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# Mitogen-Activated Protein Kinases and Hedgehog-GLI signaling in cancer: a crosstalk providing therapeutic opportunities?

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**ABSTRACT**

The Hedgehog-GLI (HH-GLI) signaling is of critical importance during embryonic development, where it regulates a number of cellular processes, including patterning, proliferation and differentiation. Its aberrant activation has been linked to several types of cancer. HH-GLI signaling is triggered by binding of ligands to the transmembrane receptor Patched and is subsequently mediated by transcriptional effectors belonging to the GLI family, whose function is fine tuned by a series of molecular interactions and modifications. Several HH-GLI inhibitors have been developed and are in clinical trials. Similarly, the Mitogen-Activated Protein Kinases (MAPK) are involved in a number of biological processes and play an important role in many diseases including cancer. Inhibiting molecules targeting MAPK signaling, especially those elicited by the MEK1/2-ERK1/2 pathway, have been developed and are moving into clinical trials. ERK1/2 may be activated as a consequence of aberrant activation of upstream signaling molecules or during development of drug resistance following treatment with kinase inhibitors such as those for PI3K or BRAF. Evidence of a crosstalk between HH-GLI and other oncogenic signaling pathways has been reported in many tumor types, as shown by recent reviews. Here we will focus on the interaction between HH-GLI and the final MAPK effectors ERK1/2, p38 and JNK in cancer in view of its possible implications for cancer therapy. Several reports highlight the existence of a consistent crosstalk between HH signaling and MAPK, especially with the MEK1/2-ERK1/2 pathway, and this fact should be taken into consideration for designing optimal treatment and prevent tumor relapse.

**Keywords:** Hedgehog, GLI, MAPK, ERK, Cancer, Signal Transduction, Signaling Integration, Targeted Therapy, Combination therapy

**Abbreviations:**

BCC, Basal Cell Carcinoma

BRAF-i, BRAF inhibitors

CCA, Cholangiocarcinoma

CSC, Cancer Stem Cell

EGF, Epidermal Growth Factor

EGFR, Epidermal Growth Factor Receptor

ERK, Extracellular Signal-Regulated Kinase

FGFb, basic Fibroblast Growth Factor

HCC, Hepatocellular Carcinoma

HH, Hedgehog

IGF1, Insulin-like Growth Factor 1

IGF1R, Insulin-like Growth Factor 1 Receptor

IHH, Indian Hedgehog

IRS1, Insulin Receptor Substrate 1

JNK, c-Jun N-terminal Kinase

MAPK, Mitogen-Activated Protein Kinase

MB, Medulloblastoma

MEK, MAPK/ERK Kinase

MMP9, Matrix Metalloproteinase-9

PDGF, Platelet-Derived Growth Factor

PDGFR, Platelet-Derived Growth Factor Receptor

PI3K, Phosphatidylinositol-3-Kinase

PTCH, Patched

RSK2, Ribosomal S6 Kinase 2

RTK, Receptor Tyrosine Kinase

SHH, Sonic Hedgehog

SMO, Smoothened

**1. Classical activation of the MAPK pathway**

Mitogen-activated protein kinases (MAPK) convert extracellular stimuli into biological functions including cell survival, proliferation, migration and apoptosis. Growth factors, cytokines, extracellular matrix, osmotic stress, reactive oxygen species as well as lipopolysaccharide may activate MAPK. These enzymes are serine-threonine kinases that include conventional (Extracellular Signal-Regulated Kinases ERK1 and ERK2, p38, c-Jun N-terminal kinases, JNK, and ERK5) and atypical (ERK4, ERK8, human orthologs of rat ERK3 and ERK7, respectively, and Nemo-like kinase, NLK) MAPK [1]. Besides being involved in a number of biological processes, MAPK play an important role in many diseases including inflammation and cancer [2-4]. The MAPK pathway consists of a MAP kinase kinase kinase (MAP3K), a MAP kinase kinase (MAP2K) and a MAPK. Stimulation of the pathway results in the eventual activation of the MAPK by dual phosphorylation of a threonine and a tyrosine residue (T-X-Y) located in the phosphorylation loop. This activation is induced by MAP2K (e.g. in case of classical MAPK, MEK1 and MEK2 for ERK1/2; MKK3 and MKK6 for p38, MKK4 and MKK7 for JNK, MKK2 and MKK3 for ERK5). Fine-tuning of MAPK activation, in strength and duration, depends on cellular context and biological processes [5,6] and is achieved by several mechanisms, including dephosphorylation by phosphatases such as dual specificity phosphatases (DUSP), kinase interaction motif protein tyrosine phosphatases (KIM-PTP) and serine/threonine protein phosphatases (e.g. PP2A). Another equally important role in MAPK regulation is that of MAPK scaffolds, such as Kinase Suppressor of Ras, KSR, that play a key role in modulating the strength and duration of MAPK activation [7].

MAPK have cytosolic as well as nuclear targets [1]. Following activation, ERK1/2 phosphorylates a large number of substrates [8]. Among ERK1/2 cytoplasmic substrates, there are death-associated protein kinase (DAPK), tuberous sclerosis complex 2 (TSC2), RSK and MNK. Nuclear targets include NF-AT, Elk-1, myocyte enhancer factor 2 (MEF2), c-Fos, c-Myc and STAT3. Proteins located at the level of cytoskeleton (neurofilaments and paxillin) or associated with membranes (CD120a, Syk and calnexin) are also target of ERK1/2. The transcription factor c-Jun is a well-described substrate of activated JNK . Additional transcription factors are phosphorylated by JNK, including p53, ATF-2, NF-ATc1, Elk-1, HSF-1, STAT3, c-Myc, and JunB [9]. Substrates of activated p38 proteins include cytoplasmic proteins such as

cPLA2, MNK1/2, MK2/3, HuR, Bax and Tau. Among nuclear targets of p38 isoforms there are ATF1/2/6, MEF2, Elk-1, GADD153, Ets1, p53 and MSK1/2 [10].

MAPK are involved in a large variety of solid and hematolgical neoplasms and, indeed, several components of the MAPK network have already been proposed as targets in cancer therapy, such as p38, JNK, ERK1/2, MEK1/2, RAF, RAS, DUSP1 and ERK5 [11,12]. Among them, alteration of the RAS-RAF-MEK1/2-ERK1/2 pathway has frequently been reported in human cancer as a result of abnormal activation of receptor tyrosine kinases or gain-of-function mutations in genes of the pathway itself [13]. Components of JNK and p38 pathways are rarely mutated in cancer compared to those of the ERK1/2 pathway. Nevertheless, alterations in JNK and p38 signaling are associated to cancer, although they may act either as oncogenes or tumor suppressors depending on the cellular context [14]. ERK5 is also involved in human cancers including those of the prostate, breast and liver [15,16].

**2. The canonical Hedgehog signaling pathway**

Initially discovered in *Drosophila*, the Hedgehog (HH) signaling is an evolutionarily conserved pathway that plays a crucial role in patterning, proliferation and differentiation during embryogenesis [17,18]. In the adult it is mostly active in stem/progenitor cells, where it regulates tissue homeostasis, repair and regeneration [19]. Canonical HH pathway activation is initiated by the binding of HH ligands, Sonic (SHH), Indian (IHH) and Desert Hedgehog (DHH), to the 12-pass transmembrane protein receptor Patched (PTCH), which reside in the primary cilium [20-22]. Upon HH binding, PTCH relieves its inhibition on the G-protein-coupled receptor-like Smoothened (SMO), which translocates into the tip of the cilium and triggers a cascade of events that promote the formation of activator forms of the GLI transcription factors (GLI-A). GLI2 and GLI3 translocate into the nucleus and induce transcription of HH pathway target genes, including GLI1 [23-25]. In absence of HH ligands, PTCH inhibits pathway activation by preventing SMO to enter the cilium. This results in phosphorylation and proteasome-mediated carboxyl cleavage of GLI2 and GLI3 to their repressor forms (GLI2/3-R) [26,27]. GLI1 is degraded by the proteasome and transcriptionally repressed, with consequent inhibition of the pathway. GLI1 acts as an activator, whereas GLI2 and GLI3 display both positive and negative transcriptional functions [26,28,29]. The GLI transcription factors activate the expression of a number of targets, including regulators of proliferation and differentiation (e.g. CyclinD1 and

D2, N-Myc, E2F1, PDGFRα, Igf2, FoxM1, FoxF1, Hes1, Igfbp3, Neogenin), survival (Bcl2), angiogenesis (Vegf, Cyr61), self-renewal (Bmi1, Nanog, Sox2), epithelial-mesenchymal transition (Snail1, Sip1, Elk1) and invasiveness (Osteopontin). The HH pathway target genes include GLI1, which further amplifies the initial HH signaling, and the HH pathway negative regulators PTCH1 and HHIP1, which restrain the HH signaling.

