT. Moreover, the mutant Smo was able to prolong the existence of proliferating GCPs up to P14, since we could generate S-cNS at this developmental stage from ND2-SmoA1, but not WT mice. But, mutant Smo was not sufficient to prevent Hh pathway shut down, as it happens for Patch-KO mice. In fact, with the exception already mentioned above, SmoA1 P21 cerebella are basically indistinguishable from the WT and cannot be a source of S-cNS any longer. Overall, these observations indicate that the mutant SmoM2 can be repressed by Patch, in a cell autonomous context, which was not expected according to current knowledge in the field.

This evidence of course raise the question of how the SmoM2 mutation affect Smo activity and Hh-signalling, and in particular what else is present in vivo to support SmoM2 activation in a different way compared to WT Smo. Of course, one possibility deals with a different sensitivity of the two molecules to Hh ligands, and future experiments will make use of S-cNS to investigate on this topic.

However, our model may also facilitate other studies concerning how the signal is transferred between Patch and Smo. It has been reported that SmoM2 is constitutively located in the primary cilium, unlike WT Smo, and this is sufficient for activation of the signaling cascade [37]. However, in recent years several papers demonstrated that the accumulation of Smo protein in the primary cilium is not sufficient per se to promote pathway activation [38-39]. Cyclopamine, a natural Smo inhibitor, promotes accumulation of both WT Smo as well SmoA1 in the cilium, despite in both cases it leads to inhibition of Hh-pathway. Thus it seems that Smo location in the cilium might be required, but not sufficient, for signaling activation. Therefore, Rohatgi R. et al. [39] proposed a two steps mechanism for Smo activation. The first step involves Smo shuttling to the cilium; in the second step full activation is achieved through yet undefined mechanisms, which however can be supported by SAG or SmoA1 mutation. As already mentioned, while our data obtained in a cell autonomous context fully support the role of SAG in this model, they seriously challenge the role of the SmoA1 mutation.

Several issues, including the structural characterization of Smo by crystallography in complex with regulatory (agonists and antagonists) support the idea that important conformational changes in particular protein domains might be involved in full activation of Smo. Moreover, it has been proposed that Patch could exert its inhibitory function by indirectly mediating Smo interactions with an endogenous agonist or antagonist, yet to be characterized [40-42]. In particular, two models have been proposed, and our data may be easily accommodated in these models.

MODEL 1: Small molecule Smo Agonist sequestered by Patch **(Fig. 15).**

This model proposes that an autocrine Smo agonist is produced by Hh-responsive cells, which has high affinity for Patch. By binding to Patch, Hh ligands would make the agonist free to reach Smo, help its translocation to the cilium and then start downstream signaling. In the absence of Patch (Patch-KO), Smo agonist would be always free to bind and activate Smo. In this case, SAG would mimic the endogenous agonist in binding and activating Smo, independent of Patch interaction with Hh ligands. By

conferring cilium localization and perhaps by enhancing Smo affinity for SAG, the constitutively inactive SmoA1 mutation could reduce the amount of SAG required for Smo activation and downstream signaling.

MODEL 2: Small molecule Smo Antagonist carried by Patch **(Fig. 16)**

This model proposes that Patch carries an antagonist onto Smo. By binding to Patch, Hh ligands would lead to Patch internalization, which in turn would lead to Smo antagonist detachment from Smo. At this point Smo would be free to localize in the cilium and to undergo further activation to start the signaling cascade. In this case, SAG binding would be able to detach Smo from the antagonist independent of Patch interaction with Hh ligands. Patch KO would make impossible for the antagonist to reach Smo, thus again leading to constitutive Hh activation. The SmoA1 mutation, in this case, could lower the affinity for antagonist binding, such that even low amount of SAG might be sufficient for full SmoA1, but not for Smo, activation.

Of course both these models are fairly speculative at the moment, but we believe that our S-cNS cell models might be a valuable tool for future investigation on this topic.

On the other hand, in this work we have also shown that the S-cNS culture might be applied to other studies on the physiopathology of the cerebellar development. In particular we also focused on the cerebellar defect detectable in the Nijmegen Breakage Syndrome (NBS) patients and in the corresponding mouse model, the NBN-CNS-del mouse. Here, cerebellar development is strongly inhibited mostly due to defects in granule cell proliferation. In this context, we have shown that inhibition of the MRN complex, obtained via NBN RNA interference or via pharmacological targeting of the MRE11 moiety, impairs MYCN- and Hh-dependent proliferation of cerebellar granule cells [32]. These results were largely interpreted in the light of MYCN-dependent replication stress and the anti-replication stress function of the MRN complex [43, 44]. We have more recently questioned whether and how cerebellar GCPs from the NBN-CNS-del mice could be grown in vitro. Interestingly, we were able to generate GF-cNS from NBN-CNS-del mice, although with much lower efficiency compared to WT animals,

which indeed suggests that neuronal cell proliferation is generally impaired in NBN deficient environment also in vitro. Surprisingly, however, we failed to grow any S-cNS. More importantly, while we could convert WT GF-cNS into S-cNS, we were unable to do so from NBN-CNS-del samples, which indicate that GCPs are far more sensitive to NBN loss compared to other cell types. Whether this reflects an higher need for the role of the MRN complex in DNA repair and replication stress control, or whether this reflects a completely different NBN function possibly specific for GCPs will be matter of further studies.

In conclusion, we believe our work provides the scientific community with a novel important tool for studying Hh-signalling and/or GCPs physiopathology in the contest of cerebellar development.

MATERIALS AND METHODS

Establishment of cultures from murine cerebellum

Explanted cerebella were collected in HBSS (GIBCO) supplemented with 0.5% glucose (Sigma Aldrich), 1 KU/ml penicillin and 0.001 mg/ml streptomycin (Sigma Aldrich), grossly grinded with serological pipette and treated with DNAse I to a final concentration of 1.28 U/ml for 30 min. Finally, cells aggregates were mechanically dissociated using pipettes of decreasing bore size to obtain a single-cell suspension. GPCs were cultured as neurospheres in selective medium after centrifugation, DMEM/F12 (GIBCO) supplemented with 0.6% glucose , 25 μg/ml insulin (Sigma Aldrich), 60 μg/ml N-acetyl-L-cysteine (Santa Cruz), 2 μg/ml heparin (Sigma Aldrich), penicillin-streptomycin and B27 supplement without vitamin A (Life technologies). [0.2-1uM] SAG (Adipogene), 20 ng/ml bFGF and EGF (Peprotech)

Clonogenic assay

Cells were pelleted and dissociated by incubation with Accutase (Sigma Aldrich) in concomitance with continuous pipetting to obtain a single cell suspension. Cells were counted with a hemocytometer and were diluted to obtain a suspension concentrated 0.200 cells/100ul per well. Cell suspension was distributed amongst a 96 well plate with corresponding treatments. Each 3-4 days medium was replenished and after 2 weeks, the number of spheres per well was recorded. Exhibited data represents averages of two independent experiments, each having 24 wells per treatment point.

