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Notch Signalling in Development and Disease:

Maml1 and Jagged1 not always on the shadow of Notch

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Index

I. Introduction

One of the most active research areas of molecular biology is represented by the study of the molecular mechanisms through which a transcriptional activator controls the transcription of specific genes in response to external or internal stimuli. In recent years, scientific research has been focusing on the elucidation of the molecular processes underlying the regulation of gene expression, mediated by transcription factors, co-activators and co-repressors. Although the beginning of transcription requires such factors, it has been observed that other molecules are necessary. Transcriptional co-activators are proteins that associate with transcription factors by regulating the specific expression of target genes. They can promote transcriptional activation through various mechanisms. For these reasons, co-activators are now recognized as key components for signalling transduction in many biological processes.

Among the several signalling pathways inside the cell we find the Notch signalling, evolutionary conserved from flies to vertebrates, that regulates a large spectrum of cellular processes, such as proliferation, differentiation and cell death. It is active during development and maintenance of self-renewing adult tissues. In recent years, different studies have highlighted the importance of Notch pathway components, that play a fundamental role in the development and/or are involved in the onset of several diseases, often with Notch-independent mechanisms.

Noteworthy, aberrant gain or loss of Notch signalling components has been directly linked to multiple human disorders, from developmental syndromes to adult-onset diseases and cancer (Kopan & Ilagan, 2009).

1. Notch signalling

The Notch signalling is an evolutionarily conserved pathway from *D. melanogaster* to vertebrates (Artavanis-Tsakonas et al., 1999), involved in cell fate differentiation and in development of different multicellular organisms. Notch signalling regulates cell survival, proliferation, apoptosis and self-renewal events (Bray, 2006). In addition, Notch receptors are intimately associated with the maintenance and fate of stem cells (Louvi & Artavanis-Tsakonas, 2012).

Morgan and Bridges discovered for the first time the *notch* gene in 1916, in *D. melanogaster* X-linked dominant mutants, characterized by “notches”, missing tissues, at the tips of the wing blades (Artavanis-Tsakonas & Muskavitch, 2010).

In *D. melanogaster* the *notch* gene encodes for a 300-kDa single pass transmembrane receptors, while in *C. elegans* two different receptors were characterized: LIN-12 and GLP-1 (Artavanis-Tsakonas et al., 1999).

In mammals, *notch* genes encode four large single-pass type I transmembrane proteins receptors that display both redundant and unique functions: Notch1, Notch2, Notch3 and Notch4 (Kopan & Ilagan, 2009). Notch proteins are constituted by an extracellular domain (NECD), involved in the interaction with DSL ligands, and an intracellular domain (NICD) responsible of the signal transduction.

The extracellular domain contains 36 tandem epidermal growth factor (EGF)-like repeats. The interaction with ligands requires some of these repeats. Moreover, many EGF-like repeats bind calcium ions, necessary for the structure and affinity of Notch in ligand binding (Cordle et al., 2008; Kopan & Ilagan, 2009). The EGF-like repeats are followed by three cysteine-rich Notch/Lin12 (LNR) repeats and a heterodimerization domain (HD). The LNR repeats together with the HD form the negative regulatory region (NRR), essential in preventing Notch activation in the absence of a ligand.

The intracellular domain contains several RAM (RBPJ association module) domains, for protein-protein interaction, a seven ankyrin repeats domain (Ank/Cdc10) with two different nuclear localization sequences (NLS) on both sides, and a transactivation domain. Instead, the C-terminal domain is characterized by the PEST domain [proline (P), glutamic acid (E), serine(S) and threonine (T)-rich motif], that presents a degron, a degradation signal, to regulate Notch stability (Artavanis-Tsakonas et al., 1999; Bertrand et al., 2012; Kopan & Ilagan, 2009; Ntziachristos et al., 2014).

The immature receptor undergoes different post-translational modifications, during maturation and trafficking to cell surfaces. Initially, Notch proteins are processed in the endoplasmic reticulum by O-fucosil transferase (O-Fut1) at the EGF-like repeats (Haines & Irvine, 2003). O-Fut1 binds a residue of O-fucose to Notch, promoting its clustering to the surface (Panin et al., 2002). After this modification by O-Fut1, the immature precursor of Notch is cleaved by a furin-like convertase at a specific site (S1), during trafficking through the Golgi complex. Notch is converted as a heterodimeric receptor, with an extracellular domain (NECD), a trans-membrane domain (NTM)

and an intracellular domain (NICD), held together by non-covalent interaction *via* a heterodimerization domain (HD). The NECD undergoes O-linked glycosylation during Notch synthesis and secretion, which is crucial for proper folding of the Notch receptor and the interaction with its ligand DSL (Delta, Serrate, Lag-2) (Hori et al., 2013). The mature receptor is transported on the cell surface and held *in situ* by non-covalent interactions (Ntziachristos et al., 2014).

The canonical Notch pathway involves trans-interactions between the receptors, expressed on the signal-receiving cells, and their specific DSL ligands, located on the signal-sending cells (Bray, 2006; Hori et al., 2013). Among the canonical ligands of Notch receptors, we find the DSL family (Delta/serrate/lag-2), highly evolutionarily conserved. Different ligands cooperate with different notch receptors to determine the cell fate.

The DSL ligands are type I transmembrane glycoproteins characterized by a DSL domain, at the N-terminal domain, involved in the binding with EGF-like repeats of Notch (Fiúza & Arias, 2007). In addition, the ligands contain EGF-like repeats in a substantially variable number between the Delta and Serrate/Jagged1 family. Finally, the serrate/jagged ligands are characterized by cysteine-rich domain (CRD) located between the transmembrane domain and EGF-like repeats (Ascano et al., 2003). Moreover, Jagged1 and 2 have almost twice as many EGF-like repeats, compared to Delta-like ligands (Dll1, Dll3 and Dll4) (Vitt et al., 2001).

