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Coordinator Prof. Giuseppe Giannini

Role of SHh modulation in neurogenesis and tumorigenesis

Candidate

Adriano Apostolico

1877582

Tutor

Prof. Enrico De Smaele

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ABSTRACT

The Sonic Hedgehog (SHh) pathway plays a major role in a variety of different processes, ranging from embryogenesis to the homeostasis maintenance of adult tissues, with Central Nervous System (CNS) being one of the most SHh signaling-dependent tissues. However, fine-tuning of this pathway is strictly required in order to avoid the insurgence of pathological conditions. SHh ligand acts indeed as a potent morphogen and mitogen. In the CNS, SHh signaling regulates the various neurogenic processes that occurs during the lifespan of mammals. Sustained SHh signaling in CNS results in the onset of various disease such as medulloblastoma and glioblastoma but, can also lead the de-regulation of the neurogenic process in the adult mammals, impairing the cognitive and behavioral processes underlying these events.

KCASH2^{KCTD21} (KCASH2, KCTD Containing Cullin-Adaptor, Suppressor of Hedgehog, 2) is a member of the KCASH protein family, originally discovered in 2011 as a negative regulator of SHh signaling pathway. KCASH2 possesses a BTB/POZ domain that enables the interaction with the E3 ubiquitin ligase Cullin-3. Through this domain, KCASH2 is able to promote interaction between Cullin-3 and the histone deacetylase 1 (HDAC1) and the sub-sequent proteasomal degradation of the latter. Given the role of HDAC1 in de-acetylating the main effector of the SHh pathway, the transcription factor GLI1 (glioma-associated oncogene 1), thus promoting its ability to translocate into the nucleus, the capacity of KCASH2 to reduce the levels of HDAC1 serves therefore as an inhibitory mechanism to turn-off the signaling cascade of SHh pathway.

In this work we examined the *in vivo* effects of KCASH2 loss on the hippocampal neurogenic process. Exploiting a reporter gene we firstly observed KCASH2 expression in the various cell populations residing in the hippocampus.

KCASH2 role as a negative regulator of SHh has been confirmed in this context. Indeed, we observed both *in vitro and in vivo* increased level of GLI1 protein and mRNA. Consistently with these findings we observed a sustained proliferation rate in the hippocampal neurogenic niche in KO mice.

In addition, we analyzed the morphology of the stem cells residing in the hippocampal neurogenic niche. By immunofluorescence analyses we noticed that KCASH2 loss leads to a marked alteration in the morphology of these cells.

The importance of Hh pathway and its cooperative crosstalk with other tumorigenic pathway in the Medulloblastoma prompted us to analyze the effects of Hh signaling inhibition on Hh-dependent Medullobalstoma cell lines. We targeted the mechanisms that lead to the activation of GLI1 transcription factor both in a SHh dependent and non-dependent fashion and observed the effects of this inhibition on cell proliferation and survival.

This evidence adds a new layer of complexity in the regulation of hippocampal neurogenesis and the involvement of SHh pathway in this process.

Furthermore, our data open new perspectives for the modulation of the Hh pathway in the treatment of Medulloblastoma.

1. Introduction

1.1. The Hedgehog signaling pathway

The Hedgehog (Hh) signaling pathway plays a major role in several biological processes, such as embryonic development, cell growth and proliferation, tissue homeostasis and repair and in the onset of several pathologies, such as cancer (Carballo et al 2018). Hh signaling was originally discovered in *D. melanogaster* by Nüsslein-Volhard and E. Wieschaus in 1980; when the authors identified the Hh gene through genetic screening for mutations that impaired the body segmentation of the flies' larvae (Nüsslein-Volhard and E. Wieschaus 1980). The core components of the Hh signaling cascade shows a high grade of similarity between *D. melanogaster* and vertebrates, while vertebrates display three Hh genes: Sonic Hedgehog (SHh), Desert Hedgehog (DHh), and Indian Hedgehog (IHh), which are thought to be the results of genome duplication processes occurred during the vertebrate's evolution (Wada e Makabe, 2006). Hh ligands act as morphogens and mitogens both in autocrine and paracrine fashion, however each Hh ligands is expressed in a tissue specific manner and has its own specific function; SHh is the most extensively studied Hh ligand, its expression is marked in the Central Nervous System, where it regulates the limbs patterning during organogenesis and the post-natal and adult neurogenic process occurring in the CNS (Yao et al 2016, Rana et al 2021). DHh is found to be mainly expressed in the gonads, and in particular by Sertoli Cells, where it signaling regulates the maturation of germ cells (O'Donnell, Smith et al. 2022). Finally, IHh is involved in the regulation of

chondrocyte proliferation during bone formation and in the development of gastrointestinal tract (Lai and Mitchell 2005, Van den Brink 2007).

Given the ability of Hh ligands to activate signaling cascades that are often implicated in cell growth and proliferation, de-regulation of this pathway has often been linked to the onset of several pathologies. The mechanisms that can lead to sustained Hh signaling are various and do not necessarily involve Hh ligands but, can revolve around the activation or the inactivation of the downstream members of the pathway. Different CNS forms of cancers are linked to Hh signaling de-regulation, as Medulloblastoma and Glioblastoma (Skoda et al. 2018, Wong et al. 2021). Known the importance of this pathway in regulating neural stem cells proliferation and differentiation, de-regulation of Hh signaling has also been linked to the development and progression of neurodegenerative and neurological diseases (Yao et al. 2016).

1.1.1. Canonical Pathway

Hh signaling pathway consists of an activated and a turned-off state (**Figure 1)**, depending on the presence of Hh ligand. When the Hh ligand is absent, the 12-pass transmembrane receptor PATCHED1 (PTCH1) inhibits the 7-pass receptor Smoothened (SMO), in this context the Suppressor of Fused (SUFU) sequesters the effectors of the signaling cascade, the GLIs family of transcription factors (GLI1, GLI2, GLI3), preventing their translocation into the nucleus and therefore inhibiting their transcriptional activity. In the turned-off state, GLIs transcription factor are subsequently phosphorylated by Protein Kinase A (PKA), casein kinase 1 (CK1) and Glycogen synthase 3β (GSK3β), marking them for proteolytic cleavage. The proteolytic cleavage of GLI2/3 results in a repressive truncated form of GLI proteins (GLIR) which translocate into the nucleus and keep silent the transcriptional state of Hh target genes

(Suchors and Kim 2022). In the presence of one of the three ligands (SHh, DHh, IHh), the repressive receptor PTCH1 is internalized, thereby relieving SMO from its inhibition. Hence, SMO translocates to the Primary Cilium (PC), a microtubule based organelle that emerges from the surface of the cell (Fabbri et al. 2019), via interaction with β-arrestin and the kinesin KIF3A (Kovacs et al 2008.). Upon translocation into the PC, SMO inhibits PKA and CK1 mediated phosphorylation of the GLIs protein. Out of three GLIs factors, the main activator GLI1 is not generally expressed when the pathway is turned-off. Instead, GLI1 expression appears to be induced by GLI2/3 transcriptional activity when the pathway is active. Furthermore, GLI1 needs to be de-acetylated to exert its activity, a process that is mediated by the Histone de-acetylase I. Ultimately, the GLIs factors in their activated form dissociate from SUFU and are able to translocate into the nucleus, where they activate the transcription of Hh related genes (Sigafoos et al. 2021, Suchors and Kim 2022, Canettieri et al. 2010).

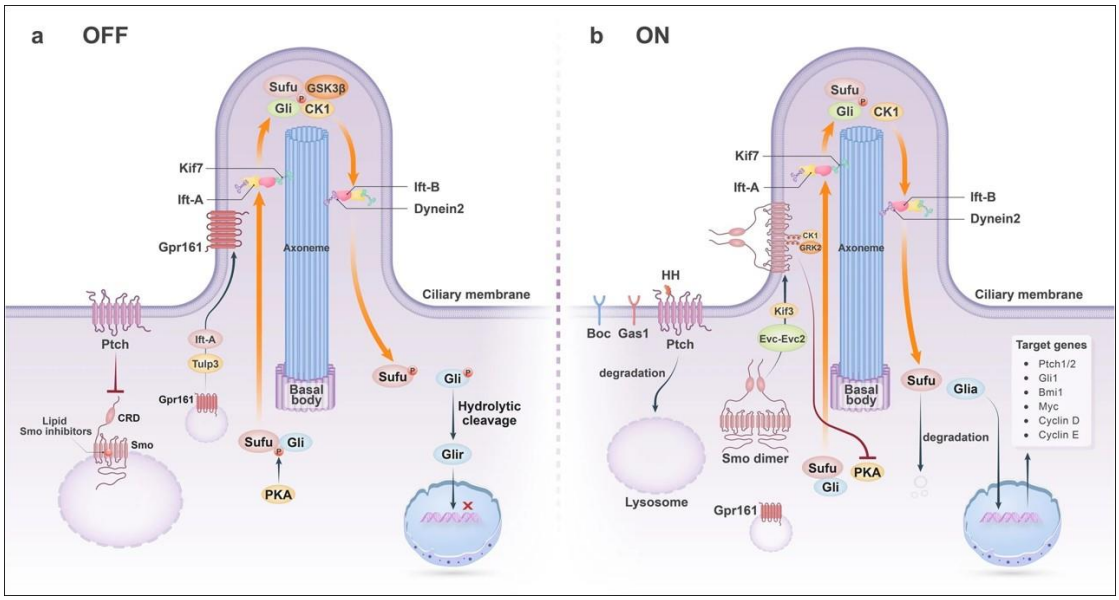

Figure 1) Hh pathway. a) In the turned-off state, SMO is inhibited by the receptor PTCH1, and the GLIs factors are proteolytically converted into the repressive form GLIR, silencing the transcription of the target genes. **b**) When Hh ligands bind to the receptor Ptch1, SMO translocates into the Primary Cilium, and release the GLIs factor

from SUFU, GLIs translocate into the nucleus and activate the transcription of Hh related genes (Image by Jing et al. 2023)

1.1.2. Non-Canonical Pathway

Canonical Hh signaling rotates around the cascade that start from the Hh ligands and continues with the downstream core component PTCH1, SMO and GLIs factors. However, Hh pathway shows an intricate interplay with several pathways, that can modulate ultimately the activity of the GLIs factors, such mechanisms are defined as "Non-Canonical" Hh signaling (**Figure 2**). These mechanisms often bypass the action of the Hh ligand and the co-receptor SMO, although SMO dependent/GLIs independent mechanisms of signaling occur (Shevde et al. 2014). The finding of a dense crosstalk between the Hh pathway and other oncogenic pathways has raised increasing interest in order to develop new treatment strategies of those tumoral forms that show increased GLI1/2 activity, such as MB (Brechbiel et al 2014.). Several pathways communicate with Hh pathway in order to activate GLI1/2 factors:

- RAS-RAF-MEK-ERK pathway: this pathway has a crucial role in cell survival and proliferation, its de-regulation is recognized as a key driver of the insurgence in many cancer types (Gòmez et al 2018.). The mitogen-activated protein kinase kinase (MEK1) is able in some form of tumors to increase GLIs factors stability and transcriptional activity trough phosphorylation, promoting their nuclear import and stabilization.

- FAK/Akt: focal adhesion kinases (FAKs) are proteins involved in intra- and inter-cellular communication, regulating processes such as migration and adhesion (Chen et al. 2022). FAKs are able to activate Akt, which in turn is

able to phosphorylate GLI1, releasing the transcription factor from SUFU repressive binding (Chen et al 2020).

- TGF-β: transforming growth factor β (TGF-β) has a dual role, acting both as a tumor suppressor or oncogene depending on the context. TGF-β shows the capacity to stimulate GLI2 and subsequent GLI1 transcription through a SMAD dependent mechanism (Dennler et al. 2020).

- Wnt/β-Catenin: depending on the context, the interplay between Wnt and HH pathway can have different outcome. β-catenin can act as a positive regulator of Gli1 transcriptional activity (Carballo et al. 2018).

Others pathway over the years have been involved in the Hh crosstalk, adding new layers of complexity in the tuning of the activity of GLIs transcription factors. The overall picture is further complicated by the presence of epigenetic interactors, such as miRNA and chromatin remodelers (Pietrobono et al 2019).

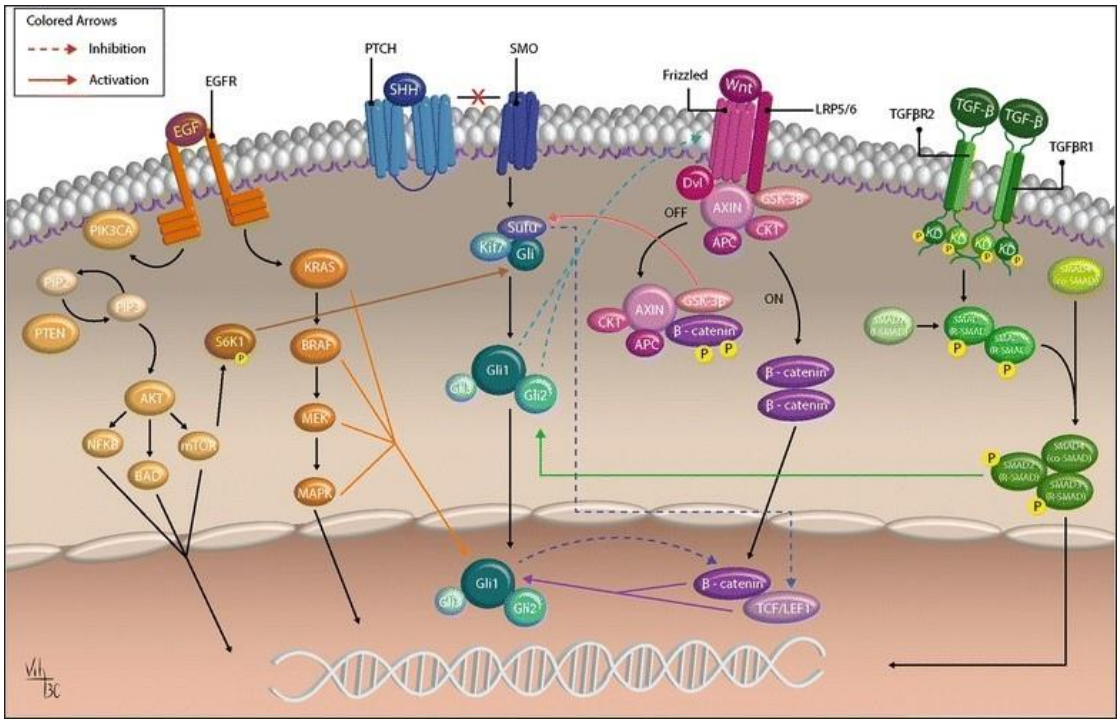

Figure 2) Hh crosstalk. Diagram representations of the existing crosstalk between Hh and others signaling pathways. Interconnections between Hh pathway and others occurs at different moments and different context in tumor development. The existing communication between these pathways poses a challenge but also gives rise to new opportunities in the treatment of several cancers (Image by Carballo et al. 2018).