Protein extraction and Western blot

Total protein extracts were obtained in RIPA buffer (0.05M Tris (pH 8), 0.150M NaCl, 0.5% Sodiumdeoxycolate, 0.1% SDS, 1% NP40) 0.05M NaF, 0.005M $NaVO_4$ with protease inhibitor tablets (Sigma Aldrich). Protein extracts (40 mg/sample) were separated by SDS-PAGE and blotted onto nitrocellulose membrane (PerkinElmer). Membranes were blocked with 5% nonfat dry milk and incubated overnight with primary antibodies at the appropriate dilutions. Antibodies were as follows: goat anti–

b-actin, rabbit anti-CCND1 and mouse anti-MYCN (Santa Cruz Biotechnology), mouse anti-Gli1, rabbit anti-PARP1 (Cell Signaling Technology Inc), rabbit anti-Myc (Sigma Aldrich), mouse anti-Nbn (Novus Biologicals), rabbit anti-Zic1 (Abcam) Immunoreactive bands were visualized by enhanced chemoluminescence (Advansta).

Immunohistochemistry

Cerebella explanted from mice were fixed in 3,7% formaldehyde (Sigma Aldrich) for 48 hours and after paraffin- embedded. Four-micrometer-thick consecutive sections were cut and processed for immunohistochemistry for Ki-67 (ThermoScientific). Solutions obtained from Dako Cytomation were used for performing immunostaining. In brief, tissue sections were deparaffinized, unmasked, blocked with avidin- biotin, and incubated with primary antibody overnight. Next day, the reaction was detected by using chromogen according to the manufacturer's instruction (Dako). The positive cells stained brown. Omission of primary antibody from the staining protocol was used as a negative control. The slides were examined under a light microscope (LEICA DM 1000)**.**

Statistical analysis

Statistical analysis was performed by a standard two-tailed Student's t test. ***$p< 0.001$ and * $p<0.05$ was considered significant.

ACKNOWLEDGMENTS

I would like to say thank you to my supervisor, the Professor Giuseppe Giannini, for his patience and his ability to share his scientific rigor, which I consider a basic pillar of research. Thank you for considering my proposals and for trying to use some spanish words.

Thank you to Marialaura Petroni for teaching in a kind and constructive way. This work is undoubtedly the result of your cooperation, I would need many years to learn all you can teach me!

To my laboratory group and especially to Francesca Sardina and Valeria Colicchia for the advices, corrections and for sharing your experience with me, being more than laboratory colleagues.

Furthermore I would like to say thank you to the people of the Molecular Oncology Laboratory for the continuative collaboration. I have been lucky to have the opportunity to work with all of you for the last three years.

Then, I want to say thank you to my family and friends for their continued support and numerous visits to Italy. And to all the friends in Rome that have become my family in recent years. You made it possible!

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FIGURE 6.

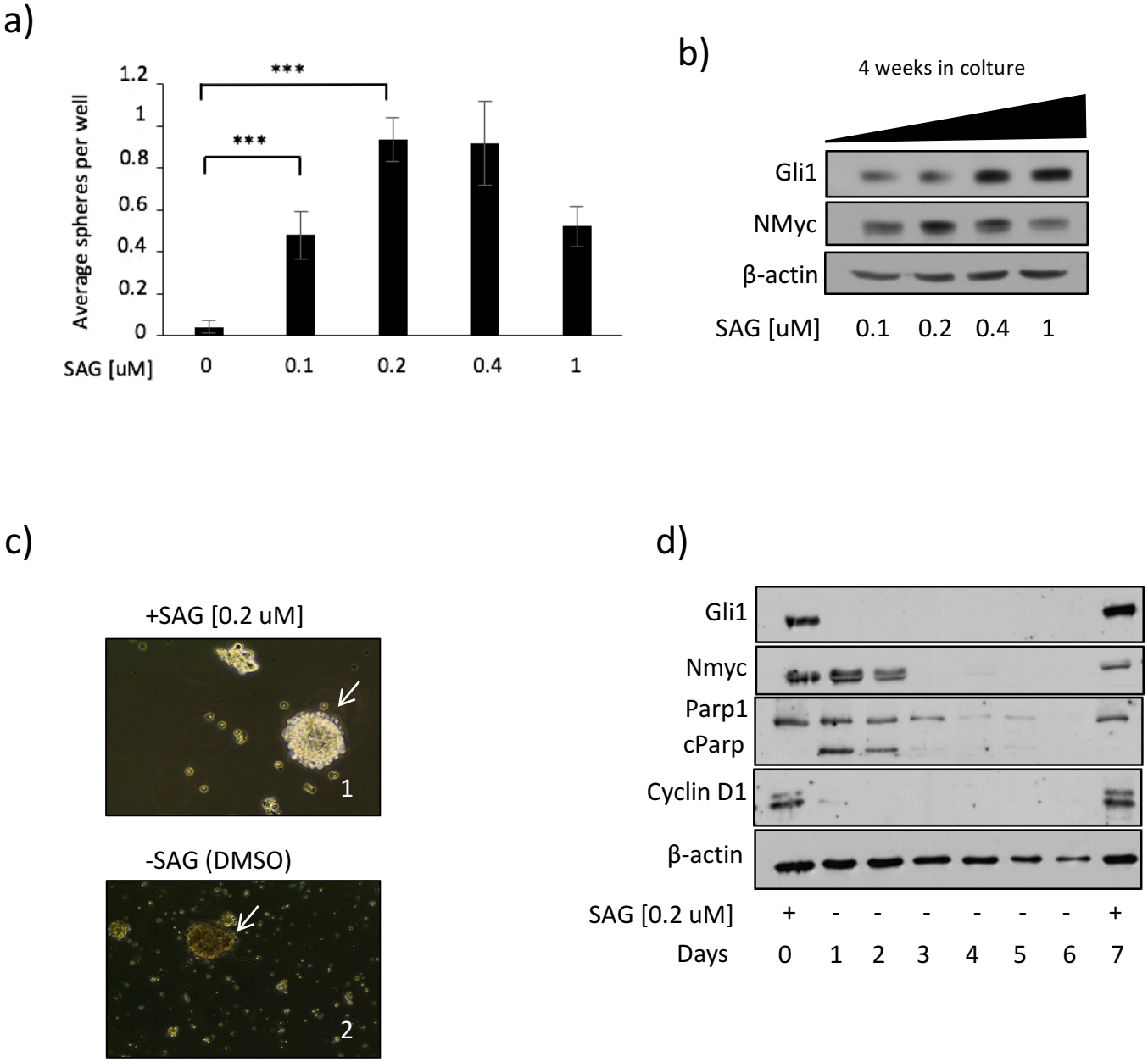
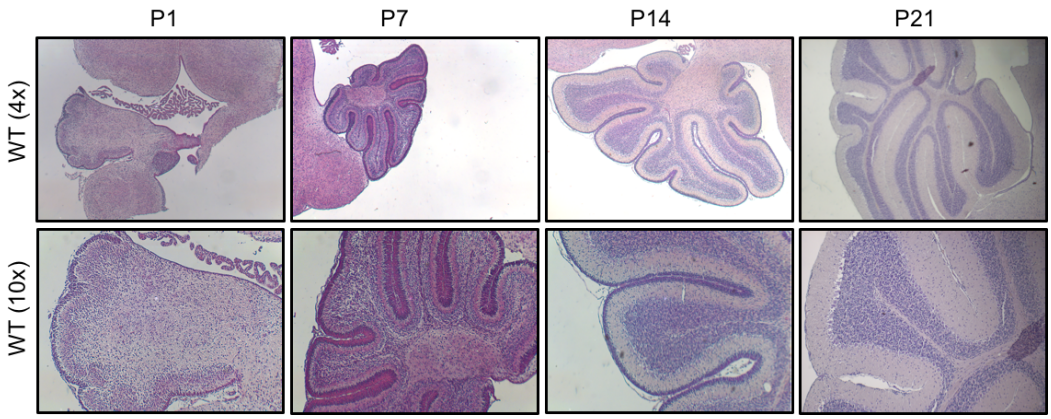