The Notch-ligand binding causes a conformational change in the receptor structure with the consequent exposure of two cleavage sites. The first cut is carried out by the family of metalloproteinase ADAM10/17, which recognize the site S2 placed in the extracellular region of Notch, determining its release (Aithal & Rajeswari, 2013). The S2 cleavage mediated by ADAM metalloproteinases stimulates the presenilin complex (PS)/γ-secretase cleavage, in the S3 site placed within the intracellular domain. These events result in the release of an intracellular domain of Notch (NICD), which translocates to the nucleus (Aithal & Rajeswari, 2013; Bertrand et al., 2012) and forms a multiprotein complex with CSL proteins (CBF1 in mammals, RBPJ in the mouse, "suppressor of hairless" in *D. melanogaster* and Lag1 in *C. Elegans*). At first, NICD binds CSL proteins with the RAM domain and, subsequently, with the Ank domain. When Notch signalling is turned off, CSL proteins inhibit transcription, together with co-repressor such as SMRT, NCoR and SHARP. The presence of Notch is able to derepress the promotor region by displacing corepressors and directly binding to CSL proteins (Kovall & Hendrickson, 2004; Wilson & Kovall, 2006). Notch requires Maml proteins and other transcriptional co-activators to drive the transcription of target genes, such as the Hes family (Bray, 2006; Kopan & Ilagan, 2009; Lai, 2004) (Fig. 1). Maml1 binds NICD and CSL only in a complexed dimer where the highly conserved N-terminal domain of

Maml1 is fitting into a molecular groove formed by the Ank domain of Notch and specific residues of the CSL protein. In addition, Maml1 recruits additional cofactor, such as p300 and CDK8, to induce posttranslational modifications. Acetylation, phosphorylation and ubiquitination events, induced by these cofactors, mediate the binding affinity and stability of Notch transcriptional complex on target genes´ promoters.

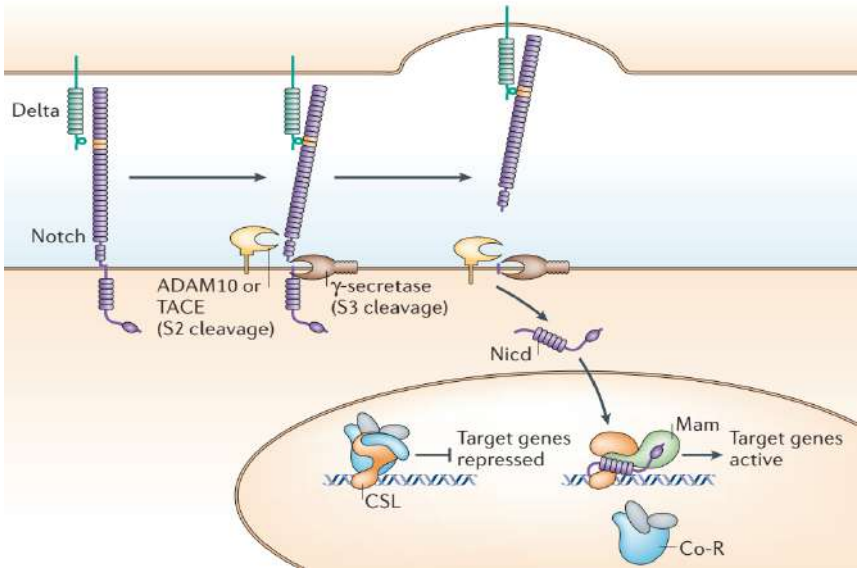


Figure 1. Canonical Notch signalling (Bray, 2006)

Notch proteins undergo several post-translational modifications (e.g acetylation, hydroxylation, methylation, phosphorylation and ubiquitination), to regulate the signalling and their response for proper tissue homeostasis (Borggrefe et al., 2016).

Different E3 ubiquitin ligases target Notch (Lai, 2002). The Itch/NEDD4/Su(dx) E3 ligases play a negative role on Notch pathway, inducing Notch ubiquitylation and degradation (Qiu et al., 2000). These events result with a down regulation of Notch target genes (Bray, 2006). Deltex, an E3 ubiquitin ligase belonging to the RING family, is recognized as a regulator of Notch signalling (Matsuno et al., 1995). Noteworthy, depending on the cellular context Deltex can either promote or inhibit Notch pathway. In *D. melanogaster* Deltex promotes Notch transcriptional activity (Matsuno et al., 1998) and induces Notch accumulation in endocytic vesicles (Hori et al., 2004; Matsuno et al., 1995). Deltex can interact with β-arrestin Kurz, and this interaction facilitates the complex formation Deltex-Notch-Kurz to induce Notch ubiquitylation and degradation (Mukherjee et al., 2005). Moreover, in mammals Deltex acts as a negative regulator in lymphoid and neuron cells (Itoh et al., 2003; Šestan et al., 1999).

Although the role played by Notch signalling is now widely discussed, the activation/shutdown of the pathway seems to be cell context-dependent (South et al., 2012). In fact, the oncogenic role of Notch receptors in the onset and progression of cancer has been discovered by the identification of intracellular domain mutations of Notch1 and Notch3, specifically in T-cell acute lymphoblastic leukaemia (Aster et al., 2008; Bernasconi-Elias et al., 2016; Palomero et al., 2006; Weng et al., 2004). In T-ALL several reports indicate the role of Notch signalling in tumorigenesis and progression both for Notch1 and Notch3 (Bellavia et al., 2000; Ferrando, 2009; Franciosa et al., 2016; Pelullo et al., 2014; Tottone et al., 2019). Aberrant activations of the Notch signalling pathway have been identified also in other types of solid tumours (i.e., pancreatic, breast, prostate, liver, cervix, lungs, ovary and colon cancer) (Diluvio et al., 2018; Ranganathan et al., 2011).

