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Wnt, Notch, and TGF-β Pathways Impinge on Hedgehog Signaling Complexity: An Open Window on Cancer

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Constitutive activation of the Hedgehog (Hh) signaling pathway is associated with increased risk of developing several malignancies. The biological and pathogenic importance of Hh signaling emphasizes the need to control its action tightly, both physiologically and therapeutically. Evidence of crosstalk between Hh and other signaling pathways is reported in many tumor types. Here, we provide an overview of the current knowledge about the communication between Hh and major signaling pathways, such as Notch, Wnt, and transforming growth factor β (TGF-β), which play critical roles in both embryonic and adult life. When these pathways are unbalanced, impaired crosstalk contributes to disease development. It is reported that more than one of these pathways are active in different type of tumors, at the same time. Therefore, starting from a plethora of stimuli that activate multiple signaling pathways, we describe the signals that preferentially converge on the Hh signaling cascade that influence its activity. Moreover, we highlight several connection points between Hh and Notch, Wnt, or TGF-β pathways, showing a reciprocal synergism that contributes to tumorigenesis, supporting a more malignant behavior by tumor cells, such as in leukemia and brain tumors. Understanding the importance of these molecular interlinking networks will provide a rational basis for combined anticancer drug development.

Keywords: Hedgehog, Notch, Wnt, TGF-β, signaling pathway, tumorigenesis

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INTRODUCTION

Signaling pathways are networks of regulatory proteins and other gene products that act in a coordinated manner to control various biological processes inside the cell. Remarkably, mutation of a single gene encoding a component of a specific pathway is able to affect related signaling cascades, triggering unbalanced crosstalk that leads to cancer development. Of note, impaired regulation of primary signaling pathways can ultimately culminate in constitutive activation of signaling effectors in the nucleus, where they act out of control, sustaining the expression of pro-tumoral target genes. To date, it is known that tumor development is characterized by deregulation of at least two major signaling pathways at the same time, which crosstalk with each other, determining the acquisition of malignant phenotypes (Petrova and Joyner, 2014).

Hedgehog (Hh) signaling is a critical pathway that mainly controls embryonic development, whereas in post-natal life, it is inactive or poorly active, playing a restricted role in stem cell maintenance and tissue homeostasis/repair (Petrova and Joyner, 2014). Since Hh signaling regulates a wide array of biological activities in various cell types, its misregulation is responsible for the development of many types of cancers. Studies show that the mutational activation of Hh signaling is a nodal point in sustaining tumorigenic programs, ranging from the tumor initiation to tissue invasion/metastasis and chemoresistance, in several different tumors.

Of note, recent studies show that Hh signaling elements talk to several other cofactors belonging to major pathways, such as Notch, Wnt, and transforming growth factor β (TGF-β), resulting in significant crosstalk between these signaling networks. The integration of several signaling pathways is a key step able to determine a more aggressive behavior of tumor cells and their resistance to pharmacological approaches. Interestingly, Notch, Wnt, and TGF-β pathways are able to promote/sustain oncogenesis through synergistic associations with Hh signaling in several types of tumors.

Here, we review a global picture of intricate protein–protein interaction networks between key components of Hedgehog, Wnt, Notch, and TGF-β signaling pathways in an unbalanced/malignant context. We also describe how these main pathways can integrate with each other and ultimately affect Hh signaling output.

THE HEDGEHOG PATHWAY

First discovered in *Drosophila*, Hedgehog signaling is an evolutionarily conserved pathway that plays a key role as a morphogenesis driver for embryonic and post-natal development. It regulates diverse cellular processes, including cell proliferation, tissue differentiation, and repair of normal tissues (Nusslein-Volhard and Wieschaus, 1980; Napolitano et al., 1999; Varjosalo and Taipale, 2008), and it is also implicated in regulation/survival of normal and malignant stem cells (Liu et al., 2006; Merchant and Matsui, 2010). Canonical Hedgehog pathway activation is characterized by the interaction of Hh ligands, Sonic (SHh), Indian (IHh), and Desert (DHh), to the Patched1 (Ptch1) receptor, which resides in the primary cilium (PC) (Rohatgi et al., 2007; Wong and Reiter, 2008). Interestingly, the PC is a key organelle that consists of microtubules emanating from the cell surface in which SHh signaling takes place, and it responds to mechanical stimuli in the micro-environment (Michaud and Yoder, 2006). In the absence of Hh ligand, Ptch localizes to the base of the PC and catalytically represses the activity of the transducer Smoothened (SMO) (Burns et al., 2018), a member of G-protein–coupled receptor-like (GPCR), by inhibiting its translocation into the PC (Denef et al., 2000) (**Figure 1A**). SMO is a seven-transmembrane-span receptor-like protein that is confined to intracellular endocytic vesicles when the Hh pathway is switched off (Taipale et al., 2002). Hh binding both causes the internalization of ligand/receptor complex from the cell surface towards lysosomes, where the proteins are degraded (Mastronardi et al., 2000), and promotes the accumulation of SMO at the cell surface (Denef et al., 2000). Once activated, SMO is hyperphosphorylated by casein kinase 1 (CK1) and G-protein–coupled receptor kinase 2 (GRK2), resulting in the release of its inhibition, and at this point, it is free to move from the base into the tip of the PC (Denef et al., 2000; Chen et al., 2011) (**Figure 1B**). Given these features, SMO is considered a positive regulator of the Hh signaling pathway because, when it is constitutively activated, it stimulates downstream components of the signaling pathway. Therefore, the ligand/receptor complex relieves the SMO