FIG 6. S-cNS are continuously dependent on SAG

a) *S-cNS clonogenic assay from P7 cerebellar explants. Cells were counted and seeded in culture using differents SAG concentrations. (***p < 0.01) (± SE)* ***b)*** *Protein extracts from S-cNS treated for 4 weeks with different SAG concentrations were analyzed by western blot for the expression of the indicated proteins.* ***c)*** *Images from S-cNS cells after 7 days in presence (1) and absence (2) of SAG.* ***d)*** *Western blot of S-cNS cells deprivated of SAG for the indicated times. Protein extracts from S-cNS taken every day during 1 week and analyzed for the indicated proteins.*

FIGURE 7.

a)



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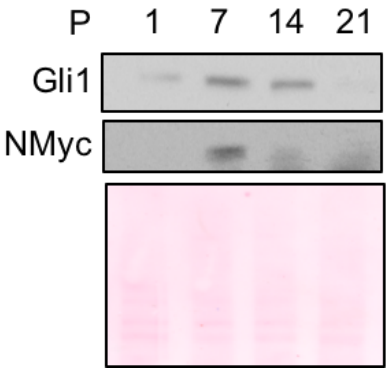


c)

WT s- cNS	P1	P7	P14	P21
+SAG	YES	YES	NO	NO
-SAG	NO	NO	NO	NO

FIG 7 . S-cNS can be obtained from P1 to P7 WT murine cerebellum

a) *Sagittal sections of murine cerebellum from postnatal P1 to P21 were stained with hematoxylin-eosin.* ***b)*** *Protein extraction from cerebellar tissue at different developmental stages (P1, P7, P14, P21) were analyzed using western blotting for the expression of indicated proteins.* ***c)*** *Table summarizes the results obtained in attempting to generate S-cNS from cerebellar explants at differents developmental stages.*

FIGURE 8.