Abnormal activation of the HH-GLI pathway is implicated in a variety of tumors, including those of the skin, brain, lungs, prostate, breast, gastrointestinal tract and blood. Multiple mechanisms of HH pathway activation have been described in cancer [30]. Ligand-independent activation is caused by loss-of-function mutations in the negative regulators PTCH1 [31,32], SUFU [33,34] or REN [35], activating mutations in SMO [36], or gene amplifications of GLI1 and GLI2 [37,38], that results in constitutive HH pathway activation. This type of HH pathway activation occurs more often in basal cell carcinoma (BCC), medulloblastoma and rhabdomyosarcoma. Ligand-dependent autocrine activation of the HH pathway has been identified in several types of cancer, including lung, pancreas, gastrointestinal tract, prostate and colon cancer, glioma and melanoma [39-48]. In this case, tumor cells secrete and respond to HH ligands and show increased HH ligands expression apparently in absence of genetic aberrations of HH pathway components. In the ligand-dependent paracrine activation of HH pathway, a mode of action that resembles the physiological HH signaling during development, HH ligands secreted by cancer cells activate HH signaling in the surrounding stroma rather than in the tumor itself. The mechanisms by which the HH signaling and the tumor stroma interact during paracrine signaling are not completely understood. Evidence supporting this mechanism has revealed from studies in human tumor xenograft models of pancreatic and colorectal cancers [49]. Similarly, the reverse paracrine HH pathway activation, in which HH ligands are secreted by the tumor microenvironment and activate the pathway in tumor cells, has been described in an experimental model of glioma [50] and in hematological malignancies, such as B-cell lymphoma and mantle cell lymphoma [51,52].

The HH signaling has also been implicated in the regulation of cancer stem cells (CSC) by promoting their self-renewal [53]. Activated HH signaling has been identified in CSCs of many solid tumors, such as glioblastoma, breast, colon, pancreatic cancer, melanoma, and hematological malignancies, including CML and multiple myeloma, and has been shown to increase tumor-initiating populations and contribute to

self-renewal, growth and tumorigenicity. Similarly, these CSC-promoting effects can be abrogated by inhibiting SMO.

Evidence of crosstalk between HH-GLI and other oncogenic signaling pathways, such as PI3K/AKT/mTOR, Notch, TGFβ, Wnt/βcatenin, has been reported in many types of cancer and extensively described by recent reviews [54-58]. Here we will focus on the interaction between HH-GLI and the final MAPK effectors ERK1/2, p38 and JNK in cancer cells. However, we also discuss findings obtained in normal cells where possible mechanisms of interaction relevant for cancer cells have been described.

Non-canonical HH signaling activation involving MAPK has also been described in normal cells and tissues. For instance, specification of oligodendrocyte progenitors by SHH is blocked by the SMO antagonist cyclopamine and PD173074, an inhibitor of FGFR [59]. During limb development induction of SHH by FGF is mediated by the ERK1/2 [60]. MAPK has been shown to modulate the expression of IHH in chondrocytic cells. MEK1/2 inhibitor UO126 decreases levels of *IHH* mRNA. Conversely, constitutively active MEK1 or MKK3 increase IHH levels, which are diminished by dominant-negative MEK1 [61]. To date, there is no evidence of crosstalk between HH-GLI pathway and non classical MAPK neither in normal nor in cancer cells.

**3. Crosstalk between HH-GLI and ERK1/2 signaling**

The first direct evidence of a crosstalk between HH and ERK1/2 signaling came from a study performed in normal NIH/3T3 cells. This report showed that activated MEK1 (S218E, S222E or Δ32-51) stimulates expression and transcriptional activity of GLI proteins, with consequent induction *PTCH1* and *GLI1* target genes, showing for the first time that ERK1/2 act upstream of HH-GLI signaling. Consistently, co-expression of activated MEK1 and GLI1 or GLI2 induces a synergistic increase in GLI transcriptional activity, that is blocked by the MEK1/2 inhibitor PD98059 [62]. Interestingly, this study identified for the first time the N-terminus of GLI1 (amino acids 1-130) as a critical region for sensing the ERK1/2 pathway. Indeed, deletion of this region produces active GLI1 protein with greatly reduced response to activation by MEK1. Nevertheless, *in vitro* kinase assays showed that GLI1 is not directly phosphorylated by ERK1/2, suggesting that the N-terminal region of GLI1 is a target for another kinase downstream of ERK1/2 [62]. A

later report identified possible MAPK consensus site, including ERK2, within the N-terminus of GLI proteins [63].

In the following Section we summarize studies providing evidence of a crosstalk between HH and ERK1/2 in different types of cancer.

### *3.1. Skin cancers*

#### *3.1.1. Basal Cell Carcinoma*

Activation of HH-GLI signaling, through genetic loss of PTCH1 or activation of SMO, occurs frequently in BCC, the most common human skin cancer [64]. Consistently, the downstream effector GLI1 is often upregulated in BCC [65]. A causal role of the activation of HH pathway in the pathogenesis of BCC has been largely demonstrated by several mouse models [66]. Consistent activation of the ERK1/2 pathway in BCC has not been reported, suggesting that activation of this pathway is not a driving event in this neoplasm. Nevertheless, ERK1/2 may be activated in BCC as a consequence of aberrant activation of upstream signaling (see below).

A crosstalk between EGFR-MEK1/2-ERK1/2 and HH-GLI signaling has been reported in keratinocytes and BCC (Figure 1). A global gene expression study in human keratinocytes with combined or single activation of EGFR and HH-GLI signaling revealed three classes of target genes: genes responding to HH only, genes responding to EGFR only, and genes responding to combined activation of both pathways [67]. Of note, the last class of genes (EGFR-HH cooperation genes) contains functional GLI binding sites in their promoters, suggesting that signaling integration occurs at the level of EGFR-HH target gene promoters. In this context, cooperation of EGFR with GLI1 and GLI2 depends on activation of MEK1/2-ERK1/2 signaling, but not on PI3K/AKT. In fact, treatment with EGFR inhibitor gefitinib and MEK1/2 inhibitor UO126 abolishes synergistic activation, whereas PI3K/AKT inhibitor LY294002 does not. Notably, MEK1/2-ERK1/2 signaling is involved in the stabilization of GLI1 and GLI2 proteins by the proteasome [67]. The same group showed that MEK1/2-ERK1/2-induced phosphorylation and activation of JUN/AP1 transcription factor is the critical event at the end of EGFR cascade, because it induces binding of activated JUN and GLI to common HH-EGFR target promoters, thereby cooperatively regulating target gene

expression and BCC transformation [68]. Of note, the beneficial effect of EGFR blockade in HH-driven BCC and pancreatic cancer models can be synergistically improved by combined targeting of both pathways [68,69]. Importantly, these studies highlight the central role of ERK1/2 in the integration of Receptor Tyrosine Kinase (RTK, see also above) and HH signaling in BCC.

HH pathway may be also upstream of MAPK and ERK1/2 activation (Figure 2). Indeed, GLI1 can lead to the induction of PDGFRα, thus resulting in the activation of the RAS-ERK1/2 pathway in BCC. Accordingly, inhibition of PDGFR or of downstream MEK1/2 decreases the proliferation of the murine BCC cell line ASZ001, that harbors constitutively active HH-GLI signaling. Similar results were obtained in HH-responsive mouse embryo fibroblasts C3H10T1/2, indicating that the signaling axis GLI1>PDGFR>ERK1/2 may be not restricted to BCC cells. The relevance of this mechanism *in vivo* is supported by a high level expression of PDGFRα in murine and human BCC [70].

The same group reported that IFNα, a local treatment option for BCC [71], prevents SMO agonist-mediated activation of ERK1/2 in BCC ASZ001 cells [72]. They also found that treatment with the MEK1/2 inhibitor UO126 induces FAS expression similarly to what happens following IFNα treatment. Interestingly, they showed that the crosstalk between IFNα and HH-GLI pathway might occur at the level of MEK1/2. Authors proposed that IFNα inhibits MEK1/2 and consequently ERK1/2 activation/phosphorylation, thus allowing the expression of Fas and the subsequent induction of apoptosis. An involvement of the MEK1/2-ERK1/2 pathway in HH-sustained survival has also been found in normal keratinocytes [73].