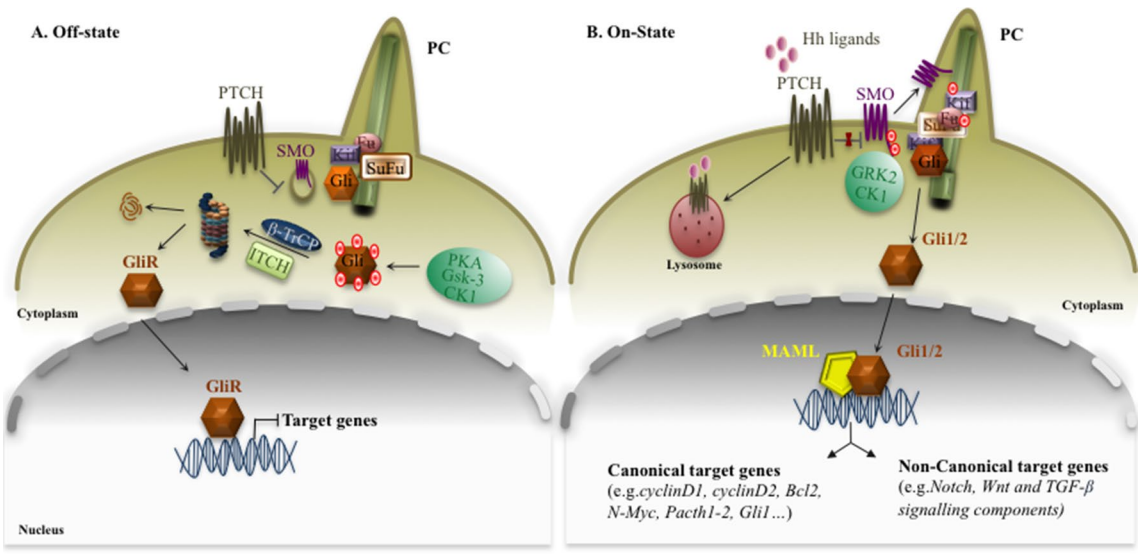

FIGURE 1 | Canonical Hh signaling. The canonical Hh signaling pathway in off (panel **A**) or on (panel **B**) state. The figure is widely discussed in the text. The red dots represent post-translational modifications: phosphorylation.

inhibition and triggers a cascade of intracellular processes that involve a dynamic association between Gli transcription factors, the final effectors of Hh signaling, and Suppressor of Fused (SuFu). Unlike SMO, SuFu is considered a tumor suppressor gene and a negative regulator of Hedgehog signaling, able to bind directly to Gli proteins to regulate their activity, processing, and localization, (Ryan and Chiang, 2012), by sequestering them in the cytosol (Ding et al., 1999; Dunaeva et al., 2003) or regulating their affinity to DNA (Pearse et al., 1999; Stone et al., 1999; Sisson et al., 2006). However, the specific mechanisms concerning Gli inactivation by SuFu are not completely understood (Carballo et al., 2018). Hh ligand binding sustains the release of Gli from SuFu that moves into the nucleus and activates Hh target genes. Altogether, these molecular events sustain the signal that is transduced from the ligand/receptor complex to the downstream transcription factors (Gli1, Gli2, and Gli3), which in turn translocate into the nucleus and regulate the expression of Hh target genes, including Gli1 itself. Prominently, Gli1 is both the downstream effector and a target gene of the pathway, representing a feedback loop that serves as a readout of Hh activity (Sasaki et al., 1999; Regl et al., 2002). Interestingly, Gli1 consists of zinc finger and C-terminal activator domains, whereas Gli2 and Gli3 also possess an N-terminal repressor domain. On the basis of these structural differences, Gli1 functions exclusively as a transcriptional activator, whereas Gli2 and Gli3 exist as full-length (FL) activator and truncated repressor (GliR) forms, displaying in this way both positive and negative transcriptional functions (Lee et al., 1997; Dai et al., 1999; Sasaki et al., 1999; Bai et al., 2004). In the absence of Hh ligand, GliFL is phosphorylated by protein kinase-A (PKA), glycogen synthase kinase-3 (GSK3), and CK1 (Price and Kalderon, 2002), and recognized by β-transducin-repeat containing protein (β-TrCP), which is able to partially degrade Gli proteins in truncated forms (Glis). At this point, Gli proteins retained at the cytoplasm by SuFu are degraded, inhibiting SHh signaling (Humke et al., 2010). These events lead to proteolytic cleavage of GliFL into C-terminally truncated repressor form, GliR, which translocates to the nucleus, where it binds to Hh target gene promoters and keeps them switched off (Goetz and Anderson, 2010). Interestingly, a recent paper shows that the Itch/β-arrestin2 complex binds SuFu and induces its polyubiquitination without any impact on stability, but sustaining the conversion of Gli3 into a repressor, which is able to switch off the signal (Infante et al., 2018).

Under basal conditions, Gli1 activity can be controlled by proteasome degradation, mediated by two distinct ubiquitin-dependent processing pathway E3 ubiquitin ligases, β-TrCP and Itch, able to inactive Gli1 in the cytosol (Lee et al., 1997; Di Marcotullio et al., 2006; Di Marcotullio et al., 2007; Di Marcotullio et al., 2011; Infante et al., 2016). On the contrary, the activation of Hh/Gli signaling is associated with a translocation of Gli1 into the nucleus, where it exerts strong mitogenic and prosurvival activities. Another mechanism able to control the Hh signaling is based on the acetylation status of Gli1 and Gli2, mediated by Histone deacetylases (HDACs). Indeed, HDAC-mediated deacetylation promotes transcriptional activation and sustains a positive regulatory loop. This mechanism is turned off by HDAC1 degradation, mediated by a Cullin3/Ren complex (Canettieri et al., 2010).

Recent studies identify Mastermind-like 1 (Maml1) as a novel positive regulator of Hh signaling. It physically interacts with Gli proteins (Gli1 and Gli2), working as a potent transcriptional coactivator (Quaranta et al., 2017; Vied and Kalderon, 2009), empowering Gli-mediated transcriptional activity. Finally, Gli1 activation induces the transcription of Hh target genes involved in key cellular processes, such as the cell cycle (Cyclin D1 and D2) (Sasaki et al., 1999), apoptosis (Bcl2) (Bigelow et al., 2004), N-Myc (Oliver et al., 2003), various transcription factors and components of the Hh pathway itself such as *Ptch1, Ptch2*, and Gli1 for negative and positive feedback loop mechanisms (canonical target genes) (Bonifas et al., 2001; Oliver et al., 2003, Vokes et al., 2007; Coni et al., 2013), and elements of other pathways such as Notch1 and Jagged1 (Stecca and Ruiz i Altaba, 2009), Hes1 (Ingram et al., 2008; Wall et al., 2009), Wnt signals (Mullor et al., 2001), and TGF-β members (Fan et al., 2010) (non-canonical target genes), suggesting that the Hh signaling pathway can integrate with elements of major signaling pathways (**Figure 1**).

HEDGEHOG MUTATIONS AND DISEASE

Mutational activation of the Hh signaling pathway is tightly linked to maintenance of tumor-initiating stem cells, tumorigenesis, and tumor invasiveness in several types of cancer (Nilsson et al., 2000; Varnat et al., 2009; Harris et al., 2012). Mutations in Hh signaling can be classified as loss of function (i.e., *Ptch1* and *SuFu*) or gain of function (i.e., *Gli1*, *Gli2*, and *SMO*), both associated with an aberrant activation of the Hh pathway (**Table 1**). Constitutive Hh signaling triggers a strong cellular mitogenic response that ultimately predisposes to abnormal proliferation leading to tumorigenesis.