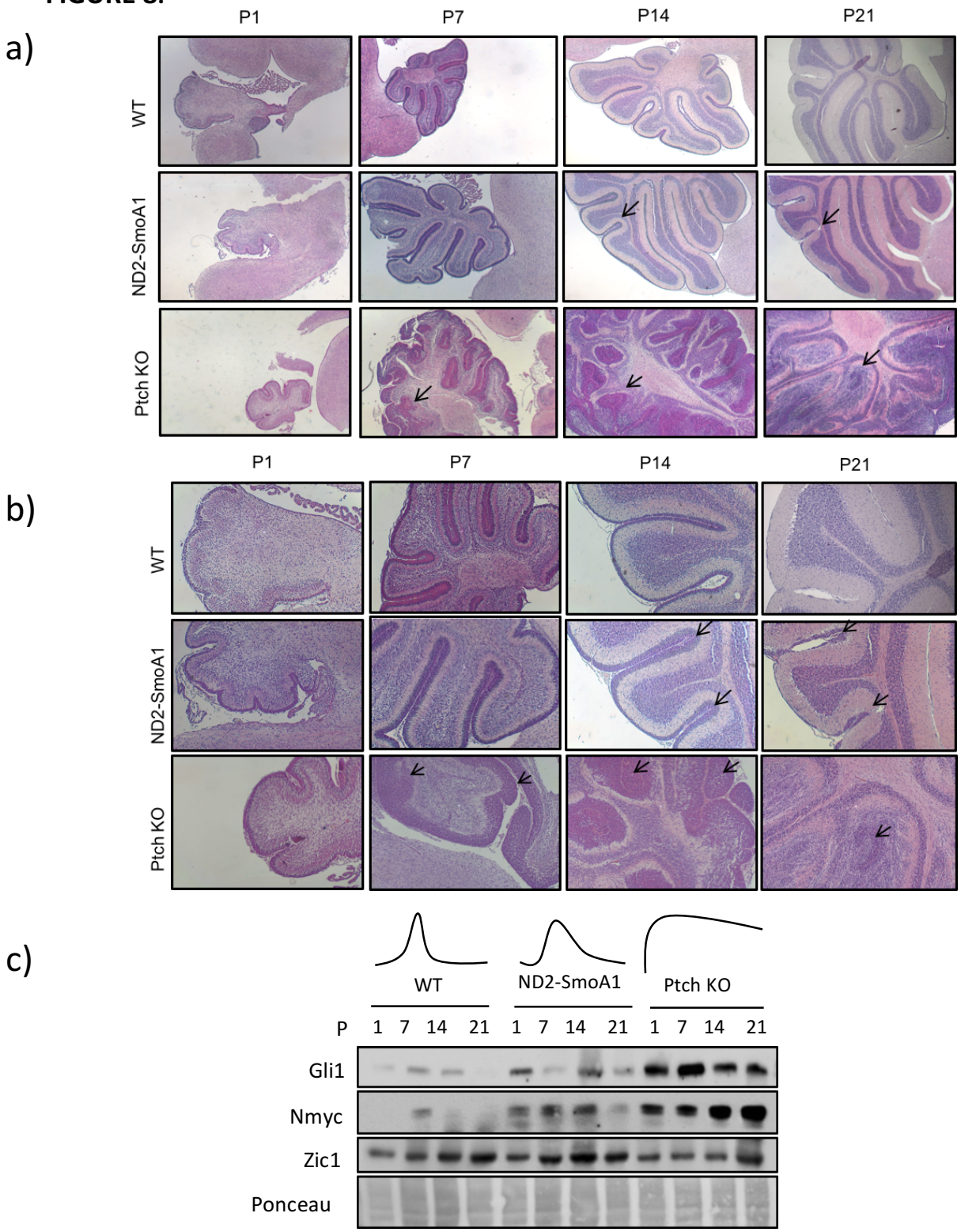
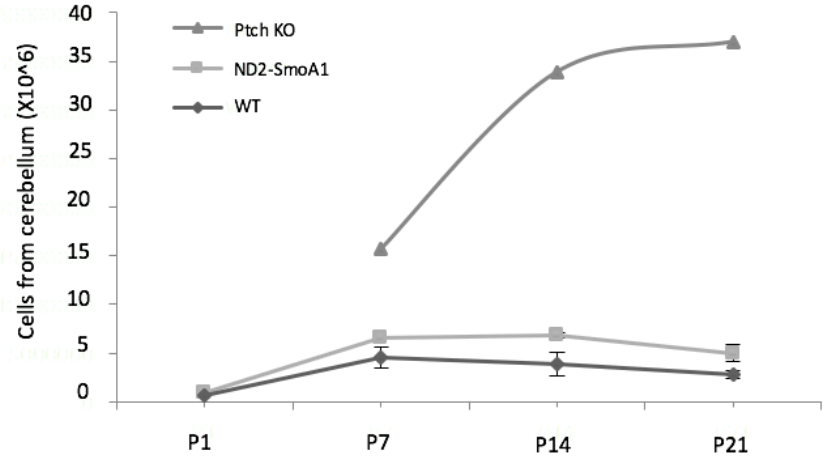


FIG 8 . Postnatal developmental features of the cerebellum in WT, ND2-SmoA1 and Ptch KO mouse models a) *Sagittal sections of WT, ND2-SmoA1 and Ptch KO mice from P1 to P21 were stained with hematoxylin-eosin. (4x) and* ***b)*** *(10x) Arrows indicate alterated regions due to the mutations.* ***c)*** *Western blot describes analysis of the expression of HH pathway proteins at different developmental stages for three murine models. The drawing represents the level of HH signaling in cerebellar development.*

FIGURE 9.

a)



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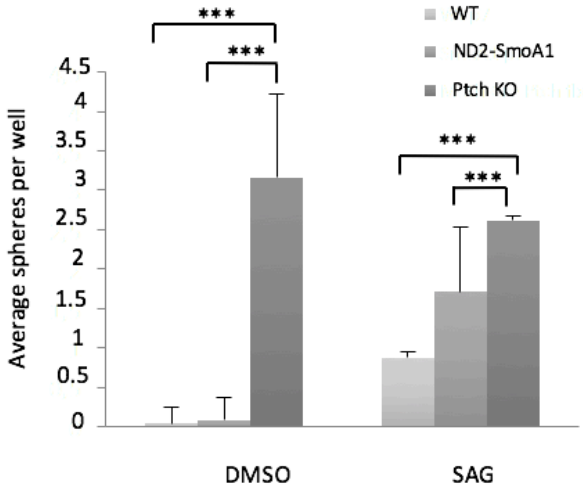
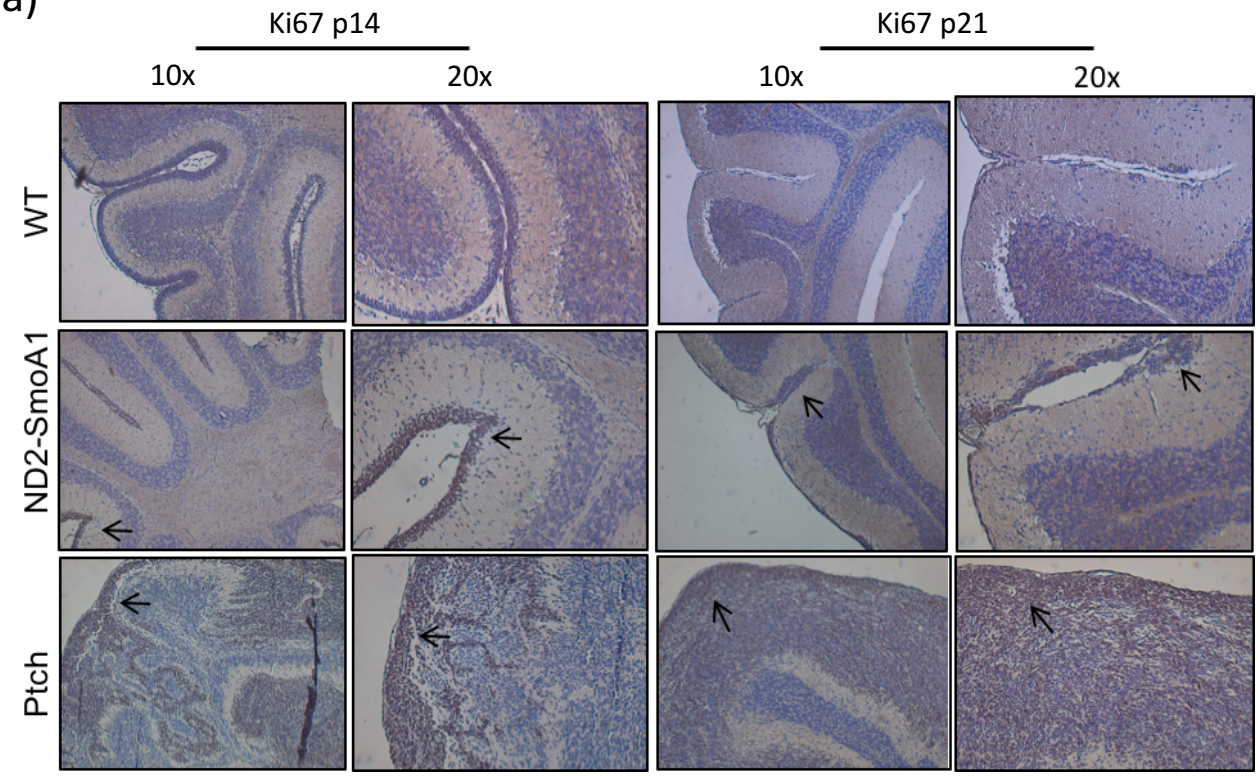