1.1.3. GLI transcription factors

GLIs proteins belong to the Kruppel-Family of zinc-finger containing transcription factors, they are the final effectors of Hh signaling pathway, responsible for the gene expression of Hh target genes. Their action result in different transcriptional outcome, given their ability to both activate and/or repress gene expression (Sabol et al. 2018). Vertebrates display three members of this subfamily, named GLI1-3, while *Drosophila* possesses a single homologue, Cubitus interruptus (Ci) (Abbasi et al 2009). GLIs factors are characterized by the presence of C2-H2 zinc fingers, these zinc fingers are responsible for the binding to the phosphate backbone of DNA and the recognition of a consensus sequence present in the promoter of GLIs target genes. All GLIs factors possess a nuclear export sequence (NES) and a nuclear localization sequence (NLS), these sequences allow the process of nuclear import/export of GLIs factor. Moreover, GLIs factor show a trans-activation domain (TAD), localized to the C-terminus, and a site which mediates the interaction with SUFU positioned in the N-terminus, responsible for the cytoplasmic sequestering of GLIs when the Hh signaling is switched-off (Pietrobono et al. 2019). As mentioned above, GLIs factor can act both as suppressor or activator of the expression of target genes, with exception made for GLI1, in fact while GLI2 and GLI3 possess both a transcriptional activator domain (TAD) at the C-terminus and a repressor domain at the N-terminus (with GLI2 generally being a weak activator and GLI3 being a repressor), GLI1 miss the N-terminus repressor domain, and is generally considered as the main activator of the expression of Hh related genes (**Figure 3**), hence, the

balance between the contribution of each single GLIs and their protein isoforms (arising from alternative splicing or proteolytical cleavage) determines the transcriptional state of GLIs related genes (Sigafoos et al. 2021, Sabol et al 2018).

GLIs target genes vary depending on the context and developmental stage, moreover the presence of splicing variants further complicates the understanding of the biological significance of the transcriptional activity of GLIs factor. Their activity has been linked with several biological processes, such as cell proliferation, stemness maintenance, epithelial to mesenchymal transition and angiogenesis. Of a note, GLIs factor can regulate their own transcription, through a mechanism of both positive and negative feedback, since GLI1 and GLI2 can activate the transcription of *Gli1* and *Ptch1* (Blotta et al. 2012). Among the other genes whose transcription that can be regulated by GLIs factors in a tumoral context there are the core stemness factors, *SOX2*, *NANOG* and *OCT4* and also *KLF4* (Sharma et al. 2015). Other genes identified as GLIs target are *cyclin D* and *cyclin E* (Duman-Scheel et al. 2002) which counteract the Retinoblastoma pathway and sustain cell proliferation and cell cycle-progression. But GLIs activity has also showed to regulate the expression of *Bcl-2,* thus affecting the pro- and anti-apoptotic balance of malignant cells (Bigelow et al. 2004).

Figure 3) GLIs transcription factors. Schematic representation of GLIs factors domains. RD (Blue) represents the repressor domain, SB (cyan) represents the SUFU binding site, ZF (green) represents the zinc finger domain, NLS (purple) represents the nuclear localization signal, NES (yellow) represents the nuclear export signal, TAD (red) represents the transcriptional activator domain (Image by Sigafoos et al. 2021)

1.2. Hedgehog pathway in development and pathogenesis

1.2.1 Tissue development and Hh signaling

Hh ligands act as along range morphogen that are involved in the formation of several tissues during the organogenesis by orchestrating processes such as cell proliferation, differentiation, and survival (Briscoe and Thérond 2013). Formation of Hh ligands gradient can be sensed by Hh responsive cells, which results in a different signaling response and transcriptional outcome (Jiang and Hui 2008). Between the Hh ligands, SHh is the most widely expressed and characterized, while IHh and DHh show tissue specific expression, although in some cases Hh ligands show overlapping functions. During the process of gut organogenesis, SHh and IHh are produced by endodermal epithelial cells and their signaling is transduced by mesenchymal stromal cell surrounding the gut tube. These mesenchymal cells are responsible for the formation of a cell cluster, from which starts the eruption of the intestinal villi (Walton and Gumucio 2021). In addition, IHh also plays an important role in skeletal development by regulating chondrocytes proliferation and differentiation (Bechtold et al. 2019). DHh seems to be majorly expressed in testis by Sertoli cells, where it regulates the testes development and the process of spermatogenesis. DHh expression has also been detected in Schwann cells, and its activity is involved in nerve sheathing. DHh signaling defects lead indeed to infertility and peripheral neuropathies (Dilower et al. 2023). Among the three Hh ligands, SHh plays a major in the development of the CNS, which is composed by the Brain and the Spinal Cord. SHh activity is responsible for the control of left-right asymmetry and dorso-ventral axis specification. There are three recognized sources of SHh during the CNS development: the notochord, the axial mesoderm and the floor plate. In the

developing neural tube SHh acts both short- and long- range forming a gradient that regulates the expression of homeodomain family proteins, including Pax, Nkx, Dx and Irx, establishing different sub-types of neural progenitor cell populations. Late in the CNS development, SHh signaling is required for the generation of Oligodendrocyte precursor cells (OPC). Notably, it has also been observed that SHh can induce OPC differentiation (Douceau et al. 2023, Martì and Bovolenta 2002). In the post-natal and adult CNS, SHh further regulates the neurogenic processes that occur under physiological and pathological conditions.

1.2.2 Hh in stemness and neurogenesis

Hh signaling shows a lower degree of extent in adults compared to the embryonic stage. Nevertheless, during adulthood, Hh signaling cascade plays a major role in controlling tissue homeostasis, regeneration and repair by modulating the behavior of the various stem cell types residing in the majority of the tissues (Zavala et al. 2017). Stem cells are defined as non-specialized self-renewing cell which possess the ability to differentiate into different cell types. Stem cells can be subdivided into embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs are actively proliferating cell which are capable to differentiate into cells belonging to the three different germ layers (mesoderm, endoderm and ectoderm), and are therefore defined as pluri-potent (Zakrzerwski et al. 2019). In contrast, ASCs exhibit germ layer restricted ability to differentiate into only one or few specialized cell types, hence being defined as mono- or multi-potent. In addition, ASCs can exist both in a quiescent or in activated state. The behavior of ASCs is coordinated by the niche where ASCs are allocated trough secreted factors that orchestrate ASCs activation, proliferation, and differentiation in order to maintain tissue

homeostasis or in response to stimuli like injuries (Hicks and Pyle 2023, Brunet et al. 2023). In the regulation of stem cells proliferation and differentiation, Hh plays a key role, interacting with other regulator elements such as SOX, FGF and HOX families, principally by regulating the activation of the local stem cells and the generation of transient amplifying daughters' cell. Hh activity has been implicated in the maintenance of homeostasis in several tissues and organs, such as epidermis, bone, gastrointestinal tract and brain (Petrova and Joyner 2014.). In the brain, Hh signaling plays a major in the regulation of neurogenesis, a process in which newborn neurons are generated from a population of precursor cell that goes through sequential phases of proliferation and differentiation. The Cerebellum, which is located in the hindbrain, is responsible for motor coordination. Hh signaling in this context is key regulator of the cerebellar granule precursor cell (GPCs) proliferation (De Luca et al. 2016). GCPCs arise from the rhombic lip and migrate along the cerebellum surface to form the external granule layer (EGL), where they undergo trough several cycles of division, after which they migrate into the inner granule layer (IGL) becoming fully differentiated and post-mitotic granule neurons. In the cerebellum, SHh is produced by Purkinje Cells which are located in the Purkinje Cell Layer (PCL) and extend towards the EGL (Wang et al. 2022) (**Figure 4**). During GPCs proliferation, Hh signaling activates the transcription of proliferative target such *cyclin-D* and *N-myc* and contemporary inhibiting anti proliferative gene expression, like *Rb1* (Kenney and Rowitch 2000, Kenney and Rowitch al. 2003). Impairment of SHh signaling during this phase leads to cerebellar hypoplasia and reduced cerebellar foliation, while hyper-activation results in delayed GPCs differentiation, extended proliferation and, ultimately, in the acquisition of

tumoral phenotype and MB insurgence (Corrales et al. 2004, Vaillant and Monard, 2009).

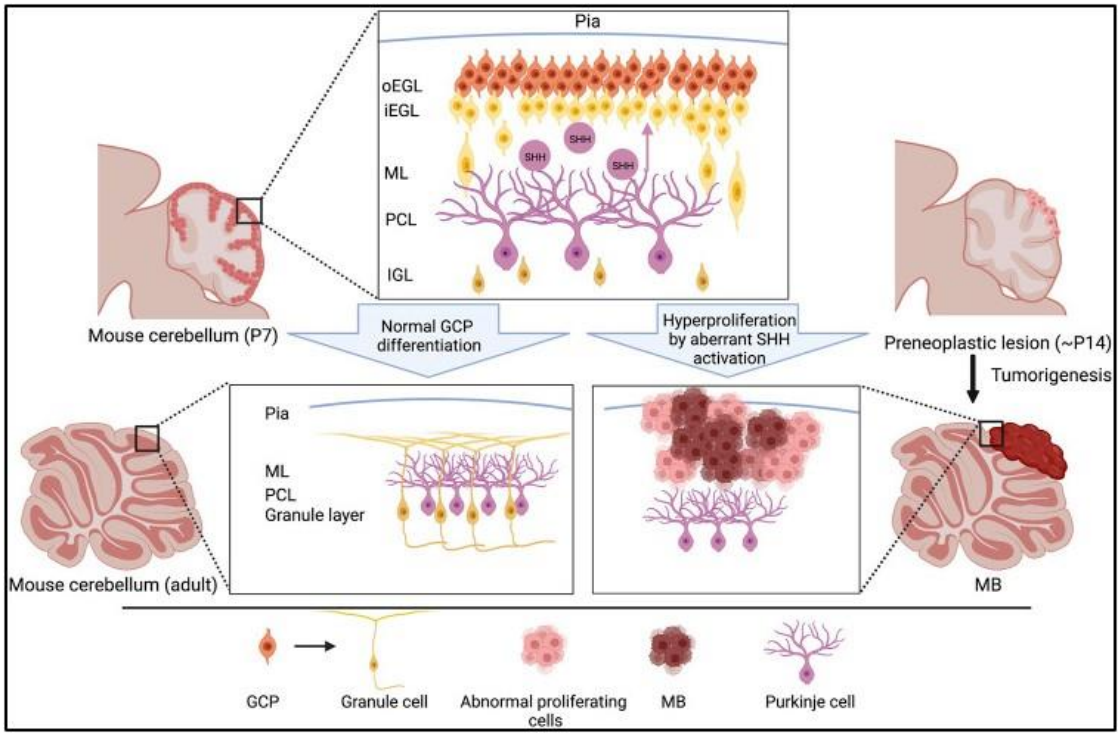

Figure 4) SHh regulates GPCs proliferation. In the cerebellum, SHh is secreted by Purkinje Cells and affects GPCs proliferation and differentiation. Under physiological conditions, GPCs exit the cell cycle and migrate toward the IGL, becoming mature Granule Cells. Sustained SHh signaling leads to GPCs hyper-proliferation and tumoral transformation (Image by Vaillant and Monard, 2009) .

The hippocampus is a region involved in memory formation, behavior and learning. In human it is located in the medial temporal lobe, while in rodents lies beneath the neocortex (Knierim 2015). The hippocampus is one of the two neurogenic niches where neurogenesis occurs in the adult mammalian brain. The neurogenic process occurs in sub-granular zone (SGZ) of the Dentate Gyrus (DG) where the hippocampal stem cells are located. These stem cells possess glia-like properties and are able to generate new neurons upon activation and maturation (Ming and Song 2012); (Whitefield and Chakravarthy 2009). Significant sources of SHh in the hippocampus are the

neuron residing in the hilus and in the DG, as well as the stem cells themselves (Gonzalez-Reyes et al. 2019, Favaro et al. 2009). SHh signaling is required in this region to ensure the maintenance and the activation of the hippocampal stem cell. When SHh signaling is reduced, the DG of the hippocampus shows a severe hypoplasia, with a concomitant reduction in the number of hippocampal stem cells (Favaro et al. 2009). On the contrary increased Hh signaling has been shown in *Ptch*$^{+/-}$ mice to lead to increased length of DG but also to a similar reduction in the number of stem cells in old mice (Antonelli et al. 2019). Surprisingly, SHh signaling has a dual role in this context: sustaining stem cell proliferation, but also inducing apoptosis. In cultured hippocampal stem cells, GLI1 overexpression induces the transcription of the DNA damage repair protein Gadd45a, which in turn leads to increased apoptosis rate in these cells (Galvin et al. 2008). By these evidence, it turns out that the intensity of the SHh signaling in this context is vital to guarantee the proper tissue functioning, SHh pathway is crucial to the proliferation process that naturally occur in this context, and its de-regulation leads to defect commonly observed in several pathologies such as cancer or neurodegenerative diseases (Yao et al 2016, Vaillant and Monard 2009).

1.2.3 Hh signaling in neurodegenerative and neurological disease

Generally, SHh signaling pathway has been considered to prevent senescence-associated disease due to its strict correlation with the neurogenic processes that are often impaired under many pathological conditions. First evidence enlightened a role for SHh signaling in the prevention on neuronal death, impaired neurogenesis and inflammatory processes that characterize neurodegenerative disease such as Alzheimer Disease (AD) (Dashti et al 2012). However, more recent findings suggest a more ambiguous role for Hh pathway in the establishment and progression of several neuropathologies. AD is the most common cause of elderly dementia, it is characterized by progressive cognitive deficits, memory impairment and behavioral disturbs (Graff-Radford et al. 2021). Hallmarks of this pathology are aggregates of β-amyloid plaques (Aβ) and neurofibrillary tangles that accumulate into the brain (Parashar et al. 2024). Dual role of SHh pathway come from evidence that show the amelioration of AD symptoms by blocking SHh signaling (e.g. with cyclopamine), which in turn leads to reduced deposition of Aβ peptides and reduced hippocampal neuron cell death (Vorobyeva et al. 2014, Li et al. 2021). In contrast to these evidence, it has been found that Aβ peptides disrupt the structure of the primary cilium, which is essential for SHh transduction, and impair the activation of the signaling cascade by co-localizing with SMO with subsequent decreasing of GLI1-dependent luciferase activity in NIH3T3 cells (Vorobyeva et Saunders 2018).

Parkinson's Disease (PD) is the second common neurodegenerative disorder, it is characterized by the degeneration and death of dopaminergic neurons in the substantia nigra and in the striatum (Yang et al. 2021). Analogously to

AD, also PD seems to affect primary cilium function, with shortening of the PC observed in the neurons cultured from sporadic PD patients. Surprisingly, PC shortening is accompanied by an increased SHh signaling, which result in mitochondrial disfunctions (Schmidt et al 2022.) Nevertheless, SHh pathway appear to play a protective role from neurotoxic induced cell death and oxidative stress in dopaminergic neurons (Ugbode et al. 2017, Ji et al. 2012). SHh acts therefore at multiple levels, potentially intervening at different stages of the development of neurological disorders. Its strict correlation with neurogenesis, mitochondrial and lysosomal functioning and metabolism makes of it a promising target for the identification of therapeutical target in the treatment of these pathologies (Chen et al. 2018).

1.2.4. Hh signaling in cancer: Medulloblastoma

Hh signaling cascade activates the transcripition of genes involved in survival, proliferation, angiogenesis stem cell-renewal (Salaritabar et al. 2019). De-regulation of Hh signaling and resulting activation has been linked to the development several tumoral forms, including basal cell carcinoma (BCC), rhabdomyosarcoma (RMS), colon-rectal cancer (CRC) and medulloblastoma (MB) (Wu et al. 2017). The overall picture, however, points at Hh pathway as a major contributor in several aspects of the tumoral establishment and progression, such as cancer stem cells (CSCs) self-renewal, immune suppression and tumor immune evasion and modulation of tumor microenvironment. Therefore, SHh is considered as a powerful contributor in the acquisition of chemotherapy resistance and in the tumor recurrence (Merchant and Matsui 2010, Giammona et al. 2023, Onishi et al. 2022). For these reason, Hh pathway has gained increased attention for the

development of therapeutical strategies in the treatment of these malignancies.

MB is most common childhood malignance, although almost the 30% of the total cases are reported in adults. MB arises exclusively in posterior fossa, which comprises cerebellum, the pons and the medulla (Millard and De Braganca 2016). MB is thought to arise mainly from an abnormal proliferation, with subsequent neoplastic transformation, of GPCs residing in the EGL, however the fact that MB occurs also in adults has questioned this hypothesis, suggesting the idea that MB could also arise from others progenitor cell types (Behesti and Marino 2009). MB is a heterogeneous group that comprises many tumoral forms with different histological features, gene expression signatures and epigenetic landscapes. The World Health Organization (WHO) has classified MBs in accord both to their histological features and gene expressions patterns (Louis et al. 2007). The histological variants of MBs are:

- Classic variant MBs. Is the most frequent type of MB, it is characterized by a high cellular density and proliferative rate.

