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Geschäftsführender Direktor: Prof. Dr. Rolf Müller
des Fachbereichs Medizin der Philipps Universität Marburg

Elucidating the functional role of Dual-specificity Tyrosine Regulated Kinase 1B (DYRK1B) in the Hedgehog Signaling Pathway

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Rajeev Singh
aus Lucknow, Indien

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1 List of Abbreviations

ADAM. ..... A Disintegrin And Metalloprotease
ATAT. ..... α-tubulin acetyltransferase 1
ATO. ..... Arsenic Trioxide
BMP. .....Bone morphogenetic protein
Boc. .....Brother of Cdo
Boi. ..... Brother of Ihog
CDK. ..... cyclin-dependent kinase
Cdo. .....Cell adhesion molecule
Cirp. ..... cold-inducible RNA-binding protein
CK1. .....Casein Kinase 1
CRD. ..... Cysteine-rich domain
DH box. ..... DYRK-homology box
DHH. .....Desert Hedgehog
Disp. ..... Dispatched
DS. .....Down syndrome
DSCR. ..... Down-Syndrome Critical Region
ERK. .....Extracellular signal-regulated kinase
FnIII. ..... Fibronectin Type III
FoxO1. .....Forkhead box protein O1
Gas1. .....Growth arrest-specific gene 1
GLI. .....glioma-associated oncogene
GPCR. ..... G protein-coupled receptor
GSK3β. ..... Glycogen Synthase Kinase 3β
HDAC6. .....Histone deacetylase 6
Hh. .....Hedgehog
HHAT. .....Hedgehog acyltransferase
HhNp. ..... N-palmitoylated Hh
HIP. .....Hedgehog interacting protein
IFT-A. .....Intraflagellar transport sub-complex A
IHH. .....Indian Hedgehog
Ihog.....Interference hedgehog
KIF7. ..... Kinesin family member 7

KRAS. ..... Kirsten rat sarcoma
MAPK. ..... mitogen-activated protein kinase
MBOAT. ..... membrane-bound O-acyltransferase
MEK. ..... MAPK/ERK Kinase
MIRK. ..... Minibrain related kinase
MT. ..... Microtubules
mTOR. ..... mechanistic target of rapamycin
NAPA. ..... N-terminal auto-phosphorylation accessory region
NPC1. ..... Niemann Pick C1 protein
NSCLC. ..... Non-small cell lung cancer
PC. ..... Primary cilium
PDA. ..... Pancreatic ductal adenocarcinoma
PI3K. ..... phosphoinositide 3-kinase
PKA. ..... Protein Kinase A
PKC. ..... Protein Kinase C
RACE. ..... rapid amplification of cDNA ends
RAF. ..... Rapidly Accelerated Fibrosarcoma
REDK. ..... Regulatory erythroid kinase
RND. ..... Resistance Nodulation Division
ROS. ..... Reactive Oxygen Species
SHH. ..... Sonic Hedgehog
SMO. ..... Smoothened
SPOP. ..... Speckle-type POZ (pox virus and zinc finger protein) protein
SRPK. ..... Serine-arginine protein kinase
SuFu. ..... Suppressor of Fused
TGFβ. ..... Transforming Growth Factor β
TULP3. ..... Tubby-like protein 3
β-TrCP. ..... Beta-transducin repeats-containing proteins

2 Summary

Hedgehog signaling (Hh) plays a crucial role in vital processes such as embryonic development or cell homeostasis. Aberrant Hh signaling is linked to formation, progression and growth of tumors.

The canonical Hh signaling cascade is initiated by binding of the Hh ligand to its receptor Patched1 (PTCH1) (Hooper and Scott 1989; Nakano et al. 1989), a transmembrane protein located in the ciliary membrane which relieves the repression of the membrane-bound G protein-coupled receptor (GPCR) Smoothened (SMO) which activates the Hh transcriptional factors, zinc finger proteins of the GLI (Cubitus interruptus (Ci) in Drosophila melanogaster) family (Hui and Angers 2011). The Hh signaling pathway is reported to activate downstream kinases which in turn lead to various cellular processes such as differentiation, polarity and proliferation.

The so-called dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs) are induced by Hh activation and a slight change in their amount could lead to large and significant effects on various cellular processes. DYRK1A as a candidate gene is responsible for the altered neuronal development and brain abnormalities in Down syndrome (DS, OMIM #190685). DYRK1B is known to be associated with the metabolic syndrome and, is commonly amplified in ovarian and pancreatic cancer (Friedman 2010a; Keramati et al. 2014b).

The results of the present work have shown, that Hh signaling induces DYRK1B and this kinase has been shown to have a regulatory kinetic effect on Hh signaling pathway, as short-term inhibition of DYRK1B kinase leads to increase in GLI protein levels and long-term inhibition has shown to deplete the protein levels of GLI. This strong fluctuation in the kinase could be detrimental in a therapeutical context, as DYRK1B has been shown to regulate PI3K/mTOR/AKT signaling pathway which is subject to strong feedback regulation and can induce oncogenic Hh signaling. Combination therapy which targets DYRK1B and other signaling pathway components such as mTOR, AKT, S6K were used to deplete growth of pancreatic and ovarian cancer cells.

Hh signaling exerts myriad functions and one of the functions is to induce acetylation of microtubules and of Acetylated Tubulin (AcTub)-dependent processes such as cell polarization or organelle transport. With my results, I find that Hh signaling increases DYRK1B levels, which inactivates Glycogen synthase kinase 3β (GSK3β) through Serine 9 (Ser9) phosphorylation, resulting in Histone Deacetylase 6 (HDAC6) inhibition and increased tubulin acetylation. In summary, I have described a mechanistic framework of how intercellular communication can impinge on cytoskeletal regulation and cell function via Hh signal transduction.

Considering these effects, I have shown that DYRK1B is one of the very crucial kinases mediating multiple signaling pathways and; thereby, it's extremely important to elucidate the mechanistic framework of its action.

3 Zusammenfassung

Der Hedgehog-Signalweg (Hh) spielt eine entscheidende Rolle bei lebenswichtigen Prozessen wie der Embryonalentwicklung oder der Zellhomöostase. Eine abweichende Signalweiterleitung ist mit der Bildung und Progression/Wachstum von Tumoren verbunden.

Die kanonische Hh - Signalkaskade wird initiiert durch Bindung des Hh- Liganden an seinen Rezeptor Patched1 (PTCH1) (Hooper and Scott 1989; Nakano et al. 1989), ein in der Ciliarmembran lokalisiertes Transmembranprotein, welches im ungebundenen Zustand die Aktivität des membrangebundenen G-Protein-gekoppelten Rezeptors (GPCR) Smoothened (SMO) reprimiert. Die Bindung aktiviert Hh-Transkriptionseffektoren, die Zinkfingerproteine der GLI Familie (Cubitus interruptus (Ci) in Drosophila melanogaster) (Hui and Angers 2011). Es wurde gezeigt, dass der Hh-Signalweg nachgeschaltete Kinasen aktiviert, die wiederum zu verschiedenen zellulären Prozessen führen. So wird beispielsweise die Expression der DYRK-Kinasen (dual-specificity tyrosine phosphorylation-regulated kinases) durch Hh-Aktivierung induziert. Bereits geringfügige Veränderungen ihrer Expression können zu großen und signifikanten Effekten auf verschiedene zelluläre Prozesse führen.

DYRK1A ist ein Kandidaten, das für veränderte neuronale Entwicklung und Hirnanomalien beim Down-Syndrom verantwortlich ist (DS, OMIM # 190685). Weiterhin ist bekannt, dass DYRK1B mit dem metabolischen Syndrom assoziiert ist und bei Ovarial- und Pankreaskrebs häufig verstärkt exprimiert wird (Friedman 2010a; Keramati et al. 2014b).

Die Ergebnisse der vorliegenden Arbeit konnten zeigen, dass der Hh-Signalweg die Expression von DYRK1B induziert, was wiederum einen regulatorischen kinetischen Effekt auf den Hh-Signalweg hat, da die Kurzzeit-Hemmung der DYRK1B-Kinase zu Erhöhung des GLI-Proteinlevels führt, wohingegen eine Langzeit-Hemmung den GLI-Proteinlevel verringert. Diese starken Schwankungen der Kinase könnten therapeutisch schädlich sein, da gezeigt wurde, dass DYRK1B den PI3K / mTOR / AKT-Signalweg reguliert, der einer starken Rückkopplungsregulation unterliegt und onkogene Hh-Signale induzieren kann. Eine Kombinationstherapie, die auf DYRK1B und andere Signalwegkomponenten wie

mTOR, AKT, S6K wirkt, wurde verwendet, um das Wachstum von Pankreas- und Ovarialkrebszellen zu verringern.

Der Hh-Signalweg steuert unzählige Funktionen. Eine dieser Funktionen ist die Acetylierung von Mikrotubuli und von AcTub-abhängigen Prozessen wie Zellpolarisation oder Organellentransport. Im Zuge dieser Arbeit konnte gezeigt werden, dass der Hh-Signalweg DYRK1B-Level erhöht, wodurch Glycogen-Synthase-Kinase 3β (GSK3β) durch Ser9-Phosphorylierung inaktiviert wird, was zu einer HDAC6-Inhibierung führt und die Tubulin-Acetylierung erhöht. In dieser Arbeit wird ein mechanistisches Zusammenspiel beschrieben, wie interzelluläre Kommunikation über die Hh Signaltransduktion auf die Zytoskelett-Regulation und Zellfunktionen einwirken kann.

In Hinblick auf die vielfältigen Die Auswirkungen von DYRK1B auf verschiedene wichtige Signalwege von Krebs und Entwicklung, die weitere Erforschung seiner Bedeutung ist von höchstem Interesse.

4 Introduction

Intercellular signaling and signal transduction underlie several aspects of development and behavior. Core intercellular signaling pathways found in animal cells include Notch, Wnt, BMP/TGFβ, Hedgehog, growth factor signaling and others (Housden and Perrimon 2014). These pathways provide a central means of communication between cells. This intercellular communication controls almost every aspect of cellular functions such as cell proliferation, migration, recognition, and differentiation (Uriu et al. 2014). Various modulators of the signaling pathways such as, the ligands, receptors, transducers, and regulators are known which provide a better understanding of the multifunctional cellular system and also serve as drug targets.

Cell-cell communication is often mediated by protein molecules within a cell that is recognized by specific receptors presenting on neighboring cells. These intracellular signaling events can occur among distinct cell types (paracrine signaling) and within the same cell type (autocrine signaling). Intracellular communication can occur via indirect interactions (autocrine, paracrine) where, the secreted ligands, such as growth factors, cytokines, and chemokines, bind to specific receptors expressed on neighboring cells and direct interactions (juxtacrine communication) where a cell-cell contact is required for triggering the signaling as ligands are expressed on the plasma membrane of neighboring cells. Various downstream signaling pathways are activated through dynamic post-translational modifications and protein-protein interactions upon activation of membrane receptors, which in turn lead to the modulation of several cellular functions (Scott and Pawson 2009).

Intercellular signal transduction also plays an important role in embryonic development by significantly affecting cell growth, differentiation, and morphogenesis. Several reports have shown that early embryogenesis and tumorigenesis share several similarities, in terms of cell invasive behavior, epigenetic regulation, protein profiling and other biological behaviors (Ma et al. 2010). The hallmark of embryonic development is ‘regulation’, the trend that cells follow to arrange themselves into organized structures whereas cancer is characterized by ‘dysregulation’ and ‘disorder’. The process of embryogenesis involves spatial and temporal activation of developmental signaling pathways (Dominic Poccia 2006). Re-activation of these embryonic signals in adult cells- due to mutations and epigenetic remodeling is one

of the major causes of cancer. Several important developmental pathways which include Wnt, Hedgehog, and Notch pathways, are mostly, dysregulated in tumor progression, initiation, and maintenance of metastatic spread and growth.

4.1 Hedgehog Signaling Pathway

The Hedgehog (Hh) Signaling pathway plays a significant role in tissue homeostasis, metabolism control, embryogenesis, and various other developmental processes. It regulates the differentiation and proliferation of cells, body patterning, stem cell maintenance, survival, and affects the oncogenic transformation and the development of tumors (Varjosalo and Taipale 2008; Gupta et al. 2010a; Briscoe and Therond 2013; Teperino et al. 2014; D'Amico et al. 2015; Lee et al. 2016b).

In 1980 Eric F. Wieschaus and Nusslein-Volhard discovered the *Hedgehog (HH) gene* during their genetic screens for mutations that disrupt the larval body plan in *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus 1980). The *Drosophila* larva is segmented, with a smooth posterior end and a bristle coated anterior end, known as denticles. This segmental patterning was shown to be affected by polarity mutants in their mutational screen. Polarity mutants led to the failed development of the posterior part, which in turn resulted in a short and spiky phenotype, resembling a Hedgehog and hence the name (Nusslein-Volhard and Wieschaus 1980; Ingham and McMahon 2001; van den Brink 2007; Varjosalo and Taipale 2008).

Three Hedgehog homologs are known to be present in vertebrates, namely: *Desert* (*DHH*), *Indian* (*IHH*), and *Sonic* (*SHH*). In mouse and humans, the three hedgehog genes are highly conserved (Marigo et al. 1995). Specific roles for all the three genes have been found (Echelard et al. 1993; Riddle et al. 1993; Roelink et al. 1994; Ingham and McMahon 2001) such as: *SHH* regulates the polarizing activity of the organizing centers located in the limb bud, the notochord, or the floor plate of the neural tube (Cohn and Tickle 1996; Jessell 2000; Singh and Lauth 2017), endochondral bone development, osteoblast differentiation and various other cellular events are regulated by *IHH* (St-Jacques et al. 1999; McMahon et al. 2003; Singh and Lauth 2017). Amongst the three, the least studied homolog is *DHH*. Several reports suggest it regulates the development of germ cells in testes and peripheral nerve sheath formation (Park et al. 2007; Singh and Lauth 2017).

The hedgehog proteins go through comprehensive and specific post-translational modifications and cleavage events producing ~45 kDa precursor protein. This precursor protein is autocatalytically cleaved and giving rise to a cholesterol modified 19 kDa NH2-terminal fragment (HhNp), which undergoes dual lipid modification and an unmodified 26 kDa COOH terminal fragment (HhC,(lacking the palmitate modification)) (Porter et al. 1996), which acts as a cholesterol transferase and also catalyzes the cleavage (Lee et al. 1994; Marti et al. 1995). The most striking feature of hedgehog proteins is the dual lipid modification of the 19 kDa NH2 terminal fragment. The modified signaling protein is covalently linked to cholesterol and palmitate groups and is poorly soluble. Palmitoylation is critical for effective long and short-range signaling. Attachment of palmitate to Hh proteins is independent of cholesterol modification and autoprocessing and is catalyzed by HHAT (Hedgehog acyltransferase). HHAT is the member of membrane-bound O-acyltransferase (MBOAT) family, a subgroup of multipass membrane proteins that catalyze transfer of fatty acyl groups to lipids and proteins. Mutations in HHAT produce a phenotype that is similar to loss of Hh function (Lee et al. 1994; Marti et al. 1995; Porter et al. 1996; Chamoun et al. 2001; van den Brink 2007; Buglino and Resh 2012). It was also recently shown that palmitoylation promotes cleavage of N-terminal amino acids by a disintegrin and metalloproteases (ADAM) family of proteases (Ohlig et al. 2011; Koleva et al. 2015). This kind of cleavage leads to the formation of active Shh multimers. The amino acid residues, if not cleaved can affect SHH, as they are shown to interact with the Zn^{2+} coordination sites on adjacent molecules and this region has been shown to interact with Patched and is known to modulate SHH activity and stability (Day et al. 1999; Fuse et al. 1999; Bosanac et al. 2009a).

Hh proteins undergo cholesterylation, a post-translational modification (Ciepla et al. 2015) at their C-termini (Porter et al. 1996) which increases their membrane affinity and restricts their dispersal (Gallet 2011). Mutant Hh proteins lacking the cholesterol modification have a longer range of distribution and signaling, which can lead to patterning defects (Li et al. 2006; Huang et al. 2007). Dispatched (Disp), a 12- pass transmembrane protein related to the bacterial RND (Resistance nodulation-cell division) family of transporters is essential for the release of Hh from cell surfaces and its long-range signaling activities. Mutants from *dispatched* retain cholesterol-modified Hh and hence show reduced signaling (Burke et al. 1999; Shirras 2000; Couso 2011).

4.1.1 Mechanism of Hh Signal Transduction

The canonical Hh signaling cascade is initiated in the target cell upon binding of the Hh ligands to 12-span transmembrane receptors, encoded by the genes *PTCH1* and *PTCH2* (Hooper and Scott 1989; Nakano et al. 1989; Goodrich et al. 1996). Two hydrophilic extracellular loops are displayed by these receptors which mediate the ligand interactions (van den Brink 2007). Ptch family members and bacterial transport proteins share extensive homology. These proteins belong to the Resistance-nodulation-cell division (RND) family and involved in the transport of various substrates across the cell membrane (Hausmann et al. 2009). The eukaryotic Ptch superfamily includes Dispatched (Disp) which maintains the discharge of Hh proteins and Niemann-Pick C1 protein (NPC1) regulating cholesterol homeostasis (Hausmann et al. 2009).

The Hh reception by Ptch is enhanced by the presence of several other Hh binding proteins at the cell surface. These additional co-receptors such as, fibronectin type III (FnIII), and immunoglobulin family of membrane proteins Ihog (Interference hedgehog) and Boi (Brother of Ihog) transmembrane proteins in *Drosophila* and Cdo (Cell adhesion molecule related/downregulated by oncogenes) and Boc (Brother of Cdo) in vertebrates are encoded by cell surface Ig/fibronectin and are the closest mammalian relatives of *Drosophila* Ihog, and Gas1 (growth arrest-specific gene 1), act as a specific surface protein and binds SHH for signaling (Tenzen et al. 2006; Kang et al. 2007; Beachy et al. 2010; Camp et al. 2010; Izzi et al. 2011). Another Hh binding protein in vertebrates is HIP (hedgehog interacting protein) a membrane glycoprotein binding to three Hh proteins with an affinity comparable to Ptch1 (Chuang et al. 2003; Bosanac et al. 2009b).

The Hh signaling cascade involves a dual function of Ptch1, as a receptor of Hh ligand and as a negative regulator of the pathway, by inhibiting the G protein-coupled receptor (GPCR) like signal transducer Smoothened (SMO) (7-pass transmembrane protein) in the non-motile primary cilium (PC), where Ptch localizes in the absence of the Hh ligand (Taipale et al. 2002; Eggenschwiler and Anderson 2007; Rohatgi et al. 2007; Roberts et al. 2016). The primary cilium, a microtubule-based organelle, which protrudes from the cell surface of most vertebrate cells, is a requisite for Hh signal transduction (Goetz and Anderson 2010).

Smoothened is a member of the Frizzled family (class F) of GPCRs and contains an N-terminal, ~14 kDa extracellular cysteine-rich domain (CRD), connected with a linker to a 7-span transmembrane (7TM) protein and an extended C-terminal tail (Ruiz-Gómez et al. 2007). Small molecule inhibitors are believed to play an important role in understanding the mechanism by which Ptch represses the pathway, by inhibiting smoothened SMO regulation. This was clarified when, there were reports showing, oxysterols, oxidized derivatives of cholesterol, bind specifically to Smo CRD and hence activate the Hh signaling pathway (Rana et al. 2013; McCabe and Leahy 2015). Various smoothened antagonists, targeting the heptahelical bundle domain of SMO, have also worked in favor of this assumption. These antagonists functionally mimic the overexpression of Ptch (Chen et al. 2002; Frank-Kamenetsky et al. 2002; Taipale et al. 2002). Recent reports have also shown that Hh signal transduction modulates cholesterylation of SMO, and thereby making SMO cholesterylation, a therapeutic target to treat Hh pathway related cancers (Xiao et al. 2017).

Gli zinc finger transcription factors mediate Hh signaling at the distal end of the pathway. *Cubitus interruptus (Ci)* is the Gli homolog in *Drosophila* and in vertebrates there are three different Gli transcription factors, GLI1, GLI2 and GLI3 (Hui and Angers 2011). In mammals, Gli1, Gli2 and Gli3 contain a carboxy-terminal activation domain but only Gli2 and Gli3 have N-terminal repressor domains (Dai et al. 1999; Sasaki et al. 1999). In the absence of the Hh ligands, Ci and Gli2/3 are proteolytically processed into repressor forms by removal of the activation domain (Aza-Blanc et al. 1997; Hsia et al. 2015). Hh regulated proteolytic processing of Ci, Gli2 and Gli3 is promoted by Protein Kinase A (PKA), Casein Kinase1 (CK1) and Glycogen Synthase Kinase 3β (GSK3β), leading to binding of an E3 ubiquitin ligase complex containing *β*-TrCP (in mammals), and hence proteasome-mediated digestion (Jiang 2006; Marks and Kalderon 2011). Albeit myriad facets of vertebrate GLI-R production remain elusive, KIF7 (Kinesin family member 7), Suppressor of Fused (SuFu) and the primary cilium are needed for adequate processing of GLI-FL (full length) into GLI-R (repressor) (Cheung et al. 2009; Liem et al. 2009; Goetz and Anderson 2010).

SuFu plays a vital role in the stabilization of GLI2/3 FL , retaining them in the cytoplasm and hence preventing their activation by nuclear translocation (Humke et al. 2010; Tukachinsky et al. 2010). When SuFu is absent GLI2-FL translocate to the nucleus and is converted into its activator form, GLI2-A (proteolytically processed) which is labile and rapidly degraded by cullin-3-based ubiquitin ligase adaptor SPOP (Chen et al. 2009; Wang et al. 2010; Jin

et al. 2016). Apart from SuFu, KIF7 plays an important role in GLI processing, although the exact mechanism is unclear it is thought to recruit PKA, GSK3 and CK1 thereby phosphorylating GLI-FL (Ryan and Chiang 2012).

Another important player in the Hh pathway is the G-protein-coupled-receptor Gpr161. It plays an essential role in Hh signaling by negatively regulating the pathway by determining Gli processing via cAMP signaling. The IFT-A complex and Tulp3 are involved in trafficking of Gpr161 to the primary cilia (Pal and Mukhopadhyay 2015). Gpr161 is mostly expressed in neural tube development and is localized in nervous system post mid-gestation period. Localization of Gpr161 has also been found in cilia and the ciliary localization is perturbed upon knockdown of Tulp3 and IFT-A complex in these fibroblasts (Mukhopadhyay and Rohatgi 2014). GPR161 double knockout mutants have shown Gli3 processing defects, which are cilia dependent and Smo independent, implying GPR161 would affect Gli3 processing. The phenotypic appearance of Gpr161 is like that of SuFu and PKA mutants. It has been shown that Sufu effects on the Hh pathway take place independent of primary cilia (Chen et al. 2009; Humke et al. 2010; Zeng et al. 2011), which indicates that Gli3 processing by Gpr161 is modulated via activation of PKA. In the absence of Shh, GPR161 is localized to primary cilium and promotes the increase in the levels of cAMP-mediated Gαs activation of adenylyl cyclase. In the presence of the ligand, Gpr161 moves away from the primary cilium preventing cAMP production and leading to pathway activation (Mukhopadhyay et al. 2013; Pal et al. 2016).

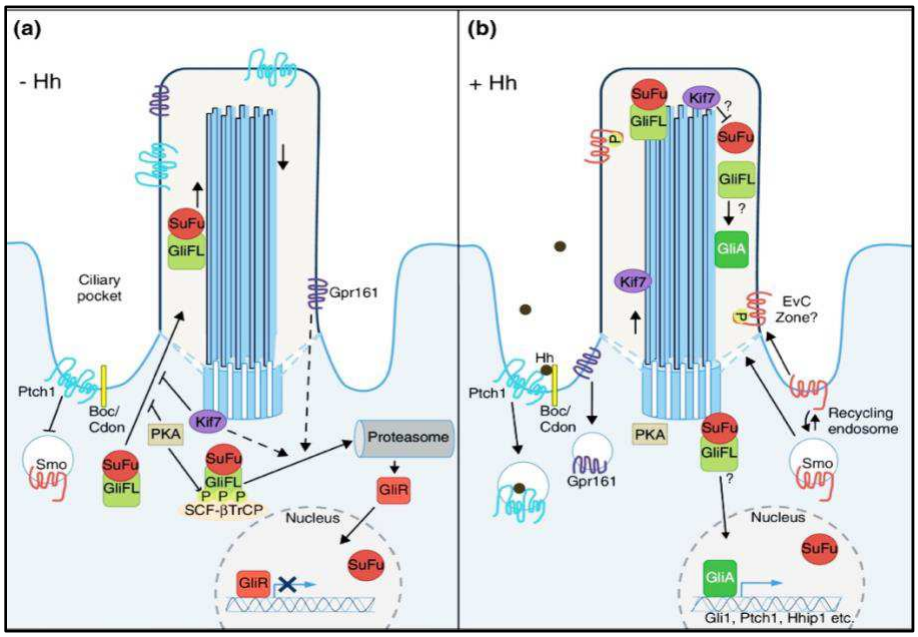

Figure1: The mammalian Hh signaling pathway. (a) In the absence of Hh ligand, the Hh receptor Patched (Ptch1) inhibits the accumulation of the signal transducer, Smoothened (Smo), on the ciliary membrane. As a result, at the base of the primary cilium, PKA and Kif7 promote proteolytic processing of the transcription factor Gli3 by the proteasome into a repressor form (GliR) that suppresses Hh target gene expression in the nucleus. In addition, Sufu stabilizes the Gli proteins and inhibits the transcriptional activity of Gli2, while PKA prohibits the accumulation of full-length Gli2 (GliFL) in the cilium. All of these events ensure silencing of the Hh pathway without the ligand. (b) The Hh ligand binds to its receptor Ptch1 and co-receptors Boc/Cdon. Ptch1 is internalized with Hh, relieving the inhibition on Smo. Smo accumulates in the ciliary membrane through both lateral transport and the secretory pathways. Phosphorylation of Smo, for instance, at the EvC zone in osteoblasts leads to its dimerization and activation. This, in turn, abrogates PKA function and promotes the movement of Sufu–Gli2/3 complexes and Kif7 to the ciliary tip and perhaps dissociation of Gli2/3 from Sufu in this process. Kif7 also facilitates the trafficking of Gli2/3 into the cilium (e.g. in chondrocytes). Accumulation of Gli2/3 at the ciliary tip is associated with the production of Gli activators (GliA), which are derived from the full-length Gli proteins. Accumulation of GliA to the nucleus enables activation of Hh target genes such as Ptch1, Gli1, and Hhip1 (Nozawa et al. 2013).

4.1.2 Hh Signaling and Cancer

The Hedgehog signaling pathway is essential for embryonic development and stem cell maintenance (Wu et al. 2017). But, aberrant activation of the pathway is also linked to various forms of cancer. Medulloblastoma (MB), basal cell carcinoma (BCC), rhabdomyosarcoma (RMS) and several other forms of cancer have documented the roles of mutated Hh pathway components (Raffel et al. 1997; Xie et al. 1998; Tostar et al. 2006). In addition, tumor microenvironment modulation by Hh signaling has been shown to be a

prominent player in breast, lung, liver, stomach, pancreas, colon and prostate cancer (Berman et al. 2003; Thayer et al. 2003; Watkins et al. 2003; Karhadkar et al. 2004; Huang et al. 2006; Mukherjee et al. 2006; Varnat et al. 2009). In light of these reports, Hh signaling becomes very important for targeted cancer therapy.

Three basic models of Hh pathway activation have been proposed (Rubin and de Sauvage 2006). Type I cancers contain an activating mutation in the Hh pathway, independent of Hh ligand and therefore inhibitors should target at or below SMO in the Hh cascade to be therapeutically effective (Gupta et al. 2010b). Type II cancers are ligand-dependent and do not display any somatic mutations in the Hh pathway. An autocrine mode of Hh signaling occurs in these cancers where Hh is produced and also utilized by the same cells or neighboring tumor cells. SMO antagonists can inhibit autocrine Hh signaling in these tumors (Gupta et al. 2010b). Type III cancers are also ligand-dependent but in these tumors, paracrine signaling works, which is motivated by the overexpression of the Hh ligand by these tumors, which are received by the stromal cells and produce reciprocal signals leading to the growth and survival of tumors. A combination therapy targeting the Hh pathway in stromal cells and drugs for the tumor cells should be used to treat this kind of cancer (Gupta et al. 2010b). A variant of this type of cancer may be a reverse paracrine signaling, where the ligand is secreted by the stromal cells and received by the tumor cells for maintaining growth and survival. Till now this mode of signaling has only been observed in hematological malignancies like leukemia, lymphoma and multiple myeloma in which stromal secreted Hh is utilized by cancerous B-cells through upregulation of Bcl2, which is an antiapoptotic factor (Dierks et al. 2007; Scales and de Sauvage 2009; Gupta et al. 2010b; Abidi 2014).

Therapeutically the Hh pathway is an important target for cancer and regenerative medicine. The primary focus has been to study the prominent role of Hh signaling in promoting cancer. Currently, vismodegib and sonidegib, which are SMO inhibitors are the only clinically approved treatments for metastatic BCC (Dlugosz et al. 2012; Burness 2015). Arsenic Trioxide (ATO), an FDA approved inhibitor of GLI1 and GLI2 transcription factors. ATO directly binds to GLI1 and GLI2, inhibiting activity and decreasing expression of canonical Shh-GLI genes. It is currently a part of several clinical trials for both solid tumors and hematological malignancies (Beauchamp et al. 2011; Amakye et al. 2013). Drug resistance and cross-resistance often follow the treatment with SMO antagonists (Atwood et al. 2013;

Sharpe et al. 2015; Danial et al. 2016). Four different acquired resistance mechanisms have been known. Type I is the resistance via mutations in SMO in metastatic medulloblastoma, which can be overcome by using SMO mutant, SMO translocation, and GLI inhibitors. Type II is the resistance to SMO inhibitor due to amplification of *GLI2* in *Ptch*-mutant medulloblastoma mouse model, which can be overcome by the use of GLI1 inhibitors. Type III is the resistance by the upregulation of PI3K-mTOR pathway, where, use of PI3K-mTOR and GLI1 inhibitors would overcome the resistance. Type IV is the resistance by the upregulation of aPKCι/λ in human basal cell carcinoma, which could be overcome by the use of aPKCι/λ inhibitors. Apart from the different mechanisms of SMO inhibitor resistance, it would be quite informative and helpful to consider and investigate other pathways that are interacting with Shh signaling so that the components of these pathways can be used in the context of combination therapy (Huang and Yang 2015).

4.2 Regulation of Hh signaling by Kinases

Protein kinases have been known to modulate the Hh signaling which includes, PKA, PKC, GRK2, MEK, ERK, AKT, and GSK3β (Wang et al. 2000; Riobo et al. 2006; Lauth et al. 2007; Chen et al. 2011; Wang et al. 2012; Zhao et al. 2016). Recent studies have pointed towards the involvement of targeting different kinases and hence aiming for a specific response.