FIG 9. Ptch KO-derived, but not ND2-SmoA1-derived, cerebellar explants allow generation of cNS

a) *Number of cells obtained from cerebella from the three different mouse models (WT, ND2-SmoA1, Ptch KO) at different developmental stages.* ***b)*** *P7 explants from WT, ND2-SmoA1 and Ptch KO cerebella were disgregated, counted and seeded in equal numbers in the absence or presence of SAG (0.2 uM). (***p < 0.001).*

FIGURE 10.

a)



b)

CULTURE	P1	P7	P14	P21	P1	P7	P14	P21
	+SAG				-SAG			
WT	YES	YES	NO	NO	NO	NO	NO	NO
ND2-SmoA1	YES	YES	YES	NO	NO	NO	NO	NO
Ptch KO	ND	YES	YES	YES	ND	YES	YES	ND

FIG 10. S-cNS generation from the three mouse models is possible in different developmental stages

a)*Sagittal sections from P14 and P21 WT, ND2-SmoA1 and Ptch KO mouse models were analyzed for Ki67 expression by immunohistochemistry. Arrows indicate Ki67+ regions.*

b) *Table summarizes the results obtained in attempting to generate S-cNS from cerebellar explants from WT, ND2-SmoA1 and Ptch KO mouse at differents developmental stages.*

FIGURE 11.

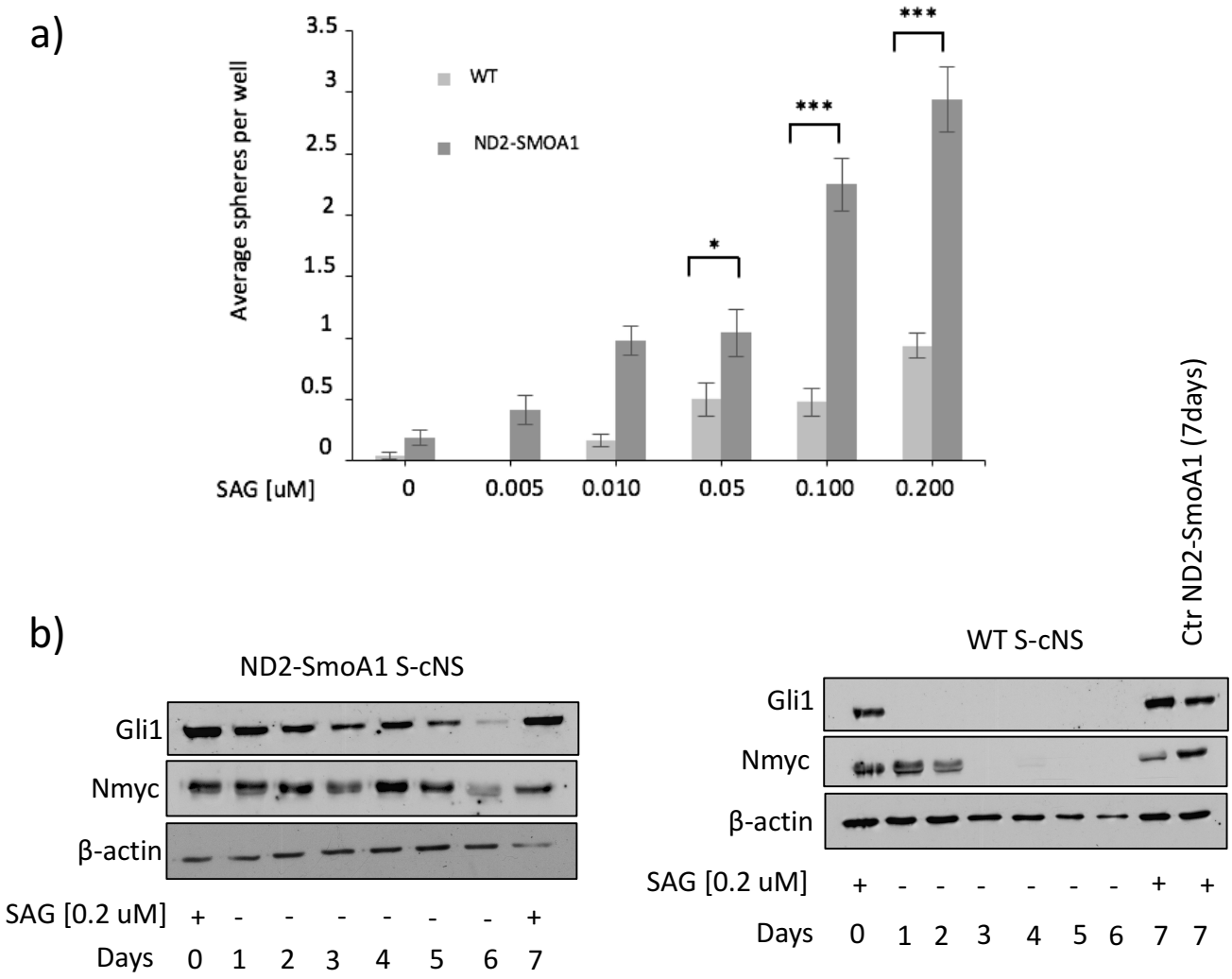


FIG 11. The SmoM2 (Trp535Leu) mutation is not a constitutively activating mutation of the HH pathway

a) *Clonogenic assay from P7 WT and ND2-SmoA1 cerebella was performed using different SAG concentrations. (***p < 0.01) (*p < 0.05) (± SE)* ***b)*** *S-cNS from WT and ND2-SmoA1 mice, were disgregated, counted , plated in equal numbers and seeded in the presence or absence of SAG. Protein extracts from S-cNS taken at different times were evaluated by western blotting for the expression of the indicated proteins.*

FIGURE 12.