In T-cell acute lymphocytic leukaemia and chronic lymphocytic leukaemia Notch1 and Notch3 activating mutations have been identified, while in diffuse large B cell lymphoma Notch2 is mutated (Bernasconi-Elias et al., 2016; Puente et al., 2011; Weng et al., 2004). These mutations result in constitutively active Notch signalling. In contrast, in chronic myeloid leukaemia novel somatic loss of function mutations of Notch have been identified, suggesting a new role for Notch signalling as a tumour suppressor (Klinakis et al., 2011). Besides, in cutaneous squamous cell carcinoma inactivating mutations in *notch1*, *notch2*, *notch3* and in other components of the pathway, such as *FBXW7* were reported (Agrawal et al., 2011; Stransky et al., 2011). On the contrary, in HPV-positive cervical cancer Notch signalling has a tumour-promoting role, with a cooperative activity between Notch1 and papillomavirus oncoproteins (Brimer et al., 2012; Rangarajan et al., 2001; Zagouras et al., 1995).

2. Mammalian Mastermind family

Transcriptional co-activators are proteins associated with transcription factors, which can represent the point of convergence of multiple signalling pathways within eukaryotic cells. This binding regulates specific gene expression during several cellular process by binding to cis-regulatory elements located upstream of the promoter of specific target genes to either activate or repress transcription.

In mammals, Maml1 (Wu et al., 2000) belongs to a family of proteins, also including Maml2 and Maml3 (Wu et al., 2002), which act as transcriptional coactivators for Notch signalling (Petcherski & Kimble, 2000). In *D. melanogaster*, *maml* gene encodes for a nuclear glutamine rich protein, that regulates Notch signalling (Wu et al., 2002).

Maml proteins are widely expressed in adult tissue with distinct expression patterns during early development in mice. Maml proteins are nuclear proteins, and they have been shown to form nuclear bodies when overexpressed in mammalian cells (Lin et al., 2002; Wu et al., 2002; Wu et al., 2000).

The first studies on *mastermind (mam)* gene were conducted by Nusslein-Volhard and Wieschaus, who identified mutations on *mam* genes in *D. melanogaster* (Lehmann et al., 1983; Nüsslein-Volhard & Wieschaus, 1980). Homozygous mutants of *mam* exhibited the same phenotype of notch mutants, i.e. hypertrophy of nervous system (Kitagawa, 2015; Lehmann et al., 1983).

The structure of basic and acidic amino acid clusters in the Mam proteins are conserved, even though the identity between the Maml1 protein and the Maml2 and 3 proteins is 19 and 30%, respectively (Kitagawa, 2015).

The conserved region between Mastermind in *D. melanogaster* and the homologous proteins in mammals (i.e. Maml1, Maml2 and Maml3) is the basic domain. The sequence identity of the N-terminal domain between Maml1 and Maml2 is 60%, between Maml1 and Maml3 50% and between Maml2 and Maml3 47%. In contrast, the sequence similarity of C-terminal domains is low, with 21% , 33% and 21% respectively (Wu et al., 2002; Wu et al., 2000).

Maml1 is a protein of 1016 amino acids. Maml1 contains two distinct transactivation domains: TAD1, a central activation domain (amino acids 75–301); and TAD2, a C-terminal activation domain essential for Notch activity in vivo (Fryer et al., 2002) (Fig. 2). The N-terminal region is characterized by a nuclear localization sequence motif (NLS, 135-141aa, PGHKKTR). While the amino acids 1-75 are involved in the binding to the Notch intracellular domains, the TAD1 interacts with p300, a histone acetyltransferase, and CDK8 (Cyclin-dependent kinase 8). The TAD2 is a glutamine rich region, required for Notch transcription *in vivo* and for the recruitment of additional factors, such as CycC:CDK8 (Fryer et al., 2002; Fryer et al., 2004).

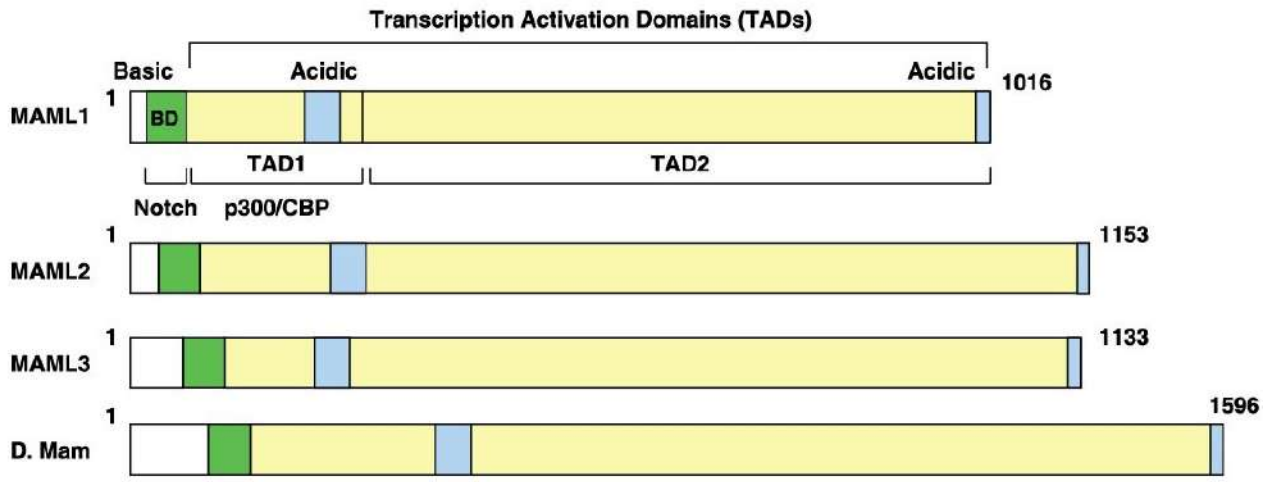


Figure 2. Structure of Mastermind proteins in *D. Melanogaster* and humans (McElhinny et al., 2008)