*3.1.2. Melanoma*

Melanoma is the most aggressive form of skin cancer, characterized by poor prognosis and high mortality. The most deregulated signaling pathway in melanoma is RAS/RAF/MEK1/2-ERK1/2. Indeed, the majority of melanoma show constitutively activated ERK1/2 due to the mutually exclusive activating mutations in BRAF and NRAS, which are present in 50% and 15-20% of cutaneous melanomas, respectively [74]. Either events lead to constitutive ERK1/2-MAPK pathway activation. BRAF inhibitors (BRAF-i) are effective in the treatment of BRAF mutant melanoma patients. However, treatment with BRAF-i is effective only for a limited time and complete clinical responses are rarely seen due to the onset of resistance. Although different

mechanisms of resistance have been described for BRAF-i, most of them lead to the reactivation of ERK1/2 or activation of the PI3K/AKT/mTOR pathway. Therefore, it is not surprising that simultaneous targeting of components of ERK1/2 and PI3K pathways can delay or overcome BRAF-i resistance [75].

Recent studies indicate that HH-GLI signaling is active in melanoma. Several components of the HH pathway are expressed in human melanoma samples [48] and high HH pathway activity is associated with decreased post-recurrence survival in metastatic melanoma patients [76]. Growth and proliferation of human melanoma cells *in vitro* and *in vivo* depends on active HH pathway. Indeed, treatment with SMO antagonists cyclopamine or sonidegib reduces proliferation of human melanoma cells and decreases human melanoma xenograft growth in nude mice [48,76,77]. Interestingly, BRAF mutant cell lines are more sensitive to sonidegib than BRAF wild type melanoma cells and combination of BRAF (vemurafenib) and Hedgehog (sonidegib) inhibitors leads to a modest but significant synergistic effect in inhibiting melanoma cell proliferation [76].

MEK1/2-ERK1/2 signaling has been shown to act upstream of HH and regulate the activity of the GLI transcription factors. For instance, oncogenic NRAS ($NRAS^{Q61K}$) and HRAS ($HRAS^{V12G}$) enhance GLI1 function, by increasing its transcriptional activity and nuclear localization. In particular, both HRAS or NRAS counteract GLI1 cytoplasmic retention by the negative regulator SUFU [48] (Figure 1). MEK1/2-ERK1/2 are likely to be the main effectors of RAS, because inhibition of MEK1/2 with UO126 reverses the effect of oncogenic RAS on GLI. A further confirmation of RAS-ERK1/2 acting upstream of HH, systemic cyclopamine treatment drastically reduces tumor growth in melanomas induced by oncogenic NRAS in a *Tyrosinase-*$NRAS^{Q61K}$*;Ink4a*$^{-/-}$ mouse model [48].

Growth factor receptor up-regulation, including that of PDGFR, is among the mechanisms underlying resistance to BRAF-i in melanoma cells [78]. Similarly to what previously observed for BCC (see above) [70], HH-GLI pathway activation induced by treatment with BRAF-i is responsible for PDGFRα up-regulation following vemurafenib treatment in human melanoma cells *in vitro*. Then PDGFRα expression through the activation of downstream ERK1/2 and PI3K determines vemurafenib resistance. Accordingly, PDGFRα or HH signaling inhibition decreases ERK1/2 phosphorylation and restores melanoma cells sensitivity to BRAF-i [79]. These findings suggest that monitoring patients for early PDGFRα up-regulation will facilitate the identification of those who may benefit from the treatment with BRAF-i in combination

with clinically approved PDGFRα or HH-GLI inhibitors and that the activation status of ERK1/2 and PI3K should be used as a read out of resistance.

### *3.2. Gastrointestinal cancers*

#### *3.2.1. Esophageal cancer*

Previous reports have shown that HH-GLI signaling is active and required for *in vitro* and *in vivo* growth of esophageal cancer cells [42,80]. Indeed, frequent inactivating mutations in PTCH1, which lead to activation of the downstream HH pathway, have been described in esophageal squamous cell carcinomas [81]. Human esophageal cancer samples often express high levels of SHH and GLI1, while normal corresponding tissue expresses low amounts. Moreover, ERK1/2 phosphorylation/activation may be detected in samples where the HH-GLI signaling is active. Consistent with the possible activation of ERK1/2 by HH-GLI, SHH-induced ERK1/2 activation is inhibited by MEK1/2 inhibitor PD98059 and by cyclopamine in TE esophageal cancer cell lines. In these cells, GLI1 is expressed thus suggesting the potential activation of HH signaling at basal levels. Stimulation with SHH enhances TE cell proliferation, and this effect is blocked by pretreatment with cyclopamine and by PD98059, indicating that loss of ERK1/2 activity inhibits HH-induced proliferation of TE cells [82].

#### *3.2.2. Gastric cancer*

Although SMO and/or PTCH1 mutations are present at low frequency in different histological subtypes of gastric tumors [83], activation and requirement of HH signaling has been demonstrated for this cancer type [42,84]. On the other hand, there are no data on the percentage of cases in which the MEK1/2-ERK1/2 pathway is activated in gastric cancers. Nevertheless, the percentage is expected to be high since many RTK as well as RAS-RAF may be activated in this malignant neoplasm [85].

An interplay between HH-GLI and ERK1/2 has also been observed in gastric cancer cells. Indeed, KRAS-MEK1/2-ERK1/2 cascade increases GLI transcriptional activity and induces the expression of HH target genes in gastric cancer cells. In agreement with a previous study performed in NIH/3T3 fibroblasts [62], the deletion of the N-terminal domain of GLI1 reduces the response to MEK1 stimulation. However,

kinase assay experiments fail to show a direct phosphorylation of GLI1 by ERK1/2 [86]. On the other hand, SHH stimulates the proliferation of rat gastric mucosal cells through ERK1/2 activation by elevating intracellular calcium concentration [87]. In addition, GANT61 and cyclopamine treatment decreases proliferation, migration and ERK1/2 phosphorylation in MKN45 gastric cancer cells [88].

*3.2.3. Pancreatic cancer*

HH-GLI signaling is an early and late mediator of pancreatic cancer tumorigenesis, as suggested by experimental studies using human pancreatic cancer cells [40] and various mouse models (see below). On the other hand, since pancreatic adenocarcinoma is the human malignancy with the highest incidence of activating KRAS mutations (70–90%), high frequency of cases with constitutive activation of the MEK1/2-ERK1/2 pathway is expected [89]. KRAS can activate HH pathway via MEK1/2-ERK1/2 in pancreatic cancer cells. In fact, expression of KRasV12 in the immortalized human pancreatic ductal epithelial cell line HPDE-c7 increases GLI1 levels and transcriptional activity. Consistently, suppression of oncogenic KRAS by siRNA inhibits GLI1 expression and activity in pancreatic ductal adenocarcinoma (PDA) cell lines with activating KRAS mutations. KRAS-mediated HH activation is suppressed by pharmacological inhibition of MEK1/2 with UO126, which decreases GLI1 protein stability in PDA [90]. Therefore, the major effector of RAS in enhancing HH-GLI activity is likely to the MEK1/2-ERK1/2 signaling module.

The interaction between RAS and GLI has been described also in various mouse models of PDA. Mice expressing endogenous levels of mutant KRAS ($KRAS^{G12D}$) and of mutant p53 ($Trp53^{R172H}$) in the pancreas show activation of HH pathway, suggesting the action of oncogenic RAS on the endogenous HH-GLI pathway during tumor development [91]. KRAS ($KRAS^{G12D}$) genetically cooperates with activated GLI2 to initiate PDA *in vivo* [92]. An additional mouse model of KRAS-induced PDA shows that SMO-independent GLI1 activation is required for survival of tumor cells and KRAS-mediated transformation [93]. KRAS also contributes to a shift from autocrine-to-paracrine signaling in PDA, by inducing SHH expression, thus leading to HH stimulation of adjacent cells, and by negatively modulating canonical HH signaling through its negative effector DYRK1B [94]. Although from these mouse models it is not possible to infer the effector of oncogenic RAS, MEK1/2-ERK1/2 are likely to be the main mediators.

The MEK1/2-ERK1/2 module might also play a role in mediating the paracrine interactions between CXCL12/CXCR4 and HH pathway (Figure 1). In fact, CXCL12 secreted by stromal cells binds to its receptor, CXCR4 on pancreatic cancer cells, initiating a downstream signaling that activates AKT and ERK1/2. This leads to accumulation of MEK1/2-ERK1/2-dependent nuclear NF-κB, that directly binds to the SHH promoter inducing its expression in pancreatic cancer cells [95].