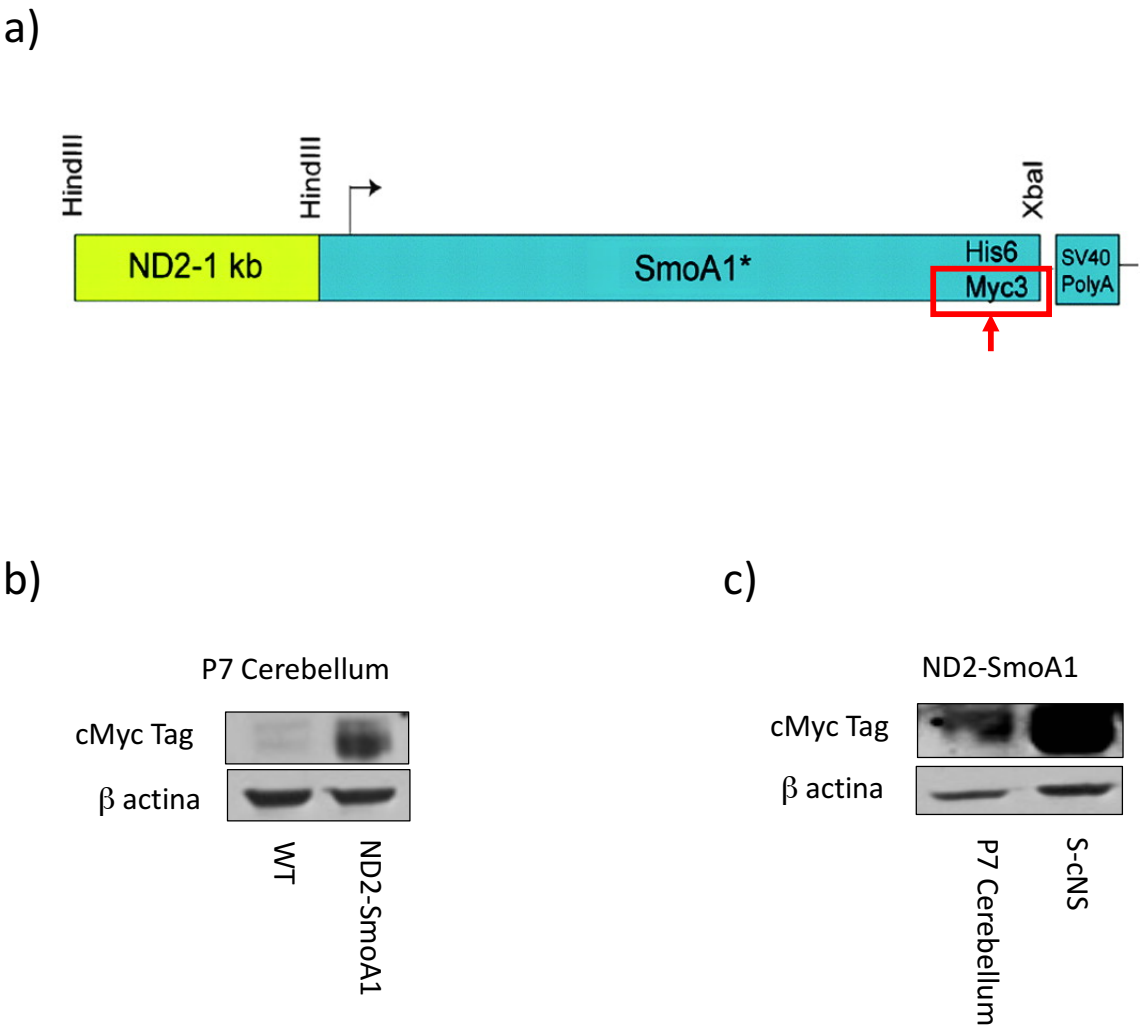
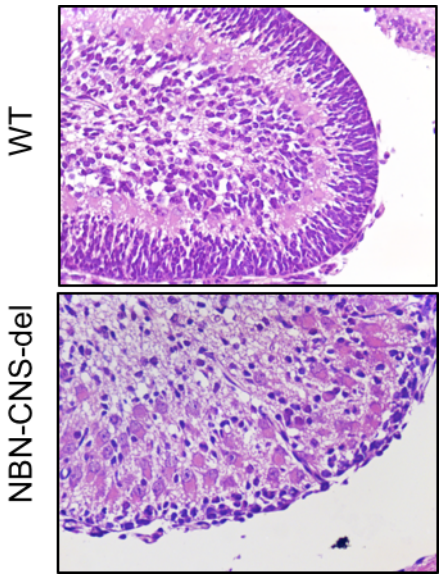


FIG 12. SmoA1 transgene is expressed in vitro in ND2-SmoA1 S-cNS cells
a) *Transgenic SmoA1 construct, carries His6 and Myc3 taggs as described [19]* ***b)*** *Western blot evaluated mouse transgene expression, analyzed by Myc tag protein expression, in cerebellar protein extracts from ND2-SmoA1 and WT mice.* ***c)*** *Expression of SmoA1 mutated protein was confirmed in ND2-SmoA1-derived S-cNS by using Western Blot for the indicated protein.*

FIGURE 13.

a)



b)

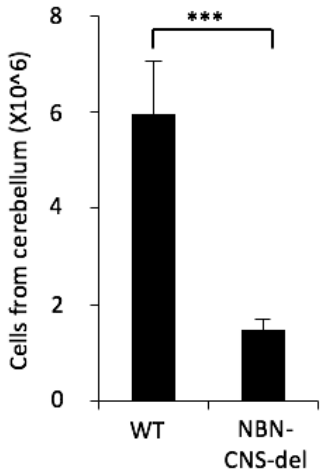


FIG 13. NBN depletion leads to reduced GC expansion during postnatal cerebellar development

a) *Sagittal sections from P5 WT and NBN-CNS-del cerebella mice were stained with hematoxylin-eosin.* ***b)*** *Number of cells obtained from P7 WT and NBN-CNS-del cerebella. (***p < 0.001).*

FIGURE 14.