- Desmoplastic-nodular. Mainly diagnosed in adult patients, this variant is associated with a better prognosis. It is characterized by packed differentiated neurocytic populations and proliferating undifferentiated cells.

- Large-cell anaplastic. This histological variant constitutes approximatively 10% of the total case of MB. Out of the total variants, large-cell anaplastic is characterized by a remarkable poor prognosis due

to high proliferation and invasiveness (Millard and the Braganca 2016, Mahapatra and Amsbaugh 2023) .

MBs are further divided into four categories according to their profile of gene expression:

- Wingless (WNT) activated. This class represents 10% of the total diagnoses of MB. WNT MBs show sustained WNT signaling, therefore this class is usually diagnosed trough immunostaining for β-catenin 1, which accumulates into the nucleus (Clifford et al. 2015).

- SHh Activated. SHh activated MBs often carries out mutations in the members of signaling cascade, such as *Ptch1*, *Smo*, *Sufu* and *Gli1/2*. This sub-class is further sub-divided depending on the status of the oncosuppressor *TP53*. Wild-type *TP53* have much better prognosis than mutant *TP53.* In addition, SHh driven MBs can show amplifications of the *N-myc* gene.

- Group 3 (non-WNT/non-SHh). This group shows the poorest prognosis among the different molecular sub-groups of MB, with a survival rate of 50%. This group show amplification of the *myc* locus.

- Group 4 (non-WNT/non-SHh). This group shows some recurrent features, such as chromosomal abnormalities involving chromosome 17 and 11. Interestingly, this group shows recurrent mutations in two antagonist epigenetic actors, *KDM6A* and *EZH2*, which respectively remove and add methyl groups from H3 histonic tails at the level of the lysine 27 (Taylor et al 2012, Archer et al. 2017).

1.2.5 Therapeutic approach in the treatment of Medulloblastoma

The integration of the histological features and genomic alterations occurring in MBs defines different groups based on overall survival rate observed. MBs are therefore classified as "very high risk" (<50% survival), high risk (50-75% survival), average risk (75-90% survival) and low risk (>90% survival) (Ramswamy et al. 2016). According to the statistics, patients with WNT-activated MBs show the best prognosis, with a nearly 100% survival rate; on the contrary patients with SHh-activated and *TP53* mutant MBs and group 3 MBs harboring *MYC* amplification are defined as "high or very high risk" MBs, with overall survival rate less than 50% (Kline et al. 2017). The current treatment of MB consists of a multimodal approach that starts by the surgical resection of the tumoral mass followed by craniospinal irradiation (CSI) and adjuvant chemotherapy. This approach is highly effective, and cures more than 75% of the cases. Nevertheless, this high ratio success comes at a high cost, since the aggressivity of the treatment, and specially the CSI usually causes neurological and neuroendocrine dysfunctions, impairing the quality of life of the patient. Furthermore, CSI is recognized as the responsible of the insurgence of secondary malignancies, promoting the invasion and metastasis of cancer cell, through the enhancement of integrin/Focal Adhesion Kinase signaling (FAK) (Kline et al. 2017, Bagchi et al. 2023, Nalla et al. 2010). Adjuvant chemotherapy is usually carried out with cytotoxic substances such as platinum compounds and alkylators. Combination of chemotherapy with CSI produces long-term toxicities, therefore the development of new therapeutical approach and targeted therapies is required to decrease the risk of side-effects observed in the patients (Thomas and Noel 2019). Clinical trials and researchers are

putting increasing efforts in the identification of new potential therapeutic targets and in the development of inhibitory molecules acting on proliferative- and invasiveness-related pathway often de-regulated in MBs. Development of Hh pathway inhibitors is the most investigated approach. SMO inhibitors Vismodegib, Sonidegib and Glasdegib have been tested for the treatment of MBs. These molecules are SMO antagonists that bind directly to the SMO receptor, preventing the initiation of the signaling cascade. However, mutations occurring downstream the SMO-PTCH1 duo, for example at the level of SUFU or GLI1/2, and cross-talk with other pathway such as EGFR/RAS/RAF/MEK reduce the sensitivity of MBs to these drugs (Gatto et al. 2022). Due to these mechanisms of resistance acquisition, other compounds have been developed, directly targeting the GLIs transcription factor, such as GANT-58 and GANT-61, Glabrescione B and Arsenic trioxide (Severini et al. 2020). However other pathways are being exploited as potential target in the treatment of MB. Tyrosine kinase inhibitors (Afatinib, Imatinib, Erlotinib etc.) are currently being investigated for their ability to block the oncogenic signaling cascades starting from tyrosine kinase receptor EGFR, with potential efficacy in inhibiting MB proliferation and migration (Luzzi et al. 2020, Geoerger et al. 2023). mTOR inhibitors have gained attention for the potential role of this pathway in the maintenance of MB stem cells (MBSCs). MBSCs are pointed as the responsible for chemotherapy resistance acquisition and MB recurrence (Aldaregia et al. 2018). PI3K/Akt/mTOR inhibitors are therefore being tested, with promising results in the treatment of recurrent MB (Luzzi et al. 2020).

1.3. Hippocampus: history, structure and functions

In the past century, post-natal mammalian brain was considered as e relatively stable structure, where neurogenesis and synapses formation were processes restricted to embryonic development and pre-natal stages of development. This idea was questioned by the discovery of post-natal neurogenesis in the rodent hippocampus by Altman and Das in 1965. After then, several studies reported events of adult neurogenesis in the adult brain of mammalians and other vertebrates. Two main neurogenic area have been identified in mammalians: the sub-ventricular zone (SVZ) of the lateral ventricles and the sub-granular zone (SGZ) in the DG of the hippocampus (Ribeiro and Xapelli 2021). Hippocampus arises in the medio-dorsal region of the telencephalon. Proper Hippocampus is divided into sub-regions, the Dentate Gyrus (DG) and the Cornu Ammonis (CA), which is further sub-divided into CA1, CA2 and CA3 (Fares et. al 2019) (**Figure 5**). Hippocampal regions are uni-directionally linked in a so called tri-synaptic circuit: axon projecting from the entorhinal cortex transmit sensory information to the neurons of the DG and the pyramidal neurons of the CA3. DG neurons have mossy fibers that end in proximity of the CA3, which in turn project their axons to the pyramidal neurons of the CA1. CA1 cells in turn project to the subiculum and the entorhinal cortex (Khalaf-Nazzal and Francis 2013). Hippocampal neurogenesis occurs at the level of the SGZ, a thin layer placed at the interface between the DG and the hilus. In the SGZ are located hippocampal stem cells, a triangle shaped cell population that express the astrocytic marker glial fibrillary acidic protein (GFAP), whose projection extend deep into the DG; for their morphology and molecular signature these cells are also named as radial glia-like stem cell (RGLCs). RGLCs are

capable of self-renewal and of generating new neuron through the lifespan of mammalian, although the rate of neurogenesis is reported to drop with aging. The signals coming from the sub-granular niche regulates the behavior of these cells, shaping the neurogenic process starting from the activation and proliferation of the RGLCs until the differentiation and synaptic integration of the newborn granule cells (Gonçalves et al. 2016). Hippocampus and hippocampal neurogenesis are known to regulate several high cognitive functions, such as memory, learning and behavior, and impairments or total ablation of hippocampal neurogenesis in mice lead to severe defects in performing tasks related to pattern separations and impairs memory formation (Anacker and Hen 2017). Furthermore, hippocampus and hippocampal neurogenesis are strongly affected by the onset of neurodegenerative and neurological disease. In AD, the deposition of Aβ-plaques starts at the level of the entorhinal cortex and continue towards the cortex and the hippocampus. In major depression disorder, a significant portion of the patient has reduced hippocampal volume and potentially exhibit reduced rates of hippocampal neurogenesis (Culig et al. 2022, Gonçalves et al. 2016); however the current difficulties to study hippocampal neurogenesis and neurodegenerative disease in humans make arduous to establish the cause-consequence relation between hippocampal dysfunction and human brain diseases.

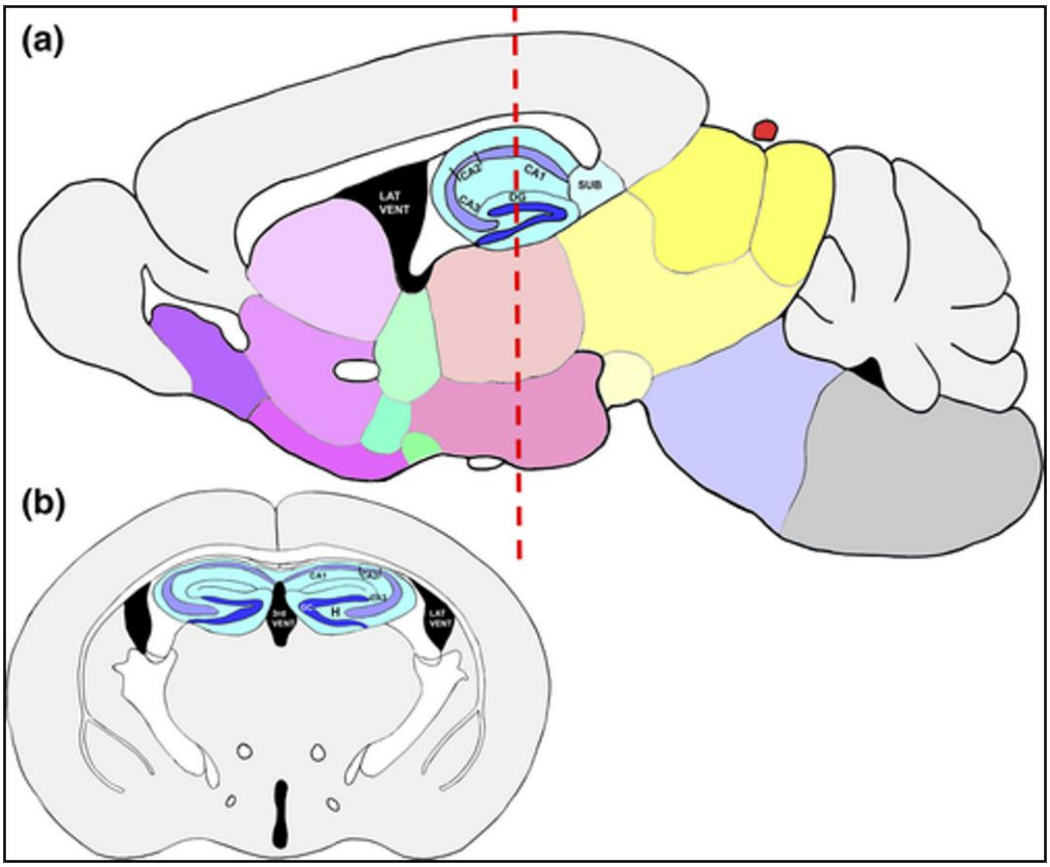

Figure 5) Brain sections of mouse hippocampus. The image show the adult mice hippocampus from two different points of view. a) Sagittal sections of adult mouse brain, SUB, subiculum, LAT VENT, lateral ventricle. b) coronal section of adult mouse brain, H, hilus, 3d VENT, third ventricle.

1.3.1 Radial glia-like cells

RGLCs are the stem cells residing in the hippocampus. RGLCs are located at the level of the SGZ of the DG. They possess a characteristic morphology, with a triangle shaped body and a single process projecting into the DG with branched end-feet extending into the inner molecular layer. These cells share some common features with astrocytes, since they express the intermediate filament protein GFAP and Vimentin; they also express the typical neural stem cells marker Nestin and Sox2 (Berg et al. 2018). The origin of these cells is still under debate, with some evidence proposing that adult hippocampal NSCs originate during the late embryonic development in the ventral hippocampus (Li et al. 2013). Clonal and lineage tracing analyses revealed that RGLCs possess self-renewal property, being able to divide both symmetrically and asymmetrically, furthermore they are considered as multi-potent stem cells, being able to generate both neurons and astrocytes *in vitro*, while *in vivo* the multipotency is restricted to neurons by the RNase III Drosha (Bonaguidi et al. 2011, Rolando et al. 2016). RGLCs can exist in two states: quiescent RGLCs (qRGLCs) and active RGLCs (aRGLCs). The rate of RGLCs neurogenic activity depends both on genetic and epigenetic stimuli. The hippocampal niche regulates the activation of RGLCs through morphogens and growth factors(BMP, Jagged, SHh), also environmental stimuli like exercise, diet and stress, are known to have an impact on neurogenesis rate (Valero et al. 2016, Mosher and Schaffer 2018). Activation of RGLCs have a profound impact on cell identity (**Figure 6)**. Transition from qRGLCs to aRGLcs determines a shift in the molecular signature of RGLCs, with a change in the profile of membrane ion transporters, cell-cell adhesion molecules and signaling pathway.

One of the hallmarks of aRGLCs is the transcription of genes involved in cell-cycle progression, protein/DNA/RNA synthesis and DNA damage control. qRGLCs rely principally on glycolysis and fatty acids oxidation as sources of energy, while aRGLCs shifts toward a metabolism oriented to oxidative phosphorylation, with an in increase in the RNA level of the mitochondrial respiratory chain complex V (Shin et al. 2015).

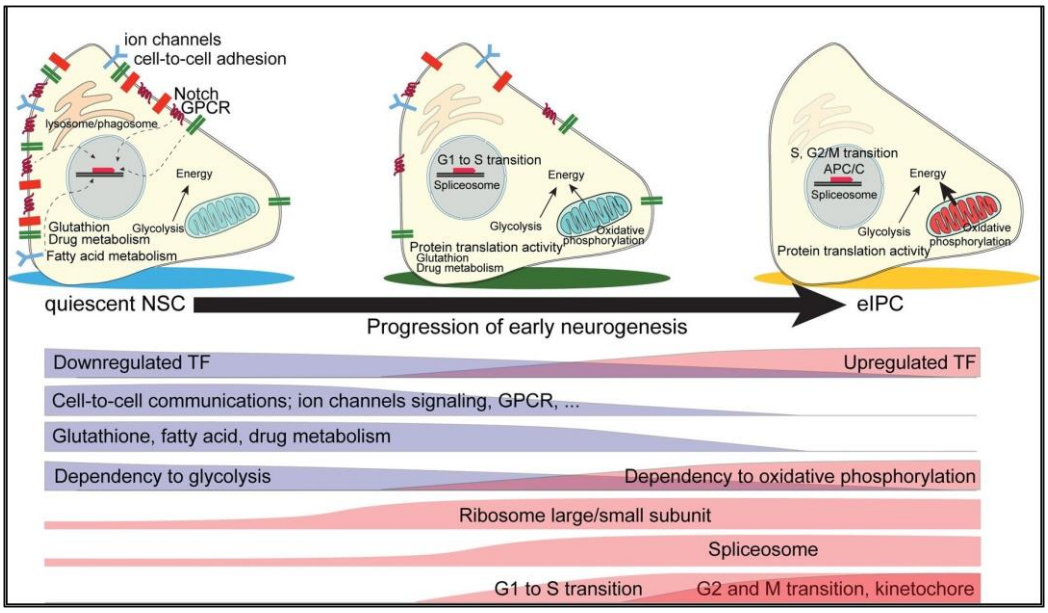

Figure 6) Activation of RGLCs. a) Early phases of neurogenesis are characterized by the alteration of the cell molecular profile at multiple levels. aRGLCs and early intermediate precursor cells (eIPC) are characterized by the entrance into cell cycle, increase of the translational capacity and by a metabolic shift from glycolysis to oxidative phosphorylation (Image modified by Shin et al. 2015)

1.3.2 Stages of hippocampal neurogenesis

Hippocampal neurogenesis is a process that consists in the generation of new granule cells of the DG. The process starts from the activation of the RGLCs and ends with the generation of a synaptically integrated mature granule cells **(Figure 7)**. Activation of the RGLCs is dependent on the signals produced by the hippocampal niche, which regulate the expression of stemness related genes as well as the expression of pro-differentiative genes. Once activated RGLCs generate a population of intermediate precursor cells (IPCs) which can be sub-divided into two population: type 2a and type 2b cells (Toda and Gage 2018). Type 2a cells still express the glia and stemness marker like GFAP and Sox2, but they do lack of the characteristic morphology of the RGLCs. The progression from type 2a to type 2b cell is marked by the loss of glial markers and by the reduction of Sox2, with a simultaneous appearance of the neuronal lineage transcription factors NeuroD and Prox1 (Kempermann et al. 2015). Neuroblast or type 3 cells arise from IPCs after a variable number of cell divisions. This population consists of a heterogeneous group of cells that shows various degrees of synapses arborization, Type 3 cell lack of the expression of any stemness marker, instead they invariably express neuronal marker (Doublecortin, PSA-NCAM), these cell migrate in the DG at short distance before further maturation (Ehninger and Kempermann 2008). After the exit from cell-cycle, immature neuron start to express the neuronal marker NeuN and the calcium-binding protein calretinin (von Bohlen und Halbach 2007). During this phase, many of the newly generated neurons undergo cell death by apoptosis (Biebl et al. 2000). The maturing surviving cells cease to express calretinin in favor of

calbindin and integrate into the DG functional connections forming new synapses (Kempermann et al 2015).