Maml proteins form DNA-binding complexes with RBPJ and Notch (Lin et al., 2002). In absence of Maml, Notch weakly binds to RBPJ. Although, in the off state of Notch signalling, Maml1 and RBPJ are not associated, upon activation of the signalling the N-terminal domain of Maml1 is necessary to form the ternary complex (Notch-RBPJ-Maml1). This interaction allows the transcription of Notch target genes (Kitagawa et al., 2001). The study of the crystal structure of the ternary complex revealed an extended groove formed by the binding of RBPJ and the Notch Ank domain. The basic domain of Maml1 is settled in this groove interacting with both proteins (Choi et al., 2012; Nam et al., 2006; Wilson & Kovall, 2006).

Although the three human Mam proteins have similar functions despite having unusual structural diversity, they show little preference among all four kinds of mammalian Notch in the presence of RBPJ (Kitagawa, 2015). The presence of Maml1 and Maml2 improves the Notch-induced activation of *hes1* as target gene, while Maml3 has a weak effect. Nevertheless, Maml3 works as a strong coactivator for Notch4 (Wu et al., 2002). Moreover, in all three proteins the N-terminal basic domain is essential for the formation of the transcriptional complex (Lin et al., 2002).

Maml1 activity and its binding with other proteins is necessary to modulate the transcriptional function of Notch (Wu & Griffin, 2004). The interaction with p300 can promote the transcription of target genes (Hansson et al., 2009). In fact, Maml1 interacts with the C/H3 domain of p300, potentiates p300 autoacetylation on Lys1499 and thereby p300 co-activator function. Maml1 enhances p300 HAT (Histone acetyl-transferase) activity directly, and this coincides with the translocation of Maml1, p300 and acetylated histones to nuclear bodies (Hansson et al., 2009). On the contrary, the recruitment of CDK8 on the complex induces the phosphorylation of Notch in the TAD and PEST domain and its degradation by Fbw7/Sel10 (Fryer et al., 2004).

To note, a proline-repeat motif in the N-terminal domain of Maml1 is important for p300-mediated acetylation of Maml1 and for the activity of the N-terminal domain *in vivo*. Three pairs of double lysine residues have been identified (Lys138/Lys139, Lys188/Lys189 and Lys278/Lys279), but only the Lys188/Lys189 were the ones most strongly acetylated (Saint Just Ribeiro et al., 2007). The proline motif in the N-terminal domain of Maml1 is the region required for the interaction with p300, both *in vitro* and *in vivo*. Nevertheless, the acetylation appears to destabilize the p300–Maml1 interaction after that p300 has been recruited by Maml1 to a target gene to acetylate histones. The residues of Maml1 involved in the acetylation are highly conserved in vertebrates. Therefore, Maml1 acetylation might be conserved throughout evolution and could harbour other functions in addition to affecting the interaction with p300 (Saint Just Ribeiro et al., 2007).

Moreover, Maml1 acetylation on Lys188/Lys189 drives the recruitment of NACK (PEAK1-related kinase activating pseudokinase 1) to the Notch ternary transcription complex and subsequent recruitment of RNA polymerase II, thereby initiating transcription (Jin et al., 2017).

To elucidate the role of Maml1 *in vivo*, *Maml1*$^{-/-}$ mice were generated (Oyama et al., 2007). It was shown that *maml1* deficient mice present a retarded growth and die before weaning. During haematopoiesis, Notch signalling plays a fundamental role during different stages. Among the Notch-dependent stages in haematopoiesis, Oyama and colleagues demonstrate that Maml1 is required for the development of marginal zone B (MZB) cells in the spleen, partially required for the development of $CD4^+CD8^+$ double-positive (DP) thymocytes from $CD4^-CD8^-$ double-negative (DN) cells. In contrast, Maml1 is not required for the development of early T cell progenitors, in T/B lineage commitment, or the generation of definitive haematopoiesis, even though Maml1 remains an essential component of the canonical Notch signalling *in vivo* (Oyama et al., 2007).

There are several reports for the role of Mastermind in human disease. Squamous epithelial neoplasms, i.e. papillomas, are caused by papillomaviruses (HPV), small encapsidated DNA viruses with double-stranded circular genomes. Generally, these kinds of neoplasms are benign, but in certain circumstances they can develop in malignancies, like squamous cell carcinoma (Brimer et al., 2012; Orth, 2006). The HPVs can control proliferation, survival and keratinocyte differentiation by three viral early open reading frames: E5, E6 and E7. The viral proteins can bind to target proteins of the host through a short acidic amphipathic helixes, that contains a LXXLL motif (Chen et al., 1998). Two different groups discovered a repression of Notch signalling mediated by BVP1-E6 and β-HPV E6 through an interaction with Maml1 (Brimer et al., 2012; Tan et al., 2012).