Notably, it is known that patients with Gorlin syndrome (GS), also called nevoid basal cell carcinoma syndrome (nevoid BCC) or basal cell nevus syndrome, are predisposed to multiple cancers, including basal cell carcinoma (BCC), medulloblastoma (MB), and rhabdomyosarcoma (Johnson et al., 1996; Jones et al., 2011). Gorlin syndrome patients carry mutations in *Ptch1* and *SuFu* genes (Hahn et al., 1996; Smith et al., 2014). Of note, *SuFu* germline mutations are present with a low frequency (Smith et al., 2014). By contrast, heterozygous germline mutations in *Ptch1*, including nonsense, missense, splicing mutations, as well as loss of heterozygosity (LOH) (Burns et al., 2018), are typical alterations in these patients, inducing different tumors (Lindstrom et al., 2006). In patients with GS, the loss of function of the *Ptch1* gene permits SMO to move into the PC, resulting in an aberrant activation of Hh signaling, which drives cellular growth of these tumors. Notably, the *Ptch1* knock-out mouse model develops tumors similar to Gorlin syndrome patients, like BCC, MBs, and sporadic rhabdomyosarcomas, providing evidence about the driver role of Hh signaling for cancer development (Hahn et al., 1996; Nitzki et al., 2011). The co-presence of *Ptch1* and *SuFu* mutations or *Gli1* amplifications is also identified in rhabdomyosarcoma patients (Bridge et al., 2000; Roberts et al., 1989). Finally, combined heterozygous *Ptch1* and *Ptch2* germline mutations are observed in newborns with rhabdomyosarcoma (Taeubner et al., 2018), suggesting that haploinsufficiency of *Ptch* is sufficient to sustain

tumor development in the absence of LOH (Nilsson et al., 2000). In addition, MB is one the most common brain tumors affecting mostly children and is a typical GS-related cancer (Jones et al., 2011; Huttner, 2012). GS patients characterized by *SuFu* mutations display a higher risk of developing MB than *Ptch1*-mutated patients. Based on the molecular features that are involved in MB pathogenesis, it is possible to distinguish four groups: SHh-activated, WNT-activated, c-Myc–activated, and Group 4 associated with isochromosome 17q (Kool et al., 2012; Rusert et al., 2014). In MB, Hh-dependent tumorigenesis may depend on an aberrant regulation of Hh ligands or a deregulation of Hh signaling with an altered expression/function of downstream components, such as loss-of-function mutations of *Ptch1* or *SUFU* (Taylor et al., 2002; Kool et al., 2012) or, conversely, activating mutations of *SMO* and *SHh* (Reifenberger et al., 1998; Zurawel et al., 2000). Of note, somatic copy number variations of *Gli1* and *Gli2* genes are found in this subgroup (Thompson et al., 2006; Northcott et al., 2009). All these mutations allow ligand-independent Hh signaling to promote cell growth and increase tumorigenesis.

Alterations in Hh signaling are also described in meningiomas, the most common primary tumor of the central nervous system (Wiemels et al., 2010). Aavikko and colleagues identified a germline *SuFu* mutation as the cause of multiple meningiomas in a single large Finnish family, suggesting that *SuFu* alterations predispose to meningiomas in addition to MBs (Aavikko et al., 2012).

BCC is a common type of skin cancer, representing almost 80% of non-melanoma skin cancer (NMSC), with an incidence that increases by 10% per year (Bakshi et al., 2017). As argued above, BCC is linked to GS and is characterized by germline mutations of *Ptch1* and *SuFu*. Accordingly, sporadic BCC presents hyperactivated Hh signaling, associated with inactivating mutations in *Ptch1* and activating mutations in *SMO*, with a high-rate frequency (Gailani et al., 1996; Reifenberger et al., 1998; Xie et al., 1998). Moreover, loss of *SuFu* function is also found in sporadic BCC (Reifenberger et al., 2005). Couvè-Privat and colleagues identify the presence of ultraviolet (UV)-specific mutations in the NH2-terminal domain of SHh in BCC patients with xeroderma pigmentosum (Couve-Privat et al., 2004). The current opinion is that during BCC genesis, SHh signaling aberrantly activated is necessary, maybe sufficient, to develop the malignancy (Teglund and Toftgard, 2010). In addition, there are numerous types of cancer associated with Hh signaling activation. Genetic aberrations on Hh signaling are found also in a subset of breast cancer, characterized by loss of function in *Ptch1* and gain of function of *Gli1* (Naylor et al., 2005; Nessling et al., 2005; Wood et al., 2007; Sinha et al., 2008; Jiao et al., 2012). A global genomic analysis from twenty-four advanced pancreatic adenocarcinomas highlighted the presence of missense mutations in *Gli1* and *Gli3* (Jones et al., 2008), and Hh signaling pathway is recognized as an early and late mediator of pancreatic cancer tumorigenesis (Thayer et al., 2003). Also, in prostate cancer is identified a *SuFu* mutation (Sheng et al., 2004). Recently, in patients with T-cell acute lymphoblastic leukemia (T-ALL), several mutations of SHh signaling are described (Dagklis et al., 2016), and loss of *Ptch1* function is able to empower T-ALL development Notch1-dependent, demonstrating that Hh pathway activation is an oncogenic driver in the molecular pathogenesis of T-ALL (Burns et al., 2018). In addition, *SMO* and *Ptch1* mutations are also found in gastrointestinal tumors (Guleng et al., 2006; Wang et al., 2013). Lee and colleagues performed a single-strand conformation polymorphism analysis and DNA sequencing in samples of colorectal and gastric cancers and identified frameshift mutations of *Gli1*, associated with a microsatellite instability (MSI) phenotype. (Lee et al., 2018). Finally, *SMO* activating mutations are involved in ameloblastomas arising in the maxilla (Sweeney et al., 2014).

Although tumors not GS-related present direct mutations in genes involved in the Hh pathway, it is not possible to identify Hh signaling as the driving force for cancer onset. In fact, activated Hh signaling is able to empower the severity of the tumors, but this pathway alone is not capable of driving cancer development. A major elucidation of the mechanisms leading to the genesis of malignancy through an aberrant activation of this pathway and/or unbalanced cross-talking with other signaling pathways could provide additional therapeutic options to limit tumor growth and relapse and to reduce drug resistance.