A number of studies reported that HH-GLI might as well act upstream of ERK1/2 in pancreatic cells. In pancreatic duct epithelial cells (PDEC) ectopic expression of SHH enhances proliferation and activates PI3K and ERK1/2. Consistent with a role for PI3K and ERK1/2 in SHH-induced PDEC proliferation, inhibition of PI3K with LY294002 or MEK1/2 signaling with PD98059 reduces the S-phase fraction while increasing the G1 fraction. The mechanism involved in the activation of ERK1/2 by SHH signaling was not clarified although an involvement of HER2 or EGFR was excluded. On the other hand, activated GLI1 does not stimulate ERK1/2 activation indicating that it is GLI-independent in PDEC cells [96]. The latter study indicates that activation of ERK1/2 by HH-GLI pathway may be involved in HH-GLI induced pancreatic tumorigenesis. Moreover, HH-GLI signaling enhances K-Ras-induced pancreatic tumorigenesis by reducing the dependence of tumor cells on the sustained activation of the MAPK and PI3K/AKT/mTOR signaling pathways. Indeed, simultaneous treatment with PD98059 and rapamycin failed to completely inhibit the growth of cells expressing KRAS and SHH, although KRAS and HH signaling determine an additive effect on cell proliferation [96].

*3.2.4. Hepatocellular carcinoma*

The HH-GLI signaling is involved in the development, invasion and metastasis of hepatocellular carcinoma (HCC) [97]. On the other hand, alteration in the ERK1/2 pathway is well documented in human HCC and phosphorylated ERK1/2 levels are significantly increased in at least 50% of HCC samples [98]. In HCC tissue samples there is a positive correlation between SHH expression and nuclear GLI1, while neither SHH nor nuclear GLI1 are found in normal liver tissue. Moreover, the amount of nuclear GLI1 positively correlates with tumor pathological grade, with the ability of the tumor to invade and metastasize, and with phosphorylated ERK1/2 and MMP9 expression [99]. Inhibition of HH-GLI pathway by KAAD-cyclopamine decreases invasion and migration of Bel-7402 HCC cells, the level of nuclear GLI1, MMP9 and p-ERK1/2.

Consistently, activation of HH-GLI pathway induces opposite effects. Furthermore, MEK1/2 inhibition with UO126 or PD98059 impairs invasion and metastasis formation of HCC cells. Therefore, HH-GLI promotes ERK1/2-dependent invasiveness by up-regulating MMP9. In this context, ERK1/2 does not seem to be an upstream regulator of the HH-GLI pathway, because UO126 and PD98059 have no effect on the GLI1 expression in HCC cells [99].

*3.2.5. Cholangiocarcinoma*

HH-GLI signaling is implicated in cholangiocarcinoma (CCA) and recently HH has been proposed as a potential target for treatment of human CCA [100]. Several pathways that activate ERK1/2 are involved in the pathogenesis and progression of CCA [101]. In CCA cells, HH-GLI and ERK1/2 pathways appear to be in parallel rather than in series, because combined inhibition of either pathways with cyclopamine and UO126 determines an additive anti-proliferative effect, particularly in cells with KRAS mutation, and induces caspase-dependent apoptosis in CCA cells [102].

*3.2.6. Colon cancer*

HH-GLI signaling is active in human colon cancer and is critical for tumor growth, recurrence, metastasis and stem cell survival and expansion [45]. In mice, *Apc* mutant epithelial cells secrete IHH to maintain an intestinal stromal phenotype that is required for adenoma development [103]. The ERK1/2 pathway is located downstream of many growth-factor receptors, including EGFR and of RAF/RAS, that are involved in the pathogenesis and progression of colon cancer [104]. A study reported that MEK1/2-ERK1/2 acts upstream of HH signaling in colon cancer cells. Indeed, inhibition of MEK1/2 with UO126 in human HT29 colon cancer cells decreases phosphorylated ERK1/2 and inhibits GLI transcriptional activity and GLI1 mRNA and protein levels [105].

***3.3. Brain tumors***

*3.3.1. Medulloblastoma*

Medulloblastoma (MB), a primitive neuroectodermal tumor of the cerebellum, is the most common malignant brain tumor in children [106]. High expression of the HH pathway occurs in approximately 25% of all MB (HH group). Activation of HH pathway in MB can occur through inactivating mutations in PTCH1 and SUFU, activating mutations in SMO, and GLI1 and GLI2 gene amplifications [33,38].

An interaction between EGFR and HH signaling occurs in the medulloblastoma cell line Daoy. EGF impairs the activation of the target GLI1 upon HH pathway activation with the SMO agonist SAG. Although EGF is able to activate ERK1/2 in these cells, MEK1/2-ERK1/2 activity is not required for inhibitory effect of EGF on GLI [107].

In murine cerebellar granule cell precursors (GCP) FGF-mediated inhibition of SHH-induced proliferation and GLI1 expression requires activation of ERK1/2. Using two different FGFR inhibitors, namely PD173074 and SU5402, it was shown that bFGF acts by means of FGFR to abrogate the effects of HH signaling. The bFGF-dependent inhibition on SHH proliferative effect depends on activation of ERK1/2 and JNK. Furthermore, bFGF inhibits the growth of medulloblastoma cells from *Ptch+/-* mutant mice [108]. The involvement of ERK1/2 was not addressed, although it is likely to occur on the basis of results obtained in GCP.

In MB arising in *Ptch+/-* mice, Chow and colleagues identified three tumor subtypes on the basis of microarray signatures. In the growth factor independent group, that contains cells capable to form spheres in culture in the absence of growth factors, high levels of phosphorylated ERK1/2 are found. No mechanism of HH-GLI ERK1/2 cross talk was identified, although this event could be explained by the occurrence of trisomy of chromosome 6. Indeed, murine chromosome 6 contains many oncogenes, including KRAS, BRAF and MET, that are activators of ERK1/2 [109].

In conclusion, although a consistent activation of the ERK1/2 pathway in MB has not been reported [110] and the evidence of a crosstalk between HH-GLI and MEK1/2-ERK1/2 is not clear, targeting MEK1/2-ERK1/2 with available molecules might be taken into consideration, especially in the HH group.

*3.3.2. Glioblastoma*

Glioma is the most frequent tumor of the central nervous system and can be classified into 4 grades, with glioblastoma multiforme being the most aggressive. *GLI1* was originally identified as a gene amplified in

malignant glioblastoma [37]. Several reports support an active role for HH signaling in glioma and glioma CSC [46,47]. On the other hand, ERK1/2 activation is likely to occur especially in gliomas driven by growth factor receptors, including EGFR, and/or activation of other pathways upstream of MEK1/2-ERK1/2 [111]. SHH mediates the activation of ERK1/2 signaling in radial glial cells during late neocortex development and in Hela cells through EGFR transactivation [112] (Figure 2).

Insulin receptor substrate 1 (IRS1) is a GLI1 transcriptional target in glioma CSC cells. In these cells, GLI1 inhibition decreases basal phosphorylation of RAF1, MEK1 and ERK1/2 and that induced by IGF-1. Therefore, GLI1 inhibition antagonized IRS1-dependent ERK1/2 activity and desensitizes glioma CSC to IGF-I stimulation [113]. The existence of an interplay between HH-GLI and IGF-I signaling on the activation of ERK/2 has also been described in adult myogenic cells [114].

In another study, inhibition of the HH-GLI pathway by Sant-1 did not alter ERK1/2 phosphorylation in glioma/glioblastoma cell lines. Moreover, Sant-1 did not alter the phosphorylation of ERK1/2 induced by guggulsterone, a Ras/NFκB inhibitor [115]. The authors suggest that the effects observed occur in the tumor bulk. Therefore, ERK1/2 seems to be downstream HH-GLI in CSC [113] but not in the bulk population [115].

### *3.4. Breast cancer*

The HH signaling is required in breast cancer and breast cancer stem cells [116,117]. Consistently, transgenic mice conditionally expressing GLI1 in the mammary epithelium develop mammary tumors [118]. Deregulation of the MEK1/2-ERK1/2 pathway occurs frequently and plays a central role in the carcinogenesis and maintenance of breast cancers, especially in the basal-like subgroup [119].