At first, Maml1 was found to be a specific interactor of BVP1-E6 and β-HPV E6. Maml1 has an acidic LXXLL motif, remarkably similar to the BVP1-E6 binding site on paxillin, a focal adhesion protein, and the cellular E3 ubiquitin ligase E6AP (Brimer et al., 2012). Also, Maml3 (but not Maml2) interacts with BPV-1 E6 (Tan et al., 2012). E6 oncoproteins associate to the C-terminal LXXLL motif of Maml1, repressing Maml1 transactivation (Brimer et al., 2012; Tan et al., 2012). The interaction is required to repress canonical Notch induced transcription of target genes. An impairment of Notch signalling causes a failure of differentiation in squamous epithelia. E6 oncoproteins disrupt specifically Notch target genes transcription as part of their cellular activity, causing a downregulation of *hes1* expression and delaying keratinocyte differentiation (Brimer et al., 2012; Tan et al., 2012).

CD44 is an integral membrane glycoprotein that interacts to hyaluronic acid and has a pivotal role in tumour growth and metastasis. Besides, CD44, being a surface membrane protein, can be regarded as a candidate in cancer stem cells (CSC) detection and isolation, specifically as a marker in epithelial CSCs (Ishimoto et al., 2011; Yae et al., 2012). Indeed, some $CD44^+$ CSCs present epithelial–mesenchymal transition (EMT) ability, and contribute to tumour progression and metastasis (Chen et al., 2011). To note, Notch signalling has a critical role in CSCs regulation and maintenance (Fre et al., 2005). In esophageal squamous cell carcinoma (ESCC), $CD44^+$ ESCC CSCs are resistant to the chemotherapy treatment with 5FU. Nevertheless, targeting Maml1 in these cells induces a reduction in cell migrations and increase the number of the cells in G1 phase (Moghbeli et al., 2019). Maml1 presence contributes to CSCs resistance to 5FU treatment. It has been suggested that Maml1 exhorts cells migration by Twist, a factor involved in EMT (Moghbeli et al., 2019).

High levels of Notch1 and its ligand Jagged1 are predictors of poor prognosis in breast cancer (Dickson et al., 2007). It is known that enhanced Notch signalling, by ligands regulation, leads to mammary tumorigenesis (Xu et al., 2012). In addition, p53 pathway has a key role in regulating the onset and progression of breast cancer (Lacroix et al., 2006). In MCF-7, a breast cancer cell line, it has been observed that overexpression of p53 induces a downregulation of Notch target genes, in a dose-dependent manner (Yun et al., 2015). The inhibitory effect was restored by overexpression of Maml1, but not p300. Notch1 and p53 complex together, and Maml1 enhances this interaction, while the Dominant negative of Maml1 (70aa of the N-terminal domain) inhibits the binding. Moreover, a chromatin immunoprecipitation assay revealed that p53 is associated in a Notch-dependent manner to the *hes1* promoter. According to the molecular model proposed by the authors, p53 acts a negative regulator of Notch by Maml1. In fact, p53 is associated to Notch transcription

complex through a Maml1-p53 interaction. This binding allows the cross-talk between Notch and p53 pathways (Yun et al., 2015).

Maml1 does not always act in the shadow of Notch. A recent publication reported Maml1 as a regulator of epithelial to mesenchymal transition (EMT), in a Notch-independent mechanism, in breast cancer (Shariat Razavi et al., 2019). Maml1 is highly expressed in MFC-7 and MDA-MB-231 breast cancer cell lines, which do not harbour Notch mutations. Overexpression of Maml1, in these cell lines, induced a suppression of mesenchymal markers, an increase in the levels of E-cadherin and a downmodulation of *Hes1*. Besides, Maml1 overexpression causes a decrease in breast cancer cell lines migration. On the contrary, Maml1 inhibition induces EMT markers and a morphological change in the cells, from epithelial to mesenchymal structure. While Notch induces EMT, a concomitant Maml1 overexpression and inhibition of Notch signalling boosts the inhibitory effect on Notch target genes, and a further increase in E-cadherin levels. On the opposite, Notch signalling induces EMT in these cell lines. These results demonstrate a novel regulatory role for Maml1, with an inverse correlation between Maml1 and EMT, in a Notch-independent mechanism (Shariat Razavi et al., 2019).

Finally, Maml1 is involved in T-cell acute lymphoblastic leukaemia (T-ALL) tumorigenesis. Knockdown of Maml1 inhibits cell proliferation and induces G0/G1 cell cycle arrest and apoptosis in different T-ALL cell lines (Cheng et al., 2019). In particular, the expression levels of genes of TRIM family were reduced, mostly TRIM59. The superfamily of tripartite motif-containing (TRIM) proteins, are induced by type I and type II interferons and are involved in innate immunity (Ozato et al., 2008). When the transcriptional factor SP1 is overexpressed, Maml1 and TRIM59 levels are restored, and promoter activation of TRIM59 is observed. Cheng et colleagues (2019) suggest the presence of a Maml1-SP1-TRIM59 axis, to regulate proliferation and survival in T-ALL. Maml1 could be used as a novel therapeutic target.

3. Jagged1, the DSL ligand for Notch pathway

Notch receptors family is involved in several processes, inside the cell, to regulate the development of multicellular organisms, in differentiation events, apoptosis or proliferation mechanisms. Notch signalling has a pleiotropic role in development and a mechanism of control is essential to maintain the normal homeostasis in the tissue. DSL (Delta/Serrate/Lag-2) family represent the canonical activators of the Notch core pathway. DSL genes encode for single-pass proteins and include Jagged-1, Jagged-2, Delta-like 1 (Dll-1), Delta-like-3 (Dll-3) and Delta-like-4 (Dll-4) (LaFoya et

al., 2016). The N-terminal domain is constituted by a DSL motif and epidermal growth factor-like (EGF-like) repeats, whose number is variable between members of the Delta and the Serrate/Jagged1 family. Moreover, Serrate/Jagged ligand present a cysteine-rich domain (CRD) between the transmembrane domain and EGF-like repeats (Fig. 3) (Ascano et al., 2003).