Protein Kinase A (PKA) is known to play key roles in many biological processes. In Hh receptive cells, PKA is involved in fate specification and in proliferation by attenuating Hh signaling. When the Hh pathway is inactive, even basal levels of active PKA can repress the Hh target genes. The important substrates of PKA are the Ci/Gli family, which can activate and repress Hh target gene expression. The basal level of PKA activity in Hh-responsive cells should be precisely regulated as increased and decreased levels of PKA activity would lead to cell proliferation negatively and can alter cell fate specification. However, the mechanism of PKA activity regulation is quite obscure, as its dependent on varying factors of different cell types, tissues, and organisms. Two different mechanisms have been proposed to address the mechanism; (1) activity of PKA is regulated by cAMP; (2) PKA activity is regulated by a protein known as Misty somites (Kotani 2012). PKA and CKI are involved in regulation of Smo accumulation at the cell surface in response to Hh. It has been shown in *Drosophila* wing disc, blockade of PKA and CKI leads to prevention of Smo accumulation upon Hh induction. Smo is phosphorylated by PKA and CKI at many

sites and phosphorylation-defective mutants of Smo are unable to accumulate at the cell surface and poorly equipped to transduce Hh signals. At the same time, it has also been shown that variants of Smo mimicking phosphorylation exhibit continuous expression at the cell surface and also able to transduce signals (Jia et al. 2004). The PKC family of proteins consists of three groups: the calcium-dependent conventional PKC isoforms (α, βI, βII, and γ), the calcium-independent novel PKC isoforms (δ, ϵ, η, and θ), and the calcium-independent atypical PKC isoforms (ζ, λ/ι) (Ron and Mochly-Rosen 1995; Brodie et al. 2004). Protein Kinase C (PKC) pathways and Hh undergo complex crosstalk and PKCdelta has been shown to inhibits Gli protein transcriptional activity and thereby alter Hedgehog signaling and negatively affect tumorigenesis in Hh associated cancers (Cai et al. 2009b). Reports have also shown, Ras-independent activation of MAPK/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK) pathway by PKCdelta (Ueda et al. 1996). And oncogenic KRAS through RAF MEK/MAPK signaling has been shown to be directly involved in the activation of the hedgehog pathway in pancreatic ductal adenocarcinoma (PDA) cells and the crosstalk between two pathways play a significant role in PDA progression (Ji et al. 2007). In general, the kinases in the CMGC (CDKs, MAPKs, GSK3s, CLKs) group have a broad spectrum of functional roles ranging from signal transduction to cell cycle regulation, RNA related processing, and intracellular communication (Varjosalo et al. 2013). Less studied candidates include the dual-specificity tyrosine regulated kinases (DYRKs) and the serine-arginine protein kinases (SRPK) (Singh and Lauth 2017). Dual specificity tyrosine phosphorylation regulated kinases (DYRKs) have shown to positively and negatively regulate Hh signaling pathway. (Mao et al. 2002; Shimokawa et al. 2008; Varjosalo et al. 2008; Lauth et al. 2010; Keramati et al. 2014b; Schneider et al. 2015a; Gruber et al. 2016; Singh et al. 2017).

4.2.1 Dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs)

The first member of the DYRK family to be discovered was YAK1 from budding yeast (Garrett and Broach 1989; Soppa and Becker 2015). DYRK family members have a characteristic sequence motif called the DYRK-homology box (DH box) (Figure 2). The mammalian DYRK subfamily is comprised of five members and they are categorized into two classes. This categorization is based on the sequence homologies within the conserved kinase domain (Becker et al. 1998; Aranda et al. 2011). Class I consists of DYRK1A and

DYRK1B (the latter is also known as a Minibrain-related kinase (Mirk)), whereas class II is made up of DYRK2, DYRK3, and DYRK4 (Becker et al. 1998; Soppa and Becker 2015).

Class I DYRK have certain distinct sequence motifs, such as a C-terminal PEST domain (a region rich in proline (P), glutamic acid (E), serine (S), and threonine (T)), which functions as a signal for rapid protein degradation (Figure 2) (Galceran et al. 2003). DYRK1A contains a poly-histidine stretch (13 consecutive histidine residues) and a serine/threonine enriched region (S/T-rich region) (Alvarez et al. 2003; Park et al. 2009). The poly-histidine stretch promotes the targeting of DYRK1A to nuclear speckles which are enriched with pre-mRNA splicing factors regulating the splicing machinery (Alvarez et al. 2003; Salichs et al. 2009). Class II DYRK kinases contain an N-terminal auto-phosphorylation accessory region (NAPA) domain, which is required for tyrosine auto-phosphorylation specifically in class II DYRKs. The NAPA domain in DYRK2 has been shown to autophosphorylate itself under *in vitro* conditions (Lochhead et al. 2005; Kinstrie et al. 2010; Aranda et al. 2011; Walte et al. 2013).

DYRK family members regulate protein stability, cell proliferation, and differentiation by phosphorylating DYRK recognition sites in target proteins. The consensus sequence motif consists of Ser or Thr followed by Pro in position +1. Furthermore, an arginine residue at position −2 or −3 relative to Ser/Thr seems to be preferred (RxxS/TP or RPxS/TP), although a considerable degree of divergence to this consensus has also been noted (Aranda et al. 2011; Soundararajan et al. 2013).

DYRK kinases contain an activation loop with a conserved YXY sequence, the phosphorylation of which leads to the activation of full enzymatic activity. Due to the distinct ability of DYRK family members to auto-phosphorylate the second tyrosine residue in order to be fully activated and then phosphorylate substrates in trans on Ser/Thr residues, they are known as Dual-specificity tyrosine-regulated kinases (Aranda et al. 2011; Soundararajan et al. 2013; Soppa and Becker 2015). The auto-activation mode by an upstream kinase is needed for the phosphorylation of the activation loop in case of MAP kinase, which is in contrast to DYRKs (Aranda et al. 2011). The tyrosine specificity of DYRKs is lost after the protein translation and the Ser/Thr specificity on target proteins remains (Lochhead et al. 2005; Alvarez et al. 2007).

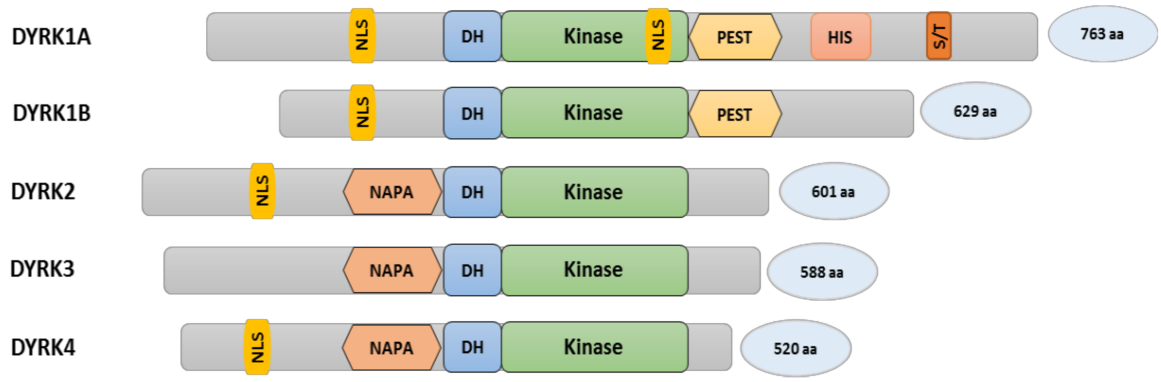

Figure 2: Schematic representation of the DYRK family of proteins: Distinct sequence motifs such as the nuclear localization signal (NLS); DYRK-homology box (DH); a motif rich in proline, glutamic acid, serine, and threonine residues (PEST); a poly-histidine stretch (HIS); a serine/threonine-rich region (S/T); a N-terminal auto-phosphorylation accessory region (NAPA); and a conserved kinase domain comprising the structural and functional features of DYRKs (Singh and Lauth 2017).

4.2.2 Class I DYRKs: DYRK1A & DYRK1B

DYRK1A Kinase:

DYRK1A is one of the most studied kinases in the DYRK family due to its connection with Down syndrome (DS, OMIM #190685). DYRK1A is mostly localized in the nucleus, but can also be found in the cytosol. DS is one of the most common genetic defects in humans and is caused by the complete or partial duplication of human chromosome 21 (trisomy 21) (Gardiner et al. 2010; Presson et al. 2013). In humans, the *DYRK1A* gene is located on chromosome 21 (21q22.13), which is part of the *Down-Syndrome Critical Region* (DSCR) (Epstein 2006). DS is generally characterized by intellectual impairment, characteristic craniofacial dysmorphologies, and congenital heart disease (Korenberg et al. 1990; Epstein 2006; Montoya et al. 2014). Despite the fact that upstream modulators of DYRK1A kinase activity exist, the prime determinant of DYRK1A protein function is considered to be its overall protein amount making it relatively sensitive to gene dosage (Litovchick et al. 2011; Tschop et al. 2011). The altered copy number of the *DYRK1A* gene in mammals or of its orthologous gene, *minibrain (mnb)* in *Drosophila*, impedes with proper development of the central nervous system (Tejedor et al. 1995). Studies on trisomy DS mouse model Ts65Dn

or cells derived from Down syndrome patients have shown that the increased expression of DYRK1A kinase affects neurogenesis and neuroblast proliferation, and results in impaired behavioral phenotypes (Contestabile et al. 2007). Behavioral and cognitive impairment and neuronal alterations in mice have been shown due to overexpression of DYRK1A (Martinez de Lagran et al. 2004; Dierssen 2012; Thomazeau et al. 2014). In contrast, a significant decrease in brain size in mice, flies, and men has been shown due to loss of function of *Dyrk1A* or *mnb* (Tejedor et al. 1995; Moller et al. 2008; Tejedor and Hammerle 2010; van Bon et al. 2016).

Studies on DYRK1A/mnb have shown that these kinases play an important role in the development of the central nervous system (Tejedor et al. 1995). The Hh signaling pathway also plays a significant role in neuronal proliferation (Ruiz i Altaba 1999). Mitogenic roles for SHH in neuronal precursor cells, in the cerebellum, neural tube and spinal cord have been reported (Rowitch et al. 1999; Wechsler-Reya and Scott 1999). Loss of function mutations in Patched which leads to the aberrant activation of HH signaling also leads to the proliferation of neural precursors (Wechsler-Reya and Scott 1999). As there have been a lot of parallel effects of Shh and DYRK1A, a plausible interaction of these pathways in neural development is foreseen.

GLI1 has been found to be one of the downstream targets of DYRK1A. Researchers have shown that the nuclear translocation and function of Gli1, an oncogenic transcription factor is mediated by DYRK1A through phosphorylation of nuclear localization signals located in the N-terminus of Gli1 (Pusapati et al. 2018). DYRK1A can retain Gli1 in the nucleus and regulate its transcription (Mao et al. 2002). DYRK1A can also dissociate the Suppressor of Fused (SuFu) (a negative regulator of Hh signaling) and Gli complex independent of the N-terminal phosphorylation of Gli1 (Schneider et al. 2015a). Several reports have suggested reduced Hh activity in DS patients, (Roper et al. 2006) which is paradoxical as per the hypothesis in which DYRK1A stimulates Hh pathway in a positive manner (Currier et al. 2012; Das et al. 2013). To support this argument, levels of Hh target genes have been compared between normal brain and the brains of DS patients and a significant reduction in the expression level was seen (Lockstone et al. 2007). Mechanistic studies revealed that DYRK1A regulates the actin cytoskeleton (Liu et al. 2009; Park et al. 2012; Schneider et al. 2015a) by functional inactivation of actin binding LIM protein (ABLIM). Novel DYRK1A

phosphorylation targets i.e. ABLIM proteins, (Schneider et al. 2015a) have been elucidated while confirming the negative regulation of Hh pathway by DYRK1A kinase.

DYRK1B Kinase:

The closest relative of mammalian DYRK1A is the DYRK1B kinase, also referred to as MIRK (Minibrain-related kinase). DYRK1B was cloned independently by two groups. One of the research groups performed RACE studies on human testis RNA (Leder et al. 1999a) and another group from a colon carcinoma cell line (Mercer and Friedman 2006b). *DYRK1B* is located on the 19q13.2 chromosome. This region is often amplified in ovarian and pancreatic cancer (Jin et al. 2007; Davis et al. 2013). This kinase has three splice variants (DYRK1B-p65, DYRK1B-p69, and DYRK1B-p75) with varying expression patterns. DYRK1B-p65 and DYRK1B-p69, are differentially expressed in mouse tissues, spleen, lung, brain, bladder, stomach and testes whereas DYRK1B-p75 is specifically expressed in skeletal muscles and differentiated adipocytes. Transcripts containing either exon 1A or exon 1B encode these variants, due to the use of separate promotors which would explain the distinct pattern of expression of these variants (Leder et al. 2003). Human DYRK1A and DYRK1B proteins are 84% identical in the N-terminal and catalytic domains but show no extended similarity in the C-terminal domain. Human and mouse DYRK1B proteins share 97% sequence similarity (Kentrup et al. 1996; Becker et al. 1998; Becker and Joost 1999; Leder et al. 1999b; Li et al. 2001). Similar to DYRK1A, DYRK1B also has an NLS sequence, a conserved kinase domain, a PEST sequence and a MAPK phosphorylation sequence in C-terminus (Leder et al. 1999a; Mercer and Friedman 2006b). DYRK1B has a similar activation mechanism as DYRK1A. DYRK1B has a limited expression in normal tissue, with highest expression seen in skeletal muscle, heart, testes, and brain (Lee et al. 2000). Myogenesis became the most studied model to elucidate the functions of the DYRK1B kinase (Lu et al. 2000; Deng et al. 2003; Mercer et al. 2005). This is due to the fact that differentiation can be followed biochemically by monitoring the expression of well-characterized proteins. The protein levels of DYRK1B are low in the dividing myoblasts. During the differentiation phase, the levels of DYRK1B increase greatly and remain unchanged in the mature myoblasts. Mirk promotes myoblast differentiation indirectly by phosphorylating class II histone deacetylases, which in turn accumulate in the cytoplasm and thereby suppression of myogenin-dependent transcription is relieved (Deng et al. 2005). Mirk promotes the survival of differentiating myoblasts by phosphorylating the CDK

inhibitor p21 and causing p21 to accumulate in the cytoplasm where it functions as an anti-apoptotic signaling molecule (Mercer et al. 2005). These studies have helped to elucidate the role of DYRK1B as a multifunctional Ser/Thr kinase which plays critical roles in muscle differentiation by executing some regulatory effects on motility, transcription, cell cycle regulation and cell survival (Mercer and Friedman 2006a).

DYRK1B phosphorylates and blocks degradation of the cyclin-dependent kinase (CDK) inhibitor p27 to maintain a quiescent state (Deng et al. 2014b). DYRK1B is highly abundant and active in normal diploid cells and in cancer cells transiently arrested in G0, or in early G1, with up to 10-fold lower levels in cycling cells (Deng et al. 2004). Mirk is known to be localized fast-twitch skeletal muscles and its inhibition leads to an increase in the amount of toxic ROS (Reactive Oxygen Species) induced in differentiating C2C12 myoblasts and postmitotic cultures of myotubes which lead to the formation of skeletal muscle. These muscles produce ROS endogenously during contractions. Cancer cells can regulate the elevated levels of ROS, which promotes many aspects of tumor development and progression by upregulating DYRK1B and amplifying *DYRK1B* gene (Deng et al. 2014b). DYRK1B has been shown to play an important role in breast cancer progression. Statistical analysis has shown that various clinicopathologic factors such as tumor size, grade, estrogen receptor status, Ki-67 (cellular marker of proliferation) expression are associated with the extent of expression of DYRK1B and overexpression results in poor prognosis. Also, DYRK1B has been shown to phosphorylate FoxO1 (Forkhead box protein O1) and hence promote its nuclear exclusion (Chen et al. 2017). FoxO1 is also subject to regulation by PI3K-AKT pathway, which phosphorylates and subsequently translocates FoxO1 out of the nucleus, suggesting the involvement of DYRK1B in the PI3K-AKT pathway (Bullock 2016). Mirk activity also increases with the exposure to chemotherapeutic drugs such as 5-FU or cisplatin (Jin et al. 2009; Hu and Friedman 2010), through stress signaling to the DYRK1B kinase activator MAPK kinase MKK3 (Lim et al. 2002). Recent reports have demonstrated that phosphorylation of S471 (site for DYRK1B autophosphorylation) contribute to DYRK1B kinase activity. Also, DYRK1B has been defined as a new substrate of ERK1/2 signaling. The ERK1/2 pathway is a key regulator of the cell cycle, promoting proliferation or cell cycle arrest (Ashford et al. 2016). Mirk is upregulated and amplified in the majority of pancreatic and ovarian cancers (Friedman 2007). Mirk has antiapoptotic functions in cancer cells in which Mirk is highly expressed such as rhabdomyosarcoma cells (Mercer et al. 2006), colon carcinoma cells (Lee et al. 2000), and HeLa cervical carcinoma

cells (MacKeigan et al. 2005). RNAi mediated knockdown of Mirk has shown to reduce the clonogenicity of pancreatic cancer cells and reducing the tumor cell number, which shows that Mirk mediates survival in these cells (Deng et al. 2006). Mirk knockout or depletion had no detectable effect on normal tissue which indicates a selective effect of Mirk kinase inhibitor on cancer cells expressing elevated levels of Mirk kinase (Ewton et al. 2011). Functional characterization of the *DYRK1B* gene revealed that the nonmutant protein inhibited the SHH and WNT pathways, thereby enhancing adipogenesis (Keramati et al. 2014a). The *DYRK1B* locus on 19q13 has been linked to type 2 diabetes (Cho et al. 2011) and is shown to play a central role in the altered pathways in metabolic syndrome (Keramati et al. 2014a). As there is a high abundance of DYRK1B in testes, one of the reports suggests that cold-inducible RNA-binding protein (Cirp) functions to positively affect the proliferation of undifferentiated spermatogonia by interacting with DYRK1B. Cirp modulates the protein levels of p27 and cyclinD1 by suppressing the kinase activity of DYRK1B and hence promoting the cell cycle progression of undifferentiated spermatogonia (Masuda et al. 2012). Adding up to the developmental roles of mirk, the zebrafish *dyrk1b* gene is shown to be important for the endoderm formation and craniofacial patterning (Mazmanian et al. 2010).

DYRK1B has been shown to positively and negatively affect Hh signaling (Singh et al. 2017). It could activate PI3K/AKT/mTOR pathway, which is an established oncogenic driver in humans. This pathway has been well characterized and recognized to play essential roles in normal cellular functions including nutrition and energy balance, protein synthesis and growth control in mammalian cells, thereby, making DYRK1B an important target in Hh signaling pathway.

4.2.3 Class II DYRKs

DYRK2, DYRK3, and DYRK4 proteins contain a canonical kinase domain located between a large N-terminal region and a short C-terminal extension and features specific to DYRK related kinases. DYRK2 and DYRK4, but not DYRK3, possess an NLS sequence and all three contain a NAPA (N-terminal autophosphorylation accessory region) domain which is absent in class I DYRKs (Aranda et al. 2011; Singh and Lauth 2017). Despite lacking NLS, DYRK3 (also known as REDK) is localized in the nucleus in hematopoietic cells (Lord et al. 2000) whereas DYRK2 is mostly cytosolic, but in conditions of genotoxic stress it

accumulates in the nucleus (Taira et al. 2007). DYRK4 displays splice variant dependent subcellular localization (Aranda et al. 2011). DYRK2 shares 46% and DYRK3 shares 43% identity with class I DYRKs in the catalytic domain respectively but lack the striking sequence motifs such as a C-terminal PEST domain (Becker et al. 1998). Tyrosine autophosphorylation by class II DYRKs requires an N-terminal auto phosphorylation accessory region (NAPA) motif, which is present in the N-terminal region of class II DYRKs only (Aranda et al. 2011). This domain provides a chaperone-like function (Aranda et al. 2011) and transiently converts class II DYRKs into intramolecular tyrosine kinases (Kinstrie et al. 2010).

DYRK2 has been shown to phosphorylate very limited number of substrates such as NFAT4, eIFB5, Glycogen synthase6, Oma-17, MEI-18 and chromatin remodeling factors SNR1 and TRX9, hence regulating calcium signaling, protein synthesis, glucose metabolism, developmental processes and gene expression (Skurat and Dietrich 2004; Nishi and Lin 2005; Gwack et al. 2006; Kinstrie et al. 2006; Lu and Mains 2007; Maddika and Chen 2009). DYRK2 has also been shown to function in DNA damage signaling pathway (Taira et al. 2007). The role of DYRK2 in human cancer remains questionable. As, overexpression of DYRK2 predicts better survival in non-small cell lung cancer (NSCLC), breast cancer and pulmonary adenocarcinoma, implying a tumor suppressor role (Yamashita et al. 2009a; Yamashita et al. 2009b). On the other hand, there are reports showing, amplification of *DYRK2* gene in esophageal/lung adenocarcinoma as well as in gastric stromal tumor implying its potential oncogenic role (Miller et al. 2003; Koon et al. 2004; Yan et al. 2016).

Signal transduction roles of DYRK3 have been reported, where DYRK3 has been shown to regulate raft-mediated endocytosis/caveolae which operate on the principles of membrane trafficking. Knockdown of DYRK3 strongly increased the dynamics of caveolar vesicles and had a minor destabilizing effect on the caveolar coat (Fujimoto et al. 2000; Pelkmans et al. 2005; Tagawa et al. 2005). The kinase-dependent differential regulation of cycling events between the cell surface and intracellular organelles probably involve changes in the cortical actin cytoskeleton and activation of microtubule-dependent motility (Mundy et al. 2002; Pelkmans and Helenius 2003; Pelkmans et al. 2005). Integral roles of caveolin-1 have also been documented for sequestering the Hh receptor complex in cholesterol-rich

microdomains of the plasma membrane, which act as a scaffold for the interactions with the Hh protein (Karpen et al. 2001).

DYRK4 is expressed in the testes of adults, but not prepubertal rats (Becker et al. 1998) and is highly restricted to step 8 spermatids (Sacher et al. 2007). DYRK4 was shown to be present in the duck ovary, and was more active or upregulated in the high egg production (HEP) ovaries, which would argue for its role in the female reproductive system but the underlying mechanism is still elusive (Tao et al. 2017).

4.3 Hh Signaling & DYRK1B kinase

The DYRK kinase family has a close regulatory connection to the Hh pathway with its five members DYRK1A, DYRK1B, DYRK2, DYRK3 and DYRK4 (Mao et al. 2002; Varjosalo et al. 2008; Lauth et al. 2010; Aranda et al. 2011; Schneider et al. 2015b; Gruber et al. 2016). In particular, DYRK1B (a.k.a. MIRK) is linked with several cancer types and is amplified or hyperactive in ovarian and in pancreatic cancer (Mercer et al. 2006; Friedman 2007; Friedman 2010b; Hu and Friedman 2010; Deng and Friedman 2014; Deng et al. 2014a). DYRK1B has been known to affect Hh signaling in both a positive and a negative manner. On the one side, DYRK1B dampens Hh signaling initiated by SMO (Lauth et al. 2010; Jacob et al. 2011; Keramati et al. 2014b), and on the other DYRK1B promotes the stability of GLI transcription factors (Gruber et al. 2016). DYRK1B has been shown to stabilize GLI1 transcription factors by activating the pro-survival PI3K/mTOR/AKT arm, a positive regulator of Gli stability (Riobo et al. 2006; Fruman and Rommel 2014; Kern et al. 2015). Hh pathway stimulation leads to an increase in DYRK1B protein levels by currently unknown post-transcriptional mechanisms suggesting a feedback loop (Singh et al. 2017). The Hh signaling has been known to have an inhibiting impact on adipocytic differentiation (Kha et al. 2004; Johnson et al. 2011; Nosavanh et al. 2015) whereas DYRK1B favors the *in vitro* differentiation into adipocytes (Keramati et al. 2014b). This is true for DYRK1B carrying mutations identified in families suffering from an autosomal dominant form of metabolic syndrome, a disease with prominent adipocytes involvement (Keramati et al. 2014b). Although the mechanistic and functional integration of DYRK1B with other signaling pathways is not elucidated, it's interesting to know that mutant DYRK1B expression reduced GLI2 levels in cultured adipocytes (Keramati et al. 2014b). Therefore, the involvement of Hh signaling can be speculated in these effects.

The cytoskeleton of eukaryotic cells is made up of filamentous protein, and it is known to provide mechanical support to the cell. Microtubules are the largest type of filament and they are composed of polymerized tubulin monomers. Tubulin contains two polypeptide subunits and dimers of these subunits come together to form long strands called protofilaments. Thirteen protofilaments come together to form long straw shaped microtubules. With addition and subtraction of tubulin dimers, microtubules are dynamic. In cells, microtubules are anchored to microtubule organization centers (MTOCs). The centrosome, basal bodies and spindle pole bodies are different forms of MTOCs. Microtubules tend to grow from the centrosome to the plasma membrane. In non-dividing cells, microtubules support the basic organization of the cytoplasm including the positioning of the organelles. Microtubules are responsible for organelle transport, mitosis, secretion, cell shape, polarization and cell migration. Various post-translational modification plays important roles in the regulation of microtubule function. Some of the examples include acetylation, phosphorylation, polyglycylation, and others (Westermann and Weber 2003; Janke and Bulinski 2011; Janke 2014).

One of the best-studied covalent modification is acetylation, which is associated with stable microtubules e.g. in primary cilia and is conserved from protists to humans. Acetylation occurs on Lys40 of the α-subunit of the α/β-heterodimer within microtubules. Functionally, microtubules facilitate the transport along their tracks by binding to specific motor proteins (Reed et al. 2006; Cai et al. 2009a; Hammond et al. 2010; Walter et al. 2012). Increased tubulin acetylation has been shown to promote MT-directed mitochondrial transport in neurons (Chen et al. 2010) and to compensate for vesicular transport deficits in a cellular model of Huntington's disease (Dompierre et al. 2007). MT acetylation is mainly governed by the opposite action of α-tubulin acetyltransferase 1 (ATAT, a.k.a. αTAT1 or MEC17), histone deacetylase 6 (HDAC6), and sirtuins2 (SIRT2) which add or remove acetyl groups from α-tubulin, respectively (Hubbert et al. 2002; Montagnac et al. 2013). Abnormal levels of this modification are linked to neurological disorders, cancer, heart disease, and other pathological diseases thereby making it therapeutically important (Singh et al. 2010; Di Martile et al. 2016; Stram et al. 2017; Tapias and Wang 2017). HDAC6 is a multifunctional protein with sequence homology to nuclear HDACs, and its involvement in the deacetylation of many non-histone proteins including tubulin (Li et al. 2012). It is involved in a wide variety of cellular processes including signal transduction, aggresome formation, stress granule

biology and gene transcription (Kawaguchi et al. 2003; Boyault et al. 2007; Kwon et al. 2007; Shan et al. 2008; Deribe et al. 2009; Mak et al. 2012; Chen et al. 2013; Dhanyamraju et al. 2014).

Although a lot is known about the enzymes regulating tubulin acetylation and different posttranslational modifications, very little is known about different signaling pathways and their effect on cytoskeletal regulation. The noncanonical Hedgehog signaling pathway has been known to modulate cytoskeletal remodeling (Brennan et al. 2012). While Hh signaling has been shown to affect the actin cytoskeleton (Bijlsma et al. 2007; Xiao et al. 2010; Schneider et al. 2015a), very little is known about its effects on MTs.

In this work, I have shown that activated Hh signaling promotes MT acetylation, cell polarization and organelle transport such as MT-dependent mitochondrial transport. Furthermore, I have shown DYRK1B, whose levels are elevated by activated Hh signaling, can also affect tubulin acetylation positively. Supporting my findings, I have also shown that overexpression of DYRK1B leads to reduced HDAC6 activity. Also, my results have shown that increased DYRK1B levels inactivate Glycogen synthase kinase 3β (GSK3β) through Ser9 phosphorylation, resulting in HDAC6 inhibition and increased tubulin acetylation. In summary, using Hh signaling as a paradigm, I describe a mechanistic framework how intercellular communication can affect cytoskeletal regulation and cell function.

4.4 Aim of the present work

Hedgehog (Hh) signaling cascade is one of the intricate signal transduction mechanisms that govern the precisely regulated developmental processes of multicellular organisms. Hh has been shown to regulate actin cytoskeleton but not much is known about its regulation of MT cytoskeleton. Reports have also shown that Hh ligands promote the phosphorylation and activation of mTOR and AKT kinases. As protein kinases are known to play a significant role in Hh signal transduction, I have focused my research on DYRK1B kinase, a member of DYRK family of kinases which, possesses both serine/threonine and tyrosine kinase activities and enhances the transcriptional activity of TCF1/HNF1A.
The aim of my present work is to elucidate the mechanistic regulation of Hh driven/mediated processes of mTOR/AKT activation and regulation of MT cytoskeleton and MT-dependent processes with the DYRK1B kinase.

5 Results

5.1 DYRK1B blocks canonical and promotes non-canonical Hedgehog signaling through activation of the mTOR/AKT pathway

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Hedgehog ligands promote the stimulation of Gli transcription factors and also promote the phosphorylation and activation of mTOR and Akt kinases, although the molecular mechanism behind this unknown (Riobo et al. 2006; Wang et al. 2012). With my results, I have tried to place DYRK1B as a mediator between Hh signaling and mTOR/AKT activation. My work has shown, how DYRK1B negatively and positively affects Hh signaling. I have targeted DYRK1B in GLI dependent cancer cells, therapeutically by combining a DYRK1B antagonist with a mTOR/AKT inhibitor resulting in a strong GLI targeting and increased cytotoxicity in pancreatic and ovarian cancer cells.