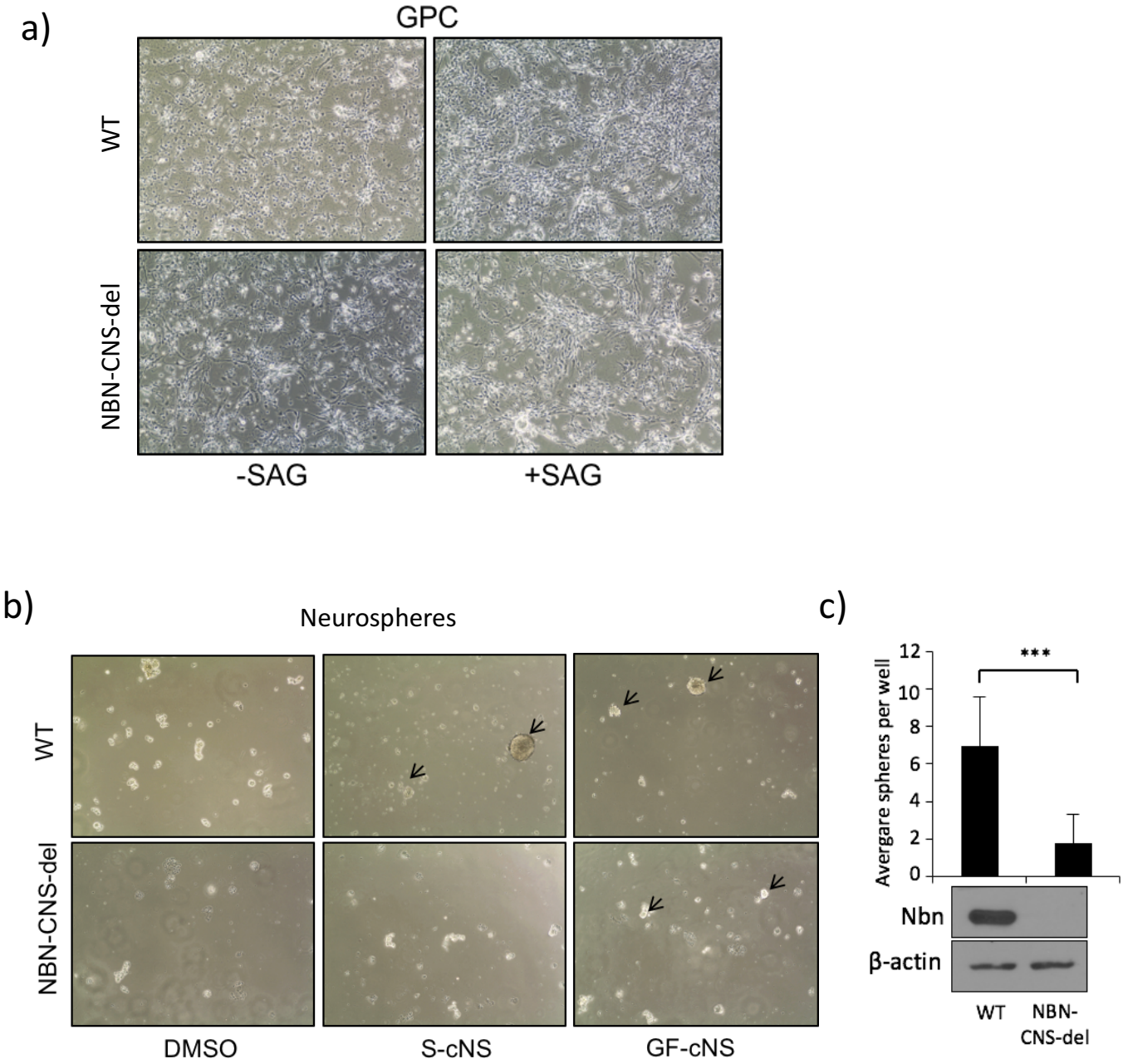


FIG 14. NBN depletion impairs GCPs proliferation

a) *P7 explants from WT and NBN-CNS-del mice were disgregated counted, and plated in equal numbers on polylisin-substrate in the presence or absence of SAG (0.2 uM).* ***b)*** *P7 cerebellar explant from WT and NBN-CNS-del mice were disgregated, counted and plated to generate GF-cNS and S-cNS in parallel.* ***c)*** *Clonogenic assay was performed for GF-cNS condition. (***p < 0.001).*

FIGURE 15.