Interestingly, the *jag1* gene encodes for a *consensus* motif PDZ-ligand (X-T/S/Y-X-V/L/I) on the C-terminal domain. It is known that PDZ-ligand motifs are characterised by few amino acids that interacts in order to form a β-sandwich structure to facilitate the protein-protein interaction (Ascano et al., 2003). Notably, it has been suggested the existence of a novel "reverse signalling mechanism" triggered by Jagged1 protein and PDZ-dependent, able to activate the Notch pathway in a non-canonical way. However, it remains unclear if the Notch signalling events take place in both the DSL-expressing and Notch-expressing cells upon receptor-ligand binding. Bidirectional signalling mechanisms have been documented such as the Eph/Ephrin pathway (Ascano et al., 2003).

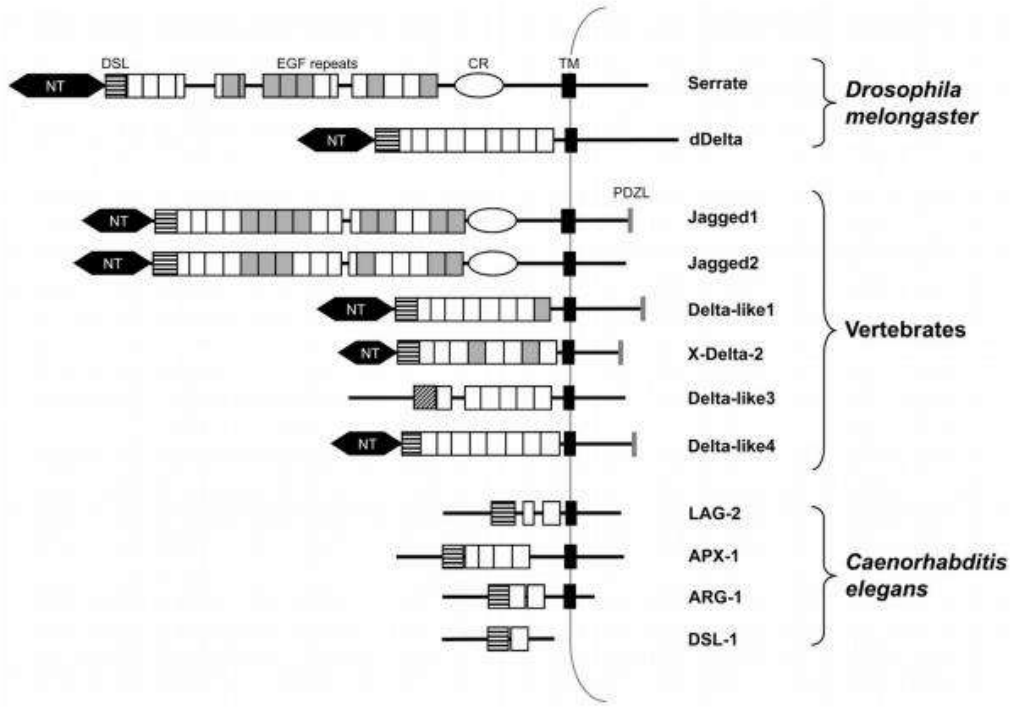


Figure 3. DSL ligands protein structure (D'Souza et al., 2008)

Notably, several evidences reported that, similarly to Notch receptors, the DSL ligands undergo the same proteolytic cleavages that result in the release of an intracellular fragment. In particular, Jagged1 is a substrate for catalytic activity of the Metalloprotease ADAM17 (A Disintegrin and

Metalloproteinase). The proteolysis mediated by ADAM17 allows the shedding of the ectodomain fragment (sJag1-ECD), generating a membrane-tethered intracellular domain (Jag1-TMICD) (LaVoie & Selkoe, 2003; Pelullo et al., 2014; Small et al., 2001). Then, the Jag1-TMICD fragment undergoes an intramembrane cleavage mediated by presenilin/γ-secretase complex activity that releases a soluble intracellular fragment (Jag1-ICD), which translocates into the nucleus (LaVoie & Selkoe, 2003). Jag-ICD is able to move into the nucleus through nuclear localization sequence (NLS) and to be part of active transcription complexes with AP1 (LaVoie & Selkoe, 2003) and RBPJ (Pelullo et al., 2014), regulating the expression of different target genes.

Literature data suggest critical role for Jag1 in the cellular fate. In fact, Jag1 mutations have been linked with several diseases, such as Alagille syndrome and in some cases also tetralogy of Fallot. Moreover, Jag1 variations are associated with multiple types of cancer (Grochowski et al., 2016). Moreover, cells with high levels of Jagged1 are able to undergo neoplastic transformation processes (Ascano et al., 2003).

The aberrant expression of Jagged1 causes tumour proliferation and transformation events. In T cell acute lymphoblastic leukaemia (T-ALL) Jag1-ICD regulates mRNA expression of Jag1 itself and Notch3, playing a central role in cellular transformation and neoplastic cell proliferation, being a link between aberrant Jag1 expression and tumorigenesis (Pelullo et al., 2014). The authors demonstrate the existence of a paracrine signalling induced by the soluble extracellular domain of Jagged1 able to activate Notch3 receptor in adjacent cells, and an autocrine signalling by Jag1-ICD to activate the transcription of target genes such as pTα (Fig. 4) (Pelullo et al., 2014).