To identify the role of the DYRK1B kinase as a positive and negative modulator of Hedgehog signaling, I have used the RNAi technique where I knocked down DYRK1B in mouse embryonic fibroblasts stably expressing Sonic Hh ligand (MEF$^{[SHH]}$ cells) (Lipinski et al. 2008)), which renders these cells constitutively signaling. In figure 1A, siRNA knockdown of endogenous *Dyrk1b* led to an upregulation of several Hh target genes (*Gli1, Ptch1, Ptch2*) when compared to control siRNA-transfected cells. A de-repression of Hh pathway activity upon *Dyrk1b* knock-down was confirmed by measuring the protein levels of GLI1 (Fig. 1A inset). Elucidating the roles of DYRK1B kinase when overexpressed, I treated NIH3T3 fibroblasts, stably transfected with empty control plasmid and V5-tagged *DYRK1B* (NIH$^{[Con]}$ and NIH$^{[1B]}$ cells; Fig. 1C) with the synthetic SMO agonist SAG (Chen et al. 2002) in order to stimulate membrane signaling. Immunoblotting for the endogenous GLI1 protein revealed that the *DYRK1B*-overexpressing cells had lost their SAG-responsiveness (Fig. 1D, E), arguing that DYRK1B prevents Hh canonical signaling. However, the basal levels of GLI1 were increased in NIH$^{[1B]}$ cells even in the absence of any stimulatory SAG, indicative of a non-canonical activation of GLI activation. Furthermore, a 130 kDa isoform of GLI1 was seen in control cells, which was not evident in 1B overexpressing cells (Fig. 1D). A 100 kDa large GLI1 isoform has previously been proposed to represent an inhibitory variant of GLI1 (Stecca and Ruiz i Altaba 2009). My results were further strengthened with the measurement of mRNA expression levels of target genes, where *DYRK1B*

overexpression blocked SAG-induced Hh signaling while at the same time it increased the basal expression of *Ptch1* (Fig. 1F). Taken together, my data suggests that DYRK1B inhibits canonical Hh signaling while it promotes non-canonical activation of the GLI1 transcription factor.

To support my results further I took human cancer cells (HeLa). Stable expression of DYRK1B increased the endogenous GLI1 protein levels (Fig. 2A) while at the same time it decreased the *GLI1* mRNA levels (Fig. 2B). This ambiguity argued for a stabilizing effect of DYRK1B on the GLI1 protein. Protein stability assays were performed in $NIH^{[Con]}$ and $NIH^{[1B]}$ cells blocking *de novo* protein synthesis with Cycloheximide. Endogenous GLI1 got degraded with a half-time ($t_{1/2}$) of approx. 3.5 h in SAG-treated control cells whereas GLI1 protein levels in SAG-treated *DYRK1B*-expressing cells were extremely stable (Fig 2C,2D). As its already known that AKT kinase has a stabilizing effect on GLI transcription factors (Riobo et al. 2006; Paul et al. 2013; Shi et al. 2015), I treated the $NIH^{[1B]}$ cells, with a pan-AKT inhibitor (GSK-690693) and GLI1 levels dropped significantly upon AKT inhibition, and could be rescued by pharmacological blockade of the proteasome (Fig. 2E). This proves that AKT mediated GLI protein stability is increased by ectopically expressed DYRK1B kinase.

Following up on the results, I wanted to find out if DYRK1B could activate the PI3K/mTOR/AKT pathway. In line with my previous findings, overexpression of DYRK1B leads to the phosphorylation (activation) of mTOR (Ser2448) and AKT (Ser473 and Thr308) (Fig. 3A, B). As, AKT^{Ser473} phosphorylation is mTORC2 dependent (Guertin et al. 2006; Shiota et al. 2006), and AKT^{Th308} phosphorylation is PDK-1 induced, my data suggests that DYRK1B directly or indirectly activates the PI3K/mTORC2/AKT signaling arm or that both phospho-sites communicate and influence each other. To investigate further, I looked at the phosphorylation status of downstream effectors of the second mTOR complex (mTORC1), which is S6-Kinase (S6K-Thr389) and the S6K target ribosomal protein S6 (S6-Ser235/Ser236). Both S6K and S6 were phosphorylated (activated), hence suggesting that DYRK1B activates mTORC1 complex. Although in high serum conditions, the phosphorylation events were less evident. (Fig. 3C, D). *DYRK1B*-expressing HeLa cells also showed similar results as the fibroblasts (Fig. 2A) for AKT and mTOR phosphorylation. My results were further strengthened by using cells which are genetically depleted of *DYRK1B* through CRISPR/Cas9 methodology (Fig. 3E). These are mammalian HAP1 cells

harboring a haploid genome (Carette et al. 2011). Interestingly, *DYRK1B*-knock out (KO) cells displayed similar results as fibroblasts and HeLa cells i.e. reduced endogenous GLI1 protein levels (Fig. 3E) and an overall reduced level of AKT (Ser473; Thr308) and mTOR (Ser2448) phosphorylation (Fig. 3F, G, and H). Taken together, my data implies that DYRK1B is an activator of the PI3K/mTOR/AKT signaling pathway.

With my results from MEF$^{[SHH]}$ cells, where signaling is continuously active I could show elevated levels of phosphorylated AKT and mTOR, which could be suppressed by inhibition of the Hh pathway with the SMO antagonist SANT (Fig. 4A, B) (Chen et al. 2002). As DYRK1B has been shown to activate the mTOR/AKT kinases, there is a strong possibility of DYRK1B mediating the effects of Hh signaling on AKT/mTOR. Two different fibroblast cell lines have shown the induction of DYRK1B levels with Hh pathway stimulation and reduced DYRK1B levels with suppressed Hh signaling (Fig. 4C). Therefore, I analyzed the effect of *Dyrk1b* knock-down on phospho-mTOR/AKT levels in MEF $^{[SHH]}$ cells. As can be seen in figures 4D and 4E, the levels of phospho-AKT^{Ser473} and phospho-AKT^{Thr308} were significantly reduced upon knock-down of *Dyrk1b*. Thereby, I could say that DYRK1B mediates the effect of Hh signaling on phosphorylation of AKT (and potentially mTOR).

The PI3K/mTOR/AKT system is subject to intense feed-back regulation. I investigated the systemic feedback regulation by analyzing AKT phosphorylation in NIH$^{[MCS]}$ and NIH$^{[1B]}$ cells upon inhibition of AKT (GSK-690693, a pan-AKT inhibitor), mTOR (KU-0063794, a dual mTORC1/2 inhibitor) and DYRK1B (AZ191, a selective small molecule DYRK1B inhibitor (Ashford et al. 2014)). As can be seen in figure 5A, all inhibitors led to a subsequent increase in phospho-AKT levels in *DYRK1B*-overexpressing cells, although they were different in amplitude. In contrast, in wild-type, NIH$^{[MCS]}$ cells, AKT, and mTOR inhibition resulted in reduced phospho-AKT levels while AZ191 led to an increase. Taken together with the previous experiments, this result strongly suggested that DYRK1B is indeed involved in a complex regulatory mTOR/AKT feedback loop. To investigate further, ShhL2 cells (a clonal NIH3T3 cell line harboring a Hh/GLI-responsive luciferase reporter construct in the genome (Taipale et al. 2000)) were used. Hh activation by SAG and AZ191 was added for different time periods and the activity of Hh signaling was recorded. As can be seen in figure 5B and 5C, when compared to the DMSO control, Hh signaling was suppressed by AZ191 in the first 24h and was then increased over controls at later time points (48-72h), suggesting a pronounced influence of feedback regulation on the kinetics of the Hh response. These data

show that the exact time point of analysis is important when determining the effects of DYRK1B. To delve deeper into the kinetics issue endogenous *DYRK1B* was knocked down in human Panc1 pancreatic cancer cells by two different approaches: 1.) In a short-term experiment (2-3d), short-interfering RNA (siRNA) was used and 2.) In a long-term experiment (6-7d), short hairpin RNA (shRNA) was applied. The acute knock-down of *DYRK1B* by means of siRNA (short-term) resulted in an increase of endogenous GLI1 levels (Fig. 5D). In contrast, the long-term knock-down of *DYRK1B* through a shRNA approach led to a suppression of GLI1 expression (Fig. 5E). To rule out potential effects of siRNA versus shRNA technology, I performed a time course experiment treating Panc1 cells for 9 d with AZ191 and determined the daily changes in GLI1 levels (Fig. 5F). Supporting my previous results, GLI1 protein levels were induced during the first 6 days, followed by a reduction afterward (7-9 d). Altogether, a prominent time-dependent impact of DYRK1B inhibition on GLI1 levels was seen.

Considering that the fluctuating kinetics could lead to upregulation of oncogenic GLI1 levels in cancer cells, with short-term treatments with DYRK1B antagonists. I, therefore, tested the combination of AZ191 (DYRK1B inhibitor) with drugs targeting mTORC1/2 (KU-0063794), AKT (GSK-690693) or S6K (PF-4708671) and measured the effects on GLI1 levels in *DYRK1B*-amplified Panc1 cells (Fig. 6A). Treatment with AZ191 alone (24h) increased the phosphorylation of AKT and the GLI1 expression, whereas co-treatment with the mTOR/AKT/S6K inhibitors significantly reduced the levels of both. In combination with AZ191 however, GLI1 levels were almost completely abrogated (Fig. 6A). To measure the cytotoxic effects of these inhibitors, I performed cell growth assays. While single treatment with AZ191, KU-0063794, AKT inhibitor and S6K inhibitor alone displayed only a moderate effect on cell growth (cytostatic), the combination of drugs was strongly cytotoxic to pancreatic cancer cells (Fig. 6B, 6C, 6D). Moreover, the results on dual DYRK1B - PI3K/AKT/mTOR/S6 inhibition was not specific to Panc1 cells as I could reproduce them in Ovcar-3 ovarian cancer cells using combinations of AZ191 and inhibitors targeting PI3K, mTOR, AKT, and S6K. This concludes that a dual targeting approach combining a DYRK1B antagonist with an inhibitor of the PI3K/mTOR/AKT pathway has a pronounced impact on the GLI1 oncoprotein and exerts strong cytotoxic effects in cancer cells.

5.2 DYRK1B regulates Hedgehog-induced microtubule acetylation

Rajeev Singh, Philipp Simon Holz, Katrin Roth, Anna Hupfer, Wolfgang Meissner, Rolf Müller, Malte Buchholz, Thomas M. Gress, Hans-Peter Elsässer, Ralf Jacob, Matthias Lauth

The post-translational modifications (PTM) of tubulin subunits are important for maintaining the physiological functions of the microtubule (MT) cytoskeleton. A lot of progress has been made in the identification of enzymes carrying out MT-PTMs although little is known on how intercellular signaling molecules and their associated pathways regulate MT-PTM-dependent processes inside signal-receiving cells. Here I show that Hedgehog (Hh) signaling, affects the MT acetylation in mammalian cells. Mechanistically, Hh pathway activity increases the levels of the MT-associated DYRK1B kinase, resulting in the inhibition of GSK3β through phosphorylation of Serine 9 and the subsequent suppression of HDAC6 enzyme activity. Since HDAC6 represents a major tubulin deacetylase, its inhibition increases the levels of acetylated MTs. Through the activation of DYRK1B, Hh signaling facilitates MT-dependent processes such as intracellular mitochondrial transport, mesenchymal cell polarization or directed cell migration. Taken together, my results provide evidence that intercellular communication through Hh signals can regulate the MT cytoskeleton and MT-dependent processes by affecting the level of tubulin acetylation.

There has been a report showing that Hh signaling promotes α-tubulin acetylation (Lee and Ko, 2016). I also found similar results in fibroblasts. After treating them with SAG, a synthetic Hh activator (Chen et al. 2002), induced levels of acetylated tubulin (AcTub) in immunofluorescence (Fig. 1A, B) and in Western blotting experiments (Fig. 1C, D) were observed. This AcTub increase correlated with the induction of the Hh pathway target GLI1 and the levels of the Dual-specificity Tyrosine (Y)-regulated kinase 1B (DYRK1B, a.k.a. as MIRK) (Mercer and Friedman 2006b) which I have previously shown to be upregulated by Hh signaling (Singh et al. 2017) (Fig. 1D). I could obtain similar results with MEFs (Fig. 1E), demonstrating that this effect was not restricted to NIH3T3 cells. To rule out a Hh-unrelated effect of the compound SAG, I investigated MEF cells stably expressing SHH ligand ($MEF^{[SHH]}$ cells) and found that pathway inhibition with the SMO inhibitor SANT (Chen et al. 2002) concomitantly reduced the levels of AcTub and DYRK1B (Fig. 1F). Based on these experiments my hypothesis was that DYRK1B could be involved in mediating all or some of the effects of Hh on AcTub. To investigate further, endogenous Dyrk1b was knocked down by RNAi in MEF cells. The SAG-mediated increase in acetylated tubulin could be fully

blocked upon removal of DYRK1B (Fig. 1G). Supporting my results further, the elevated AcTub levels in MEF[SHH] cells could be reduced by siRNA transfection targeting Dyrk1b (Fig. 1H). These experiments suggest that Hh signaling increases DYRK1B protein levels by posttranscriptional mechanisms leading to a rise in DYRK1B-mediated tubulin acetylation.

To understand the role of DYRK1B in more detail I generated NIH3T3 cells stably expressing a V5-tagged form of this kinase (NIH3T3$^{[1B]}$ cells). Interestingly, there was a striking difference in the morphology when compared to control cells (Figure 2A). Control cells were elongated and had a spindle-shaped morphology of mesenchymal cells whereas the *DYRK1B*-overexpressing cells had much smaller and rounder cell bodies with longer cellular extensions, suggesting a potential cytoskeletal effect induced by the increased *DYRK1B* expression. AcTub levels were found to be strikingly elevated in these cells (Fig. 2B, C, D), showing that the sole overexpression of *DYRK1B* can lead to AcTub induction. I could recapitulate these finding in HeLa cells stably expressing DYRK1B (Fig 2E, F, G). As HDAC6 is known as a major determinant of the tubulin acetylation status, I used the Hela$^{[DYRK1B]}$ cells to investigate whether DYRK1B expression affects HDAC6 enzyme activity when compared to control cells. I immunoprecipitated endogenous HDAC6 from control and from *DYRK1B*-expressing HeLa cell lines and subjected the precipitate to a luminometric *in vitro* deacetylase assay. Indeed, the HDAC6 enzyme activity (normalized to the amount of total HDAC6 protein precipitated) was significantly lower (by about 40 %) in *DYRK1B*-expressing cells. This difference was blunted when an HDAC6-selective inhibitor (Cay10603) was co-applied to the deacetylase assay (Fig. 2H). With these experiments, I could conclude that increased expression of DYRK1B is sufficient to elicit MT acetylation in the absence of additional Hh receptor activation. Furthermore, increased DYRK1B levels result in functional downregulation of HDAC6, a known master regulator of MT acetylation.

I wanted to investigate the mechanism by which DYRK1B and HDAC6 are related. There have been reports suggesting a stimulatory effect of Glycogen synthase kinase 3 β (GSK3β) on HDAC6 (Chen et al. 2010). I worked further to determine if Hh/DYRK1B would inhibit GSK3β, thereby indirectly leading to the suppression of HDAC6 activity. To this end, I treated the cells with SAG and could clearly see the elevated phosphorylation of GSK3ß at Ser9 (Fig. 3A) and SANT treatment reduced levels of GSK3ß (Fig. 3B) phosphorylation. To

investigate how DYRK1B affects GSK3ß phosphorylation, I knocked down Dyrk1B in continuously *SHH*-expressing cells, and this led to a clear reduction in phospho-GSK3ß levels (Fig. 3C). Adding to this, pharmacological blockade of DYRK1B with the inhibitor DYRK1B (Ashford et al. 2014), also reduced the phospho-GSK3ß levels in SAG treated cells (Fig. 3D). Moreover, stable expression of DYRK1B was enough to elevate phospho-GSK3ß levels (Fig. 3E), which suggests a close functional connection between the two players. Thereby, I performed *in vitro* kinase assays and could see that the recombinant DYRK1B was able to phosphorylate GSK3ß (Fig. 3F). My data suggest that Hh signaling, by upregulation of DYRK1B, phosphorylates GSK3ß (inactive) and hence inhibits HDAC6 enzyme activity.

It is already known that HDAC6 and GSK3β are localized to MTs (Hubbert et al. 2002; Kovacs et al. 2004; Sun et al. 2012). I wanted to find if DYRK1B is also localized to MTs. To this end I performed biochemical MT-association assay (MTaa), using control and SAG treated NIH3T3 fibroblasts, and looked at the protein fractions bound to polymerized microtubules. I could clearly see, a fraction of total DYRK1B and GSK3ß (positive control) at polymerized microtubule protein fractions (Fig. 4A). To support my observations further, I performed sub-diffraction super-resolution microscopy (GSD-Ground State Depletion microscopy) and were able to visualize transfected V5-tagged DYRK1B on endogenous MTs (Fig. 4B) in human fibroblasts (PSC). With the help of this type of microscopy, I could also visualize the localization of endogenous DYRK1B on single MT tracks in HeLa cells (Fig. 4C). As a positive control, I was able to detect endogenous HDAC6 on defined MT tracks in PSC fibroblasts (Fig. 4D). With these experiments, I could prove that DYRK1B is associated with MTs.

After looking into the mechanical aspects of Hh-DYRK1B-GSK3β-HDAC6-AcTub axis, I looked at the functional consequences of this process. To this end, I analyzed MT-dependent mitochondrial transport where tubulin acetylation had been shown to facilitate organelle motility in neurons (Chen et al. 2010). I wanted to find out if Hh signaling also affects the mitochondrial transport and to this end, I generated NIH3T3 cells stably expressing fluorescent Dendra protein fused to a mitochondrial targeting sequence derived from human cytochrome c oxidase subunit 8a (NIH[Cox8a-Dendra] cells). The mitochondrial expression of this fusion protein was verified by its perfect co-localization with MitoTracker (Fig. 5A). Also the SAG and DYRK1B dependent regulation of AcTub levels

were verified in these cells (Fig. 5B) Thereafter, I looked at the impact of Hh activation (SAG), DYRK1B inhibition (AZ191) and HDAC6 inhibition (ACY-1215 (Santo et al. 2012)) on mitochondrial transport by using live cell imaging. My results have clearly shown that overall track length of mitochondrial transport is considerably increased with SAG stimulation and HDAC6 inhibition, whereas this effect could be completely abrogated with DYRK1B inhibition by AZ191 (Fig. 5C). Adding to these results, the mitochondrial track speed was also increased with HDAC6 inhibition, and Hh activation (SAG) whereas DYRK1B inhibition by AZ191 completely abrogated this effect (Fig. 5D). Plotting the Mean square displacement (MSD), which is a measure of the deviation of the position of cells, with respect to a reference position over time, showed us increased directionality with SAG and reduced directionality with DYRK1B inhibition with AZ191. HDAC6 inhibition by ACY-1215 also led to an increased directionality, which suggests that increased tubulin acetylation enhances the mitochondrial transport (Fig. 5E). With these results, I could prove that Hh signaling promotes MT based mitochondrial transport and that DYRK1B plays a critical role in this process.

Another important MT-dependent functional process is the polarization of migrating mesenchymal cells. In this process, the microtubule organizing center (MTOC) is re-oriented towards the wound (scratch made on the confluent NIH3T3 fibroblasts). I performed the *in vitro* wounding assays in confluent fibroblast cultures and stained for the MTOC (using an α-Pericentriolar material 1 (PCM-1) antibody) and the MT cytoskeleton (αTub antibody). A scratch was made in the confluent cultures on the glass coverslip, and positive polarization towards the wound is indicated by an asterisk. As can be seen in Fig. 6A and 6B, induction of Hh signaling by SAG and inhibition of HDAC6 by ACY-1215 led to an increased polarization towards the wound. When I pharmacologically blocked DYRK1B by AZ191, I could see that it reduced the polarization process (Fig. 6C). To investigate further, I tested the impact of DYRK1B inhibition on Hh-driven scratch wound closure using live-cell imaging (Fig. 6D). In line with my previous results, SAG promoted the migration of NIH3T3 fibroblasts into an *in vitro* wound whereas DYRK1B inhibition reduced the migratory potential. This result was quantified and represented in Fig. 6E. This shows the importance of DYRK1B in Hh induced fibroblast polarization and cell motility.

6 Discussion

6.1 DYRK1B regulates Hh signaling pathway

Earlier reports on the role of DYRK1B on Hh pathway stated positive (Gruber et al. 2016) as well as negative (Lauth et al. 2010; Jacob et al. 2011; Keramati et al. 2014b) regulatory functions to this kinase. With my findings, I have bring together the previous results and clarify the regulatory role of DYRK1B in more detail. With my data, I have shown the dual and sometimes an opposing interaction of this kinase with Hh pathway: 1.) The ectopic expression of *DYRK1B* has been shown to block canonical SMO-initiated signaling, although the exact mechanism still needs to be elucidated. 2.) On the other hand, overexpressed *DYRK1B* stabilizes GLI1 by rescuing it from proteasomal degradation. DYRK1B is also known to activate the PI3K/mTOR/AKT signaling arm and there have been reports showing that AKT phosphorylates and protects GLI transcription factors from decay (Riobo et al. 2006; Shi et al. 2015). 3.) DYRK1B activates the PI3K/mTOR/AKT pathway which is subject to intense feedback regulation (Manning and Cantley 2007) and thereby the whole DYRK1B-Hh/GLI-system is subject to pronounced feedback control, which results in a strong kinetic influence on the Hh pathway output. As shown by my results, short-term inhibition of DYRK1B resulted in increased GLI protein levels whereas, long-term blockade of DYRK1B led to decreased GLI protein levels. With these findings, I would suggest, that the earlier reports, might have considered a specific part of the entire crosstalk spectrum and with these results, I could explain most, if not all the published results about the regulatory effects of DYRK1B on Hh signaling. The role of oncogenic RAS on Hh signaling has also been reported for its dubious effects (Ji et al. 2007; Stecca et al. 2007; Lauth et al. 2010; Zhao et al. 2015). And DYRK1B has been shown as a downstream effector of mutant KRAS (Jin et al. 2007) and was also discovered as synthetic lethal gene partner of mutant *KRAS* in a screen (Barbie et al. 2009), which might be explained by my results showing a connection between DYRK1B and PI3K/mTOR/AKT signaling. Adding to this the discovery of *DYRK1B* mutations in the metabolic syndrome and its involvement with PI3K signaling are fascinating.

Interestingly, I could observe the stress-induced response of DYRK1B kinase in one specific cell line tested which was able to promote GLI1 stability, even in the absence of clearly measurable *Gli1* and *Gli2* mRNA expression (Fig. 1F, S1D). Considering this effect, I could

imagine that GLI1 would be activated in various pathological events, by unrelated Hh regulators which, induce DYRK1B expression, bypassing the need for increased *GLI1/2* mRNA levels. My results have shown that ectopic expression of *DYRK1B* altered the appearance of GLI1 protein isoforms and a new 100 kDa variant was generated. Although, previous findings have also mentioned a variant of this size to be inhibitory (Stecca and Ruiz i Altaba 2009), another shorter isoform of 130 kDa seems to be activating (Amable et al. 2014). My data leads us to some of the open questions, about the shorter GLI1 isoform and would require further investigations.

There is enough evidence that Hh signaling leads to the induction of various kinases, including protein kinase B (PKB) or AKT. AKT kinase is a key mediator of PI3K signaling pathway and has two phosphorylation sites at Ser473 and Th308. My results have shown that DYRK1B is upregulated by SAG, and overexpression of this kinase leads to induced levels of mTOR and AKT phosphorylation, and depletion of this kinase by RNAi, abrogates the ability of Hh signaling to induce phosphorylation. The PI3K/mTOR/AKT pathway is one of the most frequently activated signaling cascades in human cancer (Fruman and Rommel 2014). The mTOR kinase is composed of two multi-protein complexes, mTORC1 and mTORC2. The first complex is downstream of AKT and is activated through TSC1/2 and Rheb proteins whereas the latter complex is upstream of AKT and is activated by PI3K in an unknown manner (Shimobayashi and Hall 2014). The mTORC1 complex is known for being a major regulator of protein translation and autophagy while mTORC2, amongst others, impinges on cell survival through regulation of FOXO and PKCα (Guertin et al. 2006; Shiota et al. 2006). My hypothesis that DYRK1B might regulate mTORC2, as both of them is shown to phosphorylate AKT at Ser473. Also, confirming the results of RNAi, I could show that with the knockdown of the DYRK1B kinase, phosphorylation levels of AKT at Ser473 are reduced, but it had no effect phospho-S6/S6K (read-out of mTORC1 activity). As AKT, DYRK1B has also been described as a survival kinase before (Deng et al. 2006; Mercer et al. 2006).

Altogether, my results describe a complex crosstalk between DYRK1B and Hh signaling. As per the suggested model, the effects of DYRK1B kinase on oncogenic Hh signaling are dependent on several factors, such as the expression level of DYRK1B, canonical/non-canonical Hh signaling, analysis time intervals or cell type. Due to these varying factors, which can affect the plausible outcome, if DYRK1B kinase is used in a clinical setting as a

target, I tried a combination treatment where I used pharmacological inhibitors of DYRK1B along with inhibitors of AKT, mTOR, and S6K. In *DYRK1B* amplified pancreatic and ovarian cancer cells the combination treatment yielded increased cell death and a significant reduction in GLI1 level which could direct us to have novel cancer therapy in the future.

6.2 DYRK1B regulates Hh induced tubulin acetylation

Microtubule-dependent cellular processes have been widely studied and the mechanism behind these processes is very well known. But, how different signaling pathways and extracellular ligands affect the cytoskeletal events, is largely unknown. With my results, I try to show how Hh signaling affects MT acetylation and MT-dependent processes by DYRK1B induction. DYRKs, unlike many other kinases, are mainly regulated by their overall abundance, thereby a slight increase in the amount could have large and significant effects on various cellular processes. This is shown for instance by the devastating effect of the 1.5-fold increase in DYRK1A on neuronal and brain development in Down syndrome (Trisomy 21) patients (Gardiner et al. 2010; Duchon and Herault 2016). DYRK1B kinase has been known to promote motility in ovarian cancer cells (Collins et al. 2006) which often harbor a 19q13 chromosomal *DYRK1B*-containing amplicon or display elevated levels of DYRK1B kinase expression by other means (Friedman 2013). In addition, work in pancreatic cancer has shown DYRK1B can protect the cells from the depolymerization agent Nocodazole (Deng et al. 2006). My findings, provide a mechanistic explanation for these observations and show that DYRK1B regulates MT acetylation. I have shown that the cellular DYRK1B pool is localized to microtubule cytoskeleton. Ectopic expression of DYRK1B promotes the phosphorylation of GSK3β at Ser9 position, which in turn inactivates GSK3β. GSK3β has been shown to have a stimulatory effect on HDAC6. Thereby, overexpression of DYRK1B kinase leads to inactivation of GSK3β which in turn leads to the reduction of HDAC6 activity, which regulates tubulin acetylation. GSK3β and HDAC6 are known to associate with MTs and as shown in Fig. 6F, I can speculate that these proteins form a functional unit around microtubules. The elucidation of how exactly HDAC6 is regulated by GSK3β awaits further studies.

DYRK1B is known to modulate the PI3K/mTOR/AKT signaling pathway, where feedback control mechanisms play a critical role (Singh et al. 2017). Glycogen synthase kinase 3 (GSK-3) has been shown to affect several biological processes such as metabolism gene

expression, cell fate determination, proliferation, and survival. GSK-3 activity is inhibited through phosphorylation of serine 21 in GSK-3 alpha and serine 9 in GSK3β. AKT is known to phosphorylate GSK3β, independent of the DYRK1B kinase. Due to this complex crosstalk, the final effect on tubulin acetylation is difficult to speculate, especially when feedback mechanisms are active.

My results support Hh mediated tubulin acetylation and this is in line with the previously published data (Lee and Ko 2016). With my findings, I address more mechanistic approach by integrating extracellular Hh ligands to intracellular cytoskeletal outputs. In this respect, it is interesting to note that both, Hh signaling as well as HDAC6 inhibition/MT acetylation were found to drive Interleukin-10 production, which is an anti-inflammatory cytokine and has multiple pleiotropic effects (Wang et al. 2014; Lee et al. 2016a). From previous reports, and my results, which are in line with the findings, I know that DYRK1B and HDAC6 are the regulatory components of the Hh cascade which, increases the possibility for cytoskeleton-mediated autoregulation of the pathway (Lauth et al. 2010; Dhanyamraju et al. 2014; Gruber et al. 2016). Hedgehog signaling pathway is often subject to a complex crosstalk, and my results, show that the effects on MT regulation are dependent on tubulin-PTMs, and not through motor proteins. Thereby understanding the regulation of cell migration became more complicated. Whether these processes involve canonical or non-canonical pathway activation, needs further work. Several noncanonical mechanisms have been reported to modulate Hh induced cell migration such as non-ciliary SMO and/or Gli-independent regulation of the actin cytoskeleton (Bijlsma et al. 2007; Polizio et al. 2011a; Polizio et al. 2011b; Bijlsma et al. 2012).

With my results, I have looked at the functional aspects of the microtubule regulation by post-translational modifications and have shown that Hh signaling promotes mitochondrial transport along microtubules in fibroblasts. These were considerably novel work as, before this, studies have reported MT-based mitochondrial motility in neuronal cells where long axons mediate the mitochondrial transport to distant sites for local ATP production (Saxton and Hollenbeck 2012). There have been studies showing the importance subcellular localization of mitochondria in migrating epithelial cancer cells (Desai et al. 2013). As per the central bioenergetics role of mitochondria in eukaryotic cells, several links have been suggested on metabolic regulation of mitochondrial dynamics (Mishra and Chan 2016). At the same time, studies have shown *DYRK1B* mutations in families with metabolic syndrome

(Keramati et al. 2014a) where a potential role of mitochondria would be interesting to follow up.

Altogether, with my results I have shown how ligand based Hh signaling pathway modulates the PTM of MTs and positively affect DYRK1B kinase and subsequently affect the intracellular processes like cell polarization, migration and organelle transport.

6.3 Emerging Roles of DYRKs in Embryogenesis & Hh Pathway Control

DYRK family of kinases have been highly conserved from yeast to humans. In my review, I have tried to outline the currently available knowledge on the DYRK family of kinases engaging in developmental biology, physiology, and pathology, focusing on its impact on Hh signaling. I have also discussed the significance of this family of kinases and their roles in embryogenesis and Hh signaling pathway. Several reports have linked DYRK kinases to Hh signaling, which suggests a close regulation between these kinases and Hh signaling. Henceforth, it can be hypothesized that they contribute to Hh mediated steps during embryonic development. Although a clear mechanistic picture is still missing, which would identify the intense crosstalk between DYRKs and Hh, particularly in *in vivo* settings. As certain DYRKs have a preferred expression in specific tissues (e.g., DYRK1A in neuronal and DYRK1B in muscle tissue), it is reasonable to speculate that the impact on the tissue-selective Hh pathway activity is specified by the respective DYRK enzyme. Although, some sort of functional redundancy might exist, in tissues or cell types where several DYRK kinases are expressed together. DYRK1A has been linked intensively with neuronal tissue development and it has prosurvival function and negatively regulates the apoptotic process. DYRK1B has been linked with muscle tissue and enhances the transcriptional activity of TCF/HNF1A and FOXO1. It has been also shown to promote adipogenesis. From a therapeutic point of view, this might be important for the development of small molecule inhibitors, which might lack necessary specificity and target several DYRKs simultaneously. DYRK kinase has also been known to modulate other signaling pathways, such as the NFAT (nuclear factor of activated T-cells) (Gwack et al. 2006) pathway or HIF (hypoxia-inducible factor) signaling (Lee et al. 2016c). Future work will reveal whether Hedgehog or any of the other signaling systems is particularly important for the physiological impact of DYRK kinases.