COMMUNICATION AMONG HH AND DIFFERENT SIGNALING PATHWAYS

Crosstalk Between Hh and Notch Signaling Pathways

The family of Notch receptors includes four heterodimeric transmembrane proteins (Notch1–4), which are activated upon interaction with different types of ligands [Serrate-like Jagged1 and 2 (JAG1–2) and Delta-like (Delta1, 3, and 4)], expressed by adjacent cells. The activation of Notch signaling requires the binding *in trans* between Notch receptors, expressed on the surface of signal-receiving cells, and Notch ligands, expressed on the surface of adjacent signal-sending cells. Such an interaction renders Notch susceptible to two sequential proteolytic cleavages that involve two distinct enzymes: an A-disintegrin and metalloprotease (ADAM) and Presenilin (PS)/γ-secretase complex. These events end in the release of a soluble Notch intracellular domain (NICD) (Mumm and Kopan, 2000; Fortini, 2009; Kopan and Ilagan, 2009; Palermo et al., 2014; Bellavia et al., 2018). In canonical Notch signaling, NICD disengages from the plasma membrane and proceeds towards the nucleus, where it associates with recombining binding protein suppressor of hairless J kappa (RBP-Jκ, also known as RBPSUH/CSL/CBF-1), a transcription repressor. CSL–NICD complex, reached by a member of Mastermind-family coactivators (MAML1–3) (Wu et al., 2000), induces the transcriptional activation of several target genes, for instance, *HES1/5, HEY* (Bellavia et al., 2018), *Myc* (Palomero et al., 2006; Weng et al., 2006), *cyclinD* (Joshi et al., 2009; Cohen et al., 2010), *pTalpha* (Bellavia et al., 2007b), and *Jagged1* (Pelullo et al., 2014). Notch signaling activation also occurs in a non-canonical pathway. NICD can coactivate transcription by forming a complex with the lymphoid enhancer factor 1 (LEF-1) transcription factor (Ross and Kadesch, 2001) or by binding to p50 or c-Rel in

the nucleus to enhance the activity of the transcription factor NF-κB (Nuclear Factor-kappa B) (Shin et al., 2006; Vacca et al., 2006; Kumar et al., 2014), and can modulate Tal-1 and/or Ikaros activity (Beverly and Capobianco, 2003; Campese et al., 2003; Talora et al., 2006; Bellavia et al., 2007a; Talora et al., 2008). Notch signaling, like the Hh pathway, is involved in the proliferation, differentiation, and survival of multiple tissues. In the hematopoietic system, Notch can increase the survival and self-renewal of hematopoietic progenitors (Varnum-Finney et al., 2000) and controls regulatory T-cell expansion/migration to peripheral lymphoid organs (Campese et al., 2009; Ferrandino et al., 2018a; Ferrandino et al., 2018b). Very intriguing is the observation that Notch1 and Notch3 receptors are associated with different steps of ongoing thymocyte development (Felli et al., 1999). This suggests that they work with distinct timing and with separate spatial expression, underlining the absence of a functional redundancy, an observation sustained also by structural differences (Bellavia et al., 2018). A hampered Notch signaling pathway takes place in several pathological events that range from neurodegenerative disorders to cancer (Joutel et al., 2000). Persistent uncontrolled Notch signaling is responsible for the onset/progression of several tumors, such as T-cell leukemia (Checquolo et al., 2010; Cialfi et al., 2013; Vargas Romero et al., 2015; Franciosa et al., 2016; Tottone et al., 2019), B-cell leukemia (Rosati et al., 2009; Arruga et al., 2014; De Falco et al., 2015), breast cancer (Rustighi et al., 2014; Diluvio et al., 2018), colorectal (Reedijk et al., 2008; Sikandar et al., 2010), ovarian cancer (Choi et al., 2008; Steg et al., 2011; McAuliffe et al., 2012), glioma (Catanzaro et al., 2017; Ceccarelli et al., 2019), and skin cancer (Cialfi et al., 2014; Palermo et al., 2014). Numerous gain-of-function mouse models show that mutations in Notch1/3 signaling are related to rare cases of human T-ALL (Bellavia et al., 2000; Allman et al., 2001). Although first associated with T-ALL, activating Notch mutations are identified in several subtypes of B-cell malignancies (Micci et al., 2007; Rosati et al., 2009; Kuang et al., 2013; Radojcic and Maillard, 2014; De Falco et al., 2018).

Studies show that Notch and Hh signaling pathways share the exact spatio-temporal window during T-cell development even if they play a non-redundant role in orchestrating early thymocyte differentiation and proliferation before pre-T cell Receptor (pre- TCR) signal initiation (Crompton et al., 2007; Rowbotham et al., 2007; Drakopoulou et al., 2010). These findings suggest that Hh may synergize with Notch signaling to maintain an intracellular balance through a significant integration of these signaling pathways. It is known that aberrant Hh signaling contributes to tumor cell growth and survival and cancer stem cell (CSC) maintenance in lymphomas, leukemia, multiple myeloma, and B-cell chronic lymphocytic leukemia (CLL) (Dierks et al., 2007; Lindemann, 2008; Singh et al., 2010; Decker et al., 2012; Aberger et al., 2017). To unravel the molecular mechanisms that subtend in the network of cross-talking pathways could be essential in treatment of hematological disease. Interestingly, Hh signaling is a potential therapeutic target for patients with myeloid and lymphoid leukemia, and Hh pathway inhibitors are used in many preclinical studies (Dagklis et al., 2016; Rimkus et al., 2016). Despite the importance of the Hh pathway in T-cell development and in several hematological malignancies (Mar et al., 2011), the role of the Hh pathway in T-ALL is unclear, and conflicting data are reported. A study shows that Hedgehog signaling is dispensable for T-ALL development (Gao et al., 2009), whereas other studies document T-ALL cell line sensitivity to Hh inhibitors, suggesting that this pathway may be important in T-ALL development and/or maintenance (Ji et al., 2007; Hou et al., 2014; Dagklis et al., 2016). Recently, several reports highlight activating mutations in components of the Hh signaling in T-ALL, supporting the idea that Hedgehog pathway deregulation may play a role in the onset and/or development of T-ALL. Functional analysis confirms the gain-of-function properties of two truncated SMO mutations in T-ALL (Dagklis et al., 2015). Moreover, primary T-ALL cases with high Gli mRNA levels are sensitive to Hedgehog pathway inhibition by GANT61 or vismodegib in *in vivo* xenograft models (Hou et al., 2014; Dagklis et al., 2016). Despite the numerous correlations, the existence of a direct molecular connection between Hh and Notch in T-ALL is not well documented, yet. Recently, it has been reported that loss of *Ptch1* function is able to accelerate the onset of Notch1-induced human T-ALL, demonstrating that Hh pathway mutations are driver oncogenic alterations, providing a molecular rationale for targeted therapy (Burns et al., 2018).

On the contrary, a reciprocal exclusion between Hh and Notch signaling pathways is already defined in the skin. In particular, Notch1 deficiency results in an increased expression of Gli2, along with tumor development in squamous cell carcinoma (Okuyama et al., 2008).