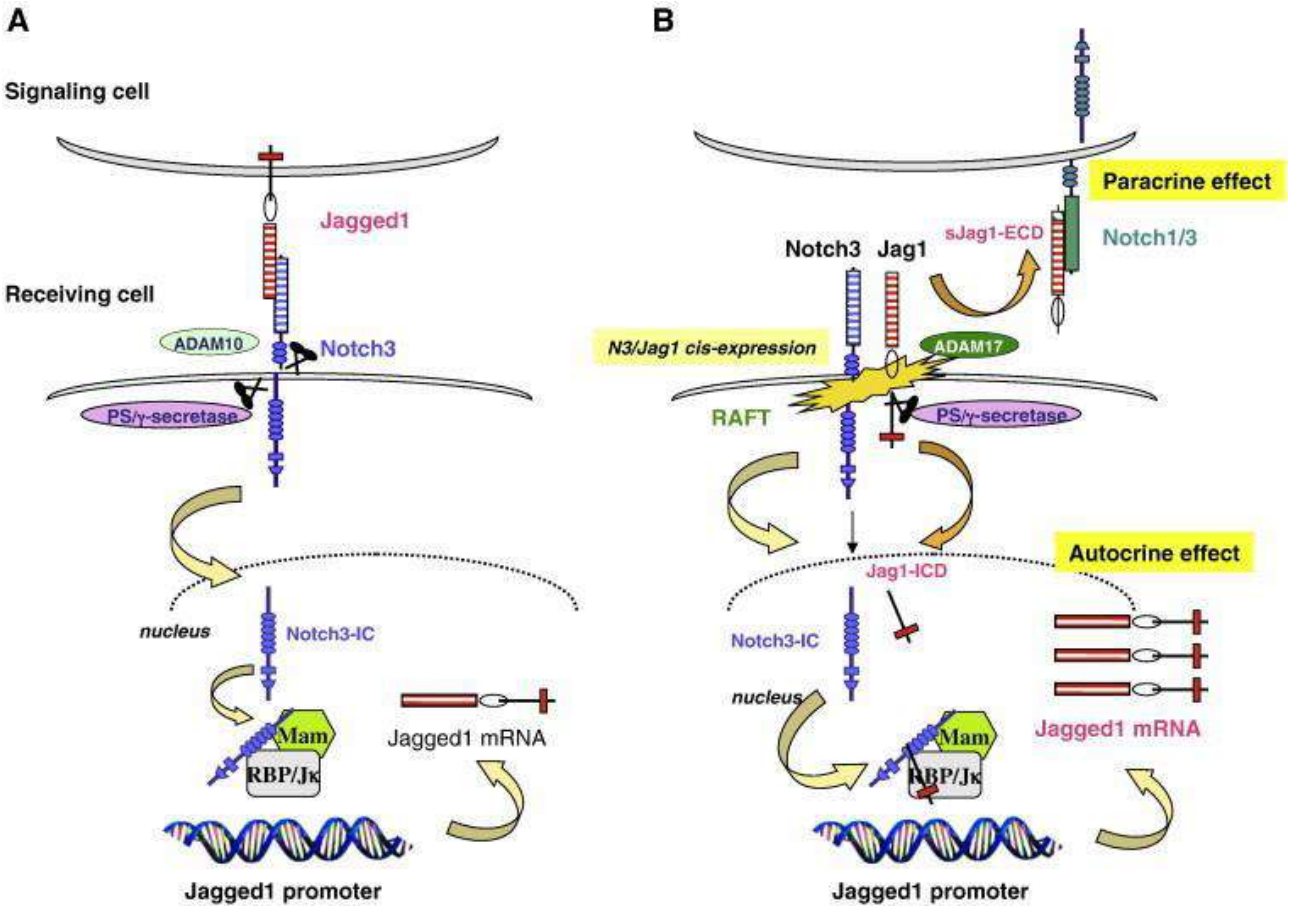


Figure 4. Representation of the positive feedback loop between Jagged1 ligand and Notch3 receptors. (A) The ligand Jagged1 activates Notch3 signalling; (B) Jagged1 signalling due to its processing by ADAM17 and PS/γ-secretase (Pelullo et al., 2014).

Recently, several studies linked the expression levels of Jagged1 with the development and/or progression of solid tumours. In breast cancer, an altered expression of the Notch pathway is caused by an aberrant activation mediated by its ligands. In particular, Jagged1 transcript and protein levels are high, compared to healthy tissue, and correlate with a poor prognosis (Cohen et al., 2010; Reedijk et al., 2005; Xu et al., 2012). The co-expression level of Jagged1 and Notch1 is associated with a reduction in overall survival in human breast cancer, predicting that a Jag1-Notch1 activation loop is promoting tumour formation and progression (Reedijk et al., 2005).

Similarly, in cervix cancer a higher expression of Jagged1 is linked with an anomalous Notch activation and Jagged1 inhibition induces a decrease in the proliferation rate *in vitro* (Veeraraghavalu et al., 2005). Moreover, Jagged1 interacts with the oncoproteins E6 and E7 of papilloma virus HPV16 (associated to cervix cancer development), to promote neoplastic transformation and tumoral growth *in vivo* (Veeraraghavalu et al., 2005). Finally, high levels of Jagged1 are found in ovarian cancer, where promote proliferation and invasiveness of cancerous cells in the intraperitoneal cavity inducing Notch3 activity (Chen et al., 2010; Choi et al., 2008).

In colorectal cancer (CRC), Notch aberrant activity lead to the development of the primary tumour rather than the metastatic one, identifying the pathway as one of the key events in the onset of the CRC (Veenendaal et al., 2008). Jagged1 role in CRC is linked to the development and progression of the tumour. It has been established by tissue microarray assays that Jagged1 is expressed at a high level in 50% of CRC patients compared to normal mucosa. In addition, *Hes1* Notch target gene is positively corelated to Jagged1 expression. In human samples of breast cancer with high Jagged1 expression, *Hes1* is also upregulated, compared to healthy controls and CRC patients with low Jagged1 expression (Guilmeau et al., 2010).