Most studies for developmental crosstalk between DYRKs and Hh stems from DYRK1A, neuronal development, and Down syndrome. Although in recent studies on DYRK1B and DYRK2 discussing the roles of Hh signaling in muscle development, adipocytic differentiation has also helped in understanding other family members of DYRKs. Recent reports involving the effects of DYRKs in metabolic syndrome and cancer have given us the cue for interesting and significant roles of these kinases in cross-talk with Hh and other signaling pathways.

7 References

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8 Appendix

8.1 List of Academic teachers

My academic teachers:

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8.3 Publications

- **Singh R**, Holz P, Roth K, Hupfer A, Meissner W, Müller R, Buchholz M, Gress T, Elsässer H, Jacob R, Lauth M. DYRK1B regulates Hedgehog-induced microtubule acetylation. (submitted)
- **Singh R**, Lauth M. Emerging Roles of DYRK Kinases in Embryogenesis and Hedgehog Pathway Control. **Journal of Developmental Biology 2017**.
- **Singh R**, Dhanyamraju P, Lauth M. DYRK1B blocks canonical and promotes non-canonical Hedgehog signaling through activation of the mTOR/AKT pathway. **Oncotarget 2017**.

DYRK1B regulates Hedgehog-induced microtubule acetylation

Rajeev Singh[1,6], Philipp Simon Holz[1,6], Katrin Roth[2], Anna Hupfer[1], Wolfgang Meissner[1], Rolf Müller[1], Malte Buchholz[3], Thomas M. Gress[3], Hans-Peter Elsässer[4], Ralf Jacob[4], Matthias Lauth[1,5]

[1] Philipps University, Institute of Molecular Biology and Tumor Research (IMT), Center for Tumor- and Immune Biology (ZTI), Hans-Meerwein-Str. 3, 35043 Marburg, Germany

[2] Imaging Core Facility, Philipps University, Center for Tumor- and Immune Biology (ZTI), Hans-Meerwein-Str. 3, 35043 Marburg, Germany

[3] Clinic for Gastroenterology, Endocrinology, Metabolism and Infectiology, Philipps University, Marburg, Germany

[4] Philipps University, Institute of Cytobiology and Cytopathology, Robert Koch Str. 6, 35037 Marburg, Germany

[5] Corresponding author: Matthias Lauth, email: lauth@imt.uni-marburg.de

[6] Equally contributing authors

Nonstandard abbreviations

DYRK1B: Dual specificity-regulated kinase 1B (a.k.a. MIRK)

Hh: Hedgehog

SHH: Sonic Hedgehog

GLI1: Glioma-associated oncogene 1

HDAC6: Histone deacetylase 6

MT: Microtubule

AcTub: Acetylated α-tubulin

SAG: Smoothened agonist

SANT: Smoothened antagonist

MTOC: Microtubule (MT) organizing center

Keywords:

Hedgehog; DYRK1B; HDAC6; Microtubules; Acetylation; organelle transport; cell migration.

Abstract

The posttranslational modification (PTM) of tubulin subunits is important for the physiological functions of the microtubule (MT) cytoskeleton. Although major advances have been made in the identification of enzymes carrying out MT-PTMs, little knowledge is available on how intercellular signaling molecules and their associated pathways regulate MT-PTM-dependent processes inside signal-receiving cells. Here we show that Hedgehog (Hh) signaling, a paradigmatic intercellular signaling system, affects the MT acetylation state in mammalian cells. Mechanistically, Hh pathway activity increases the levels of the MT-associated DYRK1B kinase, resulting in the inhibition of GSK3β through phosphorylation of Serine 9 and the subsequent suppression of HDAC6 enzyme activity. Since HDAC6 represents a major tubulin deacetylase, its inhibition increases the levels of acetylated MTs. Through the activation of DYRK1B, Hh signaling facilitates MT-dependent processes such as intracellular mitochondrial transport, mesenchymal cell polarization or directed cell migration. Taken together, we provide evidence that intercellular communication through Hh signals can regulate the MT cytoskeleton and contribute to MT-dependent processes by affecting the level of tubulin acetylation.

Introduction

The microtubule (MT) cytoskeleton is crucial for a vast number of cellular processes including signal transduction, organelle transport, mitosis and cell migration. A major mode of MT regulation is through posttranslational modification (PTM) such as acetylation, phosphorylation, polyglycylation, polyglutamylation and others [1-3]. One of the best-studied modification is acetylation, which can occur on Lys40 of the α-subunit of the α/β-heterodimer within MTs. Acetylated α-tubulin (AcTub) is often associated with stable and long-lived MTs, such as those observed e.g. in primary cilia. Although initial speculations about tubulin PTMs affecting transport velocity along MTs were questioned later, these modifications seem to determine the binding specificity of selected motor proteins and thereby facilitate the transport of certain cargoes along MT tracks [4-7]. Indeed, increasing tubulin acetylation has been shown to promote MT-directed mitochondrial transport in neurons [8] and to compensate for vesicular transport deficits in a cellular model of Huntington's disease [9]. Moreover, recent data show that acetylation protects MTs from mechanical breakage, which might affect transport processes indirectly [10, 11].

Key enzymes regulating MT acetylation are α-tubulin acetyl transferase (ATAT, a.k.a. αTAT1 or MEC17) and histone deacetylase 6 (HDAC6), which add or remove acetyl groups from α-tubulin, respectively [12-14]. HDAC6 is a multifunctional protein with sequence homology to nuclear HDACs, deacetylating many non-histone proteins such as tubulin [15]. It is involved in a wide variety of cellular processes including signal transduction [16-19], aggresome formation [20, 21], stress granule biology [22] and gene transcription [23].

Although a lot of information about the enzymes governing tubulin PTMs has been gathered in recent years, comparatively little knowledge is available about how this cytoskeletal regulation is controlled by signaling networks. This information would be of particular interest as signaling pathways are perfectly suited to sense extracellular conditions and to translate these cues into modifications of the intracellular cytoskeleton.

One example of such a signaling system is the evolutionary conserved Hedgehog (Hh) pathway. Hedgehog signaling is absolutely essential for proper embryonic development [24, 25] and its overactivation is associated with numerous forms of cancer [26-28]. In many instances during development, tissue repair or cancer, Hh ligands (Sonic Hh (SHH), Desert Hh

(DHH), Indian Hh (IHH)) signal from epithelial to neighboring mesenchymal cell types [29, 30]. Binding to the primary cilia-localized Patched1 (PTCH1) receptor releases Smoothened (SMO) from PTCH1-mediated inhibition and allows for the activation of the GLI family of transcription factors (GLI1, GLI2, GLI3) [31-35]. While Hh signaling has been shown to affect the actin cytoskeleton [36-39], very little is known about its effects on MTs.

In this work, we show that activated Hh signaling promotes the acetylation of microtubules and contributes to AcTub-dependent processes such as cell polarization, migration or organelle transport. Mechanistically, we identify the Hh-regulated DYRK1B kinase as a negative modulator of GSK3β, leading to the suppression of HDAC6 enzyme activity and an increase in tubulin acetylation. Using biochemical assays as well as super-resolution microscopy we could further show that DYRK1B, GSK3β and HDAC6 are associated to MTs. In summary, using Hh signaling as a paradigm, we describe a mechanistic framework how intercellular communication can impinge on cytoskeletal regulation and cell function.

Results

Hedgehog signaling regulates tubulin acetylation

In line with another recent report [40], we found that Hh signaling promotes the increase of α-tubulin acetylation in fibroblasts, a cell type representing a major Hh-responsive cell population *in vivo*. When NIH3T3 cells, cultured under low serum conditions, were treated with the Hh pathway-activating synthetic compound SAG (Smoothened agonist) [41], we observed an induction of acetylated tubulin (AcTub) in immunofluorescence (Fig. 1A,B) and in Western blotting experiments (Fig. 1C,D). This AcTub increase correlated with the induction of the Hh pathway target GLI1 and the levels of the Dual-specificity Tyrosine (Y)-regulated kinase 1B (DYRK1B, a.k.a. as MIRK) [42] which we have previously shown to be upregulated by Hh signaling [43] (Fig. 1D). However, *Dyrk1b* mRNA levels were not significantly affected by Hh signaling, as were the levels of AcTub regulating enzymes such as *Hdac6* and *Mec17 (αTat1)* (Fig. S1A), arguing for a post-transcriptional regulation of DYRK1B by Hh.

Furthermore, Hh-induced tubulin acetylation and DYRK1B induction was also observed in other fibroblast cells such as in SAG-treated mouse embryonic fibroblasts (MEFs) (Fig. 1E), demonstrating that this effect was not restricted to NIH3T3 cells. To rule out a Hh-unrelated effect of the compound SAG, we investigated MEF cells stably expressing SHH ligand ($MEF^{[SHH]}$ cells) and found that pathway inhibition with the SMO inhibitor SANT [41] concomitantly reduced the levels of AcTub and DYRK1B (Fig. 1F). Based on these experiments we hypothesized that DYRK1B could be involved in mediating all or some of the effects of Hh on AcTub.

In order to demonstrate that the Hh-induced AcTub increase was indeed mediated through DYRK1B, we knocked down endogenous *Dyrk1b* by means of RNAi in MEF cells. As can be seen in figure 1G, the SAG-mediated increase in acetylated tubulin could be fully blocked upon removal of DYRK1B. In support of our finding of Hh-regulated tubulin acetylation, the elevated AcTub levels previously seen in $MEF^{[SHH]}$ cells could be reduced by siRNA transfection targeting *Dyrk1b* (Fig. 1H). These experiments suggest that Hh signaling increases DYRK1B protein levels by posttranscriptional mechanisms leading to a rise in DYRK1B-mediated tubulin acetylation.

The expression of DYRK1B is sufficient for AcTub induction

Given the cross-talk between Hh signaling and tubulin acetylation, we were interested to address the role of DYRK1B in more detail. To this end, we generated NIH3T3 cells stably expressing a V5-tagged form of this kinase (NIH3T3$^{[DYRK1B]}$ cells). Intriguingly, these cells displayed a strikingly different morphology in culture when compared to control cells (Figure 2A). While control cells (NIH3T3$^{[Mock]}$) possessed the expected elongated, spindle-shaped morphology of mesenchymal cells, the *DYRK1B*-overexpressing cells had much smaller and rounder cell bodies with longer cellular extensions, suggesting a potential cytoskeletal effect induced by the increased *DYRK1B* expression. Indeed, when analyzing the levels of AcTub in these cells, we found strikingly elevated levels of acetylated tubulin (Fig. 2B,C,D), showing that the sole overexpression of *DYRK1B* can lead to AcTub induction. Intriguingly, DYRK1B-induced acetylated MT were more resistant to the depolymerizing activity of Nocodazole (Fig. S2A,B), which is in line with a previous report describing a protective function of DYRK1B against Nocodazole [44]. We furthermore investigated whether an increased *DYRK1B* expression might affect the morphology of primary cilia, a cellular organelle rich in AcTub and essential for proper Hh signal transduction [45, 46]. However, by using immunofluorescent staining with an α-detyrosinated tubulin antibody (a primary cilia marker) as well as by electron microscopy we were unable to detect obvious morphological aberrations in the (ultra)structure of primary cilia upon *DYRK1B* overexpression (Fig. S2C).

Next, we went on to investigate whether the finding of increased AcTub levels in *DYRK1B*-expressing cells was specific to fibroblasts. As can be seen in figures 2E,F,G, we could recapitulate these observation also in Hela cells stably transfected with *DYRK1B* (Hela$^{[DYRK1B]}$ cells). Since HDAC6 is known as a major determinant of the tubulin acetylation status, we used the Hela$^{[DYRK1B]}$ cells to investigate whether DYRK1B expression would diminish the overall HDAC6 enzyme activity when compared to control cells. We immunoprecipitated endogenous HDAC6 from control and from *DYRK1B*-expressing Hela cell lines and subjected the precipitate to a luminometric *in vitro* deacetylase assay. Indeed, the HDAC6 enzyme activity (normalized to the amount of total HDAC6 protein precipitated) was significantly lower (by about 40 %) in *DYRK1B*-expressing cells. This difference was blunted when a HDAC6-selective inhibitor (Cay10603) was co-applied to the deacetylase assay (Fig. 2H). Taken together, these experiments demonstrate that the increased expression of DYRK1B is

sufficient to elicit MT acetylation in the absence of Hh receptor activation. Furthermore, increased DYRK1B levels result in functional downregulation of HDAC6, a known master regulator of MT acetylation.

DYRK1B phosphorylates the inhibitory Ser9 site of GSK3β

In our attempts to decipher the mechanistic link between DYRK1B and HDAC6, we speculated that the DYRK1B kinase might directly phosphorylate HDAC6. To address this issue, we performed *in vitro* kinase assays with both proteins but failed to observe a direct phosphorylation (not shown), which prompted us to hypothesize an indirect mechanism. One example of such a mechanism would envision that DYRK1B does not phosphorylate HDAC6 directly, but instead phosphorylates an HDAC6-regulating protein. Therefore, we focused on Glycogen synthase kinase 3 β (GSK3β), which had previously been shown to regulate HDAC6 in a stimulatory manner [8]. Hence, we wanted to find out whether Hh/DYRK1B would inhibit GSK3β, thereby indirectly leading to the suppression of HDAC6 activity. To this end, we first investigated whether Hh signaling modulates GSK3β. In fact, immunoblot analyses of lysates from SAG-treated cells revealed that Hh activity promotes the phosphorylation of Ser9 (Fig. 3A), an important regulatory residue known to control phosphorylation-induced GSK3β inactivation. In contrast, blocking Hh pathway activity in continuously *SHH*-expressing cells reduced the levels of phospho-GSK3β^{S9} (Fig. 3B).

Next, we investigated whether DYRK1B plays a role in Hh-induced GSK3β phosphorylation and found that RNAi-mediated knock-down of endogenous *Dyrk1b* resulted in a concomitant reduction of phospho-GSK3β^{S9} levels in MEF$^{[SHH]}$ cells (Fig. 3C). In addition, the pharmacological blockade of DYRK1B with the selective inhibitor AZ191 [47] reduced phospho-GSK3β^{S9} and AcTub levels in SAG-treated cells (Fig. 3D). Moreover, the stable expression of *DYRK1B* (in the absence of Hh signaling) was sufficient to induce GSK3β phosphorylation (Fig. 3E), suggesting a close functional connection between these two players. Therefore, we performed *in vitro* kinase assays and found that recombinant DYRK1B was able to phosphorylate immunoprecipitated GSK3β on Ser9 under *in vitro* conditions (Fig. 3F). In summary, we could provide evidence that Hh signaling, through upregulation of DYRK1B, inactivates GSK3β by phosphorylation on the important control residue Ser9. This subsequently inhibits HDAC6 enzyme activity, leading to an increase in cellular AcTub levels.

DYRK1B is associated with the MT cytoskeleton

Since our data suggested that DYRK1B regulates tubulin acetylation and since it was known that HDAC6 and GSK3β are localized to MTs [12, 48-50], we were interested whether DYRK1B would also be associated to MTs. To analyze this issue in more depth, we performed biochemical MT-association assays (MTaa) using control or SAG-treated NIH3T3 cells in order to purify protein fractions bound to polymerized MTs. As can be seen in figure 4A, a fraction of total DYRK1B (as well as GSK3β which was included as positive control) could consistently be found in the pelleted fraction ('MT') containing polymerized MTs. Stimulation with the SMO agonist SAG led to increased levels of MT-bound DYRK1B, which was however most likely due to an overall increase in protein amount and not due to a specific recruitment to MTs.

In order to support the MT-localization of DYRK1B by an independent technical approach, we performed sub-diffraction super-resolution microscopy (GSD-Ground State Depletion microscopy) and were able to visualize transfected V5-tagged DYRK1B on endogenous MTs (Fig. 4B) in human fibroblasts (PSC). As shown in figure S3A, DYRK1B also regulates AcTub levels in these cells. In addition, super-resolution microscopy also revealed the localization of endogenous DYRK1B on single MT tracks in Hela cells (which were used as the endogenous DYRK1B levels in PSC cells were difficult to visualize by microscopy) (Fig. 4C). As a positive control of another protein previously reported to be MT-associated, we were able to detect endogenous HDAC6 on defined MT tracks in PSC fibroblasts (Fig. 4D). Taken together, using biochemical as well as microscopic techniques we could provide evidence for DYRK1B being associated with MTs, the expected subcellular localization for a MT-regulating protein.

Hh signaling enhances the intracellular transport of mitochondria

After having investigated mechanistic aspects of the Hh-DYRK1B-GSK3β-HDAC6-AcTub axis, we wanted to address the functional consequences of this chain of events. To this end, we investigated different cellular processes which have been described as being dependent on MTs and which are potentially influenced by MT-PTMs: Intracellular mitochondrial transport and mesenchymal cell polarization coupled with directed cell migration. First, we analyzed

MT-dependent mitochondrial transport where tubulin acetylation had been shown to facilitate organelle motility in neurons [8]. In order to investigate whether Hh signaling affects mitochondrial transport, we generated NIH3T3 cells stably expressing fluorescent Dendra protein fused to a mitochondrial targeting sequence derived from human cytochrome c oxidase subunit 8a (NIH$^{[Cox8a\text{-}Dendra]}$ cells). The mitochondrial expression of this fusion protein was verified by its perfect co-localization with MitoTracker (Fig. 5A). In addition, we also verified the SAG- and DYRK1B-dependent regulation of AcTub levels in these cells (Fig. 5B).

Using live cell imaging on the NIH$^{[Cox8a\text{-}Dendra]}$ cells, we first demonstrated that MT depolymerization by means of Nocodazole addition significantly reduced the overall distance (track length) and the speed of labelled mitochondria, verifying the importance of MT-dependent transport in this process (Fig. S4A,B). In addition, when we plotted the mean square displacement (MSD) rate as a quantitative measure for directionality [51], we observed a decreased directionality in mitochondrial transport, as would be expected in a situation in which the MT tracks have been destroyed (Fig. S4C).

Next, we investigated the impact of Hh activation (SAG), DYRK1B inhibition (AZ191) and HDAC6 inhibition (ACY-1215 [52]) on mitochondrial transport. We decided to measure a longer time frame (3h) than in the previous Nocodazole experiment, with less resolution in order to get an idea of physiologically meaningful intracellular distances (although this meant that we might not have recorded all short lateral movements). Nevertheless, our recordings clearly showed that, when compared to untreated control cells, SAG stimulation significantly increased the overall track length of transported mitochondria (Fig. 5C). Importantly, this effect could be completely abrogated by co-application of the DYRK1B antagonist AZ191 (Fig. 5C). In line with the hypothesized function of acetylated tubulin in organelle mobility, the mere induction of AcTub levels by the small molecule HDAC6 inhibitor ACY-1215 also led to a significant increase in mitochondrial track length. In addition, also the mitochondrial transport speed was significantly increased by ACY-1215 and there was a trend towards increased speed with SAG application. Again, pharmacological blockade of DYRK1B resulted in a clear reduction in mitochondria transport speed in SAG-treated cells (Fig. 5D). Plotting the MSD revealed that Hh signaling (SAG) increased the directed movement whereas the co-application of AZ191 completely abrogated this surplus in directionality (Fig. 5E). Moreover, inducing tubulin acetylation by

pharmacological HDAC6 inhibition (ACY-1215) also led to more directionality in mitochondrial transport, strongly suggesting that these effects are largely mediated through tubulin acetylation. In summary, we could provide evidence for Hh signaling enhancing MT-based intracellular organelle transport and for a critical role of DYRK1B in this process.

Hh promotes cell polarization and directed migration through DYRK1B

In our attempts to link Hh signaling, DYRK1B and MT-PTMs to physiological events, we next turned to another MT-dependent process: The polarization of migrating mesenchymal cells, a process which can be recapitulated in *in vitro* wounding assays. In confluent cultures of fibroblasts, cells are usually not polarized towards a particular direction and the microtubule organizing center (MTOC) can be found randomly localized around the nucleus. However, if a scratch would is applied to the cultured monolayer, cells at the border re-orient their MTOC towards the wound. This sequence of events requires, among others, inactive GSK3β and the MT-bound motor protein dynein [53-56]. In addition, cell polarization and the subsequent directed cell migration towards the wound need the stabilization of MTs [54].

Therefore, we performed *in vitro* wounding assays in confluent fibroblast cultures and stained for the MTOC (using an α-Pericentriolar material 1 (PCM-1) antibody) and the MT cytoskeleton (α-αTub antibody). As can be seen in figure 6A,B (and S5A), induction of Hh signaling by SAG led to an increase in cell polarization towards the wound. Increased cell polarization was also observed with the independent MT stabilizer ACY-1215 (HDAC6 inhibitor) (Fig. 6A,B). In line with our previous results on the involvement of DYRK1B in AcTub regulation, we found that blocking DYRK1B function with AZ191 abrogated the Hh-mediated mesenchymal cell polarization (Fig. 6C).

As the polarization of mesenchymal cells is the first step for directed migration into e.g. wounded areas, we tested the impact of DYRK1B inhibition on Hh-driven scratch wound closure using live-cell imaging (Fig. 6D). As expected, when compared to control cells, SAG promoted the migration of NIH3T3 fibroblasts into an *in vitro* wound (Fig. 6E). When AZ191 was co-administered, this increase in migratory potential was blunted, demonstrating an important role for DYRK1B in Hh-induced fibroblast polarization, cell motility and experimental wound closure.

Discussion

A considerable amount of data has been accumulated on the mechanisms of MT-dependent cellular processes such as intracellular transport and mitosis, but little knowledge exists on how extracellular ligands actually modulate cytoskeletal events. Here, we show that Hh signaling has the capability to affect MT acetylation and MT-dependent processes through induction of DYRK1B. In contrast to many other kinases, DYRKs are mainly regulated through their overall abundance. Even small changes in total DYRK amount can have significant impact on cellular functions, as evidenced for instance by the devastating effect of the 1.5-fold increase in DYRK1A levels on neuronal and brain development in Down syndrome (Trisomy 21) patients [57]. As such, we anticipate that also moderate Hh-induced DYRK1B increases could have larger effects on cellular processes, such as tubulin acetylation. We are however also aware of the fact that highly complex processes such as cell migration involve numerous regulators on several cellular levels and that the post-translational modification of tubulin most likely exerts a modifying role and is not the sole cause of these processes.

DYRK1B has previously been identified as a potent pro-migratory gene in ovarian cancer cells [58], which often harbor a 19q13 chromosomal *DYRK1B*-containing amplicon or display elevated expression of this kinase by other means [59]. In addition, work in pancreatic cancer revealed that DYRK1B can protect cells from the MT-depolymerizing agent Nocodazole [44]. Our data provide a mechanistic explanation for these observations and present evidence for the role of this kinase in regulating MT acetylation. We find that a fraction of the cellular DYRK1B pool is localized to the microtubule cytoskeleton. Functionally, it inactivates GSK3β by direct phosphorylation of Ser9, leading to the indirect suppression of HDAC6 enzyme activity, a major cellular regulator of tubulin acetylation. Since both, GSK3β and HDAC6 can also associate to MTs, it is reasonable to speculate that these proteins form a functional unit at or around MTs (Fig. 6F). The elucidation of how exactly HDAC6 is regulated by GSK3β awaits further studies.

Of note, we have previously shown that DYRK1B is able to manipulate PI3K/AKT signaling, which itself is subject to intense feedback control mechanisms [43]. As AKT can potentially phosphorylate GSK3β independently of DYRK1B, this complex network of signaling molecules could complicate the predictability of the net effect of tubulin acetylation,

particularly at time points at which feedback mechanism are still at play. Adding to this situation are somatic mutations activating the PI3K/AKT kinase arm in a constitutive manner as found in many cancer cells.

Supporting and extending previously published evidence on Hh-regulated tubulin regulation [40], our manuscript integrates this regulation into a wider mechanistic framework ranging from Hh ligands to modulation of intracellular cytoskeletal outputs. In this respect, it is interesting to note that both, Hh signaling as well as HDAC6 inhibition/MT acetylation were found to drive Interleukin-10 production [60, 61]. Furthermore, DYRK1B and HDAC6 seem to be regulatory components of the Hh cascade itself, raising the possibility for cytoskeleton-mediated autoregulation of the pathway [16, 62, 63].

Our results of Hh-mediated MT control through effects on tubulin-PTMs, and not through effects on e.g. motor proteins, add a new layer of complexity to the regulation of directed cell migration, a process involving a large number of proteins. Whether these effects utilize the canonical Hh signaling cascade or whether other 'non-canonical' mechanisms are responsible requires further investigations. Certain non-canonical mechanisms at several levels have been reported to modulate Hh-induced cell migration, such as non-ciliary SMO and/or GLI-independent regulation of the actin cytoskeleton [36-38, 64].

Another interesting finding of this study is the fact that Hh signaling promotes the transport of mitochondria along MTs in non-neuronal cells such as fibroblasts. Until now, MT-based mitochondrial motility has been mostly investigated in neuronal cells, where long axons necessitate the transport of mitochondria to distant sites for local ATP production [65]. However, there is emerging evidence that the subcellular localization of mitochondria is also important for cell migration in non-neuronal cells [66]. In addition, the first links between mitochondrial dynamics and cellular metabolism are considered [67]. In light of the fact that *DYRK1B* has recently been associated with the metabolic syndrome [68, 69], a potential role of mitochondria will be interesting to follow up. Furthermore, Hh signaling has been implicated in controlling the functionality of the T-cell immunological synapse, a structure relying, among others, on motor-protein driven mitochondrial transport [70, 71].

In summary, we present a mechanistic framework how extracellular Hh ligands can modulate the PTM status of MTs and can subsequently contribute to the regulation of intracellular MT-dependent processes such as cell polarization, migration and organelle transport.

Material and Methods

Cell lines

NIH3T3 and HeLa cell lines were purchased from ATCC. MEF and MEF$^{[SHH]}$ cells were kindly provided by Wade Bushman [72]. PSC cells were a kind gift of M. Löhr [73]. The generation of NIH3T3 cells stably expressing empty vector control or *DYRK1B* was described in [43]. All cell lines were mycoplasma-free and were cultured in Dulbecco's Modified Eagle Medium (DMEM (high Glucose plus Glutamine and Pyruvate), Invitrogen) supplemented with 10 % fetal bovine serum (FBS) and 1 % Penicillin/Streptomycin at 37°C with 5 % CO2. If not otherwise stated, serum concentrations were reduced to 0.5% during experiments for all cell types.

Small-interfering RNA (siRNA) transfection

Cells were transfected with 35 nM siRNA (Dharmacon SMARTpools and Qiagen control siRNA using RNAiMax (Invitrogen). Control siRNA (siCon) was purchased from Qiagen (All-Stars-siRNA; siAll). The mouse *Dyrk1b*-specific siRNA was an equimolar pool of four target sequences: si1b_1: AUACAGAGAUGAAGUACUA; si1b_2: GCACAUCAAUGAGGUAUAC; si1b_3: GAGAUGAAGUACUACAUAG; si1b_4: GGACAAAG*GAACUCAGGAA.* The human *DYRK1B*-specific siRNA target sequences were: si1B_3: GAGAUGAAGUACUAUAUAG; si1B_4: CGAAAGAACUCAGGAAGGA; si1B_5: GGUGAAAGCCUAUGAUCAU; si1B_6: GGACCUACCGCUACAGCAA.

RNA preparation, cDNA synthesis, qPCR

Total RNA was extracted using NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's protocol. cDNA synthesis of 1 µg total RNA was performed using iScript cDNA Synthesis Kit (Biorad) following the manufacturer's guidelines. Quantitative PCR reactions were performed using the Absolute QPCR SYBR Green Mix (ABGene). qPCR reactions were performed on 96 well qPCR plates (ABGene) using either the Mx3000P or Mx3005P qPCR systems (Agilent). Results were calculated as relative mRNA expression ($2^{\Delta\Delta Ct}$). Data was obtained from at least three independent experiments and is shown as the mean ± StDev. Primer sequences (5' to 3') for the detection of mouse *Dyrk1b* were: For-

TTGACACCTGCCCCTCCTCTAGCAC; Rev-GGCCCCCACAATATCGGTTGCTGTA. Human *DYRK1B*: For-TTGGCCAGGTGGTGAAAGCCTATGA; Rev-CAATCTGGGCCTGGTTCAGGAAAGC. Mouse *Hdac6*: For-TCCCTACAGCTTGGGGTTCTCAGCA; Rev-TCCCCAAATCCTTGTGTCAGCATCA. Mouse *Mec17*: For-TGACCGGGAGGCTCACAATGAGGTA; Rev-TGGGGCTCCACTCGCTCTTTCTGTA. All other primer sequences have been described elsewhere [16, 74-76].

Immunoblotting

Separation of lysates by SDS-PAGE was followed by subsequent Western Blot analysis. SDS-PAGE gels were blotted on Immobilon-PVDF membranes (Millipore) and incubated with the respective primary antibody, followed by an HRP-coupled secondary antibody. Detection of the HRP signal was performed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. The following primary antibodies were used: α-DYRK1A (#2771; Cell Signaling Technology (CST), Danvers, USA); α-DYRK1B (#5672; CST); α-DYRK2 (#8143; CST); α-DYRK3 (sc-390532; Santa Cruz Biotechnology, Santa Cruz, USA); α-GLI1 (#2643; CST); α-phospho-GSK3β^{S9} (#5558, CST); α-total GSK3β (#12456, CST); α-acetylated α-tubulin (AcTub, T6793, Sigma-Aldrich, St. Louis, USA); α-tyrosinated-α-tubulin; α-polyglutamylated-α/β-tubulin; α-α-tubulin (T6199, Sigma); α-Lamin B (sc-6217, Santa Cruz); α-GAPDH (#G9545; Sigma); α-β-Actin (#A5441; Sigma).

Immunofluorescence on fixed samples

Cells were seeded on cover slips and fixed with 4 % formaldehyde/PBS for 10 min at RT. After washing twice with PBS at RT for 5 min, cells were permeabilized with 0.5 % Triton-X100/PBS at RT for 5 min. For immunostaining, cover slips were blocked with 10 % serum/PBS for 1 h at RT and washed with PBS at RT for 10 min. Primary antibodies were diluted in PBS containing 10 % serum and 0.1 % saponin and incubated overnight at 4°C. After washing twice with PBS at RT for 5 min, the cover slips were incubated with fluorophor-coupled secondary antibodies diluted in PBS containing 10 % serum and 0.1 % saponin at RT in the dark for 2 h. After washing twice with PBS for 5 min and rinsing with H_2O, the cells were covered with mounting medium containing DAPI. Microscopy was performed on a Leica DMR epifluorescence and a Leica AF6000 widefield fluorescence microscope with 3D deconvolution software (Leica Microsystems, Wetzlar, Germany).