Likewise, an inverted gradient between Hh and Notch is observed in normal colon crypt development (Kosinski et al., 2007; Takebe et al., 2011; Geissler and Zach, 2012). Very little is known regarding the crosstalk mechanisms between Hh and Notch in colorectal cancer (CRC) tumorigenesis and progression. A paper shows an antagonistic crosstalk between Hh and Notch in order to give rise to proper intestine organogenesis in the mouse, but the molecular mechanism is unknown (Kim et al., 2011). Moreover, interaction between Notch and Hedgehog signaling pathways is critical in regulating self-renewal, proliferation, and differentiation of target cells, ensuring correct organogenesis. Numerous evidence supports the significant role of Hh/Notch crosstalk in cancer biology and chemotherapy-resistant CSCs (Takebe et al., 2011). Studies suggest that crosstalk between Hh and Notch signaling pathways exists and may be involved in the regulation of embryonic stem cell fate determination (Huang et al., 2012), in self-renewal and differentiation of breast cancer cells (Guo et al., 2011), and in hepatic stellate cell fate (Xie et al., 2013). In addition, a study provides evidence that Notch signaling regulates the expression of SHh in neuronal stem cells. Notch activation leads to expression of Hes3 and SHh through activation of serine/threonine protein kinase B (Akt), signal transducer and activator of transcription 3 (STAT3), and mammalian target of rapamycin (mTOR), which promote cell survival (Androutsellis-Theotokis et al., 2006). Conversely, Gli1 is able to sustain the transcription of Jagged1 and Notch1 in the brain (Stecca and Ruiz i Altaba, 2009), and Notch ligand Jagged2 (Katoh and Katoh, 2009). On the other hand, the Notch target Hes1, a repressive transcription factor, is able to suppress Hh signaling, by inhibiting Gli1 transcription in glioma and MB tumors (Schreck et al., 2010). Of note is the observation that Hes1, the principal effector

of the Notch pathway, is also a target of SHh in both in mesodermal and neural cells (Ingram et al., 2008). These data agree with the observation that in a subset of MB patients, Hes1 is upregulated and its expression correlates with shorter patient survival (Fan et al., 2004). Combined activation of Hh and Notch signaling pathways is observed in prostate cancer cells (Domingo-Domenech et al., 2012) and in the pathogenesis of pituitary adenomas (Yavropoulou et al., 2015). Furthermore, novel mechanistic insights demonstrate that Notch signaling plays a central role in left–right asymmetry, playing a crucial role in regulating cilium length (Krebs et al., 2003, Lopes et al., 2010). Interestingly, Notch signaling controls trafficking of Hh signaling mediators into the PC, sustaining the responsiveness to SHh (Kong et al., 2015, Stasiulewicz et al., 2015). Consistent with this observation, Notch and Presenilin 2, a subunit of the γ-secretase complex, localize around the PC. Notch signaling is activated by presenilin/γ-secretase activity, and the Notch intracellular domain is able to move into the nucleus to activate the transcription of several genes involved in cilium length and Hh signaling (Ezratty et al., 2011, Stasiulewicz et al., 2015). This phenomenon could be explained by the observation that the NICD/RBP-Jκ complex is able to drive the transcriptional activation of SMO and Gli2/3 in neuronal cells (Li et al., 2012) (**Figure 2**).

However, the existence of a regulatory mechanism impinging on the crosstalk between Hh and Notch in controlling tumor onset and progression needs to be better understood.

Crosstalk Between Hh and Wnt/β-Catenin Signaling Pathways

The Wnt family of secreted glycoproteins governs multiple developmental events during embryogenesis *via* the transcriptional coactivator β-catenin, and it is also implicated in adult tissue homeostasis and repair (Logan and Nusse, 2004).

In the absence of Wnt proteins, the cytoplasmic β-catenin protein is constantly degraded by the action of a β-catenin degradation complex, composed of the tumor suppressor adenomatous polyposis coli gene product (APC) (MacDonald et al., 2009), the scaffolding protein Axin, CK1, and GSK3. Together, these proteins induce post-translational modifications, resulting in a phosphorylated β-catenin, which is recognized by β-TrCP, targeted for ubiquitination and degraded by the proteasome (He et al., 2004). Altogether, these events prevent a β-catenin shift into the nucleus, and Wnt/β-catenin target genes are thereby repressed by the T-cell factor (TCF)/LEF family of proteins (Brannon et al., 1997),