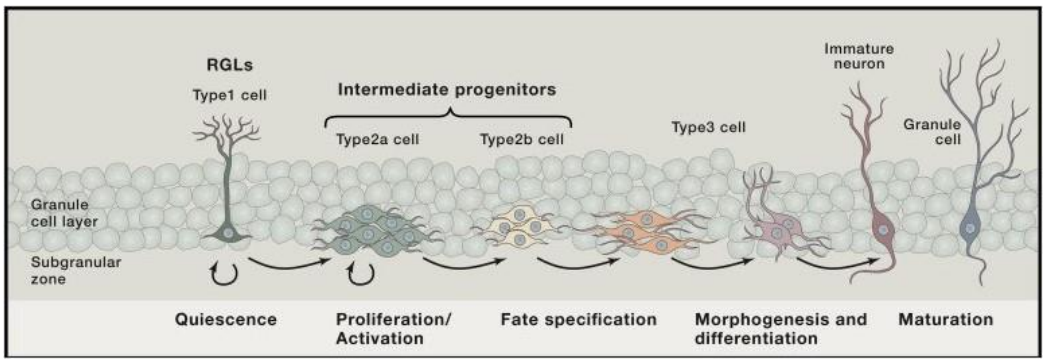

Figure 7) Schematic representation of neurogenesis stages. Hippocampal neurogenesis starts from RGLCs, which give rise to transient amplifying intermediate progenitors. After the specification of the neuronal lineage, newborn granules migrate into the DG and synaptically integrate into the hippocampal circuit.

1.3.3 Hh in hippocampal physiology

SHh signaling act as a major morphogen in the developing CNS, regulating proliferation, survival and maturation of several types of cells. Despite being generally considered active at much more low level in the post-natal brain, SHh signaling still plays a major role in adult CNS, with roles ranging from the control of neurogenesis to cell survival and differentiation. In the hippocampus SHh signaling drives stem cell pool maintenance and activation, neurons survival and axonal generation (Alvarez-Buylla et Ihrie 2014). Several sources of SHh ligand have been identified in the hippocampus trough *in situ* and reporter gene techniques: DG and hilar neurons, astrocytes and RGLCs (Favaro et al. 2009, Gozalez-Reyes et al. 2109, Eitan et al. 2016, Yao et al. 2016). The two receptors PTCH1 and SMO are differentially expressed in the hippocampus, where SMO is expressed both in the DG and the CA, while PTCH1 expression seems to be principally expressed to the CA (Qin et al. 2019). On the contrary GLI1 expression is predominantly found in RGLCs, which make up to the majority of the GLI1 positive cells in the DG, with only a minor fraction of non RGLCs GLI1 expressing cells (Bottes et al. 2021). RGLCs capacity to divide and to generate both new neurons and RGLCs is strictly dependent on the function of the primary cilia and SHh signaling. Ablation of the *Stumpy* gene, which is required for the formation of the PC, impairs GLI1 levels and leads to decreased rates of proliferation and loss of RGLCs pool in young mice (Breunig et al. 2008). On the other hand, studies exploiting the opposite strategy brought evidence that increased Hh signaling, and in particular increased GLI1 levels, leads to depletion of the RGLCs and to the drop of neurogenesis rate in the old mice (Antonelli et al. 2018).

Interestingly it appears that SHh has a significant impact also in non-proliferating neurons. Down-regulation of Hh signaling resulting from high-fat diet (HFD) in mice leads to marked induction of apoptosis in pyramidal neurons of CA3 region. Administration of SAG in HFD mice reduces the levels of apoptosis observed by regulating the levels of Bcl-2 (Qin et al. 2019). Furthermore, through non-canonical Smo/Ca^{2+} dependent mechanism, Hh signaling can trigger in hippocampal neurons the secretion of BDNF, which is a potent neurotrophic factor driving growth of GABAergic and glutamatergic synapses (Delmotte et al. 2020, Colucci et al. 2020).

1.3.4 Hippocampus and aging

Aging is a naturally occurring physiological progress that impairs physical and cognitive abilities. In the hippocampus, aging is accompanied by morphological and biological changes that affect hippocampal functions. At the level of hippocampal cyto-architecture, evidence of the reduction of the total hippocampal volume have been reported in aged rodents and human (Driscoll et al. 2006, Persson et al. 2012), furthermore aged hippocampus shows a marked reduction in synaptic plasticity with increased neuronal atrophy and a decline in the neurogenesis rate (Bettio et al 2017). The reduction of total hippocampal volume seems to be correlated with neuronal loss (Fu et al. 2015) and with the decline of the neurogenesis rate occurring during the process of aging. Aging neurons in hippocampus show a remarkable increase in the level of pro-apoptotic genes, with a concomitant reduction of autophagy related genes (Yu et al. 2017). The increase of apoptotic rate in old hippocampus neurons is the reflection of the environmental changes that

take place with aging. In both human and rodents, aging is associated with the disruption of Blood Brain Barrier (BBB), which constitute a strictly controlled gateway for the communication between the blood flow and the CNS (Knox et al. 2022). Leaking BBB allows for the entrance of neurotoxic substances in the CNS, eliciting pro-inflammatory microglia response and oxidative stress, ultimately leading to cell death (Enciu et al. 2013). Furthermore, in rodents, hippocampal neurons show an increasing age-dependent weakening of the existing hippocampal synapses, a process known as long-term depression (LTD), as a consequence of reduced neurotrophic factors like BDNF, which seems to be correlated with increasing levels of class I HDACs (Erickson et al. 2010, Bettio et al. 2017). In addition to neurons, aging affects RGLCs ability to activate and generate new neurons. The aging hippocampal niche in mice is characterized by a reduction in the number of proliferative IPCs and immature Doublecortin positive granule cells, possibly due to lower levels of growth factors, such as FGF-2, IGF-1 and VEGF, with a concomitant increase in pro-inflammatory signals like IL-1 and IL-6β produced by the resident microglia. Furthermore, RGLCs in aged subjects are more prone to become quiescent due to telomeres shortening, oxidative stress and DNA damage (Fan et al. 2017). Cellular senescence and apoptosis, impaired synaptic plasticity and reduced neurogenesis rate are common features in the aging hippocampus. These processes are linked to the cognitive defects that often occur in elder subjects, such as spatial and episodic learning and memory, and the severity of the changes coming with age in the hippocampus has been correlated with the susceptibility to develop neurodegenerative diseases (Bettio et al. 2017).

1.4. KCTD family of protein

The human family of *Potassium (K^+) Channel* T*etramerization Domain* (KCTD) comprises 25 members, while mice encode one more member. The members of this family likely arise from events of gene duplication from a common ancestor and share a common domain named BTB (Broad Complex, Tramtrak and Bric-a-brac)/POZ (poxvirus zinc finger) located at the N-terminus of the KCTD proteins. According to the more recent classification, based on the alignment of BTB domain sequences, KCTD protein can be sub-divided in 8 clades (Angrisani et al. 2021). Except for the sharing of a common BTB domain, no similarity can be found in the highly variable C-terminal region between members of the KCTD family belonging to different sub-groups (Skoblov et al. 2013). Although poorly characterized, the role of KCTD proteins has been linked to neurodevelopmental, neurological and tumoral disease. The BTB/POZ is responsible for the KCTD protein capacity to oligomerize and to bind several targets (Perez-Torrado et al. 2006,). The current evidence demonstrates that KCTD protein can regulate neurotransmitter signaling (Ivankova et al. 2013). Furthermore, group B KCTD proteins have been shown to bind the ubiquitin E3 ligase Cullin3, thereby promoting the degradation of different targets. This feature has been linked with the ability of KCTD proteins to act as tumor suppressors, regulating cell growth and tumorigenesis (Teng et al. 2019). This ability led to the identification of a new family of KCTD protein, the KCASH family (KCTD containing-Cul3 adaptors, suppressor of Hedgehog), identified due to the ability to suppress the oncogenic Hh/GLI1 signaling pathway.

1.4.1 KCASH family

The KCASH family of oncosuppressors comprises the members of the group B of KCTD proteins, that includes KCASH1^{KCTD11}, KCASH2^{KCTD21}, KCASH3^{KCTD6}. Among the KCASH family members, KCASH1^{KCTD11} was the first one identified for its capability to suppress GLI1 activity(Canettieri et al. 2010).

Further efforts in order to identify KCASH1^{KCTD11} homologues led to the discovery of the two other members of the KCASH family, KCASH2^{KCTD21} and KCASH3^{KCTD6}.

KCASH2^{KCTD21} is able to form homo- or hetero-oligomers with KCASH1^{KCTD11} and KCASH3^{KCTD6} and to bind the ubiquitin ligase E3 Cul3 and HDAC1, promoting the degradation of the class I Histone de-acetylase, while KCASH3^{KCTD6} cannot directly bind HDAC1. HDAC1-mediated de-acetylation of GLI1 determines for the transcriptional activation of the latter. Indeed, GLI1 in its acetylated form in transcriptionally inactive and unable to transduce Hh ligand signaling. Therefore, KCASH2 activity is responsible for the repression of GLI1 transcriptional activity.

KCASH2^{KCTD21} shows a high expression in mouse brain and cerebellum, where SHh pathway plays a major role in tissue development and tumorigenesis (Carballo et al. 2018). Interestingly, KCASH2^{KCTD21} genetic locus can be lost in several sporadic tumors, such as MB, Neuroblastoma, and leukemia. These findings suggest a possible prominent role for this protein in the regulation of disease insurgence

and progression (Reardon et al. 2000, Angrisani et al.2021, De Smaele et al. 2011)

1.4.2. Cerebellar phenotype of KCASH2^{KO} mouse

The discovery of KCASH2^{KCTD21} as a suppressor of the Hh/GLI1 oncogenic pathway prompted our research group to generate a KCASH2^{KO} mouse model to analyze *in vivo* the consequences of KCASH2 loss. The generation and characterization of KCASH2^{KO} mouse is described in section **Materials and Methods** paragraph **2.1.** The importance of Hh/GLI1 pathway in the insurgence of MB led us, during previous analyses, to the investigation of the cerebellar phenotype of the KCASH2^{KO} mouse. Through the enzymatic assay that take advantage of the reporter *LacZ* gene in the KCASH2^{KO} cassette, KCASH2 expression was assessed in mouse cerebellum and other regions of KCASH2^{KO} mouse brain (**Figure 8**, data not published).

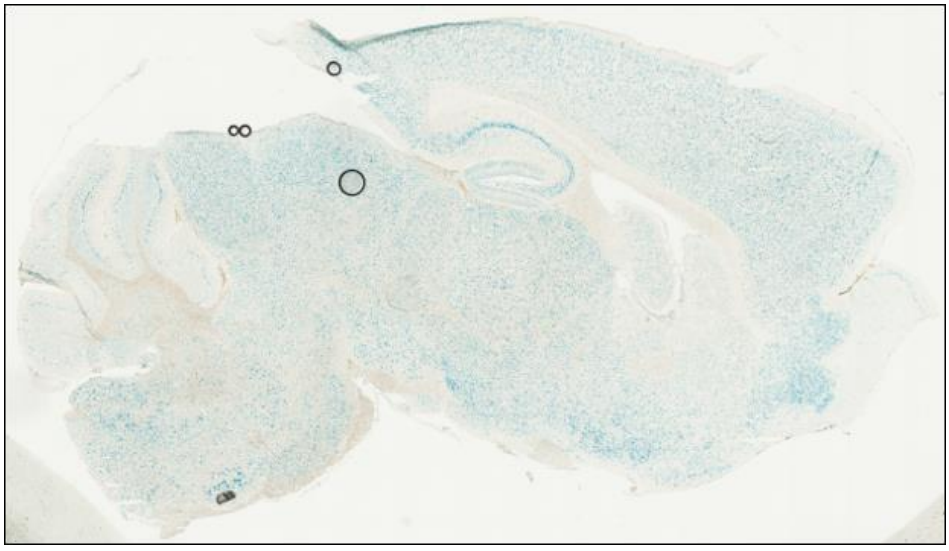

Figure 8). KCASH2 expression in mouse brain. *LacZ* gene reporter enzymatic assay highlight KCASH2 expression in the regions of KCASH2^{KO} mouse brain.

The role of KCASH2 as a negative regulator of HDAC1, and subsequently of GLI1, was assessed in the mice cerebellum in the window between P7 and P14, when the levels of GLI1 protein and mRNA are increased in comparison to wild type littermates (**Figure 9**, data not published**)**.

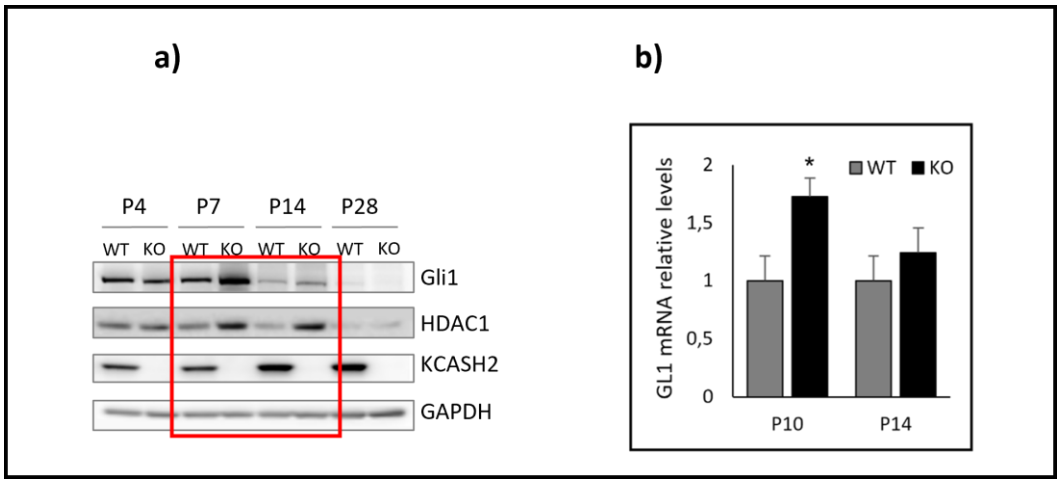

Figure 9) KCASH2 loss leads to increased levels of HDAC1 and GLI1. Western Blot (**a)** and (**b)** RT-qPCR analyses show the increase of HDAC1 and GLI1 levels at P7 due to KCASH2 absence (n=10, *p<0.05).