Microtubule-association assay (MTaa)

Fully confluent NIH3T3 fibroblasts were incubated on 10 cm culture dishes in 0.5 % FBS-containing DMEM with or without SAG (100 nM) for 48 h. Subsequently, cells were washed with warm PBS, scraped off and pelleted (300 g, 30 sec, RT), followed by resuspension in PBS/Taxol (20 µM) and incubation for 15 min at RT. After another centrifugation step (300 g, 30 sec, RT), cells were resuspended in 1 ml of room-temperature MTaa lysis buffer (1 mM EGTA, 0.05 % NP-40, 3 mM $MgCl_2$, 100 mM NaCl, 10 mM Tris pH 7.5 plus protease inhibitors) and an aliquot (400 µl) of the lysate was stored (whole cell lysate). The remaining cell lysate (600 µl) was layered on a cushion of cold MTaa lysis buffer containing 1M sucrose and centrifuged (400 g, 5 min, RT) to pellet the nuclear fraction. The supernatant was transferred to a new tube for ultracentrifugation (27.000 g, 45 min, RT) in order to pellet unwanted membrane debri. The supernatant was collected and another ultracentrifugation step was performed (100.000 g, 90 min, RT). The supernatant collected from this step was stored as cytoplasmic fraction and the pellet was taken as polymerized microtubule fraction.

Cell polarization assay

NIH3T3 cells were grown confluent on glass cover slips (24h) followed by another 24h in the presence of 100 nM SAG (0.5% FBS). Subsequently, DMSO, ACY1215 (10 µM) or AZ191 (0.5 µM) was added for 30 min followed by wounding of the confluent monolayer with a yellow pipette tip. Cells were washed once with medium, followed by addition of 3% FBS-containing medium containing SAG/DMSO/ACY-1215/AZ191 for 6 h at 37° C. Cells were fixed with 3.7% formaldehyde, stained with antibodies against α-tubulin and pericentriolar material 1 (PCM1) and mounted in Vectashield containing DAPI (Vectorlabs). Images shown in the manuscript are maximum intensity projections of 3D-deconvoluted Z-stacks taken on a Leica AF6000 widefield fluorescence microscope with 3D deconvolution software.

Ground state depletion microscopy (GSD)

GSD was performed on a Leica GSDIM Super Resolution SR microscope system according to the manufacturer's protocols using AlexaFluor488- and AlexaFluor647-labelled secondary antibodies. In some cases, the soluble cytoplasm was washed out before fixation by gently shaking the cells for 2x 5min in 1M EGTA/2.5mM GTP/4% PEG-6000/0.1M PIPES/0.2% Triton-X100 at room temperature.

Live cell measurement of mitochondrial transport

Cells were grown in chamber slides (Ibidi) in 0.5% FBS for 48h with/without SAG (100 nM), followed by addition of DMSO, ACY-1215 (10 µM) or AZ191 (0.5 µM). Live cell imaging was started approx. 30 min later. Recordings were taken on a laser scanning confocal microscope (LSCM) (Leica TCS-SP8i) with an incubation chamber tempered to 37°C. Recordings were made in 10 min intervals from several slide areas for a duration of 3h (20x objective, NA 0.75, 1024x1024 pixel, 2x average, zoom 3.0). Analysis of mitochondria motility from three cells was done in Imaris software (Bitplane, v8.2.0) using the spot algorithm. The overall movement of the cells was set to zero by using the surface algorithm before mitochondria calculations.

Statistical analysis

Unless otherwise stated, data is presented as the mean of three independent experiments ± standard deviation (StDev). Statistical significance was calculated by applying a two-tailed student's t-test. *p<0.05; **p<0.01; ***p<0.001.

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Competing interests

No competing interest declared.

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Figure legends

Figure 1: Hh signaling increases levels of DYRK1B and tubulin acetylation.

(A) Immunofluorescent AcTub (green) staining of NIH3T3 cells treated with either DMSO, Smoothened agonist SAG (100 nM) or SANT1 (200 nM). Scale bars equals 10 µm. Blue = DAPI staining.

(B) Quantification of AcTub intensities as shown in (A). Shown is the AcTub fluorescence intensity per cell measured in nine different viewing fields from 3 different experiments. The total cell number analyzed was 180-210.

(C) Immunoblot of NIH3T3 cell lysates treated with SAG. Shown is a representative blot of three.

(D) Quantification of (C) (mean of n=3 ±SD). Values were normalized to GAPDH levels or total α-Tubulin (aTub).

(E) Western blot of MEF lysates treated with SAG (100 nM, 48-72h). Shown is one blot of two.

(F) Immunoblot of MEF cell lysates stably expressing SHH ($MEF^{[SHH]}$ cells). Cells were treated with the SMO antagonist SANT to block Hh signaling. Shown is a representative blot of three.

(G) Western blot of MEF cell lysates transfected with control siRNA or with *Dyrk1b*-specific siRNA. Cells were treated with SAG (100 nM, 48h). Shown is one blot of two.

(H) Immunoblot depicting the changes in AcTub levels after RNAi-mediated knock-down of *Dyrk1b* in $MEF^{[SHH]}$ cells. Shown is one blot of two independent experiments.

Figure 2: DYRK1B expression is sufficient to augment tubulin acetylation.

(A) Bright field images of NIH3T3 cells stably expressing mock control (empty vector) or *DYRK1B*. Scale bar 50 µm.

(B) Immunoblot of NIH3T3 cells stably expressing DYRK1B or empty vector control (mock) showing the expression levels of the indicated proteins. Shown is one representative blot of four.

(C) AcTub immunofluorescence image (green) of control NIH3T3 (mock) or of cells with stable DYRK1B expression.

(D) Quantification of the results obtained in panel (C). Shown is the mean of n=5 ±SD. AcTub= Acetylated α-tubulin.

(E) Acetylated tubulin staining (green) in human Hela cells stably expressing *DYRK1B* or empty vector control (mock). Blue = Nuclei. Scale bar 100 µm.

(F) Western blot verifying DYRK1B-V5 overexpression of cells depicted in (E).

(G) Quantification of AcTub intensities of cells shown in (E). Shown is the mean of n=4 ±SD.

(H) *In vitro* HDAC6 enzyme assay (mean of n=3 ±SD). Endogenous HDAC6 protein was immunoprecipitated from Hela cells stably expressing empty vector control (mock) or *DYRK1B*. HDAC6 activity was subsequently measured with a luminometric HDAC assay. Values were normalized against western blot band intensities of immunoprecipitated HDAC6 (see inset as example). The selective HDAC6 inhibitor Cay10603 (100 nM) was used as a positive control.

Figure 3: Hh and DYRK1B inactivate GSK3β by Ser9 phosphorylation.

(A) Western blot example of n=2 independent experiments showing phospho-GSK3β^{S9} levels after SAG induction.

(B) Western blot data and the corresponding quantification (mean of n=3 ±SD) of MEF$^{[SHH]}$ cells treated with the SMO inhibitor SANT (200 nM, 48-72h).

(C) MEF$^{[SHH]}$ cell lysates transfected with control siRNA (siCon) or with *Dyrk1b*-directed siRNA (si1B). Shown is one representative blot of three.

(D) Western blot of NIH3T3 lysates. Cells were pre-treated with SAG (100 nM) for 48h prior to addition of the DYRK1B inhibitor AZ191 (AZ, 0.1 or 0.5 µM) for 3h.

(E) Quantification of Phospho-GSK3β^{S9} levels in NIH3T3 stably expressing empty vector (mock) or V5-tagged *DYRK1B* in two different serum concentrations (mean of n=3 ±SD). Inset: One representative blot.

(F) Quantification of *in vitro* kinase assays with recombinant DYRK1B enzyme and immunoprecipitated GSK3β. Shown is the mean of n=3 ±SD. Rec.1B = Recombinant DYRK1B. The inset depicts a representative blot.

Figure 4: DYRK1B is associated with microtubules.

(A) Microtubule-association assay (MTaa) of lysates derived from control NIH3T3 cells or of cells treated with SAG (100 nM, 48h). WCE = Whole cell extract; Nuc = Nuclear fraction; Cyt = Cytoplasmic fraction; MT = Polymerized MT-containing pellet. The levels of endogenous HDAC6 in control NIH3T3 cells were too low to be detected by Western blotting. Lamin B (mainly WCE + Cyt fractions) was included to demonstrate purity of fractions. Shown is one representative experiment of n=3.

(B) Super resolution images by means of Ground State Depletion (GSD) microscopy showing transiently transfected human PSC cells. Left panel: Mock-transfected; right panel: DYRK1B-V5 transfected. Red = endogenous β-tubulin. Green = V5-antibody. The orientation of microtubules is indicated as faint white dotted lines in the insets. A scale bar of 2.5 µm is given.

(C) GSD-image of non-transfected Hela cells. Red = endogenous α-tubulin. Green = endogenous DYRK1B.

(D) GSD-image of non-transfected PSC cells. Red = endogenous α-tubulin. Green = endogenous HDAC6. The soluble cytoplasm has been washed out before in this experiment.

Figure 5: Hh signaling facilitates organelle transport.

(A) Confocal image of NIH3T3 cells stably expressing a *Cox8a-Dendra2* fusion construct (NIH$^{[Cox8a\text{-}Dendra]}$; green). In addition, cells were co-stained with MitoTracker (Red). Nuclei appear in blue. Scale bar represents 10 µm.

(B) Levels of DYRK1B and acetylated tubulin in SAG (100 nM, 48h)- and AZ191 (DYRK1B inhibitor, 0.5 µM for last 2h)-treated NIH$^{[Cox8a\text{-}Dendra]}$ cells.

(C) Track length of mitochondria movement over a 3h time window in NIH$^{[Cox8a\text{-}Dendra]}$ cells. Shown is one representative experiment (20x objective) measuring three different cells of n=4 independent experiments. At least 500 events were recorded for each condition. Drug concentrations were: ACY-1215 (ACY, HDAC6 inhibitor) 10 µM; SAG 100 nM; AZ191 0.5 µM (all in 0.5%FBS).

(D) Speed of mitochondria movement over a 3h time window in NIH$^{[Cox8a\text{-}Dendra]}$ cells. Shown is one representative experiment (20x objective) measuring three different cells of n=4 independent experiments. At least 500 events were recorded for each condition. Drug concentrations were: ACY-1215 (ACY) 10 µM; SAG 100 nM; AZ191 0.5 µM (all in 0.5%FBS).

(E) Mean square displacement (MSD) over time. Shown is the MSD calculation (exponential curve fitting) of the experiment depicted in panels (C) and (D). An increase in directed transport is reflected as an increased slope.

Figure 6: Hedgehog promotes mesenchymal cell polarization and cell migration.

(A) Microscopic determination of NIH3T3 fibroblast polarization by means of MTOC (PCM1, red) and α-tubulin (green) staining. Nuclei appear in blue. The orientation of the scratch is indicated by a white dashed line. Positive polarization towards the wound is indicated by a white asterisk. For experimental details see materials and methods section.

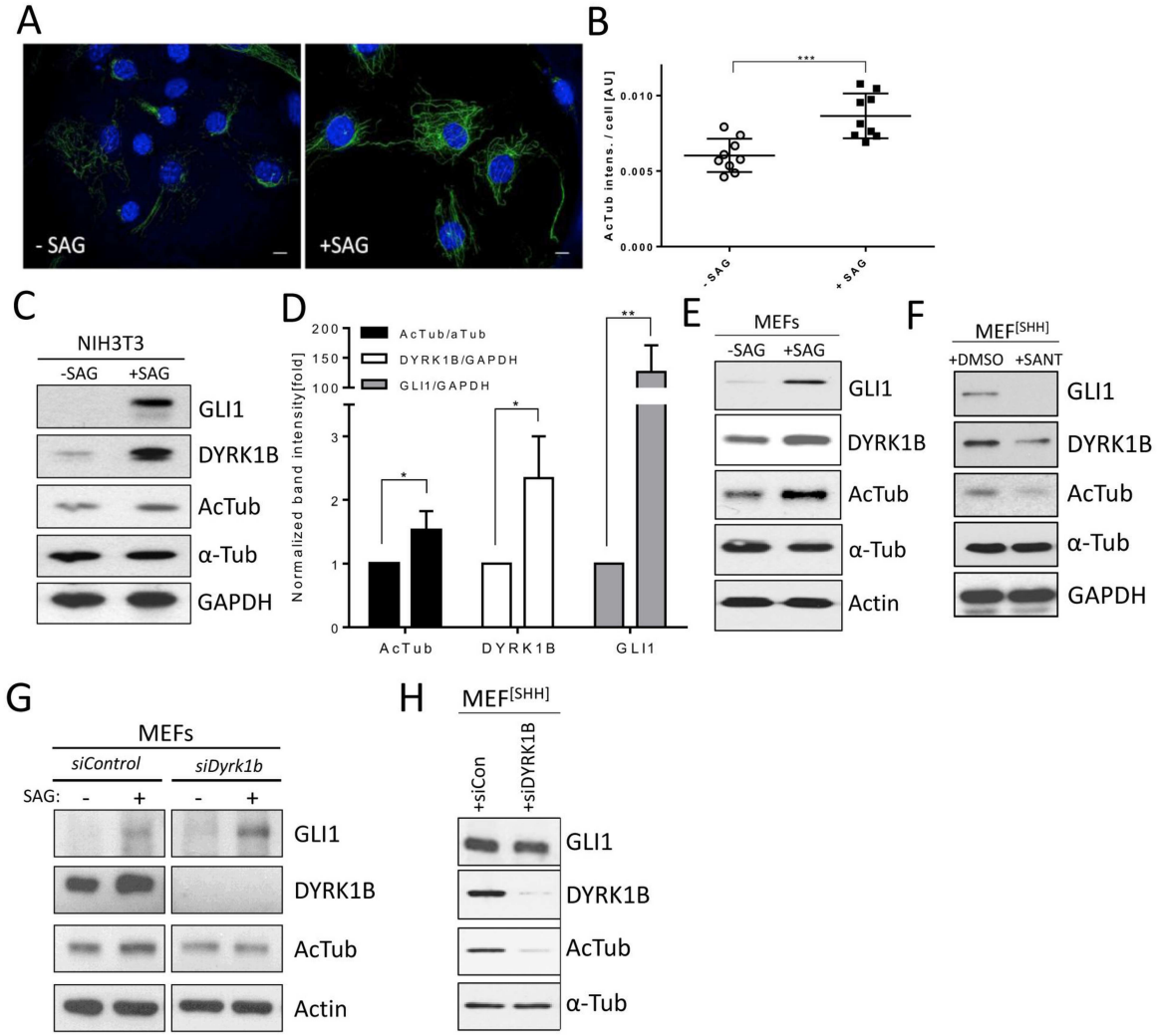
(B) Quantification of the polarization experiment depicted in (E) (mean of n=3 ±SD). SAG (SMO agonist, 100 nM); ACY-1215 (HDAC6 inhibitor, 10 µM).

(C) Fraction of polarized NIH3T3 fibroblasts, pre-treated with SAG (100 nM) for 2d, followed by scratch wounding. DMSO or AZ191 (DYRK1B inhibitor, 0.5 µM) was added 30 min before the scratch. Shown is the mean of n=3 ±SD.

(D) Example of scratch wounds in confluent NIH3T3 cultures directly after scratching (Start, left), after approx. 9.5h (Mid, middle) or at the end (approx. 20h, right panel). The border is outlined by a dashed line. AZ = AZ191 (1µM).

(E) Relative wound closure over time as assessed by live cell recording. One representative example of three is shown. AZ = AZ191 (1µM).

(F) Schematic depiction of the findings described in this manuscript. Left panel: Without Hh stimulation. Right panel: With Hh stimulation. Not shown is the possibility that Hh/SMO might also activate AKT, leading to an additional route of GSK3β regulation. DYRK1B can also functionally interact with AKT.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B
***
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0.005
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- SAG
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C
NIH3T3
-SAG
+SAG
GLI1
DYRK1B
AcTub
α-Tub
GAPDH
D
AcTub/aTub
DYRK1B/GAPDH
GLI1/GAPDH
Normalized band intensity [fold]
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150
100
3
2
1
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DYRK1B
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AcTub
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Actin
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+SANT
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G
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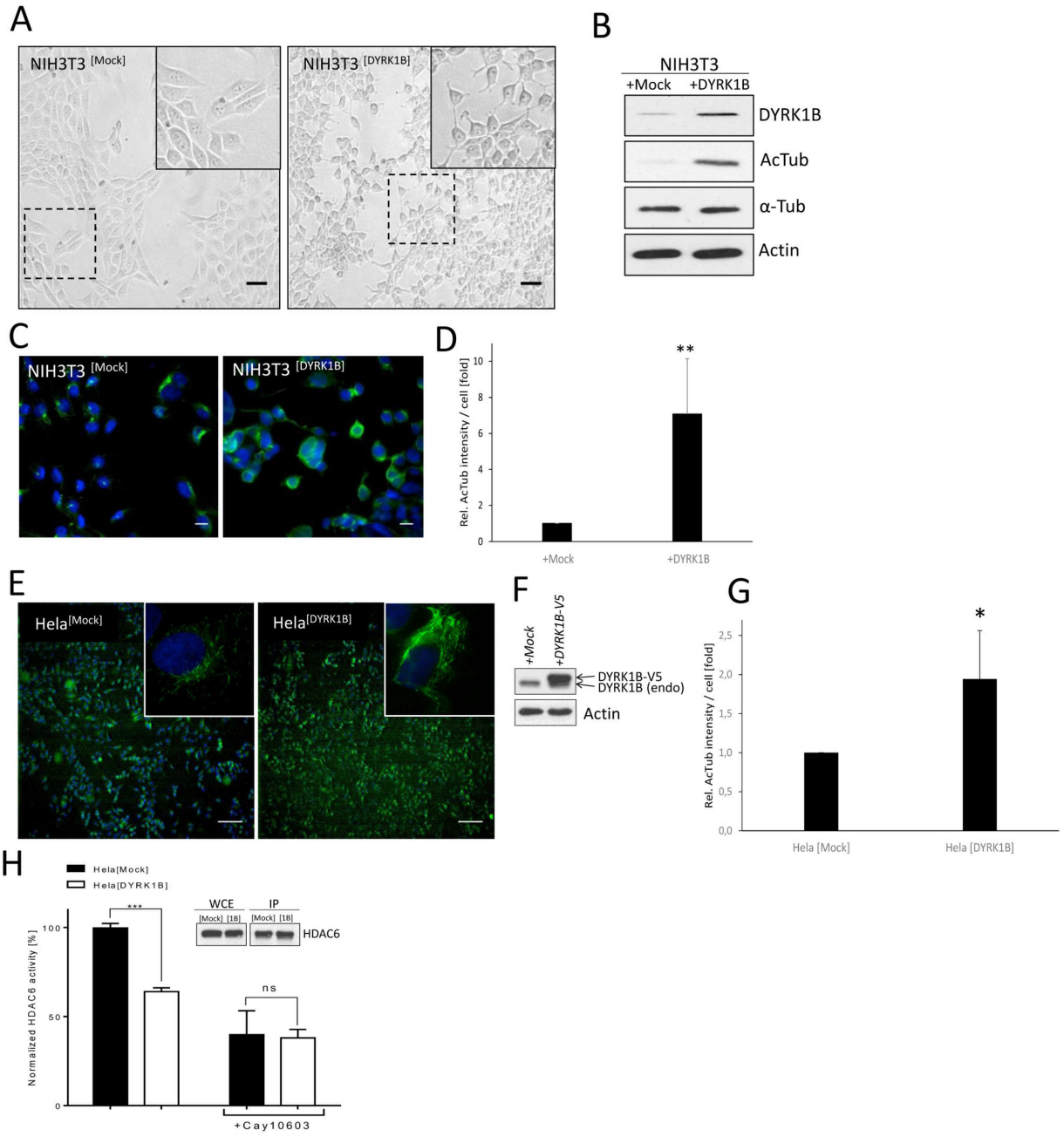
- Figure 1 -


A
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NIH3T3 [DYRK1B]
B
NIH3T3
+Mock
+DYRK1B
DYRK1B
AcTub
α-Tub
Actin
C
NIH3T3 [Mock]
NIH3T3 [DYRK1B]
D
Rel. AcTub intensity / cell [fold]
**
+Mock
+DYRK1B
E
Hela[Mock]
Hela[DYRK1B]
F
+Mock
+DYRK1B-V5
DYRK1B-V5
DYRK1B (endo)
Actin
G
Rel. AcTub intensity / cell [fold]
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Hela [Mock]
Hela [DYRK1B]
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Normalized HDAC6 activity [%]
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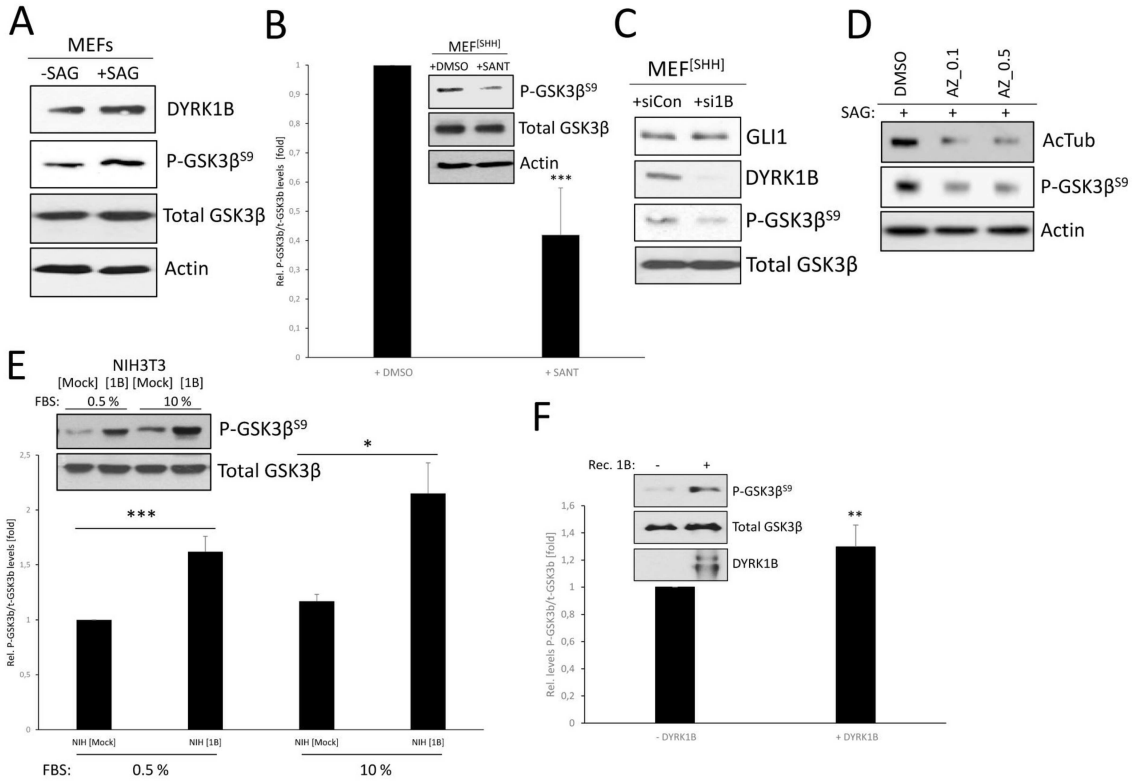
- Figure 2 -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Total GSK3β
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B
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Total GSK3β
Actin
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Rel. P-GSK3b/t-GSK3b levels [fold]
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MEF[SHH]
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GLI1
DYRK1B
P-GSK3βS9
Total GSK3β
D
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Actin
E
NIH3T3
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FBS: 0.5 % 10 %
P-GSK3βS9
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Rel. P-GSK3b/t-GSK3b levels [fold]
NIH [Mock]
NIH [1B]
NIH [Mock]
NIH [1B]
FBS: 0.5 % 10 %
F
Rec. 1B: - +
P-GSK3βS9
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DYRK1B
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Rel. levels P-GSK3b/t-GSK3b [fold]
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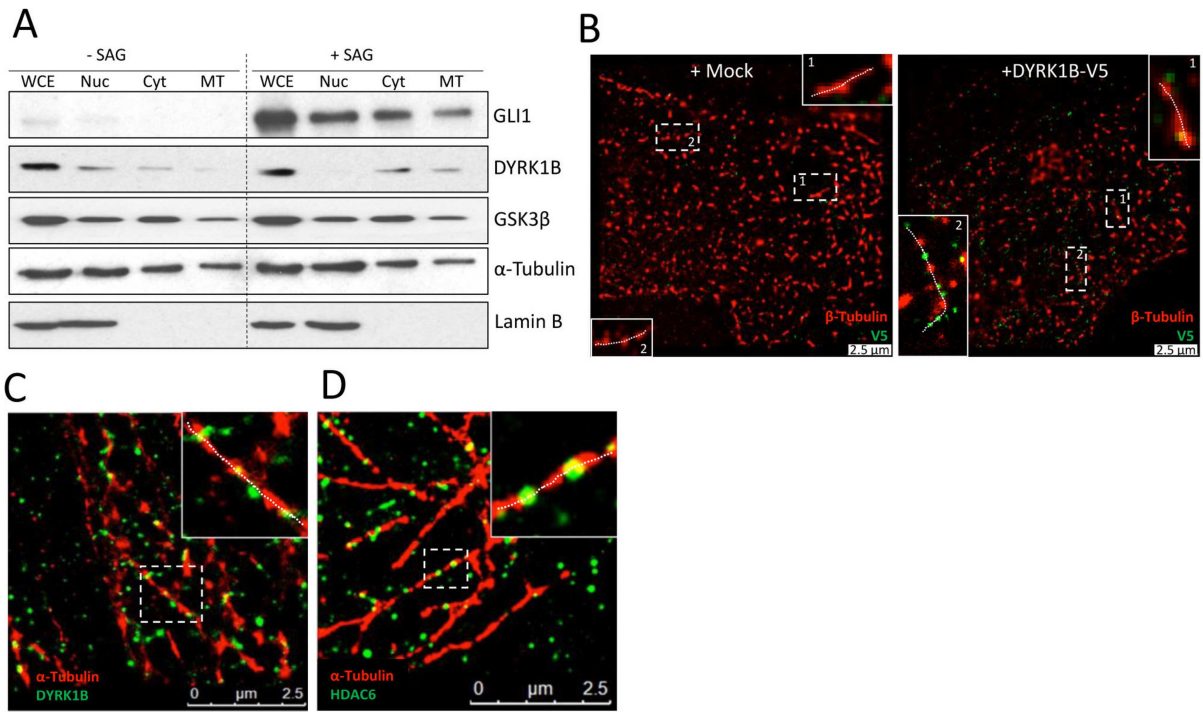
+ DYRK1B

- Figure 3 -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GLI1
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GSK3β
α-Tubulin
Lamin B
B
+ Mock
+DYRK1B-V5
β-Tubulin
V5
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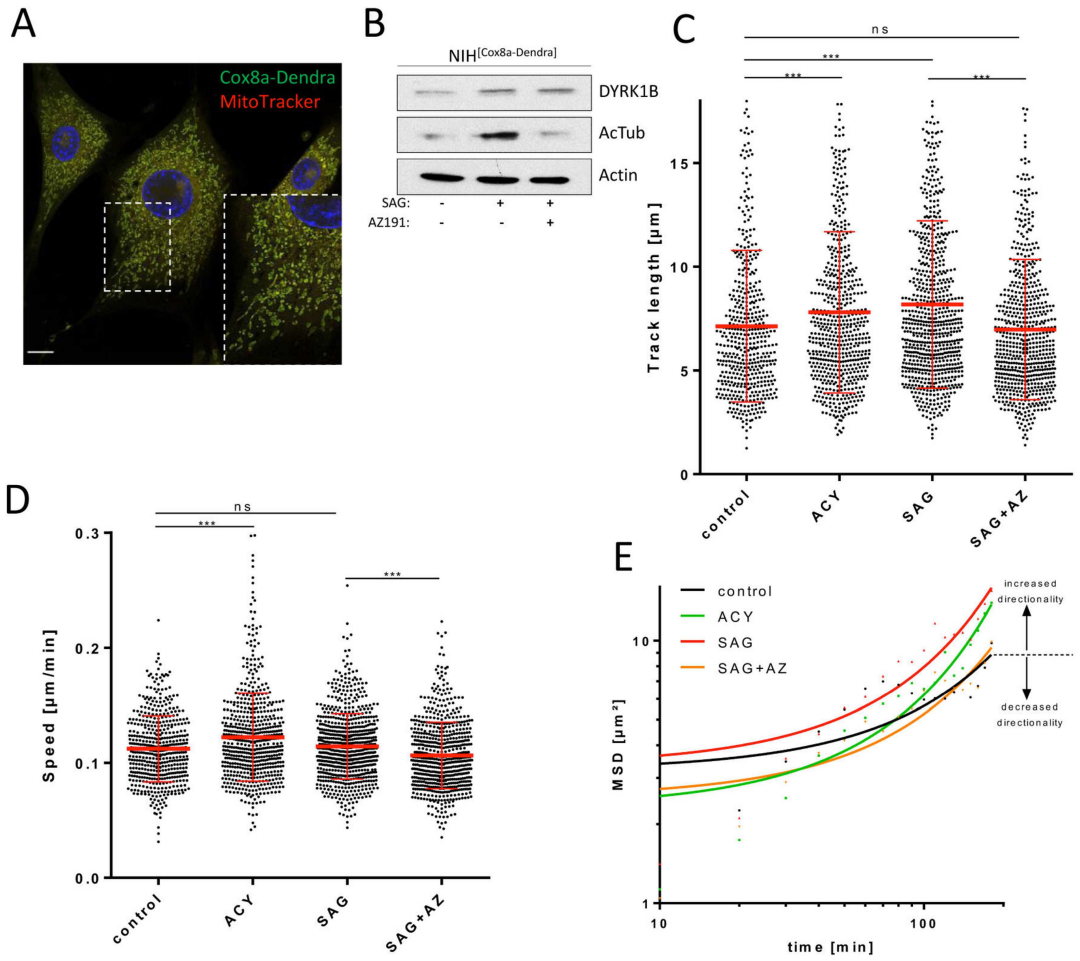
D
α-Tubulin
HDAC6
0 µm 2.5