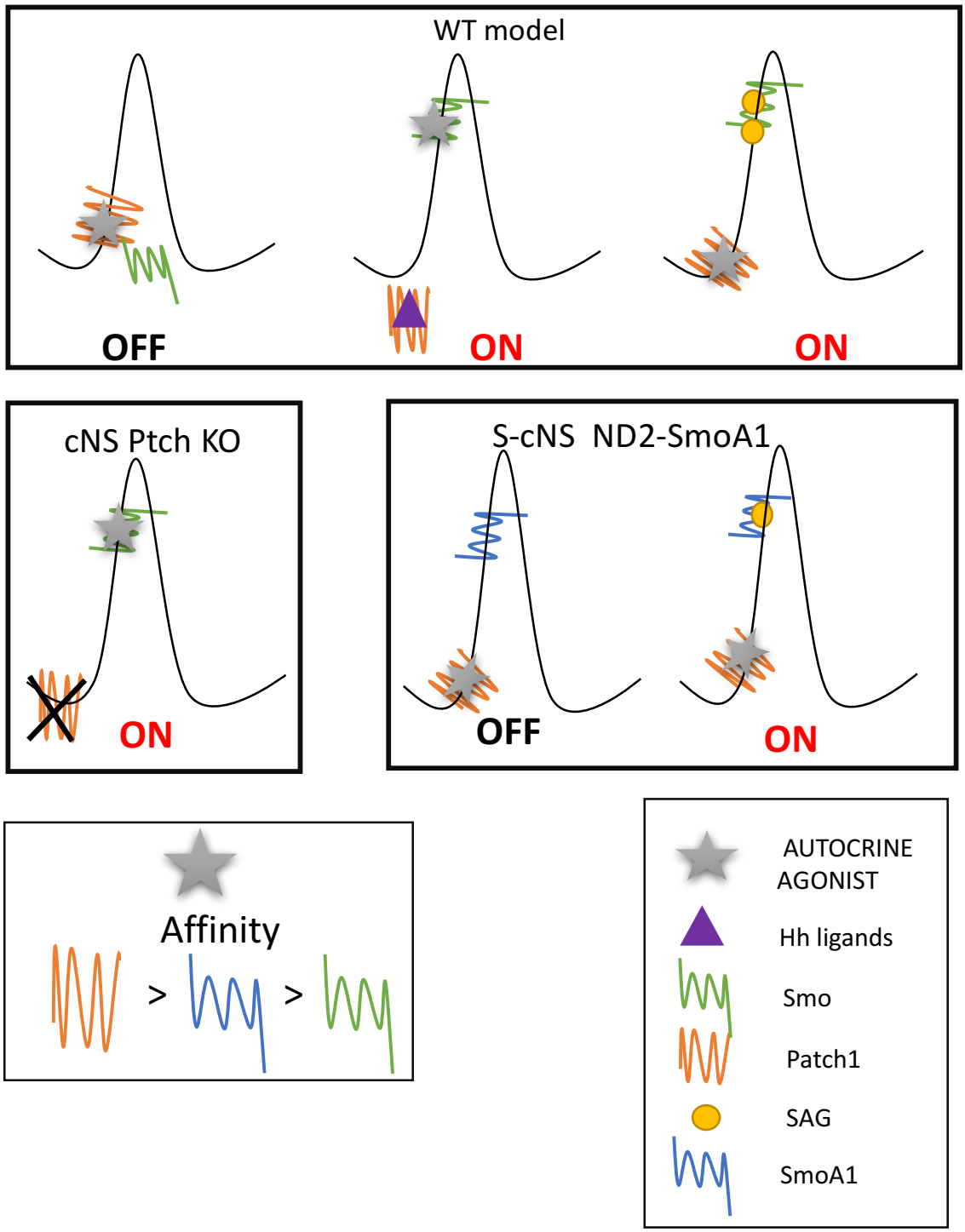


FIG 15. Small molecule Smo Agonist sequestered by Patch (Agonist Model)
In the absence of Hh ligands (magenta), autocrine agonist (grey) which has high affinity for Patch (orange), binds Patch. In the presence of Hh ligands make agonist free to reach Smo (green) , help its translocation to the cilium and then start downstream signaling. In the absence of Patch (Patch-KO), autocrine agonist is free to bind and activate Smo. SAG (yellow) would mimic the endogenous agonist in binding and activating Smo. SmoA1 mutation (blue) could reduce the amount of SAG required for Smo activation.

FIGURE 16.

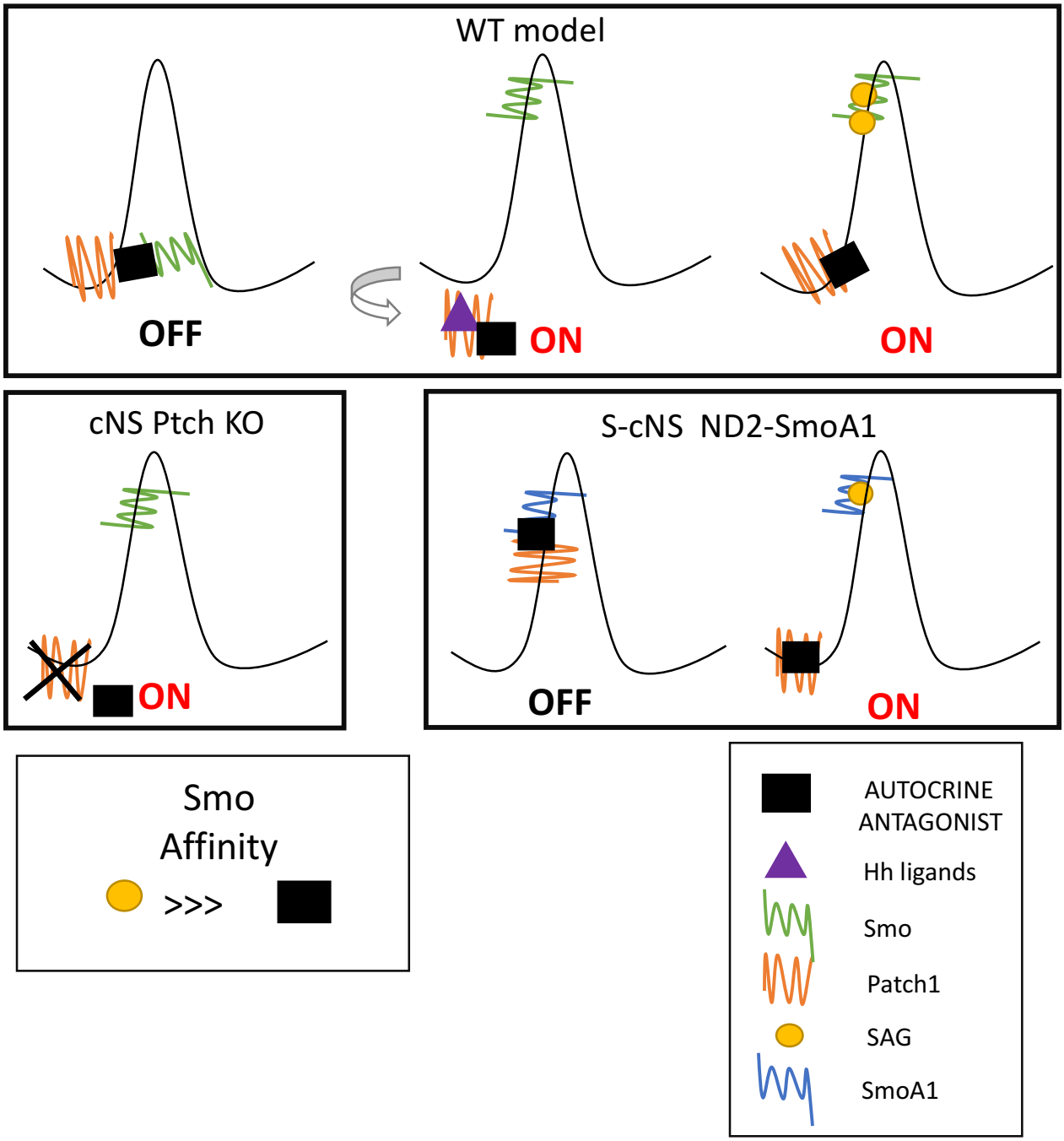


FIG 16. Small molecule Smo Antagonist carried by Patch (Antagonist Model)
Patch (orange) carries an autocrine antagonist (black) onto Smo (green). The presence of Hh ligands (magenta) would lead to Patch internalization, which allow Smo antagonist detachment from Smo. Smo would be free to localize in the cilium and to undergo further activation to start the signaling cascade. Patch KO make impossible for the antagonist to reach Smo, thus again leading to constitutive Hh activation. SAG (yellow) binding would be able to detach Smo from the antagonist independent of Patch interaction with Hh ligands. The SmoA1 mutation (blue), in this case, could lower the affinity for antagonist binding, such that even low amount of SAG might be sufficient for full SmoA1, but not for Smo, activation.