As a consequence of sustained Hh signaling, KCASH2^{KO} mice show a mild Hedgehog-dependent phenotype, with a significant increase in the thickness of IGL in adult mice (**Figure 10**, data not published) due to sustained Granule Precursor Cells proliferation and delayed cell-cycle exit.

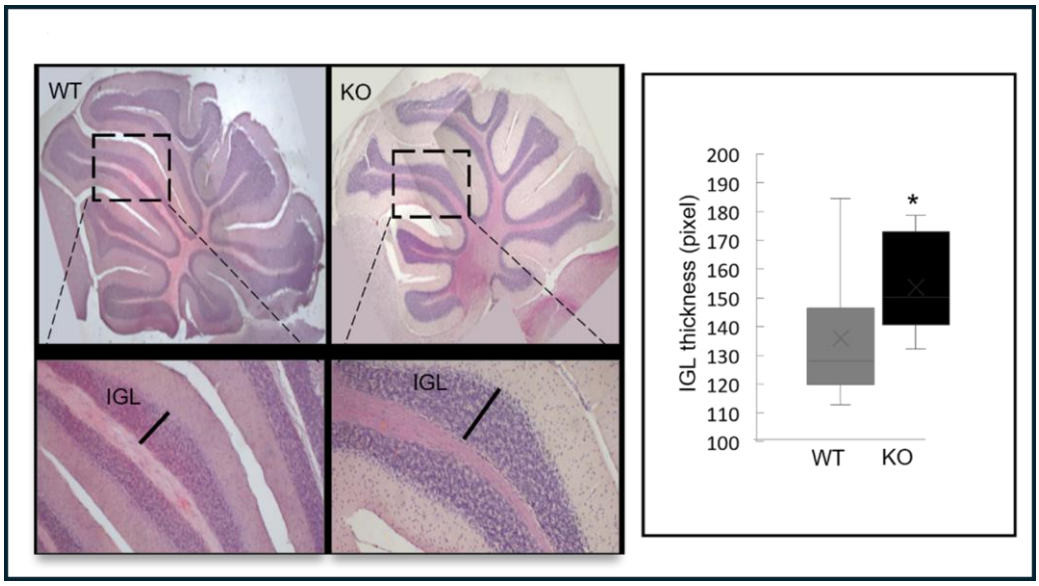

Figure 10) Morphological alterations of KCASH2^{KO} mice cerebella. Immunohistological analyses reveal a thickening of the adult KCASH2^{KO} mice IGL (n=22, *p<0.05).

1.5 AIM

Hh signaling pathway plays a key role in several biological processes, such as embryogenesis, tissue homeostasis, stem cell maintenance and cancer. In the CNS, Hh pathway plays a major role in regulating the neurogenic processes that occur during the lifespan of mammals, starting from peri-natal period until elderliness. De-regulation of Hh signaling has been however found in numerous malignancies occurring in the CNS, also in a fashion that is independent from the activity of Hh ligands, with the involvement of others tumorigenic pathways. The current therapeutical approaches for these treatments remain in some cases uneffective, and often lead to life long side effects. The main effector of Hh pathway is GLI1, a transcription factor whose activity is regulated trough different mechanisms, including phosphorylation, acetylation and ubiquitination. Among these, acetylation appears to be crucial for GLI1 in order to exert its transcriptional activity, acetylated GLI1 is indeed transcriptionally inhibited, while de-acetylation due to HDAC1 enhances GLI1 activity . The balance between acetylated and de-acetylated GLI1 forms can be regulated by KCASH2, a KCTD protein that is able to promote HDAC1 ubiquitination and degradation trough the interaction with Cul3, therefore silencing Hh signaling.

Starting from these perspectives, the present work has two main aims. The first aim is the characterization *in vivo*, using a KCASH2^{KO} mouse model, of the role of KCASH2 and Hh pathway in hippocampal neurogenesis.

The second aim is the definition of a new approach in the treatment of MB. Using single and multiple-drug strategies we pointed at GLI1

transcription factor inhibition trough both direct targeting and inactivation of cross talk between Hh and other tumorigenic pathways.

2. Materials and Methods

2.1. Mouse Strains

KCASH2HT(C57BL/6J) were generated at the facility of EMBL using the KCASH2^{KO} first (vector GenBank: JN947162.1), which abrogates KCASH2 expression through homologous recombination by inserting a cassette containing a *LacZ* reporter gene, a resistance for Neomicin and a poly-adenylation signal between the promoter of KCASH2 and the first exon. The construct was injected into fertilized egg of C57BL/6JRj mice. The recombinant mice were backcrossed with C57BL/6JRj for 10 generations in order to obtain transgenic mice with C57BL/6JRj background. Genotyping was conducted through PCR analyses on DNA extracted from the mouse tails using the following primers:

- Fw 3LR Primer: 5'- TCCCAAGCAGCTCATGTGAG - 3'
- Rv 4LR Primer: 5'- GTGGAGCCACAAAGGGTTCT – 3'

Animal maintenance and experimental procedures were performed in the facility of "Sapienza" University of Rome. All protocols were approved by the Italian Ministry of Health and performed according to the guidelines for animal care.

2.2. Cell lines

The MB-UW-228 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, United States), supplemented with 10% FBS, 1% Penicillin and Streptomycin, and 1% Glutamine. The MB-ONS-76 cell line was cultured in RPMI1640 (Sigma-Aldrich, St. Louis, United States) supplemented with 10% FBS Glutamine and 1% Penicillin and Streptomycin.

The MB-DAOY medulloblastoma cell line was maintained in Minimal Essential Medium (MEM) (Sigma-Aldrich, St. Louis, United States), supplemented with 10% FBS, 1% Sodium Pyruvate, 1% Non-Essential Amino Acids, 1% Glutamine and 1% Penicillin and Streptomycin. Mycoplasma contamination in cell cultures was routinely assessed using a PCR detection kit (Applied Biological Materials, Richmond, BC, Canada).

2.3. Drug treatment and MTT assay

The EGFR inhibitor Afatinib, the inhibitor of GLI1/2 GANT-61 and the FAK inhibitor Y15 were purchased by MedChem Express and resuspended in DMSO at the final concentration of 50mM. The MB cell lines were treated with increasing concentration of drugs (1-20μM for GANT-61, 0.5-5μM for Afatinib, 0.5-10μM for Y15), alone and in combination for 24h, 48h and 72h. At the end of each time point, 20μL of dye solution (Cell Titer 96^{R} Promega) were added to the cells in each well and incubated for 3 hours. Reaction was stopped using the solubilization/stop mix and the survival/proliferation of cell was measured recording the absorbance at 560nm. The values of absorbance were normalized to the values of the control (DMSO) at equimolar concentrations. All results are expressed as the mean of three independent experiments performed in triplicate. Interactions between drugs during combined treatment were evaluated using the method of Kern as described by Bei R. et al. work (Bei et al. 2022). Kern R Index >1 indicates a synergistic effect, R=1 indicate an additive effect, R Index<1 indicate that the combined drugs have an effect less than additive.

2.4. Radial glia-like cells primary culture

The protocol for RGLCs primary culture was performed according to Babu et al. Protocol (Babu et al. 2011). In brief, hippocampi were dissected from hemispheres of 8 weeks old mice. Hippocampi were minced and then enzymatically digested using Neural Tissue Dissociation Kit (P) (Miltenyi Biotec) following manufacturer instructions. After enzymatic dissociation, cells were resuspended in a solution of Percoll 22%, composed of Percoll 100% (Sigma-Aldrich) and Neurobasal (Gibco), and centrifuged at 450g for 15 min in order to obtain a more pure culture , after three washes in Neurobasal Medium, cells have been resuspended in complete growth medium, composed of Neurobasal Medium supplemented with 1% GlutaMax (Gibco), 2% B-27 (Gibco), 2% Penicillin and Streptomycin, 20ng/mL Epidermal growth factor and 20 ng/mL Fibroblast growth factor-2 (Peprotech) and Heparin 2μM (Sigma-Aldrich). Cells were plated at the density of 2,5 x 10^4 cell/cm^2 in Laminin (Merck Millipore, final concentrations of 5mg/mL) and poly-lysine (final concentration of 0,1 mg/mL) coated vessels. Cells were splitted once or twice a week following enzymatic dissociation by Accutase (Sigma-Aldrich) and plated in new coated vessels.

2.5. RNA extraction and rt-qPCR

Total RNA was extracted from mice hippocampi using TRIsure reagent (Bioline) following manufacturer instructions. DNaseI treatment (ThermoFisher) was performed in order to eliminate contamination from genomic DNA. Reverse transcription for cDNA synthesis was performed using the high-capacity cDNA reverse transcription kit (Meridian Bioscience). cDNA was then used to analyze relative gene-expression by performing real

time quantitative PCR (RT-qPCR) using TaqMan™ Gene Expression Assay (Applied Biosystem-Thermo Fisher Scientific). TaqMan probes used are KCASH2 (Mm01279524_s1) and GLI1 (Mm00494654_m1). All results are expressed as the mean of three independent experiments performed in triplicate.

2.6. Protein extraction and Western Blots

Cell were washed with Phosphate-Buffered Saline and lysed using denaturing SDS-urea buffer (50 mM Tris HCL pH 7.8; 10% Glycerol; 2% SDS; 10 mM EDTA pH 8.0; 100 mM NaF; 10 mM Na2P2O7; 6M Urea) added with protease inhibitors (10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1mM PMSF, 2mM Na3VO4). The lysate was then sonicated, collected and stored ad -80 °C. Quantification of the protein extract was carried out measuring the absorbance at 280 nm). Protein extract were analyzed trough Western Blot assay using the following antibodies: anti-KCTD21 [EPR12873(2)] (1:1000, Abcam, ab192259), anti-HDAC1 (1:1000, Sigma-Aldrich, H3284), anti-GLI1 (A-7) (1:500, sc-515781, Santa Cruz Biotechnology), anti-GFAP (1:2000, Z0334, Dako), anti-Sox2 (1:1000, GT1876, ThermoFisher Scientific), anti-β-actin (C4) HRP (1:1000, sc-47778; Santa Cruz Biotechnology), anti-Vinculin (7F9) HRP (1:1000, sc-73614; Santa Cruz Biotechnology).

2.7. Immunofluorescence analyses

Brain tissues were collected from mice and fixed overnight in paraformaldehyde 4%, dehydrated in sucrose 20% and embedded in OCT (Tissue-Tek). OCT blocks were cut into 10μM thick slices and stored at -80°. When needed, slices were permeabilized using PBS containing Triton-X 0.2%

for 15 minutes, slices were then blocked using blocking solution (Glicine 1M, BSA 3%, Goat Serum 5%). After blocking, slices were incubated overnight at +4° with primary antibody. After incubation with primary antibody, slices were washed three times with PBS and then incubated with secondary antibody. Slides were sealed with OCT Mounting Medium (Dako). Images were acquired at Leica DM 2500 microscope.

Primary antibodies for immunofluorescence were: anti-Ki67 (1:250, SP6, MA1-39550, Thermo Fisher), anti-Nestin (1:200, Rat 401, Abcam), anti-GFAP (1:500, Z0334, Dako) Anti-Vimentin (1:200, E-5, sc-373717), anti-NeuN (1:250, mab377, Merck Millipore). Secondary antibodies were Alexa Fluor 594 goat anti-rabbit (1:500, A11012, Thermo Fisher), Alexa Fluor 488 goat anti-mouse (1:500, A28175, Thermo Fisher).

2.8. β-galactosidase staining and double staining

Tissue slices were incubated overnight at 37° in X-gal staining solution (NP40 0,02%, 0,01% Sodium Deoxycholate, 5mM Potassium Ferricyanide, 5mM Potassium Ferrocyanide and 5% X-gal in PBS 1X) added with 5-Bromo-4-Chloro-3-indolyl β-D-galactopyranoside at the final concentration of 5mg/mL, resuspended in N-N-dymethil-formamide (Sigma, B4252). After X-gal staining, slices were washed three times in PBS and then subjected to the immunofluorescence protocol.

2.9. Statistical analyses

The experimental result are expressed as the mean±SD of three biological replicates. Differences in the mean of the experimental group were evaluated using Student's t-test. Statistical significance was set at *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3. Results

3.1. Hippocampal phenotype of KCASH2^{KO} mouse

3.1.1. KCASH2 expression in the Hippocampus

It is well known that SHh pathway regulates the neurogenic processes that occur in the hippocampus. SHh signaling is, indeed, strictly correlated with the neurogenic rate in the DG (Yao et al. 2016). This evidence, together with previous analyses that reported the expression of KCASH2 in mouse brain (De Smaele, Di Marcotullio et al. 2011) prompted us to investigate KCASH2 expression and role in the hippocampus. Taking advantage of the use of the KCASH2^{KO} transgenic mouse, which allows exploiting the expression of the exogenous LacZ gene driven by the endogenous KCASH2 promoter, we were able to monitor the physiological expression of KCASH2. The results of the enzymatic assay visualized as blue dots appearing in correspondence of LacZ activity confirms that KCASH2 is expressed all along the main regions of the hippocampus, including the DG, the different sub-regions of the CA, comprising CA1, CA2 and CA3, the crest and the stratum at P21 **(Figure 11a)**. Expression levels of KCASH2 in the Hippocampus were analyzed by RT-qPCR, performed at different stages (P10, P21 and 8 weeks) of murine hippocampal development (**Figure 11b**). Thus, we have identified that mRNA levels of KCASH2 are already present in the hippocampus at P10, increase between P10 and P21, while decreasing slightly after 21 to 8 weeks of age.

Furthermore, double-staining for X-Gal (black dots), the neuronal marker NeuN (green) and the astrocytic marker GFAP (red) confirmed the expression of KCASH2 in the GABAergic neuronal population of the DG, the pyramidal neuron of the CA and the astrocytes located in the stratum radiatum (**Figure 12)**. Wild type mice brain cryosections were used as control to confirm

staining specificity (**Figure 12a)**. It is interesting to note that X-gal dots are also present in the sub-granular zone of the DG, where neural stem cells and generally less differentiated neuronal populations reside (**Figure 12c and 12d)**. Indeed, the nuclei in the SGZ are negative for the NeuN staining. X-gal staining shows a remarkable intensity at level of the pyramidal neuron of the CA. GLI1 seems to not be expressed in these cells , although SHh signaling has been showed to protect them from High-fat diet induced apoptosis by promoting the expression of the anti-apoptotic protein Bcl-2 (Qin et al. 2019). Thus, X-Gal expression in this region suggests still unveiled functions of KCASH2 protein that need further investigation.

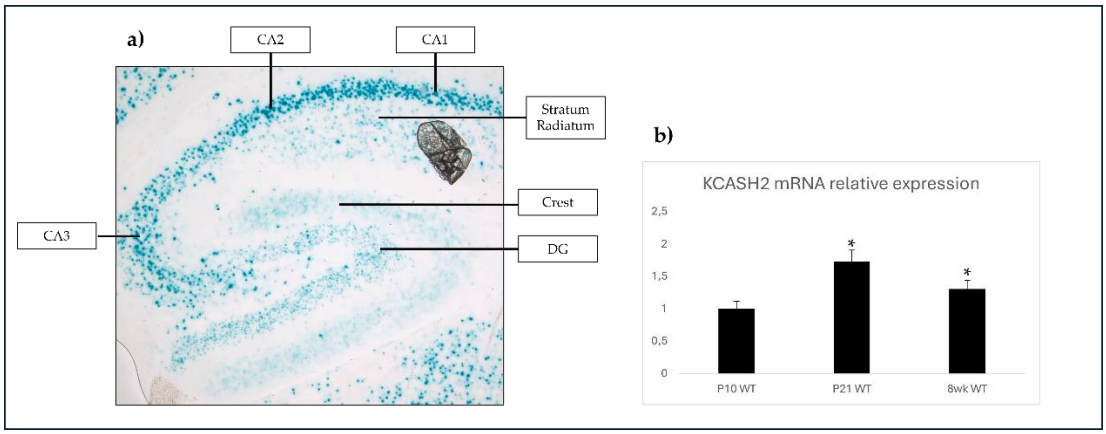

Figure 11) KCASH2 expression in the hippocampus. a) β-Galactosidase enzymatic assay performed on mouse brain sagittal cryosections reveals KCASH2 expression in the different regions of the hippocampus. **b**) RT-qPCR analyses on total RNA extracted from hippocampus show a significant increase of KCASH2 mRNA expression with age (*$p<0.05$, $n=9$).