- Figure 4 -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MitoTracker
B
NIH[Cox8a-Dendra]
DYRK1B
AcTub
Actin
SAG: - + +
AZ191: - - +
C
ns
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Track length [µm]
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5
10
15
control
ACY
SAG
SAG+AZ
D
ns
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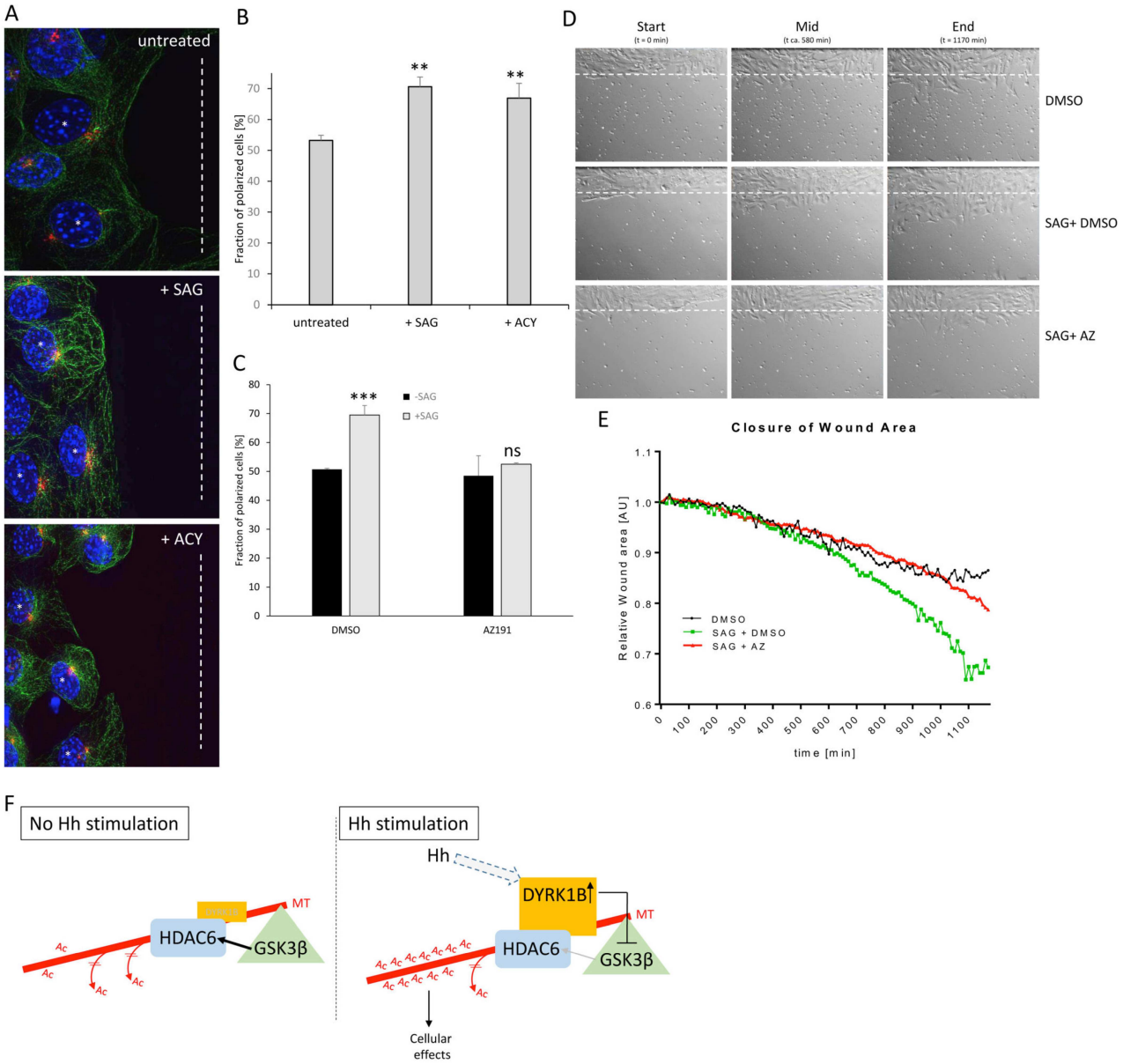
Speed [µm/min]
0.0
0.1
0.2
0.3
control
ACY
SAG
SAG+AZ
E
control
ACY
SAG
SAG+AZ
increased directionality
decreased directionality
MSD [µm²]
1
10
10
100
time [min]

- Figure 5 -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untreated
+ SAG
+ ACY
B
Fraction of polarized cells [%]
untreated
+ SAG
+ ACY
C
Fraction of polarized cells [%]
-SAG
+SAG
DMSO
AZ191
ns
D
Start
(t = 0 min)
Mid
(t ca. 580 min)
End
(t = 1170 min)
DMSO
SAG+ DMSO
SAG+ AZ
E
Closure of Wound Area
Relative Wound area [AU]
DMSO
SAG + DMSO
SAG + AZ
time [min]
F
No Hh stimulation
Hh stimulation
Hh
DYRK1B
MT
HDAC6
GSK3β
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Cellular effects

- Figure 6 -

Journal of *Developmental Biology*

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Review

Emerging Roles of DYRK Kinases in Embryogenesis and Hedgehog Pathway Control

Rajeev Singh and Matthias Lauth *

Philipps University Marburg, Institute of Molecular Biology and Tumor Research (IMT), Center for Tumor and Immune Biology (ZTI), Hans-Meerwein-Str. 3, 35043 Marburg, Germany; singhr@staff.uni-marburg.de

* Correspondence: lauth@imt.uni-marburg.de; Tel.: +49-6421-2866727

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Abstract: Hedgehog (Hh)/GLI signaling is an important instructive cue in various processes during embryonic development, such as tissue patterning, stem cell maintenance, and cell differentiation. It also plays crucial roles in the development of many pediatric and adult malignancies. Understanding the molecular mechanisms of pathway regulation is therefore of high interest. Dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs) comprise a group of protein kinases which are emerging modulators of signal transduction, cell proliferation, survival, and cell differentiation. Work from the last years has identified a close regulatory connection between DYRKs and the Hh signaling system. In this manuscript, we outline the mechanistic influence of DYRK kinases on Hh signaling with a focus on the mammalian situation. We furthermore aim to bring together what is known about the functional consequences of a DYRK-Hh cross-talk and how this might affect cellular processes in development, physiology, and pathology.

Keywords: hedgehog; GLI1; dual-specificity tyrosine-regulated kinase; DYRK; MIRK; Down syndrome

1. Introduction

1.1. The Hedgehog Signaling Pathway

The *Hedgehog (Hh)* gene was first identified in genetic screens for mutations that disrupt the larval body plan in *Drosophila melanogaster* [1]. The name Hedgehog originates from the short and 'spiked' phenotype of the cuticle of the Hh mutant *Drosophila* larvae, which resembles the spikes of a hedgehog [1,2]. The members of the Hh family of proteins have since been recognized as key mediators of many fundamental processes in embryonic development, playing a crucial role in controlling cell fate, patterning, proliferation, survival, and differentiation. Furthermore, Hh signaling also regulates the maintenance of tissue stem cells and affects oncogenic transformation and the development of tumors [3–5]. Vertebrates possess three Hedgehog homologues: Desert (*DHH*), Indian (*IHH*), and Sonic (*SHH*). All three genes have evolutionary conserved roles in body plan organization and development [2,6–8]. The polarizing activity of the organizing centers located in the limb bud, the notochord, or the floor plate of the neural tube is regulated by SHH [9,10]. IHH regulates the coordination of multiple cellular events during endochondral bone development including osteoblast differentiation [11,12], while DHH is required for the development of germ cells in testes and peripheral nerve sheath formation [13].

The Hh signaling cascade has been discussed in depth by other excellent reviews of this special issue on embryogenesis (e.g., [14–17]). Briefly, the canonical Hh signaling cascade is initiated in the target cell by the Hh ligand binding to the Patched1 receptor (PTCH1) [18,19], a 12-span transmembrane protein located in the ciliary membrane relieving the repression of Smoothened (SMO) [20,21], a 7-span transmembrane protein, which is a member of the G protein-coupled receptor (GPCR) superfamily.

This de-repression results in the activation of the Hh transcriptional effectors, the zinc finger proteins of the GLI (Cubitus interruptus (Ci) in *Drosophila melanogaster*) family [22].

Several studies have reported the modulation of Hh signaling through protein kinases, amongst others PKA, PKC, GRK2, MEK, ERK, AKT, S6K, and GSK3β all of which have been documented to play a role in Hh signal transduction [23–28]. Moreover, recent studies have outlined the importance of dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs) in the positive and negative regulation of Hh pathway activity [29–36]. This review centers on the DYRK family of kinases and their role in regulating the developmentally important Hh signaling pathway.

1.2. Protein Kinases: An Introduction

Protein kinases are central for the regulation of major cellular processes. Kinases play particularly prominent roles in signal transduction as they direct the cellular activities by the addition or removal of a phosphate group. As abnormal levels of protein phosphorylation are associated with the development of several diseases [37], it is crucial to delve deeper into the understanding of the varying mechanisms that control these phosphorylation events [38]. Eukaryotic protein kinases (ePKs) are divided into nine large groups (plus one atypical group which does not show similarity to ePKs), which are further divided into families and subfamilies [39,40]. These groups are: (1) Tyrosine kinases (TK); (2) Tyrosine kinase-like (TKL); (3) cAMP-dependent protein kinase, cGMP-dependent protein kinase and protein kinase C (AGC); (4) Calcium/calmodulin-dependent kinases (CAMK); (5) Casein kinase 1 (CK1); (6) Cyclin-dependent kinases (CDK), Mitogen-activated protein kinases (MAPK), Glycogen synthase kinase (GSK3) and CDC-like kinase (CLK) group of protein kinases (CMGC); (7) Homologs of the yeast STE7, STE11 and STE20 genes (STE); (8) Receptor Guanylate Cyclases (RGC); and (9) Others (kinases that do not fit within any of the other main kinase groups) [40–42].

2. The CMGC Group of Kinases

Due to sequence homologies in their kinase domains, CDKs, MAPKs, GSK3s, CLKs, and related kinases (CMGCs) form one big group of eukaryotic protein kinases [40]. The CMGC group consists of 62 members in total, which are subdivided further into nine families (CDK-, CDKL-, GSK-, CLK-, MAPK-, HIPK-, DYRK-, RCK-, and SRPK-families) [43]. This group is highly conserved during evolution, arguing that its members fulfill important functions from nematodes to humans. Given their involvement in cell proliferation, MAPKs and CDKs are the most studied kinases within the CMGC group and are the subject of intense research efforts in oncological research. Less studied candidates include the dual-specificity tyrosine regulated kinases (DYRKs) and the serine-arginine protein kinases (SRPK). In general, the kinases in the CMGC group have a broad spectrum of functional roles ranging from signal transduction to cell cycle regulation, RNA related processing, and intracellular communication [43].

3. The DYRK Family of Kinases

Dual-specificity tyrosine phosphorylation-regulated kinases belong to the CMGC group of kinases and contain a characteristic sequence motif called the DYRK-homology box (DH box) (Figure 1). YAK1 from budding yeast was the first member of the DYRK family to be discovered [44,45]. There are five members within the mammalian DYRK subfamily and they are categorized into two classes. Class I consists of DYRK1A and DYRK1B (the latter is also known as Minibrain-related kinase (Mirk)), while class II is made up of DYRK2, DYRK3, and DYRK4 [45,46]. The assortment of mammalian DYRKs in the corresponding classes is based on sequence homologies within the conserved kinase domain [46,47]. Certain sequence motifs can only be found in class I DYRKs, such as a C-terminal PEST domain (a region rich in proline (P), glutamic acid (E), serine (S), and threonine (T)) (Figure 1). The PEST sequence is known to act as a signal for rapid protein degradation [48]. However, to our knowledge, this function has not been formally proven in DYRKs. DYRK1A protein stability is regulated through the ubiquitin/proteasome system, but involves an N-terminal region [49]. DYRK1A is the only

family member containing a poly-histidine stretch (13 consecutive histidine residues) and a region enriched in serine/threonine residues (S/T-rich region) [50,51]. The poly-histidine stretch promotes the targeting of DYRK1A to nuclear speckles which are enriched with pre-mRNA splicing factors regulating the splicing machinery [51,52]. Other elements, such as nuclear localization signals (NLS), can be found in many DYRKs. On the other hand, only class II DYRK kinases contain a N-terminal auto-phosphorylation accessory region (NAPA) domain, which is thought to be required for tyrosine auto-phosphorylation specifically in class II DYRKs, although DYRK2 lacking the NAPA domain has been shown to auto-phosphorylate itself under in vitro conditions [47,53–55]. Further differences include the extent of the respective N- and C-termini (Figure 1). In general, DYRK family members are known to regulate protein stability, cell proliferation, and differentiation. These events are mediated by the phosphorylation of DYRK recognition sites in target proteins. The consensus sequence motif consists of Ser or Thr followed by Pro in position +1. Furthermore, an arginine residue at position −2 or −3 relative to Ser/Thr seems to be preferred (RxxS/TP or RPxS/TP), although a considerable degree of divergence to this consensus has also been noted [47,56].

The activation loop of DYRK kinases contains a conserved YXY sequence, the phosphorylation of which leads to the activation of full enzymatic activity. Members of the DYRK family auto-phosphorylate the second tyrosine residue in order to be fully activated and then phosphorylate substrates in trans on Ser/Thr residues, hence they are known as Dual-specificity tyrosine-regulated kinases [45,47,56]. The auto-activation mode of DYRKs is in contrast to MAP kinases, where an upstream kinase is needed for the phosphorylation of the activation loop [47]. The tyrosine specificity of DYRK kinases is thought to be lost once the protein is fully translated and only the Ser/Thr specificity on target proteins remains [53,57].

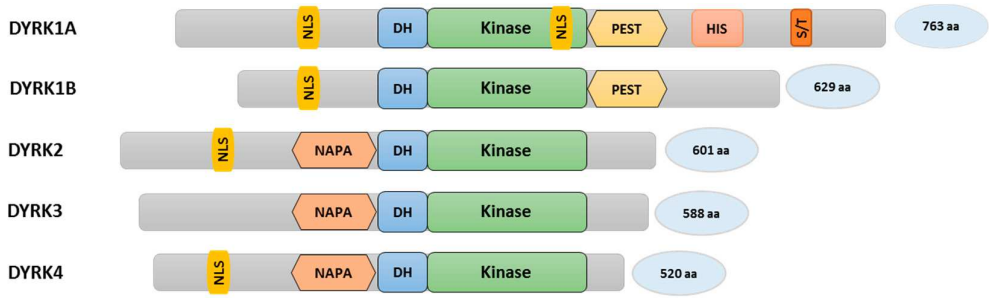

Figure 1. Schematic representation of the DYRK family of proteins: Distinct sequence motifs such as the nuclear localization signal (NLS); DYRK-homology box (DH); a motif rich in proline, glutamic acid, serine, and threonine residues (PEST); a poly-histidine stretch (HIS); a serine/threonine rich region (S/T); a N-terminal auto-phosphorylation accessory region (NAPA); and a conserved kinase domain comprising the structural and functional features of DYRKs.

4. Class I DYRKs: DYRK1A and DYRK1B

4.1. The DYRK1A Kinase

DYRK1A is a nuclear kinase, but can also be found in the cytosol. It represents the most studied member of the DYRK family, which is due to its presumed involvement in the Down syndrome (DS, OMIM #190685). DS is one of the most common genetic defects in humans with an estimated incidence of about 1 in 1000 live births worldwide and is caused by the complete or partial duplication of human chromosome 21 (trisomy 21) [58,59]. In humans, the *DYRK1A* gene is located on chromosome 21 (21q22.13), which is part of the so-called *Down-Syndrome Critical Region* (DSCR) [60]. Genes present within the DSCR (21q22.1–22.3 encompassing 33 genes) are thought to account for the development of DS, characterized by a general intellectual impairment, characteristic craniofacial dysmorphologies, and congenital heart disease [60–62].

Despite the fact that upstream modulators of DYRK1A kinase activity exist [63,64], the prime determinant of DYRK1A protein function is considered to be its overall protein amount making it very sensitive to gene dosage. An altered copy number of the *DYRK1A* gene in mammals or of its orthologous gene, *minibrain (mnb)* in *Drosophila*, impedes with the proper development of the central nervous system [65]. Different studies with the trisomic DS mouse model Ts65Dn or cells derived from Down syndrome patients [66] have shown that an increased kinase expression affects neurogenesis and neuroblast proliferation, and results in impaired behavioral phenotypes. Genetic overexpression of *Dyrk1A* in mice leads to behavioral and cognitive impairment and neuronal alterations [67–69]. In contrast, loss of function of *Dyrk1A* or *mnb* results in significant brain size reduction in mice [70], flies [65,71], and men [72].

Intriguingly, a recent study identified *DYRK1A* loss-of-function mutations which are associated with impaired dendritic and spine growth, cortical development, and the pathophysiology of autism [73]. The exact mechanisms underlying DYRK1A's effects on dendritogenesis and neurogenesis remain open, but might involve its role in actin regulation [36,74–77]. Furthermore, DYRK1A has functions in synaptogenesis and synaptic vesicle endocytosis [74,78]. Haplo-insufficiency of *DYRK1A* is associated with the development of autosomal dominant mental retardation-7 (MRD7) (OMIM #614104), a syndrome characterized by primary microcephaly, facial dysmorphism, and behavioral problems [79]. Also, *DYRK1A* expression might be epigenetically misregulated in the William-Beuren region duplication syndrome (WBS) (OMIM #609757). WBS phenotypes commonly include craniofacial anomalies and cognitive deficits ranging from mental retardation to autism [80,81].

The documented evidence of DYRK1A functioning in brain development suggests that it interacts with embryonic signaling pathways such as Hedgehog, which is known to be crucial for neuronal specification in the neural tube, hippocampal neural stem cell maintenance, and the development of the cerebellar cortex [82,83]. Mice with a genetic *Shh* knockout present with Cyclopia [84], and inactivating mutations in the human *SHH* gene cause holoprosencephaly (OMIM #236100), a common form of structural malformation of the developing brain hemispheres [85–87]. In contrast, human patients suffering from the Hh-activating Gorlin syndrome (Basal Cell Nevus Syndrome, OMIM #109400) have an increased brain size [88].

4.2. DYRK1A as a Regulator of (Neuronal) Hedgehog Signaling

The fact that both Hh signaling and the DYRK1A kinase have such important roles in embryonic brain development suggests that they might be functionally linked. The exact interplay between DYRK1A and Hh signaling seems to be complex and stimulatory, and inhibitory functions have also been described (Figure 2). Indeed, suppression of Hh pathway activity was seen in cerebellar cells derived from a Down syndrome mouse model [89]. Furthermore, some morphological as well as functional deficits could be ameliorated by the application of a synthetic SMO agonist or by genetic Hh pathway activation [90,91], suggesting a too low level of Hh signaling in DS. Subsequent mechanistic studies revealed that increased levels of the DSCR-localized DYRK1A kinase can dampen Hh signaling [92], most likely through its effect on the actin cytoskeleton and on actin-regulated transcriptional regulators [36]. For instance, DYRK1A can phosphorylate the F-actin stabilizing ABLIM proteins and thereby functionally exert a negative impact on the actin cytoskeleton and on actin-modulated transcriptional co-factors such as MAL (MKL1, MRTF), which also modulate the Hh pathway [36]. This mechanism might explain why DS cerebellar cells display a limited response to Purkinje cell-derived SHH (Figure 2). However, this finding was unexpected as previous reports had proposed a direct activating function of DYRK1A on GLI1. Specifically, DYRK1A can phosphorylate amino acid residues in the N-terminus critical for the nuclear import of GLI1 [31,32,36,92] (Figure 2). As a result, the impact of DYRK1A on Hh signaling might be context-dependent and might also be dictated by the exact mode of pathway activation (ligand/receptor-triggered versus direct GLI1 activation). Further investigations are certainly needed to clarify this point.

Hypothetically, a physiological connection between Hh signaling and DYRK1A might also exist in the case of neural stem cell (NSC) division. In general, stem or progenitor cells can undergo symmetric or asymmetric types of cell division in order to generate progeny [93]. Hh signaling preferentially supports symmetric cell divisions [94,95]. DYRK1A has also been implicated in signaling aspects during asymmetric versus symmetric neural stem cell division, although the details await further investigation [96,97]. In general, Hh pathway activity has been associated with brain size (see above), which might be caused by its positive effects on neural stem cell pools. Intriguingly, truncation of DYRK1A results in the stimulation of kinase activity [98] and *DYRK1A* gene truncations have been found in human microcephaly [72]. Finally, it is interesting to note that Down syndrome patients have a reduced risk of developing solid cancer. The fact that DYRK1A is capable of suppressing canonical Hh signaling might contribute to its described potential as a tumor suppressor [99–103], in addition to other Hh-independent proposed mechanisms [104,105].

In addition to the neuronal effects of DYRK1A, one report exists describing the Hh-related impact of DYRK1A and its physical interactors HAN11 and mDia1 in cultured sebocytes [106]. Overexpression of either HAN11 or mDia1 suppressed GLI1 nuclear localization and activity in reporter assays and slowed the growth of these cells. In the murine embryo, *Han11* is expressed in the developing limb bud (E10.5), together with *Gli1* and *Ptch1*. It is possible that the actin-regulating formin mDia1 is functionally linked to the aforementioned DYRK1A-ABLIM-Actin-MAL-GLI axis, but experimental proof is lacking at this point.

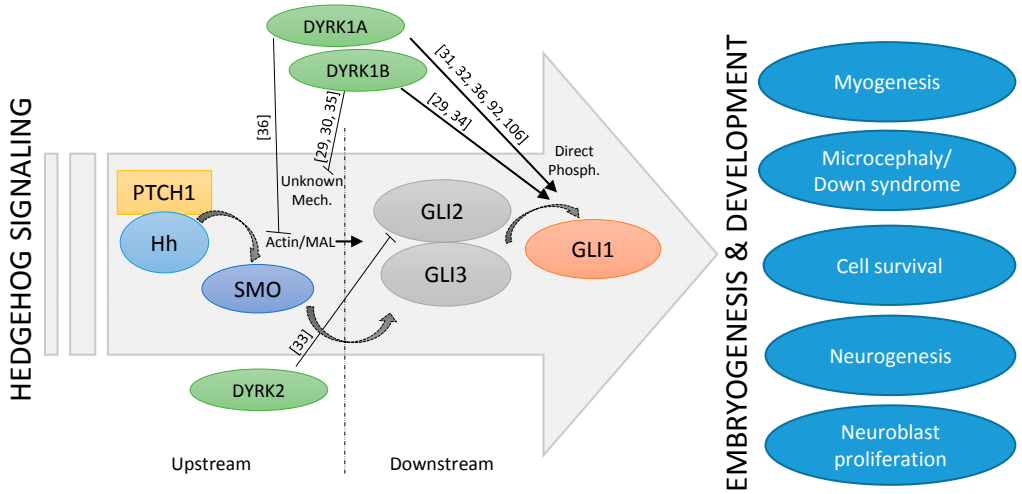

Figure 2. Schematic depiction of the cross-talk between Hh signaling and DYRK kinases.

Three DYRK kinases (DYRK1A, DYRK1B, DYRK2) have been described as regulators of upstream (above GLI transcription factors) and downstream (at the level of GLI transcription factors) Hh signaling. As Hh pathway modulators, they might control important embryogenic and developmental processes, such as myogenesis, neurogenesis, or the pathophysiology of Down syndrome. Literature references are given in square brackets.

4.3. The DYRK1B Kinase

The closest relative of mammalian DYRK1A is the DYRK1B kinase, also referred to as MIRK (Minibrain-related kinase). The human *DYRK1B* gene is located on chromosome 19q13.2, a region often amplified in ovarian and pancreatic cancer [107,108]. This kinase has three splice variants (629aa (p69), 601aa (p66), and 589aa (p65)) and is expressed in abundance in human skeletal muscle and testes [109]. Human DYRK1A and DYRK1B proteins are 84% identical in the N-terminal and catalytic domains but show no extended similarity in the C-terminal domain. Human and mouse DYRK1B proteins share

97% sequence similarity [46,110–113]. In many different cell types, DYRK1B can be found both in the nucleus and in the cytoplasm [114].

4.4. DYRK1B in Developmental and Physiological Processes

The observation that DYRK1B is strongly expressed in skeletal muscle argues for a physiological role in muscle function and/or development. Indeed, DYRK1B levels have been shown to be comparatively low in myoblasts, but to increase significantly upon the induction of differentiation [115]. DYRK1B favors myoblast fusion and the subsequent expression of differentiation markers [116]. Furthermore, DYRK1B supports the survival of muscle progenitor (C2C12) cells in culture and of cells from muscle-related tumors such as rhabdomyosarcoma [117,118]. The pro-differentiating effects of DYRK1B on myoblasts are opposite to the effects which Hh signaling exerts on muscle stem cells (satellite cells) and on C2C12 progenitor cells [119]. Here, Hh promotes cell division and blocks differentiation along the myogenic lineage, thereby maintaining the progenitor cell pool. Although it is not clear whether the influence of Hh or DYRK1B occurs exactly at the same developmental stage, currently available data would suggest a primarily antagonistic relationship between these two pathways. Recent work has identified a complex regulatory relationship between DYRK1B and Hh. While DYRK1B dampens Hh signaling initiated by SMO, it also promotes the stability of the GLI1 transcription factor on the other side [29,30,34]. The latter might be mediated by DYRK1B-induced stimulation of the pro-survival PI3K-AKT signaling pathway, a known positive regulator of GLI stability [23,120] (Figure 2). In addition, at least in cultured fibroblasts, Hh pathway stimulation increases DYRK1B protein levels by currently unknown post-transcriptional mechanisms [29], suggesting a feedback loop.

Another example of physiological cross-talk between DYRK1B and Hh might be the differentiation of mesenchymal progenitor cells into adipocytes. Hh signaling has a generally inhibiting impact on adipocytic differentiation, usually redirecting cellular fate towards the osteogenic lineage [121–123]. In contrast, DYRK1B favors the in vitro differentiation into adipocytes [35]. This holds particularly true for DYRK1B carrying mutations which were identified in families suffering from an autosomal-dominant form of metabolic syndrome [35], a disease with prominent adipocyte involvement. The mutations found result in misfolding of the DYRK1B protein and in intracellular aggregation [124]. It remains to be clarified how these mutations affect the functional integration of DYRK1B into other signaling pathways, but it is intriguing to note that mutant DYRK1B expression reduced GLI2 levels in cultured adipocytes [35]. It is therefore reasonable to speculate that the suppression of Hh pathway activity contributes to these effects.

5. The Class II DYRKs

5.1. The DYRK2, DYRK3, and DYRK4 Kinases

Compared to the DYRK class I members, the class II DYRKs (DYRK2, DYRK3, DYRK4) contain a larger N-terminal region and a shorter C-terminal domain. DYRK2 and DYRK4, but not DYRK3, possess an NLS sequence and all three contain an NAPA (N-terminal autophosphorylation accessory region) domain which is absent in class I DYRKs [47]. The NAPA domain provides a chaperone-like function and transiently converts class II DYRKs into intramolecular tyrosine kinases [54]. Despite lacking an apparent NLS, DYRK3 (also named REDK) is localized in the nucleus in hematopoetic cells [125], whereas DYRK2 is mostly cytosolic, but under conditions of genotoxic stress, it accumulates in the nucleus regulating p53 [126]. DYRK4, which is currently the least studied DYRK family member, displays splice variant-dependent subcellular localization [47].

In contrast to DYRK1B, which has been described as an oncogenic kinase in numerous cancer types, DYRK2 can also exert opposite functions and can display tumor suppressive traits. This is brought about by DYRK2's ability to activate p53-dependent apoptosis following DNA damage [126,127] and by negatively controlling the protein stability of well-established oncogenes such as c-MYC

or c-JUN [128]. Phosphorylation-dependent regulation of proteasomal degradation seems to be a recurrent mechanism employed by many if not all DYRK kinases [129–134].

5.2. Class II DYRKs in Development

In zebrafish, *DYRK2* has been shown to be expressed in lateral somites (mesodermal blocks around the anterior-posterior axis of the developing embryo) and adaxial cells (muscle precursor cells that are adjacent to the notochord and part of the presomitic mesoderm) at an early stage of embryogenesis [135]. Co-localization of *Dyrk2* mRNA and *myogenic differentiation factor D (MyoD)* mRNA was seen in muscle progenitor cells in the posterior compartment of somites. Here, DYRK2 might positively regulate fast twitch muscle differentiation in the early stages of embryonic development [135]. Although the link has not yet been experimentally verified, it is intriguing to note that in contrast to DYRK2, Hh signaling promotes the formation of slow-twitch fibers in zebrafish [136]. Furthermore, mammalian DYRK2 has been shown to negatively regulate Hh pathway activity by phosphorylating and degrading GLI2 [33] (Figure 2). It is therefore reasonable to speculate that DYRK2, through its negative influence on Hh signaling, might impact on the slow/fast-twitch fiber differentiation during muscle development.

A similarly antagonistic relationship between DYRK2 and Hh signaling might also play a role in *Drosophila*, which encodes three DYRKs: Minibrain/Dyrk1A, DmDyrk2, and DmDyrk3. Recent reports have shown that *DmDyrk2* is expressed in the developing third antennal segment, an anatomical structure responsible for smell, and in the morphogenetic furrow of the developing eye, where it contributes to the development of the visual system [137]. In addition, Hedgehog is a known regulator of morphogenetic furrow progression and ommatidial cell differentiation in the *Drosophila* eye disc [138,139]. If, in analogy to mammals, DmDYRK2 also regulates Hh signaling, it is intriguing to hypothesize that functional DYRK2-Hh cross-talk is involved in the specification of the *Drosophila* eye.

In comparison to class I DYRKs and DYRK2, the class II family members DYRK3 and DYRK4 show a very restricted expression profile with the strongest expression in erythroid progenitors and testes, respectively [140,141]. As can be assumed from this expression pattern, DYRK3 is involved in erythropoiesis. While *Dyrk3*$^{-/-}$ mice surprisingly present without a hematological phenotype, they develop increased numbers of red blood cells under conditions of anemia, suggesting that DYRK3 functions as a negative regulator of erythropoiesis [140]. Therefore, small-molecule DYRK3 inhibitors might be of interest to ameliorate anemic conditions. Although Hh ligands (mostly DHH and IHH) have also been shown to regulate erythropoiesis [142–144], it currently remains unclear whether cross-talk between DYRK3 and Hh signaling contributes to this process.

Significantly more work has been done on the function of DYRK3 on the cellular and molecular level, albeit a clear link to Hh signaling has so far not been established. Specifically, DYRK3 impinges on stress-associated mTOR signaling [145], as well as on endocytosis dynamics [146]. Endocytic sorting of SHH and PTCH1 in clathrin-coated vesicles is also critical for proper Hh signaling in signal producing and receiving cells [147–149]. However, whether DYRK3 is indeed involved in these steps awaits further experimentation.

Of all the DYRK kinases discussed so far, the least is known about DYRK4. The expression of this family member is strongly restricted to testicular tissue, with a strikingly selective peak of expression in step VIII spermatids [46,141], suggesting a role in male fertility. Surprisingly, however, analysis of *Dyrk4* null animals revealed no aberrant sperm phenotype or defects in male fertility [141]. DYRK4 was shown to be present in the duck ovary, and was more active or upregulated in the high egg production ovaries, which would suggest a hitherto unrecognized role in the female reproductive system, at least in some species [150]. Desert Hh (DHH) signaling also occurs in testes and, at least in certain species, also in ovaries, but the involvement of DYRK4 in DHH-mediated processes is unclear at the moment [151–153].