A crosstalk between HH-GLI and ERK1/2 has been also described in breast cancer cells. Cyclopamine inhibits the proliferation of breast cancer cell lines, either estrogen dependent or independent, and decreases the expression of CCND1. This effect is partially prevented by UO126, indicating that HH pathway inhibition requires an active MEK1/2-ERK1/2 module to produce a decrease in proliferation [120]. Furthermore, SHH activates ERK1/2 in a SMO-independent manner in MCF10A normal mammary cells. Authors suggested an involvement of GRB2- or p85β-recruited proteins in the activation of ERK/2 [121].

### *3.5. Urologic tumors*

#### *3.5.1. Renal carcinoma*

ERK1/2 pathway is constitutively active and sustains tumor growth in clear cell renal carcinoma cells [122]. Similarly, HH signaling is active in tumors, as suggested by elevated expression of SMO and GLI transcription factors in tumors compared to corresponding normal tissues. In clear cell renal carcinoma cells, irrespectively of VHL status, inhibition of HH-GLI pathway with cyclopamine decreases AKT and ERK1/2 phosphorylation. No mechanism of interaction was proposed [123].

#### *3.5.2. Prostate cancer*

HH and MEK1/2-ERK1/2 pathways are both involved in prostate cancer [43,44,124]. EGF signaling has been shown to increase the invasive capability of $ARCaP_E$ human prostate cancer cells via upregulation of pERK1/2 and of GLI1. Enhanced invasiveness is reversed by inhibition of GLI1 *in vitro* with GANT61. Authors hypothesized that the mediator of EGF and HH pathway crosstalk is ERK1/2 through an unknown mechanism [125].

### *3.6. Hematological malignancies*

#### *3.6.1. Leukemia*

HH pathway components are commonly expressed across different acute T-cell leukemia (T-ALL) cell lines. In particular, GLI1 is activated by both canonical HH signaling (via SMO) and by non-canonical activation, through the PI3K/AKT and MEK1/2-ERK1/2 pathways. Consistent with a non-canonical activation of HH downstream of SMO, inhibition of SMO is less effective than targeting GLI in inducing cell death. The GLI inhibitor GANT58 reduces proliferation and induces cell death in T-ALL cells. Perifosine, a PI3K/AKT inhibitor, decreases GLI1 protein in part through AKT/GSK3b, and inhibition of MEK1/2 with PD98059 enhances this effect. Interestingly, combination of AKT inhibitor and GANT58 has a synergetic therapeutic role in the treatment of T-ALL. Combination of HH and MEK1/2 inhibition was not investigated, although of potential interest [126].

MEK1/2-ERK1/2 pathway may be aberrantly activated in chronic lymphocytic leukemia (CLL) [127]. SMO inhibition with cyclopamine, LDE-225 or IPI-926 decreases the number of CLL cells in culture and induces apoptosis in a subset of CLL samples. Responsiveness correlates with elevated GLI1 and PTCH1 transcript levels and the presence of trisomy 12 (interestingly, the gene locus for human DHH and GLI1 is present on chromosome 12), whereas no other karyotype correlates with responsiveness. All CLL with trisomy 12 display constitutive HH pathway activation driven by autocrine DHH ligand secretion, which could be blocked by anti-HH antibody 5E1. Moreover, PTCH1 activates ERK in a SMO-independent manner. In this case either ERK1/2 or ERK5 could be involved (see also Section 4) because antibodies for pERK in immunohistochemistry do not distinguish between pERK1/2 and pERK5. Consistently, ERK1/2 activation could be prevented by the 5E1 HH-blocking antibody but not by SMO inhibitors [128] (Figure 2). These results beg for a possible clinical development of HH ligand-blocking antibodies. Indeed, these therapeutic tools could effectively block canonical (SMO-dependent) and non-canonical (SMO-independent) HH signaling and might therefore be more effective than SMO inhibitors for the treatment of human CLL or other cancer types with ligand-dependent HH pathway activation.

HH-GLI components are expressed in the HL60 human acute myeloid leukemia cell line. In these cells, cyclopamine induces dose- and time-dependent apoptosis, cell cycle arrest and monocytic differentiation. Moreover cyclopamine determines an inhibition of AKT and ERK1/2, indicating that AKT and ERK1/2 are downstream to HH-GLI pathway. However, how GLI transcription factor activity sustains the activation of ERK1/2 in HL60 cells was not addressed [129].

Significant upregulation of PTCH1, Frizzled2, Lef1, CCND1, p21 and downregulation of HOXA10 and HOXB4 transcripts in CD34+ cells distinguish blast crisis from chronic phase CML. Ectopic SHH increases STAT phosphorylation (Y705) and activation in chronic phase CML primary blasts but not in those from blast crisis patients. On the other hand, no ERK1/2 or AKT activation/phosphorylation is detected. Moreover, cyclopamine, that is able to prevent SHH-induced STAT3 phosphorylation, is ineffective on constitutive ERK1/2 and AKT phosphorylation, indicating that HH-GLI does not sustain the activation of these two pathways in CML, at least for what concern the chronic phase [130].

*3.6.2. Multiple myeloma*

The HH signaling has been shown to maintain a tumor stem cell compartment in multiple myeloma (MM) [131]. More recently, it was shown that constitutively active MEK1 increases half-life of GLI2 and enhances its nuclear translocation, decreasing ubiquitination of GLI2 protein (Figure 1). RSK2, a protein kinase downstream of MEK1/2-ERK1/2 cascade, mimics the effect of MEK1 on GLI2 stabilization. MEK1 and RSK2 fail to augment the half-life of GLI2 lacking GSK3β phosphorylation sites, suggesting that MEK1/2-RSK stabilizes GLI2 by controlling targeting GSK3β-mediated phosphorylation and ubiquitination of GLI2. The significance of MEK1/2-RSK stabilization was demonstrated in experiments showing that activation of MEK1/2-RSK parallels higher GLI2 protein level in several MM cell lines compared to normal B cells. Inhibition of RSK function using SL0101 accelerates GLI2 degradation and reduces the expression of GLI2 target genes in MM cells. Interestingly, combined treatment with RSK inhibitor SL0101 and GLI inhibitor GANT58 leads to a synergistic decrease of apoptosis in MM cells [132].

**4. Crosstalk between HH-GLI signaling and ERK5**

To date no crosstalk has been reported between HH-GLI and ERK5 pathway. However, MEF2C, a well known target of ERK5 [133], has been shown to activate the expression of GLI2 by binding to its promoter during cardiomyogenesis *in vitro* [134]. Moreover, it is worth pointing out that the majority of experimental data presented in Section 3 have been performed with small molecule kinase inhibitors targeting the ERK1/2 pathway, and these molecules inhibit also MEK5-ERK5. Indeed, PD98059 and U0126, which were initially identified as MEK1/2-specific inhibitors, also affect the MEK5-ERK5 pathway [135-137]. Moreover, PD184352 (CI-1040), another MEK1/2 inhibitor, decreases the activity of MEK5-ERK5 pathway although at lesser extent [136].

**5. Crosstalk between HH-GLI signaling and JNK**

JNK are protein kinases that regulate many physiological processes, including inflammatory responses, morphogenesis, cell proliferation, differentiation, survival and death. It is increasingly apparent that persistent activation of JNK is also involved in cancer development and progression [138]. Few evidences of an interaction between JNK proteins and HH-GLI pathways have been identified. Below are listed the papers

that refer to a crosstalk between JNK and HH signaling in cancer cells or in normal contexts where possible mechanisms of interaction have been described.

Deregulation of HH signaling pathway in epidermal keratinocytes is a primary event leading to the formation of BCC (see above). Overexpression of SHH in HaCaT keratinocytes grown in organotypic cultures induces a basal cell phenotype and increases invasiveness. This behavior is linked to increased EGFR activation, JNK phosphorylation and MMP9 expression. However the effective role of JNK in these SHH-induced effects was not addressed [139]. On the other hand, bFGF can antagonize the effects of SHH in cerebellar GCP and mouse medulloblastoma cells. FGF-mediated inhibition of SHH-induced proliferation occurs in a JNK-dependent manner [108].

Treatment with lipotoxic agents determines JNK-dependent SHH expression, via AP-1, in human hepatocellular carcinoma HUH-7 cells silenced for caspase 9, but not in parental control cells. Moreover, caspase 9 deprived cells are more resistant to the lipotoxic effect of fatty acids. These results may suggest how in nonalcoholic steatohepatitis, a condition that might predispose to HCC, ballooned hepatocytes that express lower level of caspase 9 compared to neighboring normal cells may escape cell death [140].