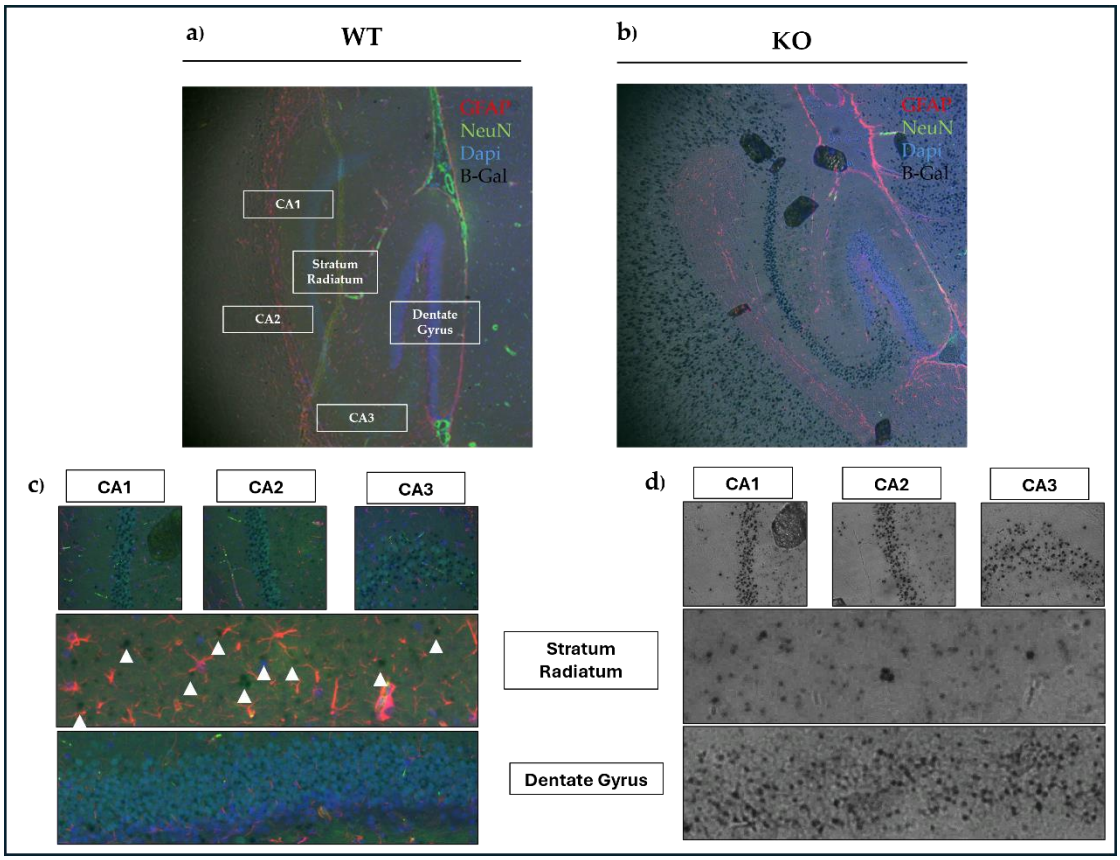

Figure 12) X-gal/immunofluorescence combined staining. Double staining for X-gal (black) and neuronal marker NeuN (green) and astrocytic marker GFAP (red) on P21 mouse brain WT (**a)** and KO (**b)** cryosections. **c, d)** Magnification of the different regions of the KO mouse hippocampus, **c)** shows the merge of the double staining for X-gal and immunofluorescence channels, **d)** shows the X-gal dots acquired in the brightfield in the same regions.

3.1.2. KCASH2 loss alters proliferation rate inside the dentate Gyrus

KCASH2 has previously been described as a negative regulator o the Hh/GLI1 pathway (De Smaele et al. 2011). Given the role of this signaling pathway in sustaining the neurogenesis in the hippocampus, we aimed to analyze the proliferation rate inside the DG and the modulation of Hh signaling.

To this purpose we analyzed the levels of hippocampal neurogenesis through immunofluorescence analyses on mouse brain cryosections at P21. Through the staining for the proliferative marker Ki67, we were able to detect a significant increase in the proliferative rate in the KCASH2^{KO} mice DG (**Figure 13a and 13b)**. The Ki67 positive cells were principally located at the level of the sub-granular zone, where hippocampal stem cells and precursor cells are located, but proliferative cells were also found in the hilus, which is inhabited by astrocytes and non proliferative interneurons. Therefore, the staining in the hilus could potentially underline reactive and proliferating astrocytes, although this hypothesis need further investigations. Consequently, we pointed to assess the levels of Hh signaling in the Hippocampus. Since GLI1 can activate its own transcription, we decided to analyze GLI1 mRNA levels in order to analyze the intensity of Hh activity. By RT-qPCR analyses we detected therefore an increase in the levels of GLI1 in KO mice analyzed at P10, P21 and 8 weeks, although the increase was statistically significant only at 8 weeks, we observed a general tendency in all the three time points observed (**Figure 13c**), confirming the role of negative regulator of Hh signaling for KCASH2.

The major population of GLI1 expressing cells in the DG are the RGLCs, but sparse GLI1 positive cells can be also found in the striatum and in the intermediate precursor cell generated by RGCLs activation and differentiation (Bottes et al. 2021, Hill et al. 2019).

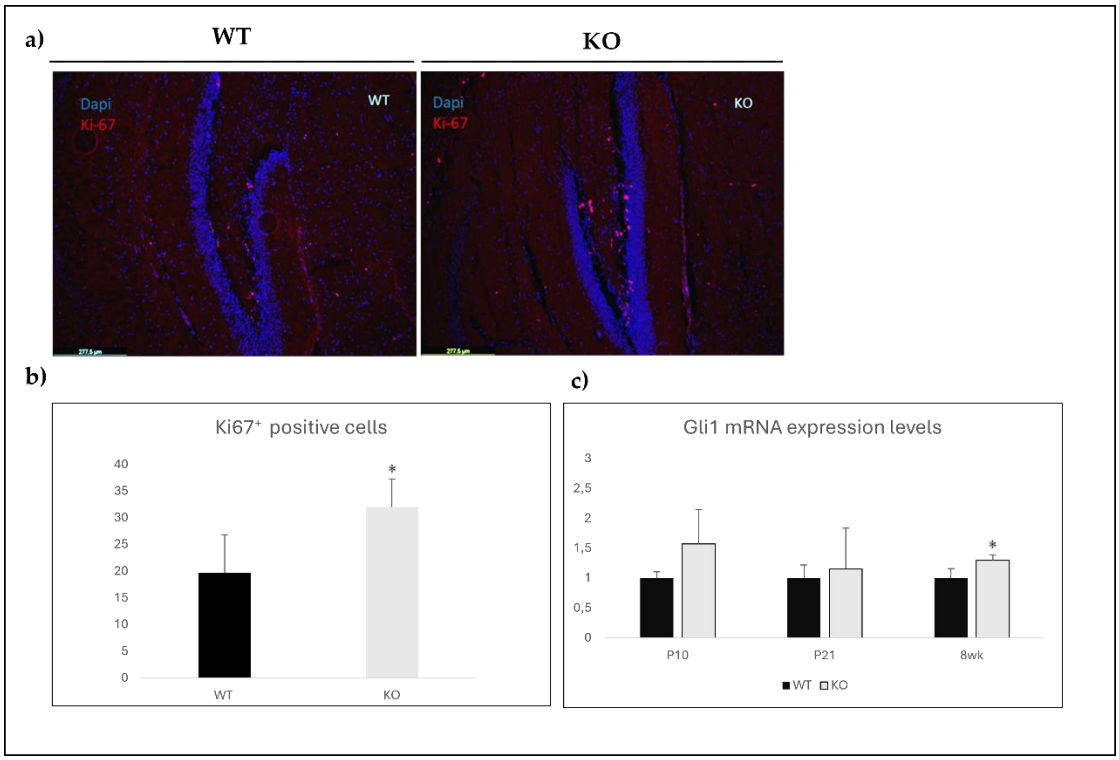

Figure 13) KCASH2 loss leads to sustained proliferation rate and increased Hh/GLI1 signaling in the Hippocampus. a) Immunofluorescence staining on P21 mouse brain cryosections for the proliferative marker Ki67, **b)** statistical analyses show an increase in the overall proliferation rate observed in the DG (*p<0.05, n=6). **c)** RT-qPCR analyses on total RNA from mice hippocampus show a significant increase in GLI1 mRNA levels at 8 week (*p<0.05, n=18).

3.1.3. Hh signaling is up-regulated in KCASH2^{KO} RGLCs

RGLCs are the hippocampal stem cell of the hippocampus, located at the level of the SGZ. RGLCs can activate and become proliferating, generating intermediate precursor cells that goes through several cell-cycle division before further differentiating in neuroblast and then in mature neurons.

Up-regulation of Hh/GLI1 signaling has been showed to directly promote the neurogenesis rate in the hippocampus by regulating RGLCs activation and sustaining intermediate precursor cells proliferation (Ahn and Joyner 2005, Lai et al. 2003).

To evaluate KCASH2 expression in RGLCs and modulation of HDAC1 and GLI1 protein levels, we cultured RGLCs from mouse hippocampi at 8 weeks of age. As expected, KCASH2^{KO} RGLCs show increased level of GLI1 and HDAC1. Furthermore, cultured RGLCs express the radial glia marker GFAP and neural stem cells marker Sox2, which appear to not be modulated due to KCASH2 absence (**Figure 14a**). It must be noted that cultured RGLCs do not show the typical morphology of RGLCs *in vivo*, on the contrary they show a bipolar elongated shape (**Figure 14b)**, although the differece in the observed morphology could be a consequence of the culture conditions and/or the absence of a hippocampal niche.

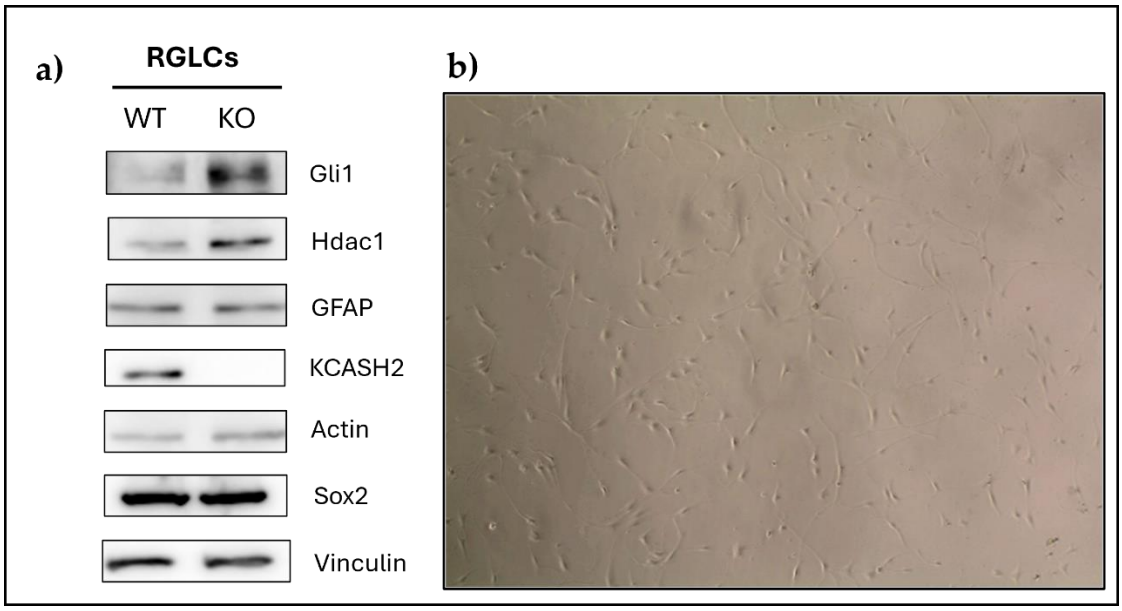

Figure 14) KCASH2 loss leads to up-regulation of Hh/GLI1 signaling in cultured RGLCs. a) Western Blot analyses confirm KCASH2 expression in RGLCs, together with the radial glia marker GFAP and neural stem cell marker Sox2. KCASH2 absence determine an increase in the levels of HDAC1 and GLI1 proteins. **b)** 10X Magnification of cultured RGLCs cells.

3.1.4. RGLCs show altered morphology in KO mice

The first macroscopical change occurring in activated RGLCs is the loss of radial glia like morphology and the generation of early intermediate precursor that still express radial glia marker GFAP and Vimentin and neural stem cells markers as Sox2 and Nestin (Kempermann et al. 2015).

Given the role of SHh pathway in the regulation of RGLCs activation and proliferation, we aimed to assess if Hh/GLI1 signaling up-regulation due to KCASH2 absence could affect the morphology of RGLCs. To this aim we performed GFAP/Nestin immunostaining on brain cryosections at P10 and 8 weeks, and we observed a marked dys-regulation and a general shortening of the intermediate filament morphology, with the severity of the phenotype being remarkable at 8 weeks in KO mice, where RGLCs still show GFAP and Nestin expression, but seem to have completely lost the radial morphology (**Figure 15a and 15b**).

To confirm the characteristics observed in KO mice were specific for GFAP and Nestin filament or the dys-regulation observed could affect the general morphology of the RGLCs, we also performed immunostaining for Vimentin, another radial glia intermediate filament marker (Gubert et al. 2009). Similarly, to what has been observed for GFAP/Nestin filaments, Vimentin filaments also exhibit a variable but marked degree of dys-regulation and shortening (**Figure 15c and 15d**).

The phenotype observed could potentially suggest that, in comparison to WT RGLCs, KCASH2^{KO} RGLCs cells are more likely to be activated due to sustained GLI1 activity, with sub-sequent loss of radial glia morphology.