6. Conclusions

DYRK kinases are highly conserved during evolution from yeast to humans. Due to the evolutionary diversification, DYRKs might represent the requirement of more critical and specialized functions in vertebrates or might have contributed to this diversification. Multifaceted roles of DYRK kinases have been discussed in this review and their importance in various developmental processes has been stated. As of now, three of five mammalian DYRK kinases have been functionally linked to Hh signaling (DYRK1A, DYRK1B, DYRK2), arguing for a close regulatory connectivity to the developmentally important Hh system. Hence, although DYRK kinases are not absolutely required for Hh signaling, they function as modulators and it is therefore reasonable to hypothesize that they contribute to many Hh-driven steps during embryonic development. Unfortunately, however, more work is needed to provide a clear picture of the exact and tissue-specific cross-talk between DYRKs and Hh, particularly in in vivo settings. As certain DYRKs have a preferred expression in specific tissues (e.g., DYRK1A in neuronal and DYRK1B in muscle tissue), it is reasonable to speculate that the impact on the tissue-selective Hh pathway activity is specified by the respective DYRK enzyme. In other tissues or cell types, where several DYRK kinases are expressed together at comparable levels, a certain degree of functional redundancy might exist, particularly for the class I DYRKs. These questions are important to address in the future in light of the development of small-molecule inhibitors which might lack the necessary specificity and target several DYRKs simultaneously. Complicating the developmental interpretation is the fact that DYRK kinases also modulate other, non-Hh signaling systems, such as, e.g., the NFAT (nuclear factor of activated T-cells) [154] pathway or HIF (hypoxia-inducible factor) signaling [155]. Future work will reveal whether Hedgehog or any of the other signaling systems is particularly important for the physiological impact of DYRK kinases.

Most evidence for developmental cross-talk between DYRKs and Hh stems from studies on DYRK1A, neuronal development, and the Down syndrome. In addition, available data encourage speculations on DYRK1B and DYRK2 modulating Hh signaling in muscle development and on the involvement of DYRK1B in adipocyte differentiation. In light of recent reports strengthening the concept of Hh-pathway modulation by DYRKs in pathological conditions such as metabolic syndrome or cancer, it will be interesting to see whether future research unveils more cross-talk between this group of kinases and the Hh system in physiological processes. In this review, we have tried to outline the currently available knowledge on the DYRK family of kinases engaging in developmental biology, physiology, and pathology, focusing on its impact on Hh signaling.

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Research Paper

DYRK1B blocks canonical and promotes non-canonical Hedgehog signaling through activation of the mTOR/AKT pathway

Rajeev Singh[1], **Pavan Kumar Dhanyamraju**[1], **Matthias Lauth**[1]

[1]Philipps University Marburg, Institute of Molecular Biology and Tumor Research (IMT), Center for Tumor- and Immunobiology, 35043 Marburg, Germany

Correspondence to: Matthias Lauth, **email:** lauth@imt.uni-marburg.de

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ABSTRACT

Hedgehog (Hh) signaling plays important roles in embryonic development and in tumor formation. Apart from the well-established stimulation of the GLI family of transcription factors, Hh ligands promote the phosphorylation and activation of mTOR and AKT kinases, yet the molecular mechanism underlying these processes are unknown. Here, we identify the DYRK1B kinase as a mediator between Hh signaling and mTOR/AKT activation. In fibroblasts, Hh signaling induces DYRK1B protein expression, resulting in activation of the mTOR/AKT kinase signaling arm. Furthermore, DYRK1B exerts positive and negative feedback regulation on the Hh pathway itself: It negatively interferes with SMO-elicited canonical Hh signaling, while at the same time it provides positive feed-forward functions by promoting AKT-mediated GLI stability. Due to the fact that the mTOR/AKT pathway is itself subject to strong negative feedback regulation, pharmacological inhibition of DYRK1B results in initial upregulation followed by downregulation of AKT phosphorylation and GLI stabilization. Addressing this issue therapeutically, we show that a pharmacological approach combining a DYRK1B antagonist with an mTOR/AKT inhibitor results in strong GLI1 targeting and in pronounced cytotoxicity in human pancreatic and ovarian cancer cells.

INTRODUCTION

Hedgehog (Hh) signaling is an important regulatory system in embryonic development, stem cell biology and tumorigenesis [1–3]. Mechanistically, Hh ligands (Sonic Hh (SHH), Indian Hh (IHH), Desert Hh (DHH)) bind to Patched (PTCH1, PTCH2) receptors, thereby de-repressing the transmembrane protein Smoothened (SMO). Activated SMO results in the generation of transcriptionally competent forms of the transcription factors GLI2 and GLI3, which enter the nucleus and initiate target gene expression. Well-established target genes include e.g. *PTCH1* and *GLI1*, which regulate the Hh pathway in a negative and positive manner, respectively, and are often utilized as surrogate read-outs for general pathway activity. Such transcriptional feed-back loops are frequently encountered in physiologically important signaling pathways and serve to fine-tune the entire system. In addition, non-transcriptional regulatory inputs through e.g. kinases have been documented and it is well known that Hh signaling promotes the phosphorylation of e.g. MEK, ERK and AKT kinases [4–7]. These mechanisms are often exploited in cancer cells in 'non-canonical' modes of signaling, leading to e.g. Hh ligand/receptor-independent activation of GLI transcription factors [8–11].

One group of enzymes with close regulatory connection to the Hh pathway is the DYRK (Dual-specificity and Tyrosine(Y)-regulated kinase) kinase family with its five members DYRK1A, DYRK1B, DYRK2, DYRK3 and DYRK4 [12–17]. In particular, DYRK1B (a.k.a. MIRK) is linked to the development of several cancer types and can frequently be found amplified or hyperactive in ovarian and in pancreatic cancer [18–23]. While some studies described a negative role for DYRK1B in Hh signal transduction [14, 24, 25], others have documented a stimulatory function for this kinase [15]. In our present study, we aimed to shed light on this issue and to clarify the role of DYRK1B in Hh signaling. We could observe that DYRK1B has opposing roles on the Hh

output depending on the site of integration in the pathway: While it suppresses canonical (membrane-initiated) Hh signaling, it promotes the non-canonical (downstream) stabilization of GLI1. The latter effect is mediated by the hitherto unknown ability of DYRK1B to activate the PI3K/mTOR/AKT pathway, which is known to stabilize GLI proteins [7, 26]. The PI3K/mTOR/AKT pathway is one of the most frequently activated signaling cascades in human cancer [27]. The mTOR kinase can be found in at least two multi-protein complexes, termed mTORC1 and mTORC2. The first complex is downstream of AKT and is activated through TSC1/2 and RHEB proteins whereas the latter complex is upstream of AKT and is activated by PI3K in an unknown manner [28]. The mTORC1 complex is well established for being a major regulator of protein translation and autophagy while mTORC2, amongst others, impinges on cell survival through regulation of AKT, FOXO and PKCα [29, 30].

Here, we present data that endogenous Hh signaling augments DYRK1B levels, and that blocking this increase abrogates the Hh-induced stimulation of mTOR/AKT signaling. The intense crosstalk between Hh signaling, GLI and mTOR/AKT is further complicated by mTOR/AKT being subject to strong feedback control through mTORC1 and S6K. In turn, we observed that blocking DYRK1B function by RNAi or small molecule inhibition resulted in a time-dependent impact on GLI1 levels and Hh pathway output. Continuing from these mechanistic findings, we could furthermore demonstrate that a pharmacological therapy combining the targeted inhibition of DYRK1B with that of PI3K/mTOR/AKT has strong effects on Hh/GLI signaling and on cell growth of *DYRK1B*-amplified pancreatic and ovarian cancer cells.

RESULTS

Differential effects of DYRK1B on Hh signaling

We and others have previously identified the DYRK1B kinase as a negative regulator of Hh signaling in different cell types [14, 24, 25]. In contrast, a recent report described DYRK1B as a positive modulator of the Hh cascade [15], prompting us to reevaluate the role of this kinase in more detail. To this end, we have begun our studies by knocking down endogenous *Dyrk1b* in mouse embryonic fibroblasts stably expressing Sonic Hh ligand (MEF$^{[SHH]}$ cells [31]), which renders these cells constitutively signaling. As can be seen in Figure 1A, an RNAi pool of four different siRNA sequences designed against endogenous *Dyrk1b* led to a significant upregulation of several Hh target genes (*Gli1, Ptch1, Ptch2*) when compared to control siRNA-transfected cells. A de-repression of Hh pathway activity upon *Dyrk1b* knock-down was confirmed by measuring the protein levels of GLI1 (Figure 1A inset). Because DYRK1B had been previously linked to the serum-induced RAS-RAF-MEK pathway [32, 33], which could potentially affect its interaction with Hh signaling, we verified our results using different serum conditions (Figure 1A, 1B). However, using low (0.5%) or high (10%) serum conditions gave almost identical results. Furthermore, testing the four siRNA sequences individually confirmed a de-repression of Hh target gene expression in three out of four cases (Supplementary Figure S1A), arguing for a negative role of endogenous *Dyrk1B* on ligand-induced Hh signaling in fibroblasts. When Hh signaling was blocked by means of a ligand neutralizing antibody (5E1) or by pharmacological SMO inhibition (SANT), *Dyrk1b* knockdown no longer led to increased pathway activity, suggesting that *Dyrk1b* knockdown can modulate active Hh signaling but cannot elicit Hh signaling on its own (Supplementary Figure S1B).

Next, we went on to elucidate the function of this kinase when overexpressed. Therefore, we stably transfected NIH3T3 fibroblasts (a Hh-responsive cell line frequently used in the analysis of the Hh pathway) with an empty control plasmid or with a construct expressing V5-tagged *DYRK1B* (NIH$^{[Con]}$ and NIH$^{[1B]}$ cells; Figure 1C). Treating these cells with the synthetic SMO agonist SAG [34] to stimulate membrane signaling and immunoblotting for the endogenous target gene product GLI1 revealed that the *DYRK1B*-overexpressing cells had lost their SAG-responsiveness (Figure 1D, 1E), arguing that this kinase blocks the signal transmission from SMO to GLI. However, the basal levels of GLI1 were increased in NIH$^{[1B]}$ cells even in the absence of any stimulatory SAG, indicative of a non-canonical activation of GLI activity. Furthermore, we noted that *DYRK1B*-overexpressing cells displayed a GLI1 isoform of about 100 kD in addition to the most abundant 160 kD full-length isoform. Besides this 160 kD variant, control cells possessed a less abundant 130 kD isoform, which was not evident in NIH$^{[1B]}$ cells (Figure 1D). A 100 kD large GLI1 isoform has previously been proposed to represent an inhibitory variant of GLI1 [35]. In agreement with our hypothesis of non-canonical GLI1 activation, we found that the DYRK1B-induced increase in GLI1 levels was largely insensitive to SMO inhibition (Supplementary Figure S1C). Furthermore, the measurement of the mRNA expression levels of Hh target genes (*Gli1, Ptch1*) revealed that *DYRK1B* overexpression blocked SAG-induced Hh signaling while at the same time it increased the basal expression of *Ptch1* (Figure 1F). In contrast, the basal expression of the two major activators of the pathway, *Gli1* and *Gli2*, was drastically reduced in NIH$^{[1B]}$ cells and was close to the technical detection limit, whereas *Gli3* levels were unaffected (Figure 1F and Supplementary Figure S1D). Taken together, our data suggest that DYRK1B inhibits PTCH/SMO-initiated (canonical) Hh signaling while it promotes downstream (non-canonical) activation of the GLI1 transcription factor.

DYRK1B promotes GLI1 stability

We verified the findings made in fibroblasts by overexpressing *DYRK1B* in human cancer cells. In line

with our previous observations, stable *DYRK1B* expression in HeLa cells increased the levels of endogenous GLI1 protein (Figure 2A) while at the same time it decreased the *GLI1* mRNA levels (Figure 2B). The fact that GLI1 protein levels were increased upon *DYRK1B* transfection despite its mRNA being decreased argued for a stabilizing effect of DYRK1B on the GLI1 protein. To address this possibility, we performed protein stability assays in NIH$^{[Con]}$ and NIH$^{[1B]}$ cells blocking *de novo* protein synthesis with Cycloheximide. As can be seen in Figure 2C and 2D, endogenous GLI1 was degraded with a half-life ($t_{1/2}$) of approx. 3.5 h in SAG-treated control cells whereas GLI1 protein levels in SAG-treated *DYRK1B*-expressing cells were extremely stable and were only minimally affected over the entire time course of the experiment. Previously, AKT kinase has been shown to promote GLI

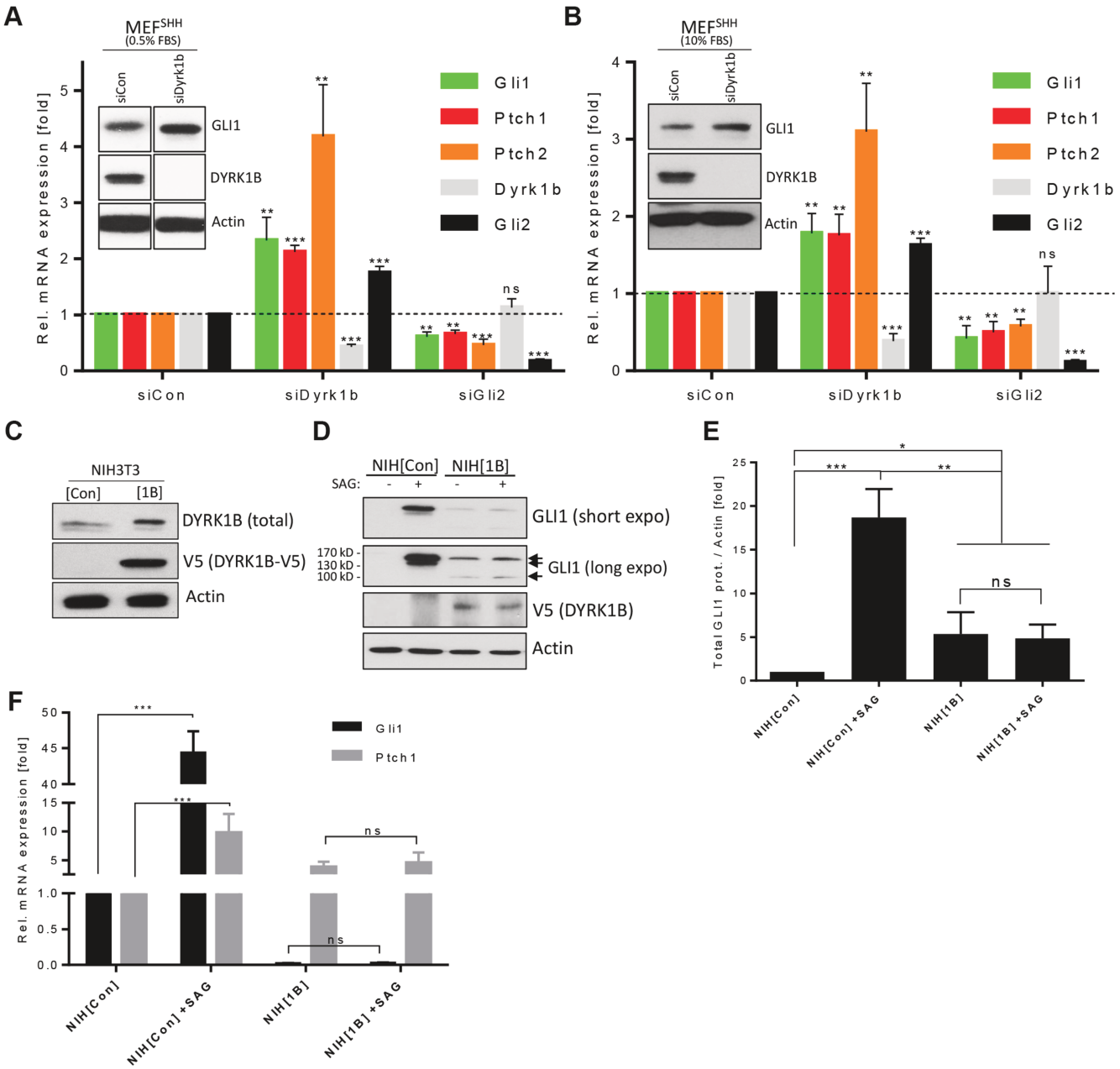

Figure 1: Differential effects of DYRK1B on Hh/GLI signaling. (**A**) Hh target gene expression in siRNA-transfected mouse embryonic fibroblasts stably expressing SHH (MEF$^{[SHH]}$). Shown is the mean ± SD of n = 3. Cells were cultured in 0.5% FBS-containing media. The inset shows a Western blot of the same experiment (samples were run on the same membrane with intervening lanes cut out). ns = non significant. (**B**) The same experiment as in (A), but performed in 10% FBS-containing media. (**C**) Immunoblot of lysates from NIH3T3 cells stably harboring an empty control (mock; NIH$^{[Con]}$) or a *DYRK1B-V5* (NIH$^{[1B]}$) expression plasmid. (**D**) Western analysis of NIH$^{[Con]}$ and NIH$^{[1B]}$ cells treated with SAG (100 nM) for 48 h. Note the three different GLI1 isoforms (arrows). (**E**) Quantification of the sum of all GLI1 bands (normalized against Actin) depicted in (D). Shown is the mean ± SD of n = 3. (**F**) Quantitative PCR of Hh target gene expression (*Gli1, Ptch1*) in SAG-treated NIH$^{[Con]}$ and NIH$^{[1B]}$ cells. Shown is the mean ± SD of n = 3.

stability [7, 26, 36]. Therefore, we treated the NIH[1B] cells, which possess stabilized GLI1 (Figure 2C, 2D), with a pan-AKT inhibitor (GSK-690693). In line with a GLI-stabilizing role of AKT also in these cells, GLI1 levels dropped significantly upon AKT inhibition, an effect which could be rescued by pharmacological blockade of the proteasome (Figure 2E). In summary, we could show that ectopically expressed DYRK1B kinase promotes an increase in GLI1 protein stability and that this effect is likely mediated through AKT.

DYRK1B activates the PI3K/mTOR/AKT pathway

Since our data suggested that AKT might play a role in the GLI1-stabilizing impact of DYRK1B, we analyzed the levels of activated (phosphorylated) AKT and mTOR. Interestingly, an induction of phosphorylation on mTOR-Ser2448 and on AKT-Ser473 and Thr308 could be observed in response to elevated *DYRK1B* expression (Figure 3A, 3B). As AKT^{Ser473} is exclusively phosphorylated by mTORC2 [29, 30], these data suggest that DYRK1B directly or indirectly activates this multi-protein complex. In addition, the PDK1-induced phosphorylation of Thr308 in AKT was also increased, suggesting that DYRK1B might activate the entire PI3K/mTORC2/AKT signaling arm or that both phospho-sites communicate and influence each other. In order to investigate whether the second mTOR complex (mTORC1) was also activated, we measured the phosphorylation status of one of its major targets, S6-Kinase (S6K-Thr389) and the S6K target ribosomal

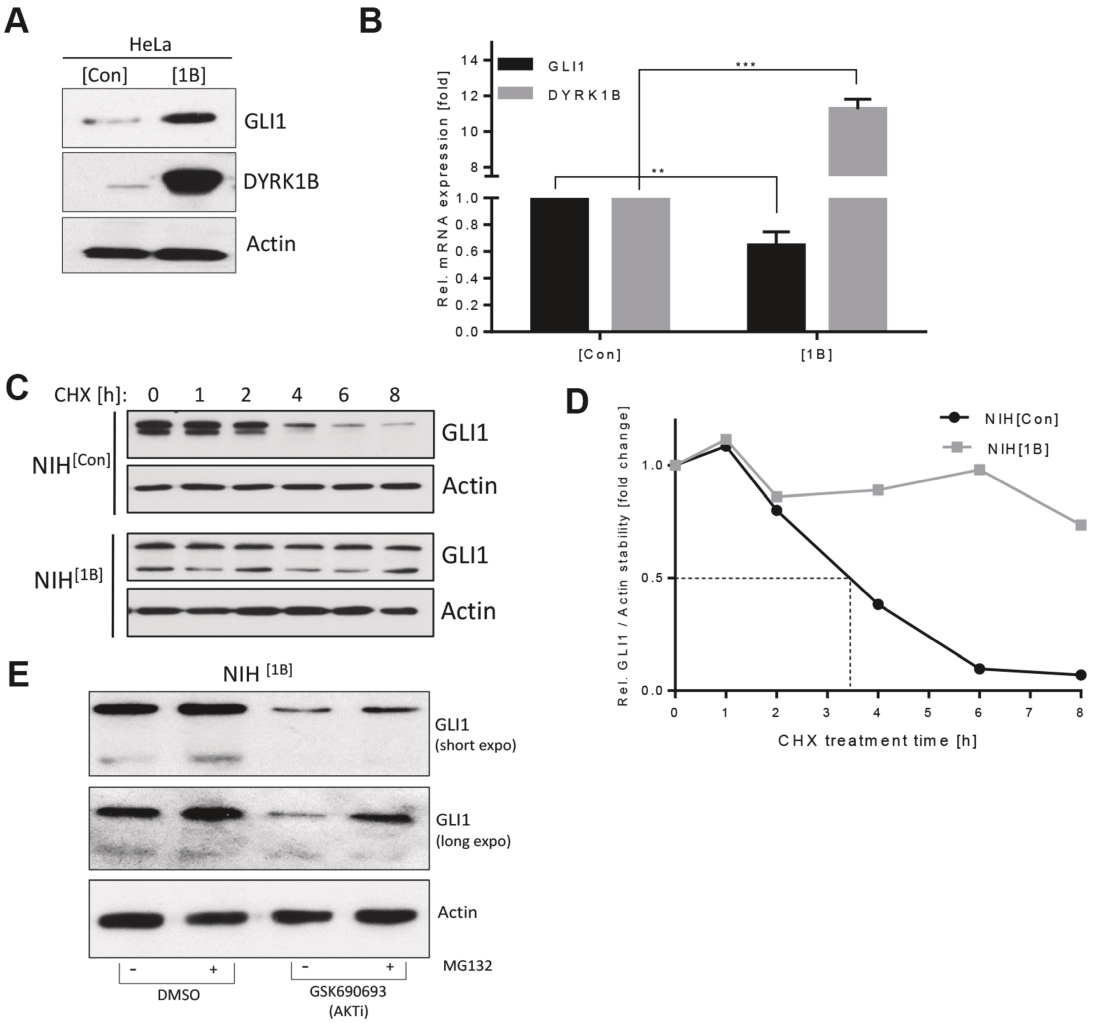

Figure 2: DYRK1B promotes non-canonical GLI1 stabilization. (**A**) Detection of GLI1 and DYRK1B by immunoblotting of HeLa cells stably transfected with empty control (mock; HeLa[Con]) or *DYRK1B* plasmid (HeLa[1B]). (**B**) *GLI1* and *DYRK1B* mRNA expression in HeLa[Con] and HeLa[1B] cells. Shown is the mean ± SD of $n = 3$. (**C**) GLI1 protein stability experiment using Cycloheximide (CHX 100 µg/ml for the indicated times). NIH[Con] and NIH[1B] cells were both pre-treated with 100 nM SAG overnight before addition of Cycloheximide (in continued presence of SAG in order to assure comparability). (**D**) Quantification of the results depicted in (C). Shown is the mean of two independent experiments. (**E**) Levels of endogenous GLI1 in NIH[1B] cells as measured by immunoblotting. Cells were pre-treated with MG132 (20 µM) or DMSO for 1 h, followed by parallel co-treatment with the pan-AKT inhibitor GSK-690693 (10 µM) for 12 h.

protein S6 (S6-Ser235/Ser236). In agreement with the induction of mTORC2/AKT activity, also the mTORC1 complex was activated by DYRK1B, as evidenced by stimulated phosphorylation of S6K and S6. However, this effect was less evident under high serum conditions, when basal levels of phospho-S6K and phospho-S6 are quite high (Figure 3C, 3D). In order to verify that DYRK1B overexpression also induces phosphorylation of PI3K/AKT pathway members in human cells, we analyzed stably *DYRK1B*-expressing HeLa cells (Figure 2A) for AKT and mTOR phosphorylation. In line with our data obtained in fibroblasts, *DYRK1B*-expressing cells displayed elevated levels of $mTOR^{S2448}$ and $AKT^{S473/T308}$ phosphorylation (Supplementary Figure S2A).

Next, we were eager to investigate the effects on mTOR/AKT in cells genetically depleted of *DYRK1B* through CRISPR/Cas9 methodology (Figure 3E). Here, we made use of mammalian HAP1 cells harboring a haploid genome, which facilitates the efficacy of CRISPR-based approaches [37]. Interestingly, *DYRK1B*-knock out (KO) cells displayed reduced endogenous GLI1 protein levels (Figure 3E). Furthermore and in congruence with our previous findings, serum-stimulated *DYRK1B*-KO cells demonstrated an overall reduced level of AKT (Ser473; Thr308) and mTOR (Ser2448) phosphorylation (Figure 3F–3H and Supplementary Figure S3A). Furthermore, in line with mTOR favoring cell growth and proliferation [28], *DYRK1B*-KO cells proliferated significantly slower than the parental *DYRK1B* wildtype cells (Supplementary Figure S3B). Taken together, our data imply that DYRK1B is an activator of the PI3K/mTOR/AKT signaling pathway.

DYRK1B contributes to Hh-induced mTOR/AKT activation

Continuing from these observations, we went on to elucidate the link between these phosphorylation events and Hh signaling. First we verified literature data that fibroblasts respond to Hh pathway stimulation with activation of AKT and mTOR (Supplementary Figure S4A). Similarly, continuously signaling $MEF^{[SHH]}$ cells displayed elevated levels of phosphorylated AKT and mTOR, which could be suppressed by inhibition of the Hh pathway with the SMO antagonist SANT (Figure 4A, 4B) [34]. As our previous data suggested that DYRK1B activates the same set of kinases, we wondered whether DYRK1B was involved in mediating the effect of Hh signaling on AKT/mTOR. Indeed, stimulating the Hh pathway led to an elevation of DYRK1B protein levels and, conversely, suppressing active Hh signaling reduced DYRK1B levels in two different fibroblast cell lines (Figure 4C). We therefore went on to analyze phospho-mTOR/AKT levels in $MEF^{[SHH]}$ cells after *Dyrk1b* knock-down. As can be seen in Figure 4D and 4E, the levels of phospho-AKT^{Ser473} and phospho-AKT^{Thr308} were significantly reduced upon knock-down of *Dyrk1b*. The levels of phospho-mTOR remained constant, which was surprising in light of the reduced AKT^{Ser473} (a selective mTORC2 substrate) levels after *Dyrk1B* knock-down. However, as the antibody detects the entire mTOR pool, this might reflect a differential effect of endogenous DYRK1B on mTORC2 versus mTORC1. Supporting our data of reduced Hh-induced AKT (mTOR) phosphorylation in the absence of DYRK1B in MEF cells, we could obtain similar results using NIH3T3 cells (Supplementary Figure S4B). In summary, our data suggest that Hh signaling induces the phosphorylation of AKT (and potentially mTOR) through DYRK1B.

The kinetics of DYRK1B-mediated GLI regulation

Our data thus far implied that DYRK1B stimulates the mTOR/AKT pathway, which subsequently promotes GLI stabilization. The PI3K/mTOR/AKT system is subject to intense feed-back regulation, resulting in e.g. pronounced upregulation of phospho-AKT in the case of mTORC1 inhibitors, which has also created difficulties with the clinical use of this compound class [27, 28]. We were therefore interested to see how the Hh pathway would be regulated over time after DYRK1B inhibition. First, we investigated the systemic feedback regulation by analyzing AKT phosphorylation in $NIH^{[MCS]}$ and $NIH^{[1B]}$ cells upon inhibition of AKT (GSK-690693, a pan-AKT inhibitor), mTOR (KU-0063794, a dual mTORC1/2 inhibitor) and DYRK1B (AZ191, a selective small molecule DYRK1B inhibitor [38]). As can be seen in Figure 5A, all inhibitors led to a subsequent increase in phospho-AKT levels in *DYRK1B*-overexpressing cells, although they were different in amplitude. In contrast, in wildtype $NIH^{[MCS]}$ cells, AKT and mTOR inhibition resulted in reduced phospho-AKT levels while AZ191 led to an increase. Taken together with the previous experiments, this result strongly suggested that DYRK1B is indeed involved in a complex regulatory mTOR/AKT feedback loop.

Next, we turned to ShhL2 cells (a clonal NIH3T3 cell line harboring a Hh/GLI-responsive luciferase reporter construct in the genome [39]) and pre-treated these cells with SAG to activate Hh signaling. Then, AZ191 was added for different time periods and the activity of Hh signaling was recorded. As can be seen in Figure 5B and 5C, when compared to the DMSO control, Hh signaling was suppressed by AZ191 in the first 24 h and was then increased over controls at later time points (48–72 h), suggesting a pronounced influence of feedback regulation on the kinetics of the Hh response. These data show that the exact time point of analysis is important when determining the effects of DYRK1B.