Whisenant and colleagues identified a D-site, a MAPK-docking site, within residues 290-296 of GLI3 protein and found that phosphorylation of Ser343 by JNK1-3 was D-site-dependent. Computational analysis suggest that D-sites are present in GLI1 and GLI2 and that therefore JNK proteins may bind to and directly phosphorylate GLI proteins [63].

In another study, using chemoresistant cancer cell lines and their respective parental cells, namely human chronic myelogenous leukemia K562-K562/A02 cells and human epidermoid carcinoma KB-KB/VCR cells, it was shown that SMO may activate GLI through Gαi, Gβγ-JNK signaling axis, thereby promoting the GLI-dependent acquired chemoresistance [141].

**6. Crosstalk between HH-GLI signaling and p38**

p38 kinases play a prominent role in regulating the production of pro-inflammatory cytokines. There are no data about a crosstalk between HH signaling and p38 in cancer. However, a series of studies suggest potential interactions in normal cells. For instance, a reciprocal crosstalk between p38 MAPK and HH signaling has been shown in primary astrocytes [142], the most abundant glial cells in the brain that protects

neurons against oxidative stress [143]. Indeed, p38 MAPK modulates the expression of downstream targets the HH signaling and, in turn, HH pathway induces phosphorylation of p38, but not that of JNK nor ERK1/2. Using RNAi and a constitutively-active mutant authors show that SHH-mediated p38 MAPK signaling and subsequent GLI1 gene transcription requires G-protein receptor kinase 2 [142].

Interestingly, p38 might also mediate the activation of the GLI by the G protein $G_{13}$ in a SMO-independent manner. The α subunit of Gα13 promotes activation of GLI transcription factors in normal C3H10T1/2 and pancreatic cancer cell lines. Although the exact mechanism was not reported, authors proposed p38 as a possible mediator of this activation, because p38 inhibitor SB202190 impairs $G_{13}$-stimulated GLI transcriptional activity by 40–60%. Based on this model, SMO-independent activation of GLI is achieved through the 7-transmembrane receptors $CCK_A$, which couples with $G_{13}$. Therefore, activated $G_{13}$ might promote activation of GLI through p38 [144].

## 7. Rationale for combining HH and ERK1/2 inhibitors

### 7.1. Inhibitors of the HH pathway

Three major modes of HH pathway inhibition have been exploited therapeutically: SMO inhibition, GLI inhibition and disruption of HH/PTCH interaction (Table 1). After cyclopamine (and its more effective derivative KAAD-cyclopamine), a naturally compound inhibitor of SMO [145,146], several more potent and specific SMO inhibitors have been developed. They include vismodegib, approved for the treatment of locally advanced or metastatic BCC, BMS-833923, saridegib (IPI-926), sonidegib/erismodegib (LDE-225), PF-04449913, LY2940680, LEQ506 and TAK-441 [147]. SMO inhibitors are particularly effective against MB and BCC harboring SMO or PTCH mutations. However, despite promising preclinical results, SMO inhibitors have yielded little clinical benefit in tumors not harboring mutations in components of the HH pathway. The poor clinical performance of SMO inhibitors beyond BCC and MB may be due, at least in part, to crosstalk between HH and other oncogenic signaling pathways (as reported above), that may significantly alter clinical response to HH pathway inhibition and limit efficacy.

The use of SMO inhibitors has been associated with the acquisition of resistance, mostly described in medulloblastoma, as a consequence of *(i)* mutations in human *SMO* (D473H) and the matching mutation in

mouse (D477G), observed during vismodegib treatment; *(ii)* amplification of downstream HH target genes, such as *GLI2* and *CyclinD1*, reported for both vismodegib and sonidegib; *(iii)* upregulation of other oncogenic signaling, such as PI3K/AKT pathway, observed during LDE-225 treatment; *(iv)* increased expression of adenosine triphosphate (ATP)-binding cassette transporter (ABC) such as P-glycoprotein, leading to increased drug efflux, observed during saridegib treatment [148-150]. Agents such as GANT58/GANT61 [151] and HPI 1-4 [152] act by blocking GLI processing, activation and/or transcriptional activity. Recently, other GLI inhibitors have been reported, including ATO (arsenic trioxide) [153], an already approved therapeutic for acute promyelocytic leukemia, the anti-pinworm pyrvinium [154] and glabrescione B, a small molecule that interferes with the binding of GLI to the DNA [155]. HH pathway can be blocked also by disrupting the interaction between HH proteins and PTCH, using anti-HH monoclonal antibody 5E1 [156] or the macrocyclic small-molecule robotnikinin [157], both exhibiting antitumor activity.

**7.2. ERK1/2 inhibitors**

Several RAF and MEK1/2 inhibitors have been developed and many are in clinical trials [158] (Table 1). On the other hand, efforts in the identification of ERK1- and ERK2-selective inhibitors have been scarce. This is partly due to the earlier assumption that MEK1/2 are the only known activators of ERK1/2 and therefore the ERK1/2 cascade might be efficiently blocked by MEK1/2 inhibitors. However, many evidences suggested to develop ERK1/2 inhibitors: i) the necessity of targeting components of the same pathway due to different possible outcomes in different molecular contexts (i.e. different types of cancer or different patients with the same type of cancer); ii) negative feedback loops elicited following treatment with inhibitors of RTK/RAS/MEK1/2-ERK1/2 cascade; iii) occurrence of ERK1/2 activation as a resistance mechanism to RAF and MEK1/2 inhibitors; iv) activation of ERK1/2 as a consequence of inhibition of PI3K [159,160]. More importantly, ERK1/2 activation induced by treatment with BRAF-i may lead to secondary malignancies as it is well known for squamous cell carcinoma and recently reported for secondary chronic lymphocytic leukemia [161,162]. The above mentioned reasons resulted, very recently, in the development of ERK1/2 inhibitors [3]. SCH772984 (Merck/Schering-Plough) is an ATP-competitive ERK1 and ERK2 inhibitor [163]. This molecule exerts its inhibiting activity by two mechanisms: inhibition of ERK1 and

ERK2 intrinsic kinase activity and prevention of phosphorylation of ERK1 and ERK2 by MEK1/2. SCH772984 has been shown to work in tumor cell lines and mice models resistant to RAS-MEK inhibitors. Specifically, MK-8353 (formerly SCH900353; ClinicalTrials.gov Identifier: NCT01358331), a clinical grade analogue of SCH772984, is currently being tested in Phase 1 clinical trials. BVD-523 (Biomed Valley Discoveries) is employed in Phase 1 trials in patients with advanced malignancies and Phase 1/2 trials for patients with acute myelogenous leukemia or myelodysplastic syndromes. Another available ERK1/2 inhibitor is RG7842 (GDC0994; Genentech/Roche). Nevertheless, properties and clinical activities of the above mentioned ERK1/2 inhibitors are not publicly available yet.

**7.3. Combination of HH and MEK1/2-ERK1/2 inhibitors**

ERK1/2 and HH-GLI pathway are involved in several aspects of cancer, ranging from tumor initiation to promotion of invasiveness. In this review we extensively described several contexts in which the HH-GLI pathway and ERK1/2 interact in a diverse array of processes relevant for cancer, including cell proliferation, escape from apoptosis, cell migration, local invasiveness as well as metastasis formation. Several inhibitors targeting SMO and MEK1/2-ERK1/2 have been developed and are available. However, preclinical and clinical studies have revealed that the application of single-agent SMO or BRAF inhibitors might not be as broad as expected and, more importantly, is often associated to mechanisms of resistance. Tables 2 and 3 report examples of possible future directions for preclinical and clinical studies focusing on combination treatment of HH-i and MEK1/2-ERK1/2-i. Along this line, a recent report showed that combination of BRAF and HH inhibitors produces a modest but significant synergistic effect in inhibiting melanoma cell proliferation [76]. This suggests that a combined therapy targeting both HH signaling and BRAF or downstream molecules, i.e. MEK1/2-ERK1/2, might be beneficial in patients with mutated BRAF and activated HH signaling. This could apply also to other types of cancer, which depend on an active HH pathway and harbor mutations in BRAF or activation of downstream MEK1/2-ERK1/2. Moreover, treatment with SMO inhibitors might also partially prevent resistance to BRAF inhibitors. In fact, SMO inhibitor LDE-225 has been shown to restore and increase the sensitivity of melanoma cells to BRAF inhibitors [75]. Nevertheless, combination treatment targeting HH and MEK1/2-ERK1/2 should be taken into consideration

also in cases in which the two signaling pathways act in parallel, such as for cholangiocarcinoma or chronic myeloid leukemia [102,130].