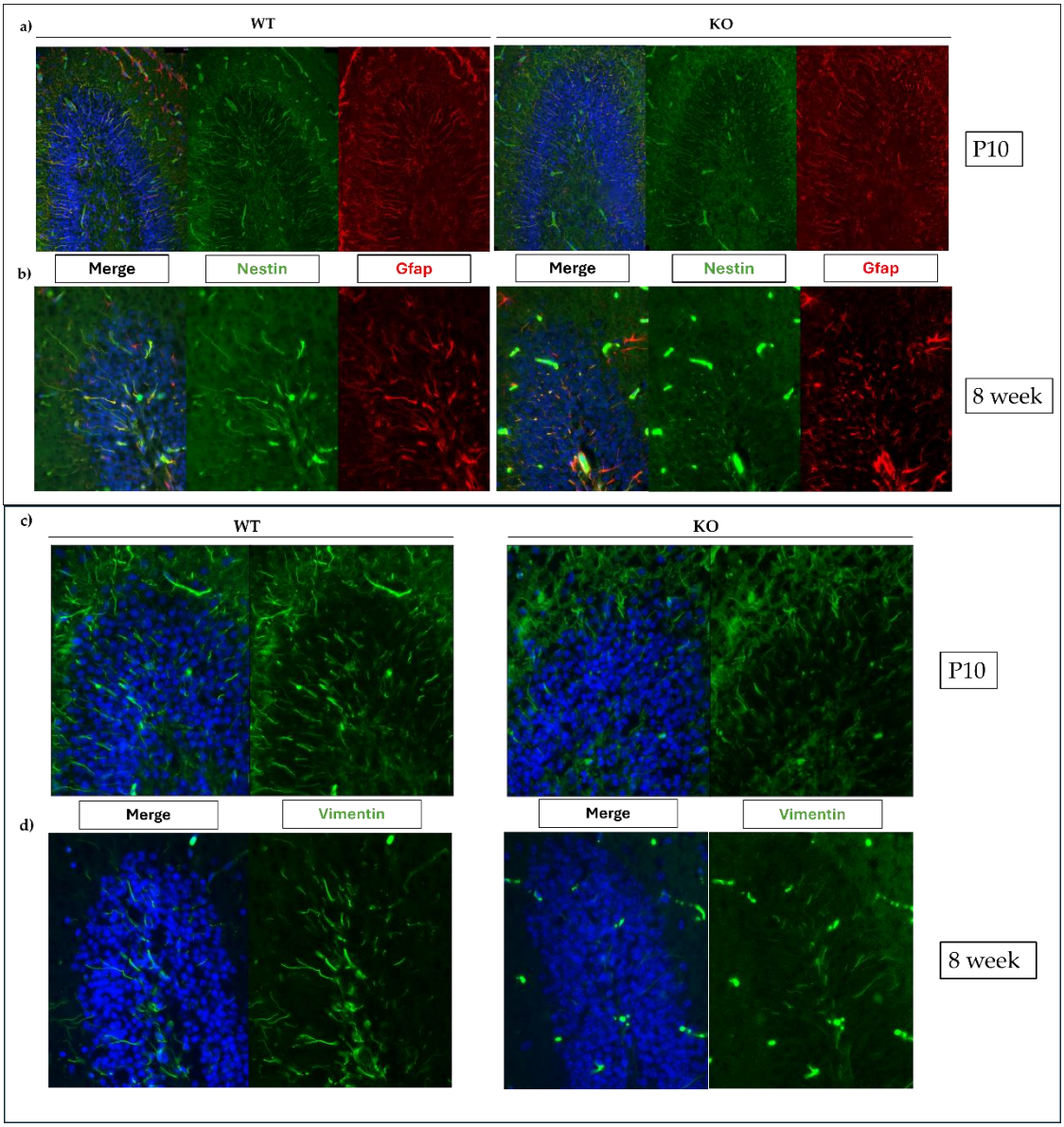

Figure 15) KCASH2^{KO} RGLCs show altered GFAP/Nestin morphology. Immunofluorescence staining on mouse cryosections at P10 (**a)** and 8 week (**b**) show general dysregulation of RGLCs intermediate filaments GFPA and Nestin morphology in KCASH2^{KO} mice. (**c, d)** Vimentin staining on brain cryosections at the same time-points reveal the same dys-regulation observed in the GFAP/Nestin staining.

3.1.5. Discussion

In adult mammalians Hh/GLI1 signaling plays a fundamental role in tissue homeostasis and adult stem cells proliferation. In CNS, SHh pathway is indeed fundamental to sustain adult hippocampal neurogenesis, which consists in the generation of new neurons starting from adult neural stem cell (or RGLCs), a process that is likely to occur throughout the lifespan of individuals and that has been linked to higher cognitive functions such as learning and behavior (Yao et al. 2016). GLI1 activity, the main activator of the pathway, is modulated through several post-translational modifications, such as phosphorylation, ubiquitination and acetylation (Gulino et al. 2012). Of particular note acetylation/de-acetylation interplay appears to play a fundamental role in regulating GLI1 activity, with only the de-acetylated form of the transcription factor being transcriptionally active. De-acetylation and sub-sequent activation of GLI1 can be mediated by the class I HDAC1 (Canettieri 2010). Previous work identified KCASH2^{KCTD21}, a member of the KCTD protein family, as a negative regulator of the Hh pathway. KCASH2 is able to bind the ubiquitin ligase E3 Cul3 and HDAC1, promoting the degradation of the latter, thereby suppressing GLI1 transcriptional activity (De Smaele et al. 2011).

Given the importance of SHh signaling in adult hippocampal neurogenesis, the first part of the present work aimed to characterize *in vivo* the hippocampal phenotype of KCASH2^{KO} mouse.

Exploiting the presence of an exogenous gene reporter in the KCASH2^{KO} cassette, we have been able to detect KCASH2 expression in the different regions that compose the hippocampus. By combination of β-galactosidase enzymatic assay and immunofluorescence staining assessed KCASH2 expression in the Dentate Gyrus, both in the Granular Layer and the Sub-

Granular zone, and the Cornu Ammonis. Adult neurogenesis in the Hippocampus occurs at the level of the Dentate Gyrus, and in particular the Sub-Granular zone, therefore KCASH2 expression in this context suggests a possible mechanism to shut down GLI1 activity during the differentiation process from neural stem cell to mature neurons. KCASH2 function in Cornu Ammonis is still uncovered, and its unlikely to involve GLI1 regulation, since there seems to be no GLI1 expression in this region (Hill et al. 2019), therefore KCASH2 role in CA pyramidal neurons could potentially revolve around the interplay with the HDACs.

The role of KCASH2 in suppressing Hh signaling prompted us to investigate neurogenesis rate in the hippocampus. Indeed, we found that KCASH2^{KO} mice exhibit a higher level of GLI1 hippocampal mRNA relative expression, coherently with that, we observed that KCASH2 loss leads to enhanced proliferation rate in the SGZ of the KO mice. We further confirmed *in vitro* in cultured neural stem cells that KCASH2 loss leads to increased GLI1 and HDAC1 protein level, confirming the role of KCASH2 as negative regulator of Hh signaling also in this context. In the hippocampus GLI1 activity has been shown to regulate neural stem cells and intermediate precursor cells proliferation, thus it is still unclear if the increased proliferation rate observed in KCASH2^{KO} hippocampus reflects an increase in the proliferation of the neural stem cells or instead is due to enhanced proliferation of downstream precursors. Nevertheless, GLI1 signaling has been showed to promote hippocampal stem cell activation, a process in which the stem cells lose their radial glia morphology before acquiring the identity of early intermediate precursor (Kempermann et al. 2015). Interestingly, we found that KCASH2 loss determines a general shortening of the radial process of RGLCs in KO

mice, suggesting that loss of KCASH2, with sub-sequent increase of GLI1 levels, could determine an increase in the ratio of activated RGLCs.

These evidences taken together highlight the role of KCASH2 regulating hippocampal neurogenesis trough the modulation of GLI1 activity.

Potentiation of hippocampal neurogenesis trough SHh modulation is already a well-studied approach, and has been demonstrated to play an important role in different pathological disorders (Chen et al. 2018). However, constitutive increase in the levels of Hh signaling has its own drawbacks, leading to depletion of RGLCs and ultimately impairing cognitive abilities (Antonelli et al. 2018). Of notice, it seems that GLI1 exerts anti-proliferative action in hippocampal stem cells when its levels are too high (Galvin et al. 2008), therefore it seems that fine balance of Hh signaling is required to ensure proper hippocampal functions. Furthermore, KCASH2 has been described as a negative regulator of HDAC1 (De Smaele et al. 2011), a feature also observed in hippocampal stem cells. Class I HDAC are responsible for the down-regulation of BDNF, a neurotrophic factor produced by neurons that stimulates synaptic plasticity and whose expression negatively correlate with age, thereby the increase in the HDAC I levels could potentially accelerate aging and cognition impairment (Fan et al. 2017).

KCASH2 could therefore represents a potential target for a short-term potentiation of hippocampal neurogenesis, while its germinal or early loss could potentially impair hippocampal generation of new neurons and cognitive abilities in the long-term. Further work will be required in this to assess if KCASH2 loss determines a change in the morphology of KCASH2^{KO} mouse hippocampus and if the de-regulation of Hh/GLI1 signaling can potentially affect cognitive abilities in adult and/or elder mice.

3.2. Hh signaling inhibition in Medulloblastoma

3.2.1. Single-drug treatment

Medulloblastoma (MB) is the most common childhood malignancy, molecular sub-grouping of identify four different categories of MB, among these SHh/activated MB account for the 30% of the total case of MB (Cambruzzi 2018). SHh plays indeed a pivotal role in the proliferation of Granule Precursor Cells in the cerebellum, and de-regulation of SHh/GLI1 signaling is recognized as a major driver of tumoral transformation in these cells. Furthermore, several pathways co-operate with SHh signaling in order to activate GLI1 and promote MB progression and invasiveness. Growing evidence point at EGFR signaling as a fundamental contributor in MB proliferation, with members of EGFR family being frequently expressed in MB (Bodey et al. 2005). Besides that, downstream kinases of the EGFR pathway are able to activate GLI1 trough phosphorylation (Carballo et al. 2018). MB is also an aggressive tumor, with a high incidence of secondary malignancies, a process which seems to be regulated by the action of the FAK, a class of tyrosine kinase involved in several process such as epithelial to mesenchymal transition and cell-cell adhesion (Dawson et al. 2021). In some cases FAK have also been showed to positive modulate GLI1 activity via Akt activation (Chen et al. 2023).

Given these evidence, we aimed to assess the efficacy of three different drugs, GANT-61 (a GLI1/2 inhibitor), Afatinib (EGFR family receptor inhibitor) and Y15 (FAK inhibitor), on the survival of three cell-lines, DAOY, ONS-76 and UW-228. All the three cell lines tested are described as SHh/activated where DAOY and UW-228 have *TP53* mutated, while ONS-76 possess *TP53* wild type (Ivanov et al. 2016).

The cell lines tested were treated with increasing concentrations of the different drugs for 24h, 48h and 72h. The results on cell survival were then assessed by MTT assay at the end of each time point and results were normalized to the values of the controls (DMSO) (**Figure 16**).

The effect of GANT-61 on cell survival was significant at the highest concentration tested after 72h of treatment in all cell lines (20μM for DAOY and ONS-76, 10μM for UW-228), at lower doses, GANT-61 did not significantly affect ONS-76 survival, lower concentration effect showed doses and cell-lines dependent effects (**Figure 16a)**

Y15 effect on cell survival was strongly dependent on the cell line the dose and the time point. In general, significant reduction of cell survival was observed in all the three cell lines at the highest dose after 72h of treatment. However, Y15 treatment significantly reduced ONS-76 and UW-288 cell survival at lower doses also at earlier time points (**Figure 16b)**.

Among the drug tested, Afatinib showed the highest consistency in reducing the cell survival of the cell lines tested, with significant results at the concentration of 2.5μM and 5 μM after 48h and 72h of treatment when compared to the control. At lower concentrations, the effect was dependent on the cell line and on the time point (**Figure 16c**).

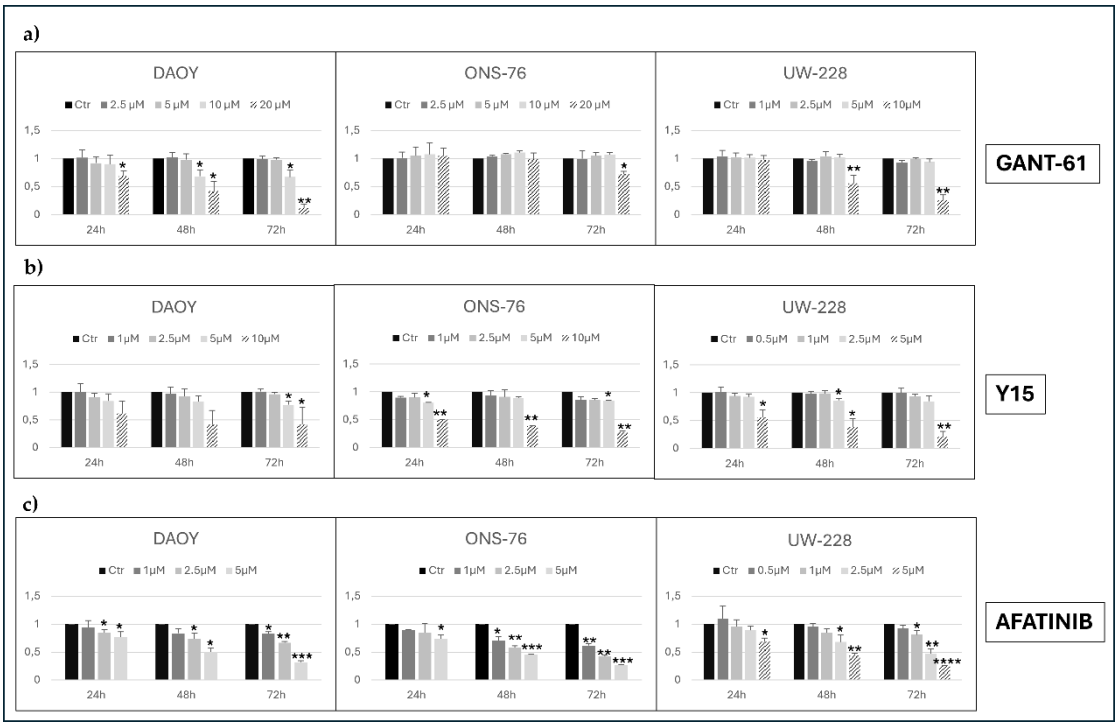

Figure 16) Effect of single-drug treatment on MB cell lines survival. The survival of MB cell lines was assessed by MTT assay after 24h, 48h and 72h of treatment with (**a)**GANT-61, (**b**) Y15 and (**c)** Afatinib. The percentage of surviving cell was calculated by normalizing the values of absorbance at 560nm to the values of controls (DMSO). The results are expressed as the mean ± SD of three independent experiments performed in triplicate. Statistical significance obtained by single-drug treatment was calculated using Student's t-test. ($^{*}p<0.05$,$^{**}p<0.01$, $^{***}p<0.001$,$^{****}p<0.0001$).

3.2.2. Multiple drug treatment

After performing single drug treatment, we aimed to the determine the efficacy of combined treatment on cell survival to highlight potential synergistic effects.

To this purpose, MB cell lines were treated for 24h, 48h and 72h with three different combinations of drugs, GANT-61 combined with Afatinib, Afatinib combined with Y15, GANT-61 combined with Y15.

At the end of each time-point, cell survival of MB cell lines was assessed by MTT assay and the results were normalized to the values of the controls (DMSO) (**Figure 17)**.

Synergistic inhibition of the combination tested on MB cell lines survival was calculated employing the method of Kern, where an R Index >1 indicate a synergistic effect, R=1 indicate an additive effect, while R<1 indicate an effect less than additive. (**Figure 18**).

Combination of GANT-61 and Afatinib affected DAOY cell-survival after 48h and 72h at all the combination tested, while in ONS-76 and UW-228 the effect was significant with the two highest combination of the drugs after 48h and 72h (**Figure 17a)**. R Index for the three cell lines treated with GANT-61+ Afatinib shows a synergistic interaction between the two compounds in DAOY cell, with approximatively R values of 3 at 72h for GANT 5μM+Afatinib 2.5μM. R index value for ONS-76 shows a synergistic interaction for GANT and Afatinib at 20μM and 5μM respectively after 24h and 48h of treatment. GANT-61 5μM and Afatinib 2.5μM synergistically reduce UW-228 cell survival after 24h and 48h of treatment (**Figure 18a**).