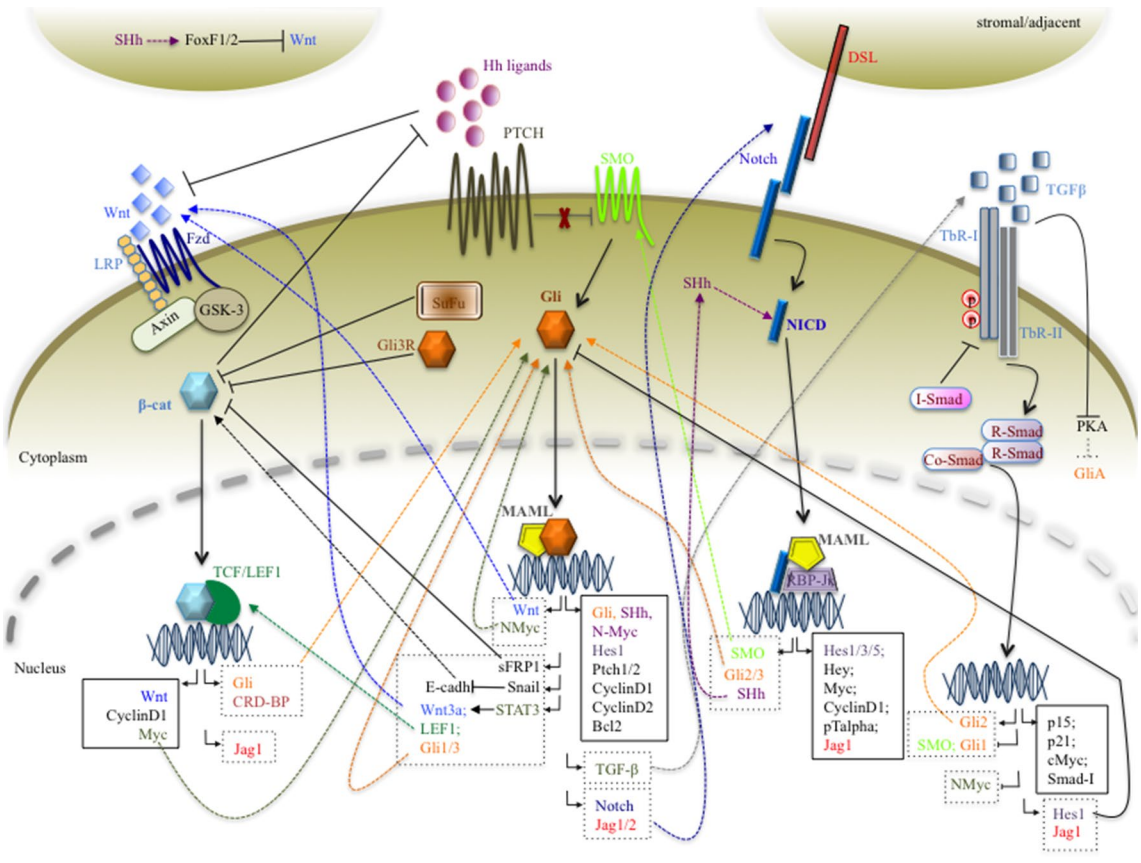

FIGURE 2 | A schematic picture of the interlinking networks between Hh, Wnt/β-catenin, Notch, and TGF-β pathways inside a cancer cell. In the picture are represented the signals deriving from major signaling pathways that converge on the Hh signaling and *vice versa.* The figure is largely discussed in the main text. The black arrows represent the main signaling pathways; the dashed and truncated arrows indicate the positive and negative crosstalks between the pathways. The continuous and dashed rectangles contain canonical and non-canonical target genes, respectively.

associated with the transcriptional repressor Groucho/Transducin-like enhancer protein (TLE) (Cavallo et al., 1998). The Wnt/β-catenin pathway is activated when a Wnt ligand binds to the seven-pass transmembrane Frizzled (Fz or Fzd) receptor, linked to (Brannon et al., 1997) its coreceptor, the low-density lipoprotein receptor-related protein (LRP). Upon the employment of the scaffolding protein Dishevelled (Dvl), the Wnt–Fz–LRP complex induces the recruitment of the Axin complex to the receptors. These events lead to inhibition of Axin-mediated β-catenin phosphorylation and thereby to the stabilization of β-catenin, which accumulates and moves to the nucleus. Once there, β-catenin converts the TCF/Groucho/TLE repressor complex into a transcriptional activator complex that activates the expression of Wnt target genes, such as Wnt components (MacDonald et al., 2009), c-Myc (He et al., 1998), Cyclin D1 (Tetsu and McCormick, 1999), and the Notch ligand Jagged1 (Rodilla et al., 2009) (**Figure 2**). Oncogenic mutations in canonical Wnt signaling determine a constitutive activation of the pathway in a ligand-independent manner, which is linked to human congenital disorders, cancers, and other diseases (Clevers, 2006). Moreover, a recent review summarizes the wide range of epigenetic modifications of the Wnt signaling pathway that affects the development of a variety of tissues and organs, producing dramatic phenotypes and sustaining tumor formation (Wils and Bijlsma, 2018).

Recent studies show that Hh and Wnt signaling pathways can regulate each other, affecting their transcriptional output in specific cellular contexts in which they normally operate (**Figure 2**). Firstly, a crosstalk between Hh and Wnt signaling pathways is described to be involved in the onset/progression of BCC. In fact, aberrant Hh signaling activation, the key step in the tumorigenic program leading to BCC (Nilsson et al., 2000), determines the transcriptional activation of *Wnt* genes through Gli transcription factors (Bonifas et al., 2001; Katoh and Katoh, 2009). Moreover, the existence of a positive feedback loop between Hh and Wnt is present during epithelial transformation, where Gli1 activates Snail, which in turn maintains Gli1 expression through Wnt/β-catenin signaling (Li et al., 2007).

Several reports demonstrate the existence of two-way communication between Hh and Wnt. On one hand, β-catenin is able to directly enhance the luciferase activity of Gli-responsive elements (Maeda et al., 2006) and to induce the post-transcriptional stabilization of Gli mRNAs, by upregulating CRD-BP, (coding region determinant-binding protein), an RNA-binding protein (Noubissi et al., 2009). On the other hand, Gli1 is able to induce the activation of Wnt2b, Wnt4, and Wnt7b, which in turn trigger Wnt/β-catenin signaling by promoting the stability of β-catenin itself (Li et al., 2007). In keeping with these experimental data, the inhibition of Hh signaling by cyclopamine reduces β-catenin/TCF transcriptional activity, induces E-cadherin expression, and reduces invasion in CRC (Qualtrough et al., 2015). Moreover, inhibition of SHh signaling causes a reduction in Wnt-mediated transcriptional activity mediated by Gli3R, able to block the active form of the Wnt transcriptional effector, β-catenin, by physically interacting with the carboxy-terminal domain of β-catenin, a region that includes the transactivation domain (Ulloa et al., 2007).

Conversely, several data suggest an antagonism between Hh and WNT in CRC. In fact, a CRC cell line exposure to recombinant SHh results in the nuclear exit and membrane accumulation of β-catenin, consistent with its role in forming adherens junctions (Yoshimoto et al., 2012) and controlling epithelial–mesenchymal transition (EMT) (Varnat et al., 2010). In addition, it has been shown that Gli1/2 regulates the expression of secreted Frizzled-related protein sFRP-1 with a subsequent cytoplasmic accumulation of Wnt/β-catenin (He et al., 2006). All these data agree with a clinical study that demonstrated the reverse association of Gli1 and β-catenin in human samples of CRC. This study also showed that the overexpression of exogenous Gli1 determines a reduction of nuclear β-catenin in CRC cell lines (Akiyoshi et al., 2006).

Moreover, it reported that IHh, required for normal colonic epithelial differentiation (Ramalho-Santos et al., 2000), is also able to antagonize the proliferative Wnt signaling in crypts by abrogating endogenous β-catenin/TCF signaling (van den Brink et al., 2004). Consistently, an analysis of patients with APC mutations showed the loss of IHh expression in dysplastic epithelial cells present in adenomas, suggesting that IHh expression is downregulated in response to constitutive β-catenin/TCF signaling (Fu et al., 2014). In addition, Gli1 upregulates SHh expression, which is secreted from and acts on stromal cells, able to respond to SHh, enhancing Foxf1 and Foxf2 expression, which inhibit mesenchymal expression of Wnt5a and lead to suppression of β-catenin (Ormestad et al., 2006).

A controversial communication between Hh and Wnt signaling is highlighted in MB. In fact, on one hand, it is reported that SuFu is able to bind β-catenin and to export it from the nucleus and thereby to repress β-catenin/TCF–mediated transcription. Loss of SuFu function not only is associated with an increased risk of MB but also results in over-activity of both SHh and Wnt signaling pathways (Taylor et al., 2004). On the other hand, the activation of Wnt/β-catenin signaling specifically decreases the proliferation of SHh-dependent cerebellar granule cell precursors (GCPs) (Poschl et al., 2014) and of SHh-MB cells, by inducing Gli to proteosomal degradation upon a direct interaction between β-catenin and Gli proteins (Zinke et al., 2015).