In light of future clinical trials combining SMO and MEK1/2-ERK1/2 inhibitors, it will be equally important to develop sensitive biomarkers of activation of HH-GLI and MEK1/2-ERK1/2 pathways to identify the subset of cancers that will likely respond to such inhibitors and to monitor the efficacy of the therapy. In this respect, efforts should be made to develop specific and reliable antibodies to detect activated GLI1 and pERK1/2 in immunohistochemistry. Moreover, the detection of HH-induced activated RTK, such as PDGFRα [75], might be another possible biomarker to identify patients who might benefit from treatment with HH-GLI and MEK1/2-ERK1/2 inhibitors.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**FIGURE LEGENDS**

**Figure 1. Modes of activation of HH signaling by the MAPK ERK1/2 pathway.** Canonical activation of the HH signaling occurs through binding of HH ligands to PTCH receptor. Consequently, PTCH releases its inhibition on SMO. Activated SMO promotes an intracellular signaling cascade that leads to the translocation of activated forms of GLI ($GLI^A$) into the nucleus, where they induce transcription of target genes. MAPK ERK1/2 can activate HH signaling by: 1) enhancement of GLI activity by RAS-MEK1/2-ERK1/2 signaling, as shown in melanoma and pancreatic cancer cells [48,90]; 2) increase in GLI2 stability and activity by MEK1/2-ERK1/2-RSK2 signaling, as suggested in multiple myeloma [132]; 3) synergistic promotion of cooperative HH-EGFR target genes, as shown in keratinocytes and BCC [68]. In this case,

activation of EGF signaling induces RAS-RAS-MEK1/2-ERK1/2 signaling, leading to activation of GLI1 and/or JUN/AP1 transcription factors; 4) transcriptional activation of *SHH*. Here CXCL12 secreted by stromal cells binds to its receptor, CXCR4, on pancreatic cancer cells. This initiates a downstream signaling that activates AKT and ERK1/2, leading to accumulation of MEK1/2-ERK1/2-dependent nuclear NF-κB, that directly binds *SHH* promoter inducing its expression [95]. Abbreviations: CXCL12, CXC ligand 12; CXCR4, CXC chemokine receptor type 4; EGF, Epidermal Growth Factor; EGFR, Epidermal Growth Factor Receptor; ERK, Extracellular Signal-Regulated Kinase; FGFb, basic Fibroblast Growth Factor; HH, Hedgehog; MEK, MAPK/ERK kinase 1; PDGF, Platelet-Derived Growth Factor; PDGFR, Platelet-Derived Growth Factor Receptor; RSK2, Ribosomal S6 kinase; PTCH, Patched; SMO, Smoothened; SHH, Sonic Hedgehog.

**Figure 2. Modes of activation of the ERK1/2 pathway by HH signaling.** Classical activation of ERK1/2 MAPK may occur in different manner. Here only activation induced by growth factor receptors via RAS/RAF/MEK1/2 is depicted being the most studied mechanism in reports involving HH-GLI-induced ERK1/2 activation. Arrows indicate activating pathways that are known (although not reported in the picture for simplicity). Broken lines indicate activating signaling whose intermediates are unknown. Red lines indicate identified GLI transcriptional targets (see below). The HH pathway may activate ERK1/2 by several mechanisms: 1) following SMO activation, in a GLI-independent manner, via unknown signaling events. These effects are blocked by cyclopamine [72,82]; 2) following GLI activation via unknown mechanisms; it is not known whether transcriptional activity of GLI is involved [88]; 3) following expression of GLI-dependent target genes that activate downstream ERK1/2, such as PDGFR and IRS1 [70,79,113]; 4) through PTCH in a SMO-independent manner [128]; 5) transactivation of EGFR by SHH has also been described [112]. Abbreviations: EGFR, Epidermal Growth Factor Receptor; ERK, Extracellular Signal-Regulated Kinase; $GLI^A$, GLI activator forms; IGF1, Insulin-like Growth Factor 1; IGF1R, Insulin-like Growth Factor 1 Receptor; IRS1, Insulin receptor substrate 1; HH, Hedgehog; SHH, Sonic Hedgehog; MEK, MAPK/ERK kinase; PDGF, Platelet-Derived Growth Factor; PDGFR, Platelet-Derived Growth Factor Receptor; PTCH, Patched; SMO, Smoothened; MMP9, Matrix metalloproteinase-9.

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**Table 1. HH-GLI and MAPK inhibitors cited in the text.**

| Inhibitors | Alternative name | Pathway inhibited | Target | References |
|---|---|---|---|---|
| Robotnikinin | | HH-GLI | SHH | [157] |
| 5E1 HH-blocking Ab | | HH-GLI | SHH | [156] |
| Cyclopamine | | HH-GLI | SMO | [145,146] |
| KAAD-cyclopamine | | HH-GLI | SMO | [164] |
| Vismodegib | GCD-0449 | HH-GLI | SMO | [165] |
| Sonidegib | Erismodegib/LDE-225 | HH-GLI | SMO | [166] |
| Sant1-4 | | HH-GLI | SMO | [167] |
| BMS-833923 | | HH-GLI | SMO | [168] |
| Saridegib | IPI-926 | HH-GLI | SMO | [169] |
| PF-04449913 | | HH-GLI | SMO | [170] |
| LY2940680 | | HH-GLI | SMO | [171] |
| LEQ-506 | | HH-GLI | SMO | NCT01106508* |
| TAK-441 | | HH-GLI | SMO | [172] |
| GANT-58,61 | | HH-GLI | GLI | [151] |
| HPI1-4 | | HH-GLI | GLI | [152] |
| ATO | | HH-GLI | GLI | [153] |
| Pyrvinium | | HH-GLI | GLI | [154] |
| Glabrescione B | | HH-GLI | GLI | [155] |
| Vemurafenib | PLX4032 | ERK1/2 | $BRAF^{V600E}$ | [173] |
| PD98059 | | ERK1/2/5 | MEK1/2/5 | [135,136,174] |
| UO126 | | ERK1/2/5 | MEK1/2/5 | [135,136,175] |
| PD184352 | CI-1040 | ERK1/2/5 | MEK1/2/ | [136,176] |
| SCH772984 | | ERK1/2 | ERK1/2 | [163] |
| MK-8353 | SCH900353 | ERK1/2 | ERK1/2 | NCT01358331* |
| BVD-523 | | ERK1/2 | ERK1/2 | NCT01781429* |
| RG7842 | GDC0994 | ERK1/2 | ERK1/2 | Robarge et al. 2014** |
| SL0101 | | ERK1/2/5 | RSK | [177] |
| SB202190 | | p38 | p38α | [178] |
| Guggulsterone | | several | NFkB | [179] |
| PD173074 | | several | FGFR1 | [180] |
| SU5402 | | several | FGFR1,VEGFR2 | [181] |
| Gefitinib | ZD1839 | EGFR | EGFR | [182] |
| Perifosine | D21266 | PI3K/AKT | AKT | [183,184] |
| LY294002 | | PI3K/AKT | PI3K | [185] |
| Rapamycin | Sirolimus | PI3K/AKT | mTOR | [186,187] |

Abbreviations: ATO, Arsenic trioxide; EGFR, Epidermal Growth Factor Receptor; ERK, Extracellular Signal-Regulated Kinase; FGFR, Fibroblast Growth Factor Receptor; MEK, MAPK/ERK Kinase; mTOR, Mammalian Target of Rapamycin; NFkB, Nuclear Factor kappa B; PI3K, Phosphatidylinositol 3-Kinase; RSK, Ribosomal S6 Kinase; SHH, Sonic Hedgehog; SMO, Smoothened; VEGFR, Vascular Endothelial Growth Factor Receptor.
*ClinicalTrials.gov identifier number was added in absence of published references; **Robarge K, Schwarz J, Blake J, Burkard M, Chan J, Chen H, et al. Discovery of GDC-0994, a potent and selective ERK1/2 inhibitor in early clinical development. Proceedings of the 105th Annual Meeting of the American Association for Cancer Research; 2014 Apr 5-9; San Diego, CA. Philadelphia (PA): AACR; Cancer Res 2014;74:Abstract nr DDT02-03.