In order to analyze the issue of kinetics further, we knocked down endogenous *DYRK1B* in human Panc1

pancreatic cancer cells by two different approaches: 1.) In a short-term experiment (2–3 d), short-interfering RNA (siRNA) was used and 2.) In a long-term experiment (6–7 d), short hairpin RNA (shRNA) was applied. The acute knock-down of *DYRK1B* by means of siRNA (short-term) resulted in an increase of endogenous GLI1 levels (Figure 5D and Supplementary Figure S5A). In contrast, the long-term knock-down of *DYRK1B* through a shRNA approach (Supplementary Figure S5B, S5C) led to a suppression of GLI1 expression (Figure 5E and Supplementary Figure S5D, S5E) and reduced levels of phosphorylated AKT and mTOR (Supplementary Figure S5F). To corroborate these findings and to rule out potential effects of siRNA versus shRNA technology, we

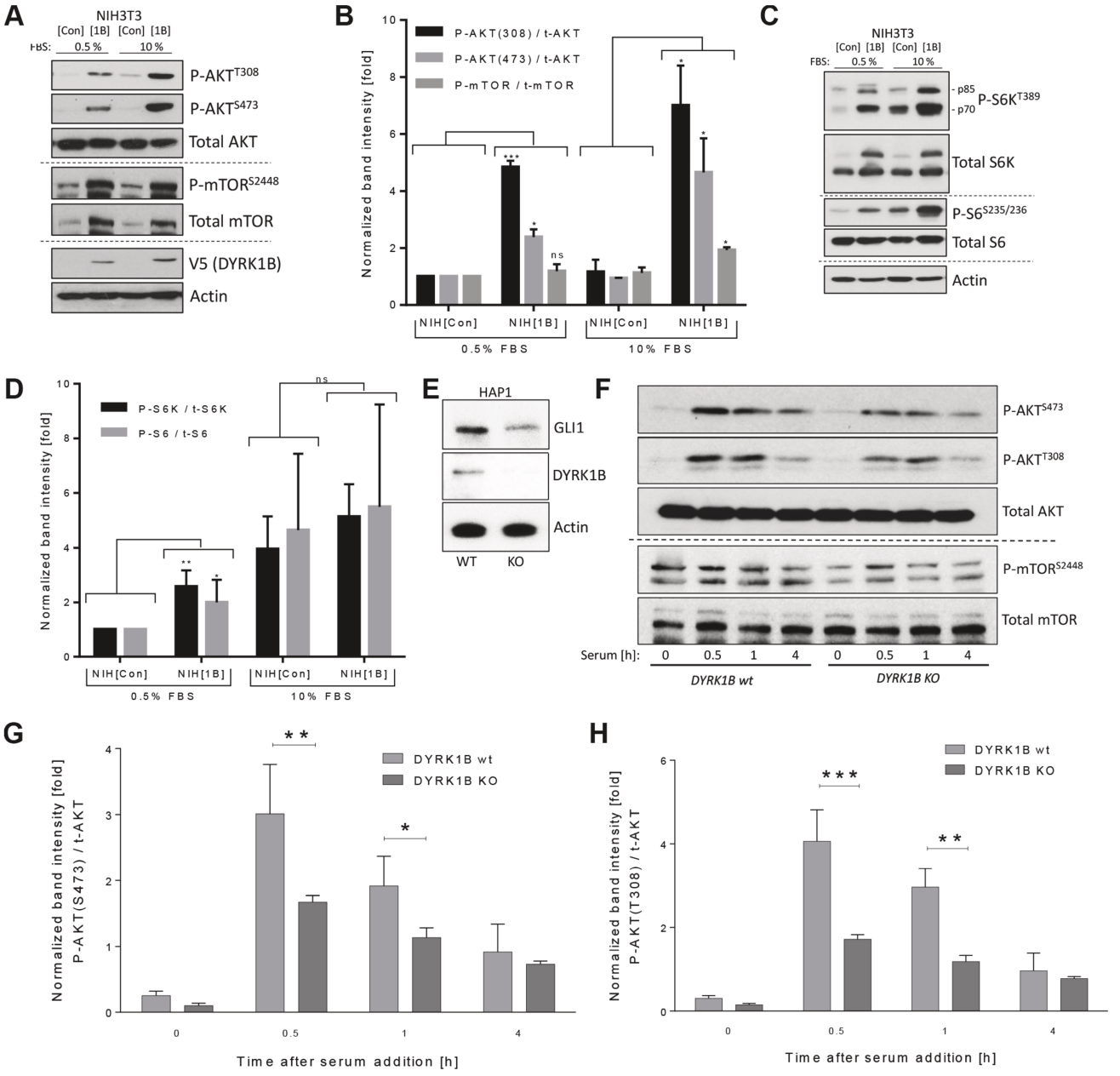

Figure 3: DYRK1B activates the mTOR/AKT pathway. (**A**) Detection of AKT and mTOR proteins by immunoblotting of NIH$^{[Con]}$ and NIH$^{[1B]}$ cell lysates. (**B**) Quantification of the results depicted in (A). Shown are band intensities of the phosphorylated proteins (e.g. P-AKT(308)) normalized to the respective total protein (e.g. t-AKT); mean ± SD of n = 3. (**C**) Detection of S6-Kinase (S6K) and S6 proteins by immunoblotting of NIH$^{[Con]}$ and NIH$^{[1B]}$ cell lysates. (**D**) Quantification of the results depicted in (C). Shown are band intensities of the phosphorylated protein normalized to the respective total protein; mean ± SD of n = 3. (**E**) Western blot using lysates from wildtype (wt) and from *DYRK1B*-knock out (KO) HAP1 cells. (**F**) Kinetics of AKT and mTOR phosphorylation in *DYRK1B* wt and KO HAP1 cells. Cells were starved in 0% FBS-containing media for 24 h before addition of 10% serum (FBS) for the indicated times. (**G**) Quantification of AKT^{Ser473} phosphorylation as depicted in (F). Shown is the mean ± SD of n = 3. (**H**) Quantification of AKT^{Thr308} phosphorylation as depicted in (F). Shown is the mean ± SD of n = 3.

performed a time course experiment treating Panc1 cells for 9 d with AZ191 and determined the daily changes in GLI1 levels (Figure 5F and Supplementary Figure S5G). In line with our previous findings, GLI1 protein levels undulated during this time frame and were induced during the first 6 days, followed by a reduction below the DMSO control levels afterwards (7–9 d). In summary and in agreement with DYRK1B impinging on the strongly feedback-regulated mTOR/AKT kinase system, we could observe a prominent time-dependent impact of DYRK1B inhibition on GLI1 levels.

Targeting DYRK1B in GLI-dependent cancer cells

From a therapeutic point of view, the fluctuating kinetics of GLI1 levels following a DYRK1B inhibition are problematic as suboptimal or short-term treatments with DYRK1B antagonists might result in concomitant upregulation of oncogenic GLI1 in cancer cells. This might be particularly true if these cells express high levels of *DYRK1B*, such as many pancreatic and ovarian cancer cells. We therefore tested the combination of AZ191 (DYRK1B inhibitor) with drugs targeting mTORC1/2 (KU-0063794), AKT (GSK-690693) or S6K (PF-4708671) and measured the effects on GLI1 levels in *DYRK1B*-amplified Panc1 cells (Figure 6A and Supplementary Figure S6A–S6D). Treatment with AZ191 alone (24 h) increased the phosphorylation of AKT and the GLI1 expression, whereas co-treatment with the mTOR/AKT/S6K inhibitors significantly reduced the levels of both. The effect was most clearly seen with the dual mTORC1/2 inhibitor (KU-0063794), which on its own had little effect on GLI1. In combination with AZ191 however, GLI1

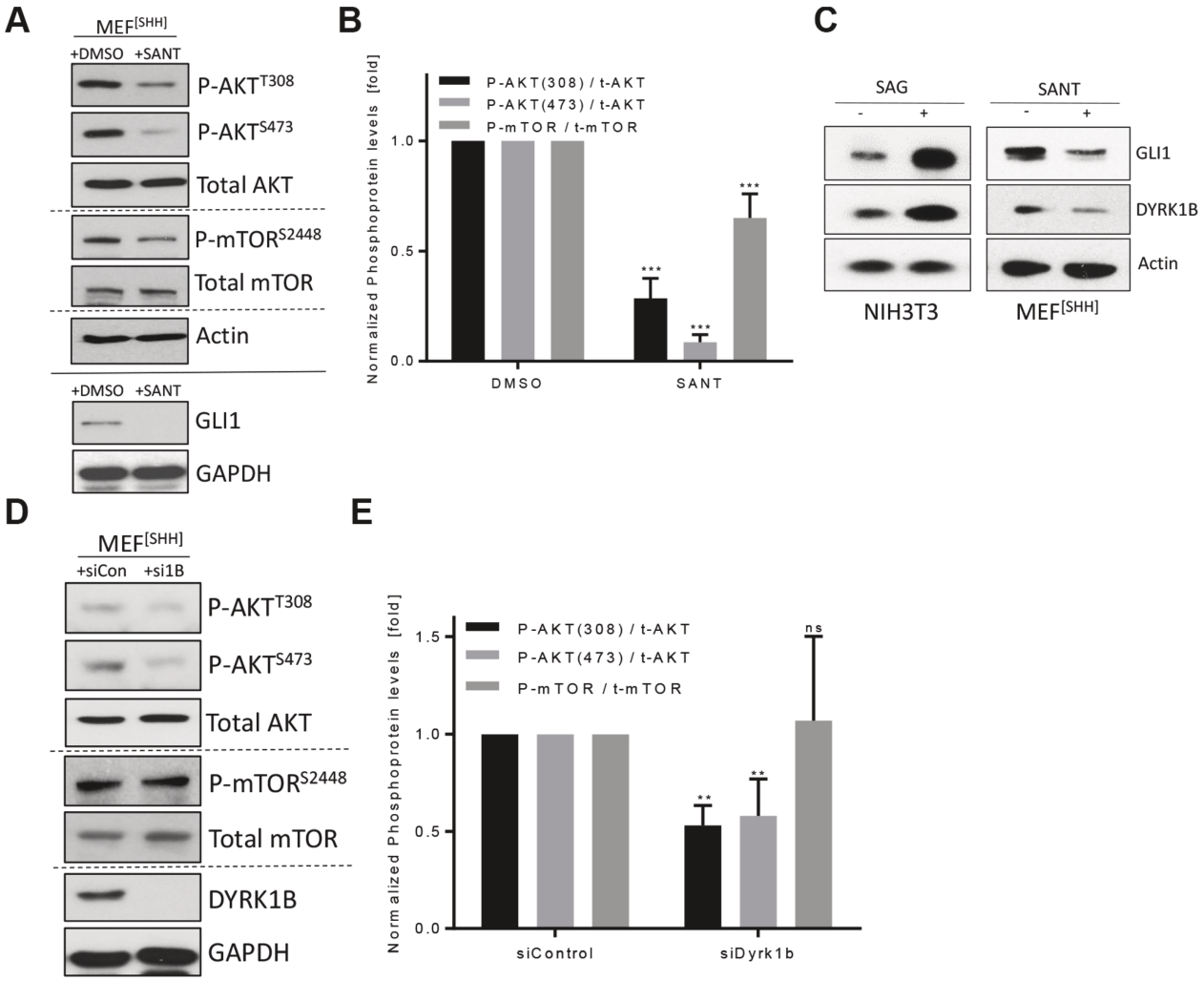

Figure 4: DYRK1B contributes to Hh-induced phosphorylation reactions. (**A**) Detection of AKT and mTOR proteins by immunoblotting of MEF[SHH] cell lysates. Cells were treated with DMSO or SANT (0.2 μM) for 2–3 d before lysis. (**B**) Quantification of the results shown in (A). Shown is the mean ± SD of $n = 3$. (**C**) Levels of endogenous GLI1 and DYRK1B protein as measured by Western blotting of NIH3T3 or MEF[SHH] cell lysates. Treatment (48 h) as indicated. (**D**) Detection of AKT and mTOR proteins by immunoblotting of MEF[SHH] cell lysates after transfection with control siRNA (siCon) or with a pool of four different *Dyrk1b*-specific RNAi sequences (si1B). (**E**) Quantification of the results shown in (D). Shown is the mean ± SD of $n = 3$.

levels were almost completely abrogated (Figure 6A and Supplementary Figure S6A–S6D). As Panc1 cells grow in a GLI1-dependent manner [13, 15, 40], we next turned to cell growth assays to measure the cytotoxic impact of these inhibitors. While single treatment with AZ191 or KU-0063794 alone displayed only a moderate effect on cell growth (cytostatic), the combination of both drugs was strongly cytotoxic to pancreatic cancer cells (Figure 6B). Comparable results were obtained with the combination treatment of AZ191 plus AKT inhibitor (Figure 6C). The S6K antagonist was quite effective as monotherapy and a significant additional effect of AZ191 was therefore not possible to detect (Figure 6D). Intriguingly, knock-down of *GLI1* significantly reduced the cell growth retardation seen with the various inhibitors, highlighting the importance of GLI1 in mediating many of the observed anti-proliferative effects (Supplementary Figure S7A–S7E). Moreover, the results on dual DYRK1B-PI3K/AKT/mTOR/S6K inhibition were not specific to Panc1 cells as we could reproduce them in Ovcar-3 ovarian cancer cells using combinations of AZ191 and inhibitors targeting PI3K, mTOR, AKT and S6K (Supplementary Figure S8A–S8E).

Taken together, we propose that a dual targeting approach combining a DYRK1B antagonist with an inhibitor of the PI3K/mTOR/AKT pathway has a pronounced impact on the GLI1 oncoprotein and exerts strong cytotoxic effects in cancer cells.

DISCUSSION

Previous findings on the role of DYRK1B in the Hh pathway were inconclusive and positive [15] as well as negative [14, 24, 25] regulatory functions were ascribed to this kinase. Here, we attempted to bring together these differing results and clarify the role of DYRK1B in more detail. Our data reveal a complex interaction of this kinase with mammalian Hh/GLI regulation showing dual and sometimes opposing effects: 1.) The ectopic expression of *DYRK1B* potently blocked canonical SMO-initiated signaling. The underlying mechanism of this negative regulation requires further investigations. 2.) In contrast, overexpressed *DYRK1B* enhanced the protein stability of GLI1 by preventing its proteasomal degradation. This stabilizing effect is most likely executed through AKT, which we found to be activated by DYRK1B and which is known to phosphorylate and protect GLI transcription factors from decay [7, 26]. The exact mechanism of AKT stimulation by DYRK1B is currently unknown and requires

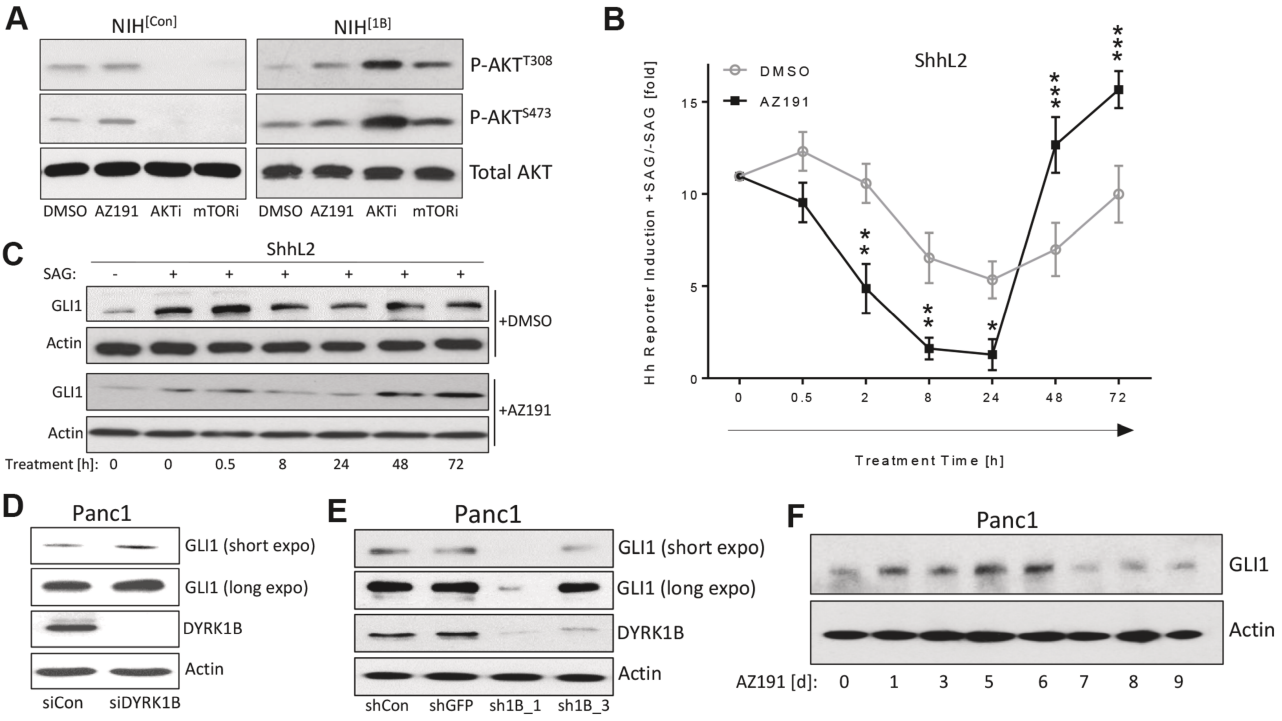

Figure 5: Kinetics of DYRK1B-induced effects. (**A**) Detection of AKT phosphorylation in NIH[Con] and NIH[1B] cells. Treatment was in 0.5% FBS for 24 h. DYRK1B-inhibitor (AZ191; 1 μM); AKT-inhibitor (AKTi: GSK-690693; 10 μM); mTOR-inhibitor (mTORi: KU-0063794; 1 μM). (**B**) Time course experiment in ShhL2 cells (Hh reporter cells). Cells were plated and 24 h later, SAG (100 nM) was added for another 24 h. Then, DMSO or AZ191 (1 μM) was added to the wells (keeping SAG as well) for the indicated treatment times. The statistical significances were calculated comparing DMSO vs AZ191-treated samples. Shown is the mean ± SD of $n = 3$. (**C**) GLI1 Western blot of ShhL2 lysates as shown in (A). (**D**) GLI1 protein levels in Panc1 cells transfected with control siRNA (*siCon*) or *DYRK1B*-specific siRNA pool (*siDYRK1B*). Cells were harvested 3d after transfection. (**E**) GLI1 protein levels in Panc1 cells transfected with control shRNA plasmids (*shCon, shGFP*) or *DYRK1B*-specific shRNA plasmids (*sh1B_1, sh1B_3*). Cells were harvested 7d after transfection. (**F**) Levels of endogenous GLI1 in Panc1 cells treated for the indicated times with 1 μM of AZ191.

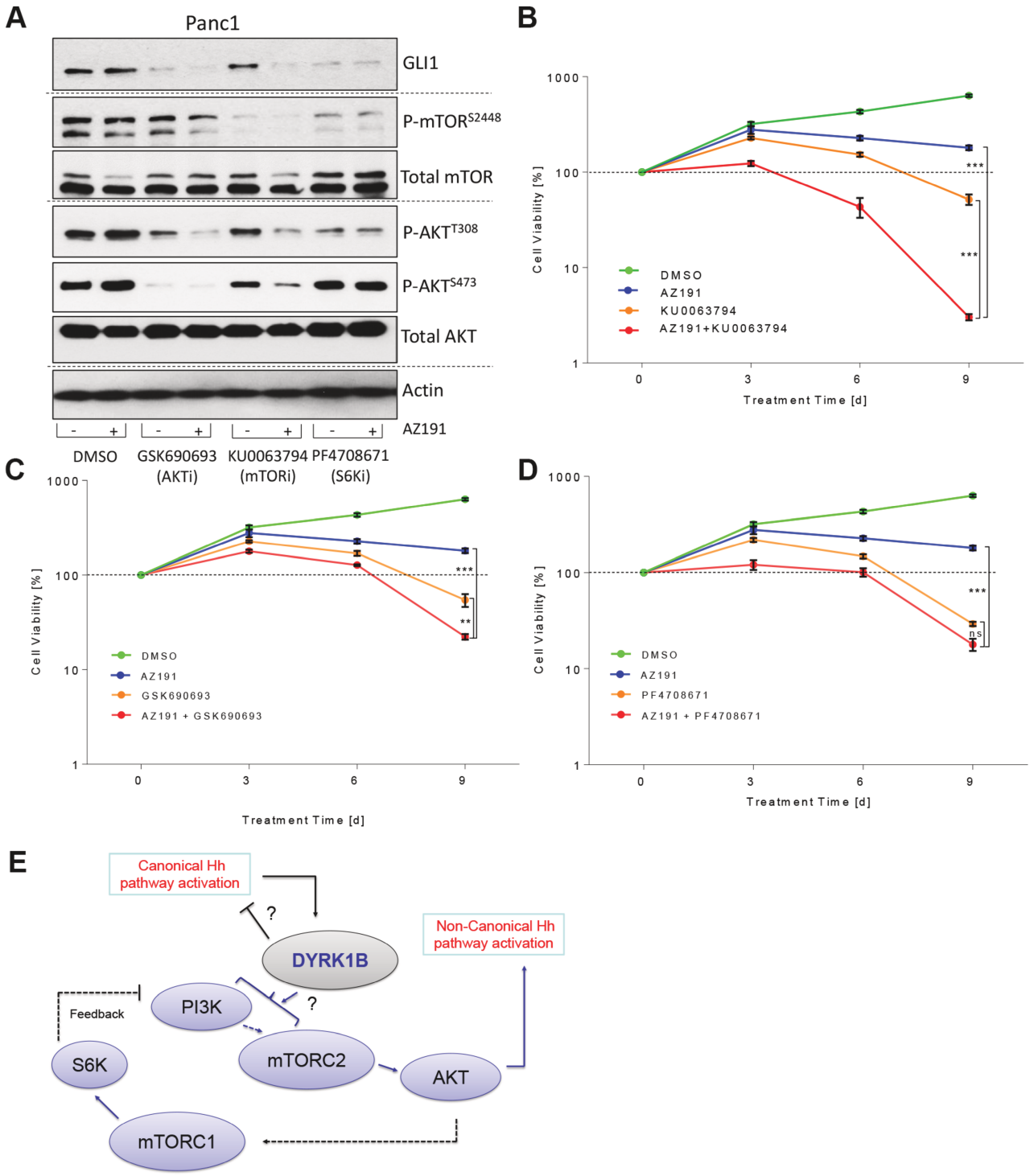

Figure 6: Improving the therapeutic targeting of DYRK1B. (**A**) Immunoblot showing the levels of GLI1, AKT and mTOR proteins in Panc1 cells upon treatment (24 h, 0.5% FBS) with various inhibitors. GSK-690693 (pan-AKT inhibitor): 10 μM; KU-0063794 (dual mTORC1/2 inhibitor): 1 μM; PF-4708671 (S6K1 inhibitor): 10 μM. (**B**) Panc1 growth curve (mean ± SD of $n = 3$). Cells were treated with DMSO, AZ191 (1 μM), KU-0063794 (dual mTOR inhibitor; 1 μM) or combinations as indicated. (**C**) Panc1 growth curve (mean ± SD of $n = 3$). Cells were treated with DMSO, AZ191 (1 μM), GSK-690693 (pan-AKT inhibitor; 10 μM) or combinations as indicated. (**D**) Panc1 growth curve (mean ± SD of $n = 3$). Cells were treated with DMSO, AZ191 (1 μM), PF-4708671 (S6K1 inhibitor; 10 μM) or combinations as indicated. (**E**) Graphical depiction of our findings on DYRK1B-mediated regulation of Hh pathway activity. Solid and punctate lines depict direct and indirect interactions, respectively. The exact mechanism of PI3K/mTORC2 activation by DYRK1B requires further investigation.

future work. 3.) Because of DYRK1B's ability to activate the PI3K/mTOR/AKT pathway, the whole DYRK1B-Hh/GLI-system is subject to pronounced feedback control, resulting in a strong influence of kinetics on the actual Hh pathway output. Therefore, short-term inhibition of DYRK1B resulted in an enhancement of Hh signaling whereas long term blockade of DYRK1B function was associated with suppression of GLI1 levels. We believe that these findings can explain many, if not all, published effects of DYRK1B on Hh/GLI signaling and suggest that most previous studies might represent only one specific aspect of the entire crosstalk spectrum. A comparable controversy attributes to the role of oncogenic RAS on Hh signaling [11, 14, 41, 42] and it is interesting to note that DYRK1B has been described as a downstream effector of mutant KRAS [32]. Moreover, our connection presented here between DYRK1B and PI3K/mTOR/AKT signaling might explain why DYRK1B was discovered in a large screen identifying synthetic lethal gene partners of mutant *KRAS* [43]. In addition, its involvement in PI3K signaling and the discovery of *DYRK1B* mutations in the metabolic syndrome are intriguing [25, 44, 45].

Strikingly, we could observe that, at least in one specific cell line tested, the stress-induced DYRK1B kinase was able to potently stimulate GLI1 protein stability even in the absence of clearly measurable *Gli1* and *Gli2* mRNA expression (Figure 1F, Supplementary Figure S1D). Thinking about possible routes of GLI1 activation in several pathological situations, it is tempting to speculate about Hh target gene induction being potentially triggered by completely Hh-unrelated regulators inducing *DYRK1B* expression, bypassing the need for increased *GLI1/2* mRNA levels. Moreover, in one cell line, ectopic *DYRK1B* expression altered the appearance of GLI1 protein isoforms and promoted the generation of a shorter variant of about 100 kD. Although a variant of this size has been suggested to be inhibitory [35], another shorter isoform of 130 kD seems to be activating [46]. How DYRK1B is generating this shorter GLI1 isoform, how general the effect is and what the role of this shorter GLI1 variant might be warrants further investigations.

It is well established that Hedgehog signaling induces numerous kinases including AKT, but the underlying mechanism has been elusive. Here, we could demonstrate that SMO activation results in upregulation of DYRK1B and that depletion of this kinase by means of RNAi abrogates the ability of Hh signaling to stimulate AKT phosphorylation. As this also applies to Ser473 phosphorylation of AKT, a faithful read-out of mTORC2 functionality [29, 30], we hypothesize that DYRK1B might be involved in mTORC2 regulation. In line with this assumption, we found that siRNA against *Dyrk1b* reduced the levels of phospho-AKT^{Ser473} (marker for mTORC2 activity), but had little repressive effect on phospho-S6/S6K (read-out of mTORC1 activity) (not shown). With respect to the activation of the pro-survival kinase AKT, it is interesting to note that DYRK1B has actually been described as a survival kinase before [21, 47].

In summary, we could describe a surprisingly complex crosstalk between DYRK1B and Hh signaling. According to our model, the exact net result of DYRK1B's impact on the Hh pathway might be dependent on DYRK1B expression level, canonical/non-canonical Hh signaling, time point of analysis and/or cell type. In a clinical situation aiming to target the DYRK1B survival kinase, considering all these different aspects will be impossible. Therefore, we have tested a combination treatment targeting DYRK1B and the mTOR/AKT pathway in a proof-of-principle study. Using *DYRK1B*-amplified pancreatic and ovarian cancer cells, co-targeting both kinases resulted in a significantly reduced GLI1 level and in increased cell death induction which could help to design new cancer therapies in the future.

MATERIALS AND METHODS

Reagents

Smoothened agonist SAG was purchased from Calbiochem. SANT (SANT-1); KU-0063794, GSK-690693, PF-4708671 and MG132 were from Sigma and AZ191 was from SelleckChem. Cycloheximide was purchased from Biomol.

The monoclonal Hedgehog neutralizing antibody (5E1, supernatant), developed/deposited by T.M. Jessell/S. Brenner-Morton was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

Cell lines

NIH3T3, HeLa, ShhL2, Panc1, OvCar3 cell lines were purchased from ATCC. All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM (high Glucose plus Glutamine and Pyruvate), Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin at 37°C with 5% CO_2. MEF$^{[SHH]}$ cells were kindly provided by Wade Bushman [31]. *DYRK1B* wildtype and knock-out haploid HAP1 cells (#HZGHC000363c010) were purchased from Horizon Genomics (Vienna, Austria). If not otherwise stated, serum concentrations were reduced to 0.5% during experiments for all cell types.

Generation of stable cells

The coding sequence of human *DYRK1B* (isoform a) was PCR amplified and cloned into pEF6/V5-His using TOPO cloning (Invitrogen) yielding pEF-DYRK1B. NIH3T3 and HeLa cells were transfected with empty vector or with pEF-DYRK1B and cell clones surviving Blasticidin selection were pooled.

Luciferase reporter assays

ShhL2 cells were plated in triplicate and were grown to full confluence in solid white 96-well plates with clear bottom. Subsequently, cells were treated in 5% FBS-containing medium with 100 nM SAG plus the respective compounds for the indicated times. Cells were lysed in Passive Lysis Buffer (Promega) and Firefly and Renilla Luciferase activity were measured using an Orion L microplate luminometer (Berthold Detection Systems) using Beetle- and Renilla-Juice reagents (both PJK).

Immunoblotting

Separation of lysates by SDS-PAGE was followed by subsequent Western Blot analysis. SDS-PAGE gels were blotted on Immobilon-PVDF membranes (Millipore) and incubated with the respective primary antibody, followed by an HRP-coupled secondary antibody. Detection of the HRP signal was performed using Pierce ECL Western Blotting Substrate (Thermo Scientific) according to the manufacturer's protocol. The following primary antibodies were used: α-DYRK1B (#5672; Cell Signaling Technology (CST)); α-GLI1 (#2643; CST); α-total AKT (#9272; CST); α-phospho-AKT^{Ser473} (#9271; CST); α-phospho-AKT^{Thr308} (#13038; CST); α-phospho-mTOR^{Ser2448} (#5536; CST); α-total mTOR (#2983; CST); α-V5 (#R960-25; Invitrogen); α-GAPDH (#G9545; Sigma); α-Actin (#A5441; Sigma).

Small-interfering RNA (siRNA) and short hairpin RNA (shRNA) transfection

Cells were transfected with 35 nM siRNA (Dharmacon SMARTpools and Qiagen control siRNA using RNAiMax (Invitrogen). Control siRNA (siCon) was purchased from Qiagen (All-Stars-siRNA; siAll). The *Dyrk1b*-specific siRNA target sequences were: si1B_1: AUACAGAGAUGAAGUACUA; si1B_2: GCACAUCAAUGAGGUAUAC; si1B_3: GAGAUGAAGUACUACAUAG; si1B_4: GGACAAAGGAACUCAGGAA: The mouse *Gli2*-specific and the human *DYRK1B*-specific siRNAs have been described before [14, 48].

Short-hairpin RNA (shRNA) target sequences in pLKO. 1-puro backbone (Mission, obtained through Sigma) were as follows: shCon (SHC002; scrambled control): CAACAAGATGAAGAGCACCAA; shGFP (SHC005; targeting EGFP): TACAACAGCCACAACGTCTAT; shDYRK1B_1: GACCTACAAGCACATCAATGA; shDYRK1B_3: CACGGAGATGAAGTACTATAT; shGLI1_1: CATCCATCACAGATCGCATTT. Panc1 cells were transfected on day 0 in 10% FBS media. Medium was changed to 0.5% FBS media and Puromycin (2 μg/ml) was added on day 1 (cells were kept like this until day 6). After recovery for 1 d in medium without selective pressure, cells were harvested on day 7 (for shDYRK1B) or day 9 (for shGLI1).

RNA preparation, cDNA synthesis, qPCR

Total RNA was extracted using NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's protocol. cDNA synthesis of 1 μg total RNA was performed using iScript cDNA Synthesis Kit (Biorad) following the manufacturer's guidelines. Quantitative PCR reactions were performed using the Absolute QPCR SYBR Green Mix (ABGene). qPCR reactions were performed on 96 well qPCR plates (ABGene) using either the Mx3000P or Mx3005P qPCR systems (Agilent). Results were calculated as relative mRNA expression ($2^{\Delta\Delta Ct}$). Data was obtained from at least three independent experiments and is shown as the mean ± StDev. Primer sequences (5′ to 3′) for the detection of mouse *Dyrk1b* were: For-TTGACACCTGCCCCTCCTCTAGCAC; Rev-GGCCCCCACAATATCGGTTGCTGTA. Human *DYRK1B*: For-TTGGCCAGGTGGTGAAAGCCTATGA; Rev-CAATCTGGGCCTGGTTCAGGAAAGC. All other primer sequences have been described elsewhere [13, 48–50].

Statistical analysis

Unless otherwise stated, data is presented as the mean of three independent experiments ± standard deviation (StDev). Statistical significance was calculated by applying a two-tailed student's *t*-test. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

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CONFLICTS OF INTEREST

None.

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