Combined treatment of Afatinib and Y15 showed cell dependent efficacy, in DAOY cells, combination of Afatinib and Y15 achieved significant reduction of cell survival at all the combination tested except for Afatinib 1μM+Y15

1μM, a similar trend was observed also in ONS-76 where the effect on the cell survival was significant at all combination tested after 72h. In UW-228 combination of Afatinib and Y15 significantly decreased cell survival at the highest dosage after 24h, 48h and 72h (**Figure 17b**). R index shows synergistical interactions of Afatinib and Y15 in DAOY cells at all three time points, with the exception of Afatinib 1μM+ Y15 1μM. In ONS-76, combination of Afatinib and Y15 shows an R index <1 for almost all the concentrations employed, with a slightly synergistic effect for Afatinib 5μM+Y15 10 μM after 72h treatment. Similarly, in UW-228 combination of Afatinib 5μM+ Y15 5μM shows a synergistic effect after 24h and 48h treatment, while other concentrations have an additive or less than additive effect (**Figure 18b).**

Combination of GANT-61 and Y15 achieved significant reduction of cell survival in DAOY cells at the concentrations of GANT 10μM+ Y15 5μM and GANT 20μM+ Y15 10μM at all the time points analyzed. In UW-228 and ONS-76, only the highest concentrations employed achieved significant results at all the time points (GANT 20μM+ Y15 10μM for ONS-76, GANT 10μM+ Y15 5μM for UW-228) (**Figure 17c**). R index for DAOY cells shows synergistical interactions for GANT-61 and Y15 at the concentrations of GANT 10μM+ Y15 and GANT 20μM+ Y15 10μM, and an R Index>1 for GANT 5μM+ Y15 2.5μM after 48h and 72h of treatment. R index analyses for UW-228 and DAOY reveal no synergistic effect of combination of GANT-61 and Y15 with the highest concentrations used showing a less than additive effect (**Figure 18c**).

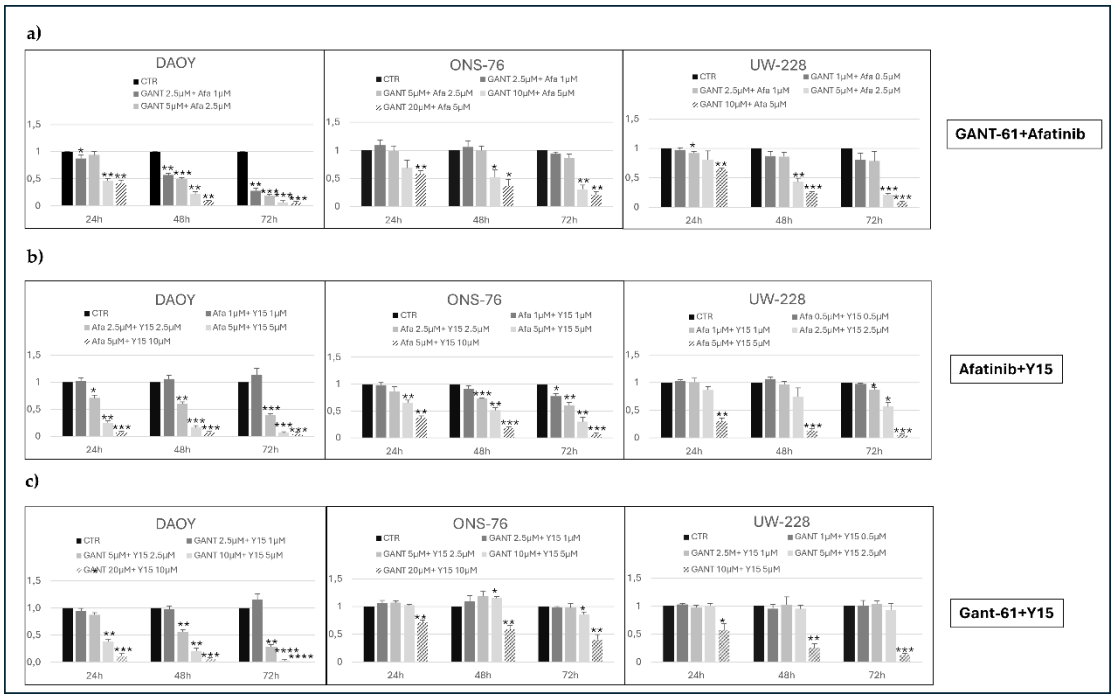

Figure 17) Effect of combined drug treatment on MB cell lines survival. The survival of MB cell lines was assessed by MTT assay after 24h, 48h and 72h of treatment with (**a)** GANT-61+Afatinib, (**b**) Afatinib+Y15 and (**c)** GANT-61+Y15. The percentage of surviving cell was calculated by normalizing the values of absorbance at 560nm to the values of controls (DMSO). The results are expressed as the mean ± SD of three independent experiments performed in triplicate. Statistical significance obtained by single-drug treatment was calculated using Student's t-test. ($^{*}p<0.05$,$^{**}p<0.01$, $^{***}p<0.001$,$^{****}p<0.0001$).

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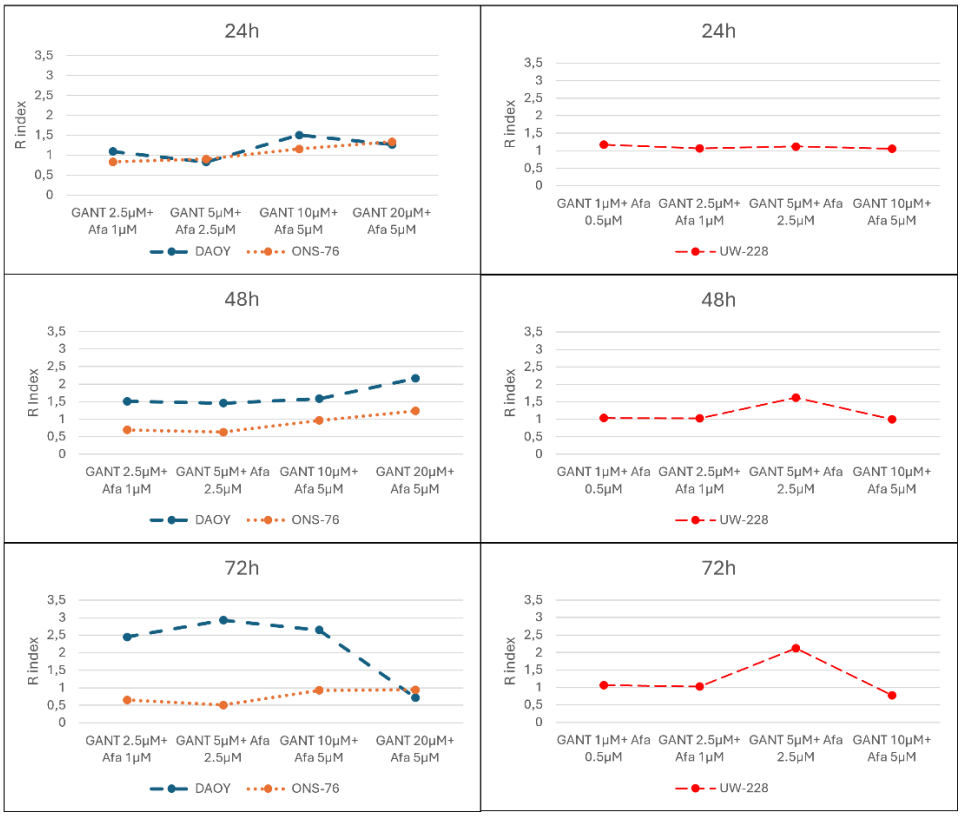
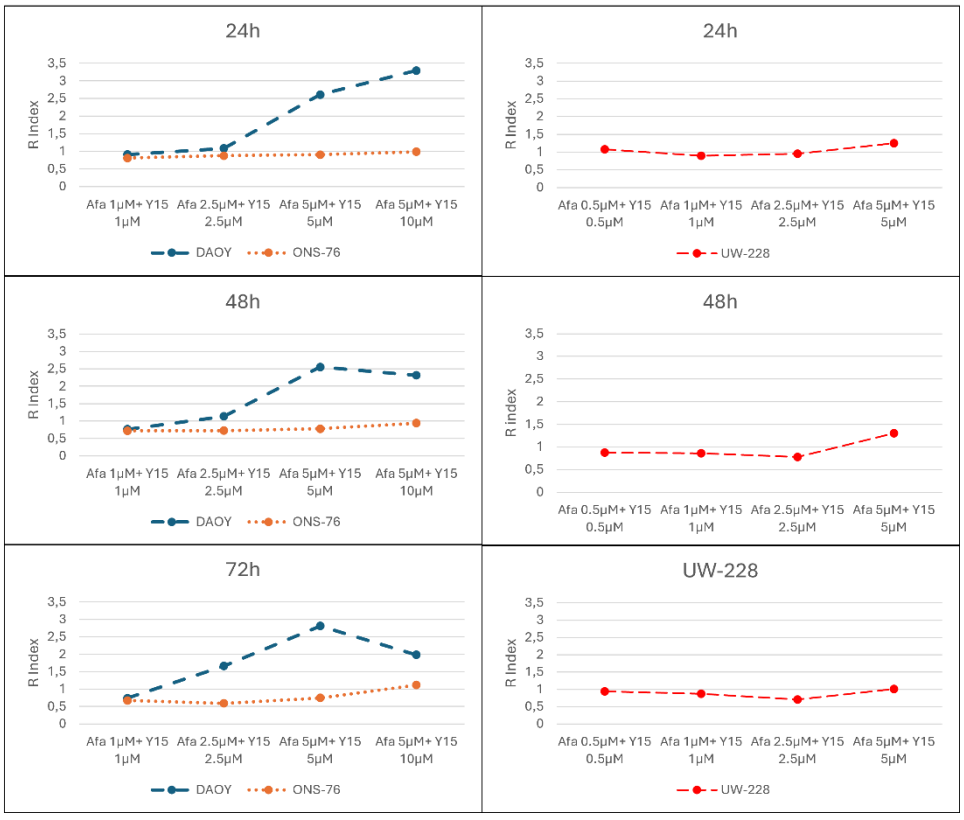
GANT-61+Afatinib

a)



Afatinib+Y15

b)



c)

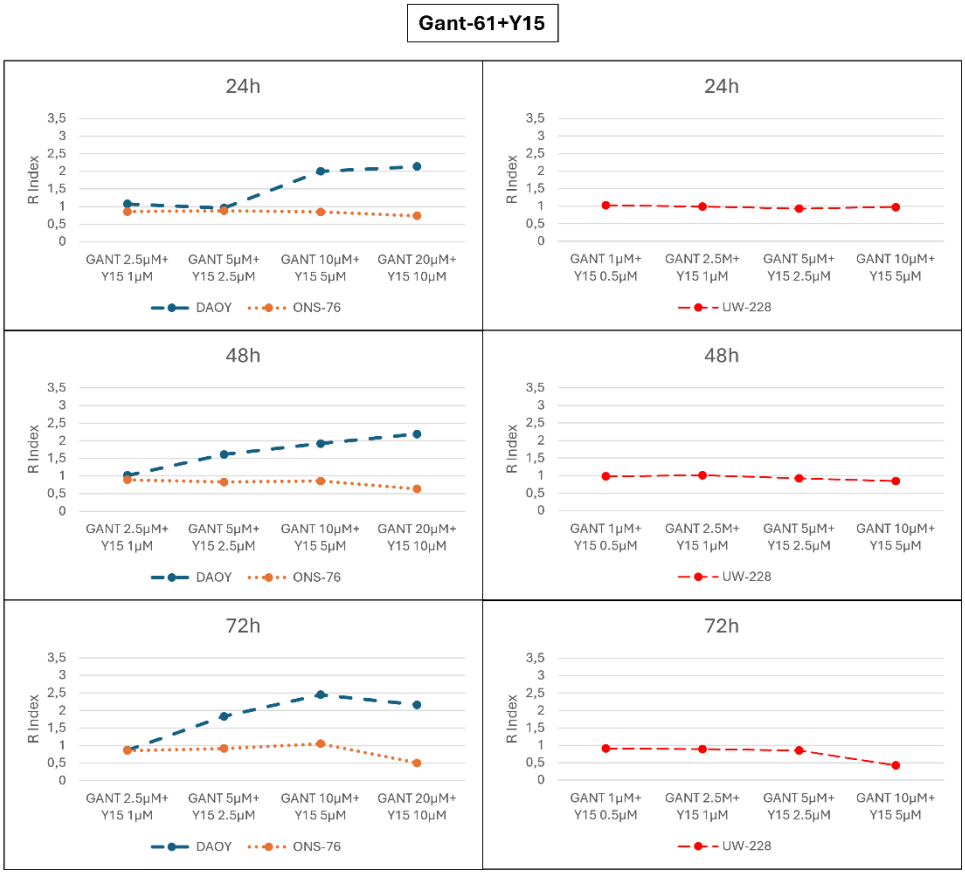


Figure 18) Interaction between GANT-61, Afatinib and Y15. The interactions between the drugs testes were assessed using the method of Kern. The graphics show the Kern R Index value obtained by the combination of (**a)** GANT-61 and Afatinib, (**b)** Afatinib and Y15, (**c)** GANT-61 and Y15. R values >1 indicate a synergistic effect, R=1 indicate an additive effect, R<1 indicate a less than additive effect.

3.2.3 Discussion

Medulloblastoma is the most common form of CNS juvenile tumor. Despite an overall high cure rate, current multimodal and aggressive approach in the treatment of MB usually leads to life-long morbidity and insurgence of secondary malignancies. SHh/activated represent a third of the total case of MB, with de-regulation of SHh pathway occurring at different levels, leading to increased activity of the transcription factor GLI1/2. Furthermore, many oncogenic pathways are recognized to cooperate with Hh/GLI1 pathway, enhancing tumoral proliferation, progression and metastasis (Sigafoos et al. 2021).

In the present work we tested three different drugs, GANT61, Afatinib and Y15 alone and in combination, to assess their effect in inhibiting the proliferation of SHh/activated MB cell lines.

Gant-61 is a small molecule that inhibits the transcription factor GLI1/2 by blocking their DNA binding ability (Severini et al. 2020).

Afatinib is a second generation of tyrosine kinase inhibitors that irreversibly blocks the member of EGFR family. Afatinib is effective against wild type and mutated form of EGFR members (Sullivan and Planchard 2017).

Y15 is an inhibitor of Focal Adhesion Kinase (FAK) (Ma 2011), these kinases have been recognized as responsible for the formation of secondary malignancies by enhancing migration and invasion of MB cells.

At the present moment, there are no studies reporting the combined use of these drugs in MB, although single drug treatment has demonstrated the anti-tumoral effects of these compounds in MB or other tumoral forms (Bei et al. 2022, O'Brien et al. 2014, Luzzi et al. 2020).

Our results indicate that EGFR inhibition alone exerts the strongest anti-proliferative effect on the cell lines tested, however GLI1/2 and FAK inhibition

also achieved significant results at higher concentrations. It is possible that single pathway inhibition could be only partially effective in reducing MB cell lines survival due to cooperative crosstalk between SHh and EGFR/FAK pathways (Mengelberger et al. 2012, Chen et al. 2023). Indeed, combined treatment achieved greater reduction of cell lines survival, especially when EGFR inhibition was combined with GLI1/2 or FAK inhibition. Interactions analyses showed the synergistic effect of GANT-61 combined with Afatinib, and Afatinib combined with Y15, while effect of combination of GANT-61 and Y15 showed a more cell line dependent effect.

The current results highlight the effects of combined treatment on MB cell lines survival, however SHh/GLI1 pathway and FAK are recognized as major contributors in tumoral invasion and metastasis, regulating epithelial to mesenchymal transition and cell adhesion/migration (Sulzaimer et al. 2014, Avery et al. 2021), therefore is possible that contemporary inhibition of GLI1/2 and FAK could exerts effects beyond cell survival/proliferation, e.g. affecting the ability of malignant cells to migrate and/or form metastasis; further work in this direction is required to assess the effects of the different combined approaches. Furthermore, future experiments will evaluate the efficacy of GANT-61, Afatinib and Y15, alone and in combination, on the cell-cycle distribution and the apoptosis rate. Therapeutical efficacy of the different combinations of drugs *in vivo* will be evaluated on xenografted tumor cell lines in mice.

Taken together, these results lay the basis for definition of a new therapeutic approach in the treatment of MB, involving the simultaneous inhibition of different oncogenic pathways that are frequently de-regulated in this tumor.

6. References

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