**Table 2. Experimental *in vitro* and *in vivo* models in which the MEK1/2-ERK1/2 module acts upstream of HH-GLI signaling.**

| Mechanism of action | Tumor type | Biological effects/function | Therapeutic implications* | References |
|---|---|---|---|---|
| MEK1/2 > ERK1/2 > GLI2/1 | Keratinocytes | Increased GLI transcriptional activity, protein stability (GLI2 more than GLI1) | EGFRi + HHi | [67] |
| EGFR > MEK1/2 > | Keratinocytes | EGFRi (gefitinib) and HHi (GANT61 | MEK1/2-ERK1/2i + | [68] |

| | | | | |
|---|---|---|---|---|
| ERK1/2 > JUN/AP1 > GLI | /BCC | or Cyc) synergistically reduce mouse BCC cell growth | HH-GLIi | |
| RAS > RAF > MEK1/2 > GLI (counteracting SUFU) | Melanoma | Increased GLI transcriptional activity and nuclear localization | MEK1/2-ERK1/2i + HH-GLIi | [48] |
| RAS > RAF > GLI | Melanoma | $BRAF^{V600E}$ inhibition decreases GLI1 expression | | [77] |
| RAS > RAF > GLI | Melanoma | Combination of SMOi (sonidegib) and BRAFi (vemurafenib) synergistically decreases melanoma cell proliferation | MEK1/2-ERK1/2i + SMOi | [76] |
| $KRAS^{G12D}$ > GLI2 | PDA mouse model | $KRAS^{G12D}$ cooperates with Gli2 in PDA initiation *in vivo* | | [92] |
| KRAS > GLI1 | PDA mouse model | GLI1 is required for KRAS-mediated transformation of PDAC cancer cells | | [93] |
| KRAS > RAF > MEK1/2 > GLI1 | PDA | UO126 decreases GLI1 protein stability and suppresses anchorage-independent growth | MEK1/2-ERK1/2i + HH-GLIi | [90] |
| KRAS > MEK1/2 > ERK1/2 > GLI | Gastric cancer | KRAS enhances GLI transcriptional activity | MEK1/2-ERK1/2i + HH-GLIi | [86] |
| RAS > RAF > ERK1/2 > GLI1 | Colon cancer | Cell death and DNA damage; MEK1/2i (UO126) decreases GLI1 expression | | [105] |
| EGF —I HH | Medulloblastoma (Daoy) | EGF down-regulates HH target genes (including GLI1) independently of MEK1/2 and PI3K | | [107] |
| bFGF —I HH | Cerebellar GCP/ Medulloblastoma | bFGF promotes GCP differentiation and blocks *Ptch+/-* medulloblastoma cell proliferation | | [108] |
| EGF > ERK1/2 > GLI1 | Prostate cancer | EGF signaling increases invasion *in vitro;* GLI inhibition (GANT61) can reverse the enhanced invasive effect induced by EGF | MEK1/2-ERK1/2i + HH-GLIi | [125] |
| MEK1/2 > ERK1/2 > GLI | Acute T-cell leukemia | MEK1/2 (PD98059) enhances down-regulation of GLI1 protein by AKT inhibition | | [126] |
| MEK1 > RSK2 > GLI2 (via GSK3β inhibition) | Multiple myeloma | Combination of GLIi (GANT58) and RSKi (SL0101) synergistically increases apoptosis | MEK1/2-ERK1/2i + GLIi | [132] |

Abbreviations: AP1, Activator protein 1; BCC, Basal Cell Carcinoma; Cyc, cyclopamine; EGF, Epidermal Growth Factor; EGFR, Epidermal Growth Factor Receptor; ERK, Extracellular Signal-Regulated Kinase; bFGF, basic Fibroblast Growth Factor; GCP, Granule cell precursors; GSK3β, Glycogen Synthase Kinase 3β; HH, Hedgehog; MEK, MAPK/ERK Kinase; PDA, Pancreatic Ductal Adenocarcinoma; PI3K, Phosphatidylinositol 3-Kinase**;** Ptch, Patched; RSK, Ribosomal S6 Kinase; SUFU; Suppressor of Fused; EGFRi, EGFR inhibitors; HHi, Hedgehog inhibitors; HH-GLIi, Hedgehog-GLI inhibitors; SMOi, Smoothened inhibitors; GLIi, GLI inhibitors; MEK1/2-ERK1/2i, MEK1/2 or ERK1/2 inhibitors. Symbols: >, activation; —I, inhibition. *Suggestions for possible future preclinical and clinical studies have been reported.

**Table 3. Experimental *in vitro* and *in vivo* models in which the HH-GLI signaling has been shown to act upstream of ERK1/2.**

| Mechanism of action | Tumor type | Biological effects/functionss/functions | Therapeutic impl |
|---|---|---|---|
| HH-GLI > PDGFRα > ERK1/2 | BCC | Proliferation *in vitro* | pERK1/2 as a reac MEK1/2i-ERK1/2 resistance |
| HH-SMO > MEK1/2-ERK1/2 | BCC | Survival *in vitro*; MEK1/2-ERK1/2 inhibition by IFNα determines apoptosis | IFNα+HH-GLIi to induce apoptosis |
| BRAFi > HH/GLI > PDGFRα > | Melanoma | RAFi chemoresistance, proliferation *in vitro* | BRAFi+PDGFRαi |

| | | | |
|---|---|---|---|
| ERK1/2 | | | a read out of BRA |
| HH-SMO > MEK1/2-ERK1/2 | Esophageal cancer | Cell proliferation *in vitro* | pERK1/2 as a read<br>HH/GLIi+MEK1/ |
| HH-GLI > ERK1/2 | Gastric cancer | Human cell line *in vitro* | pERK1/2 status as<br>inhibition |
| HH > ERK1/2 (EGFR-, HER2-, GLI-independent) | Pancreatic tissue/cancer | PDEC growth *in vitro*. HH reduces MEK1/2-ERK1/2-dependent tumor cell growth *in vitro* | HH-GLIi and/or M<br>tumorigenesis; K- |
| HH-GLI > ERK1/2 > MMP9 | Hepatocellular carcinoma | Invasion *in vitro* | MEK1/2-ERK1/2i<br>(aggressive/invasi |
| HH-GLI // ERK1/2 | Cholangiocarcinoma | Human cholangiocarcinoma cell lines *in vitro* | MEK1/2-ERK1/2i |
| PTCHi > ERK1/2 | Medulloblastoma | *In vitro* sphere formation** | Combined HH-GL<br>(HH subgroup) |
| HH-GLI > IGF1-IRS1 > MEK1/2-ERK1/2 | Glioma (CSC) | *In vitro* CSC proliferation, clonogenicity, invasion**; resistance to temozolomide | MEK1/2-ERK1/2i<br>to overcome resist |
| HH-GLIi no effect on ERK1/2 | Glioma (bulk) | GS enhances the inhibitory effect of SANT-1 (HHi) on cell growth while activating ERK1/2 | |
| | Breast cancer | ERK1/2 is required for inhibition of proliferation by cyc *in vitro* | |
| HH-GLI > ERK1/2 | Clear cell renal carcinoma | Cyc decreases ERK1/2 phosphorylation | MEK1/2-ERK1/2i |
| HH > PTCH > ERK1/2 (SMO independent) | chronic lymphocytic leukemia | ERK1/2 is involved in HH-GLI-dependent growth in patient-derived cells | MEK1/2-ERK1/2i<br>SMOi resistance |
| SMO > ERK1/2 | Acute myeloid leukemia | Cyc inhibits cell growth, induces monocytic differentiation and ERK1/2 dephosphorylation** | |
| HH-GLI // ERK1/2 | Chronic myeloid leukemia | | MEK1/2-ERK1/2i |

Abbreviations: BCC, Basal Cell Carcinoma; CSC, Cancer Stem Cells; Cyc, Cyclopamine; EGFR, Epidermal Growth Factor Receptor; ERK, Extracellular Signal-Regulated Kinase; EGFR, Epidermal Growth Factor Receptor; GS, Guggulsterone; HER2, Human Epidermal Growth Factor Receptor 2; HH, Hedgehog; IFNα, Interferon α; IGF1, Insulin-like Growth Factor 1; IRS1; Insulin Receptor Substrate 1; MEK, MAPK/ERK Kinase; MMP9, Matrix Metalloproteinase-9; PDEC, Pancreatic Duct Epithelial Cells; PDGFRα, Platelet-Derived Growth Factor Receptor α; PTCH, Patched; SMO, Smoothened; MEK1/2-ERK1/2i, MEK1/2 or ERK1/2 inhibitors; HH-GLIi, HH-GLI inhibition or inhibitors; SMOi, Smoothened inhibitors; anti-HHAb, anti-Hedgehog blocking antibodies (see the text for details). Symbols: >, activate; //, parallel signaling. *Suggestions for further preclinical and clinical studies have been reported; **a direct dependence of this biological effect on ERK1/2 activity has not been addressed.