Of interest is the demonstration that the mutations in Wnt signaling are recently described in about 15% of MB, corresponding to a minority, and defining a subset of patients with improved outcomes (Swartling et al., 2010). Nevertheless, N-Myc, a known target gene of the Wnt pathway, is also related to a subtype group of MB (Kool et al., 2008), suggesting its important role in the MB pathogenesis. Moreover, several groups report that SHh promotes the expression and post-transcriptional stabilization of N-Myc in mice (Kenney et al., 2003, Thomas et al., 2009).

To date, loss-of-function and gain-of-function mutations in known regulators of the Hh signaling pathway have been elucidated in hematological malignancies (Campbell and Copland, 2015), and the involvement of an autocrine and/or paracrine Hh signaling has been also demonstrated in multiple myeloma (Blotta et al., 2012), lymphoma (Dierks et al., 2007), and colon cancers (Bian et al., 2007; Yoshikawa et al., 2009). Genetic and epigenetic mutations in Wnt signaling components are also identified in leukemia (Staal et al., 2016). Intriguingly, the ability of Hh to crosstalk with Wnt is suggested by

Sengupta and coworkers, who demonstrated that Hh signaling *via* Stat3 activation gives rise to the expression of Wnt3a, Lef1, Gli1–3, and other target genes in the chronic phase of chronic myeloid leukemia (CML) (Sengupta et al., 2007). Although it has been reported that the Hh inhibitor cyclopamine and the WNT inhibitor quercitin suppress the growth of leukemia cells (Kawahara et al., 2009), direct crosstalk between Hedgehog and Wnt/β-catenin in hematological disorders has not yet been well evaluated (**Figure 2**).

Crosstalk Between Hh and TGF-β Signaling Pathways

Mammalian TGF-β family members include more than 35 structurally related secreted proteins, including TGF-βs *stricto sensu*, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), glial-derived neurotrophic factors (GDNFs), Nodal, Lefty, and the Müllerian inhibitory substance/anti-Müllerian hormone (MIS/AMH) (Zi et al., 2012). Members of the TGF-β family play fundamental roles during embryonic development and in maintenance of tissue homeostasis since they regulate diverse cellular processes, such as proliferation, differentiation, migration, and extracellular matrix synthesis (Verrecchia and Mauviel, 2007).

Family members of TGF-β *stricto sensu* are identified in three isoforms: TGF-β1, TGF-β2, and TGF-β3. They signal *via* cell-surface receptors, which consist of specific hetero-oligomeric complex of type-I and type-II serine/threonine kinase receptors (TbR-I and TbR-II). Typically, TGF-β signaling initiates with the binding to and subsequently activation of TbR-I and TbR-II. Smad proteins are the initial responders and transduce the signal from the TGF-β receptor activation process. Smads comprise a family of structurally similar proteins with different functions: receptor-regulated (R-Smads), common mediator (Co-Smads), and inhibitory (I-Smads). After ligand binding, the TbR-II receptor phosphorylates TbR-I, which in turn phosphorylates and activates R-Smads (e.g., Smad2 and Smad3). Then, the R-Smads bind to Co-Smads (e.g., Smad4), and this complex translocates to the nucleus, where it regulates the transcription of TGF-β–responsive genes, involved in cytostatic and/or apoptotic events (p15 and p21) (Pardali et al., 2000; Seoane et al., 2001; Siegel and Massague, 2003), proliferation (c-Myc) (Chen et al., 2002), and the transcription of elements of other signaling pathways, such as Hes1 and Jagged1 (Zavadil et al., 2004). On the contrary, the I-Smads exert a negative feedback effect by competing with R-Smads for interaction with the receptor. Therefore, the I-Smads (e.g., Smad7) associate with ligand–TbR-I receptor complex and interfere with phosphorylation of R-Smads (e.g., Smad3), by preventing their interaction with activated TbR-I. Since the expression of Smad7 is induced by TGF-β1, in turn, it inhibits TGF-β signaling by a negative feedback system (Wahl, 1994, Moustakas et al., 2001). Several publications display a crosstalk between TGF-β and Hh signaling pathways in cancer (**Figure 2**). In the onset of BCC, GLI2 is identified as an early target gene of the TGF-β/SMAD cascade, independently by Hedgehog signaling activity (Dennler et al., 2007). Interestingly, Gli1 is induced by TGF-β in a Gli2-dependent manner, and such an induction is not inhibited by cyclopamine, an SMO antagonist, demonstrating that Hh signaling is not required (Dennler et al., 2007). Concomitantly, studies show that TGF-β inhibits PKA activity, which may contribute to increasing the pool of Gli activator proteins (Pierrat et al., 2012). In addition, Hh signaling induced the expression of TGF-β members. In detail, in the mouse model of SMO-mediated BCC, Fan and colleagues identify TGF-β2 as an Hh target gene (Fan et al., 2010). Thus, the crosstalk between Hh and TGF-β may activate a vicious circuitry, able to promote and amplify downstream targets of Gli1/2, which in turn sustains the activation of TGF-β and Hh itself (**Figure 2**).

During the development of the cerebellum, the GCPs express BMPs. It is reported that in GCPs, BMP-2 and -4 antagonize the proliferative effects of Hh through SMAD5. In addition, BMPs downregulate Hh signaling proteins such as SMO and Gli1. Moreover, it is suggested that BMP-2 inhibits proliferation and promotes cell differentiation of GCPs, by downregulating the oncogene N-Myc (Alvarez-Rodriguez et al., 2007).

It is well described that the architectural structure of the colon is controlled by a gradient of WNT, Hh, BMP, and Notch signaling (Geissler and Zach, 2012). In particular, BMP signaling exerts its function in the differentiated compartment at the top of the crypt, while it is relatively inactive in early compartments at the bottom of the crypt, due to the presence of the BMP inhibitor, Noggin (Syed, 2016). Thus, the Hh and BMP overlapping signaling pathways in the crypt regulate stem cell self-renewal, proliferation, and differentiation, but the existence of a direct regulation between them is not defined yet. However, loss of phosphorylation of Smad1, Smad5, and Smad8 is observed in 70% of sporadic colon cancer (Kodach et al., 2008). Loss of Smad4 or BMPRII function is the likely mechanistic basis for inactivated BMP signaling in sporadic colon cancer. In fact, several reports support the hypothesis that TGF-β/BMP signaling is involved in invasion/metastasis events in tumor cells (Zavadil and Bottinger, 2005).

Although TGF-β is proposed to be a potent negative regulator of hematopoiesis (Fortunel et al., 2000; Fortunel et al., 2003) and the loss of TGF-β signaling is reported in several leukemias (Le Bousse-Kerdiles et al., 1996; DeCoteau et al., 1997; Jakubowiak et al., 2000; Imai et al., 2001; Wolfraim et al., 2004), the disruption of TGF-β signaling alone is not sufficient to induce leukemia (Datto et al., 1999, Yang et al., 1999; Larsson et al., 2003). Thus, its role in leukemogenesis and its ability to crosstalk with other signaling pathways remain largely unknown.

CONCLUSION

Biological processes such as stem cell self-renewal, cell growth, and differentiation are orchestrated by a number of major signaling pathways that integrate with each other to modulate cell outcomes in response to several intracellular signals.

The picture in **Figure 2** schematically summarizes the intricate molecular crosstalk between Hh, Notch, Wnt, and TGF-β,

TABLE 1 | Mutations of HH signaling

	Gene	Locus	Disease	Inheritance	Mutations	Reference
Loss of function			Gorlin syndrome	Germline	Insertion deletion	Hahn et al., 1996; Lindstrom et al., 2006
			Rhabdomyosarcoma	Germline	Missense	Bridge et al., 2000; Taeubner et al., 2018
			Medulloblastoma	Germline Somatic	Insertion Deletion Frameshift LOH	Raffel et al., 1997; Reifenberger et al., 1998; Zurawel et al., 2000; Thompson et al., 2006; Kool et al., 2014; Rusert et al., 2014
	Ptch1	9q22.32	Basal cell carcinoma	Somatic	Missense LOH	Gailani et al., 1996; Reifenberger et al., 2005
			Leukemia	Germline Somatic	Missense	Dagklis et al., 2015; Burns et al., 2018
			Breast cancer	NA	LOH	Naylor et al., 2005; Wood et al., 2007; Sinha et al., 2008
			Gastric–intestinal cancer	NA	Frameshift Missense	Wang et al., 2013
			Gorlin syndrome	Germline	Missense Frameshift	Fan and Eberhart, 2008; Fujii et al., 2013
			Rhabdomyosarcoma	Germline	Missense	Taeubner et al., 2018
	Ptch2	1p34.1	Leukemia	NA	Transition	Dagklis et al., 2015
			Gorlin syndrome	Germline	Nonsense Missense deletion	Smith et al., 2014
			Rhabdomyosarcoma	Germline	LOH	Bridge et al., 2000
			Medulloblastoma	Germline Somatic	Missense deletion Truncating LOH	Taylor et al., 2002; Thompson et al., 2006; Rusert et al., 2014; Kool et al., 2014
	SuFu	10q24.32	Leukemia	Somatic	Missense	Burns et al., 2018
			Basal cell carcinoma	Somatic	Missense	Reifenberger et al., 2005
			Prostate cancer	Somatic	LOH deletion Nonsense	Sheng et al., 2004
			Meningioma	Germline	Missense LOH	Aavikko et al., 2012
			Pancreatic cancer	Somatic	Missense	Jones et al., 2008
	Gli3	7p14.1	Leukemia	Germline Somatic	Missense	Dagklis et al., 2015; Burns et al., 2018
			Colorectal cancer	NA	Missense	Wood et al., 2007
Gain of function			Rhabdomyosarcoma	Germline	Amplification	Roberts et al., 1989
			Medulloblastoma	Somatic	Amplification	Thompson et al., 2006; Northcott et al., 2009
			Leukemia	Somatic	Missense	Dagklis et al., 2015; Burns et al., 2018
	Gli1	12q13.3	Breast cancer	Somatic	Amplification	Nessling et al., 2005; Wood et al., 2007; Jiao et al., 2012
			Pancreatic cancer	Somatic	Missense	Jones et al., 2008
			Colorectal cancer	Somatic	Frameshift	Lee et al., 2018
			Medulloblastoma	Somatic	Amplification	Northcott et al., 2009; Rusert et al., 2014; Kool et al., 2014
	Gli2	2q14.2	Leukemia	Somatic	Missense	Burns et al., 2018;
			Medulloblastoma	Somatic	Missense	Reifenberger et al., 1998; Rusert et al., 2014; Kool et al., 2014
			Basal cell carcinoma	Somatic	Missense	Reifenberger et al., 1998; Reifenberger et al., 2005
	SMO	7q32.1	Leukemia	Somatic	Frameshift	Dagklis et al., 2015
			Gastric–intestinal cancer	NA	Missense Insertion	Wang et al., 2013
			Ameloblastoma	Somatic	Missense	Sweeney et al., 2014
			Medulloblastoma	NA	Amplification	Reifenberger et al., 1998; Kool et al., 2014
	SHH	7q36.3	Basal cell carcinoma	Somatic	Missense	Couve-Privat et al., 2004

which establishes a network of protein–protein interactions able to affect gene expression programs across the pathways. The cartoon clearly represents the complexity of molecular interlinking inside the tumor cell, which is underlying the severity of the neoplasia phenomenon. The pathogenesis of malignancies is characterized by dysregulation of at least two pathways at the same time, proving a functional advantage to neoplastic cells, influencing relapse and drug responsiveness. We need to unravel the intricate signaling networks between the major pathways to prevent tumor formation and/or to contribute to the development of novel anticancer treatment modalities. Aberrant activation of the Hh pathway is tightly associated with cancer development. Notably, a plethora of stimuli activate multiple signaling cascades in tumor cells, which can impinge on Hh signaling and affect its output, by direct protein–protein interaction or by regulating indirectly the expression of key

components of the signaling. Conversely, the Hh pathway can also have an impact on other pathways, determining a mutual signaling interference, a phenomenon underlying an unbalanced network in cancer cells. The Hh signaling pathway is considered an important molecular cross point able to integrate/synergize with other major signaling cascades, such as Wnt, TGF-β, and Notch. Therefore, **Figure 2** presents an overview concerning direct or indirect molecular mechanisms that sustain Hh cross-talking with major signaling pathways. Molecular sharing between Hh and the Wnt/β-catenin, Notch, or TGF-β signaling pathway suggests that two major signaling networks crosstalk with each other during oncogenesis. Therefore, a combined involvement of these signaling pathways in early stages of tumorigenesis as well as in the metastatic process in several types of cancers suggests the need to combine novel targeted inhibitors with standard antineoplastic therapy to enhance therapy efficacy.

This complex scenario could open a huge window of opportunity for the development of new therapeutic drugs for multiple cancers.

AUTHOR CONTRIBUTIONS

MP, SZ, and DB wrote the paper. MP, SZ, and FN prepared the figures. SC, IS, and DB reviewed drafts of the paper. All authors read and approved the final manuscript.

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