In particular, it has been shown that Jagged1 expression levels correlate with differentiative parameters and CRC staging. Specifically, high levels of Jagged1 are associated with the C and D levels of Dukes staging system (i.e. involvement of lymph nodes, widespread metastases) (Gao et al., 2011). Recent studies reported Jagged1 as a target gene of Wnt/β-catenin pathway, inducing its up-regulation, required for intestine tumorigenesis (Rodilla et al., 2009). In the CRC the Notch signalling is downstream of the Wnt pathway. The β-catenin/TCF complex activates the Notch signalling through a direct activity on Jagged1 that leads to a deregulated signal transduction (Pannequin et al., 2009; Rodilla et al., 2009).

In CRC context it has been demonstrated that, the deletion of one Jagged1 allele is sufficient to significantly reduce the size of tumours in the APC mutant background, associated with a reduction in the amount of active Notch1. The authors observed that Notch inhibition mediated by Jagged1silencing results in a decrease in the migratory and invasive properties of tumour cells. Jagged1 is overexpressed in most of the CRC cases and potentially responsible for the constitutive activation of Notch signalling (Dai et al., 2014).

It has been stated that Jagged1 is able to cooperate with APEX1 (apurinic-apyrimidinic endonuclease 1). Specifically, APEX1 is a positive regulator of Jagged1 expression that correlates with an active Notch signalling. The axis APEX1/Jag1/Notch induces metastasis markers, such as MMP-2 and MMP-9 (Kim et al., 2013).

Finally, laboratory data have highlighted the role of ADAM17 in promoting the development of cancer stem cell (CSC) in the CRC by the Notch1 dependent signal transduction pathway (Wang et al., 2016). The regulation of CSC tumour phenotype by ADAM17 is known in the literature, also in different tumour contexts (Chen et al., 2013; Kamarajan et al., 2013). Wang and collaborators (2016) have demonstrated, in the context of colorectal cancer, that the release of the extracellular

soluble fragment of Jagged1 and 2, mediated by ADAM17, is able to modulate Notch-dependent signalling in adjacent cells, inducing the tumour phenotype of CSC (Wang et al., 2016).

Therefore, the role of Jagged1 in the regulation of various cellular events mediated by both the protein “full length” and the intracellular fragment (Jag1-ICD) are known. Notch and its ligands are processed by the same molecular machinery, hence, the regulated intramembrane proteolysis of both receptor and ligand may play important, potentially competitive roles in cell signalling (Pelullo et al., 2019; Pelullo et al., 2014).

II. Aim of the studies

The Notch signalling is a signal transduction pathway essential for cell proliferation and differentiation, and for the correct development of multicellular organisms. The intracellular domain of Notch receptors controls the transcription of the target genes through the presence of different co-factors such as ligands, whose presence permits the activation of the pathway, and transcriptional co-factors, that allows the recruitment of other factors to the transcriptional complex to modulate Notch activity. However, several experimental evidences report that these factors also play a key role in development and differentiation. In addition, aberrant alterations in these components are implicated in the onset of pathological conditions.

In this thesis, I report novel observations about the pathway of Notch, by paying attention in particular to Maml1 and Jagged1 proteins and their impact on development/differentiation processes and their role in tumour proliferation and metastatic processes. The experimental units have the following objectives:

1) ***Maml1 as a novel co-transcription factors of Gli1, able to empower Shh signalling (Quaranta et al., 2017)***

   Maml proteins (i.e. Maml1, Maml2 and Maml3) are recognized as transcriptional co-factors for Notch signalling, enhancing Notch-induced activation of target genes (Kitagawa, 2015; Petcherski & Kimble, 2000). There are several reports for the role of Maml1 as co-activator in other cell signalling pathways, including p53 (Zhao et al., 2007), MEF2C (Shen et al., 2006), β-catenin (Alves-Guerra et al., 2007), EGR1 (Hansson et al., 2012), NF-κB (Jin et al., 2010) and Runx2 (Watanabe et al., 2013) in a Notch-independent manner.

   In silico analysis of Maml1 expression in human tissue revealed that Maml1 is more abundant in the cerebellum than in other tissues, suggesting an important role in this context. Sonic hedgehog (Shh) signalling plays a key role in cerebellum development. In particular, Shh promotes and sustains GCPs proliferation and normal cerebellum foliation (Dahmane & Ruiz-i-Altaba, 1999; Lewis et al., 2004; Wallace, 1999). An aberrant regulation of Hh signalling alter the development of granule precursors cerebellar (GCPs) inducing malignant transformation (i.e. Medulloblastoma).

Based on these observations we sought to examine if Maml1 has a role in the activation of Shh pathway and analyse the possible implications for the proliferation of GCPs and cerebellar development.

2) ***Maml1 as a negative regulator of Itch (Zema et al., manuscript in preparation)***

Itch is an E3 ubiquitin ligase that belongs to HECT (Homologous to the E6 associated protein Carboxyl-Terminus) E3 ligase family. Mechanisms of regulation of Shh pathway involve different post-translational modification, such as ubiquitylation processes. Itch activity is able to modulate, together with the adaptor Numb, the ubiquitylation and proteasomal degradation of Gli1 (Di Marcotullio et al., 2011; Di Marcotullio et al., 2006). The control of Gli protein degradation could play a role in preventing tumorigenesis events. Interestingly, Maml1 induces protein stability for different transcriptional factors (i.e. p53, NF-κB, EGR1) with direct or indirect events Maml1-mediated. We already described a Maml1-dependent up-regulation of Gli1 transcript levels; thus, the aim of this study is to determine whether in Shh pathway Maml1 plays the dual role of transcriptional co-factor and post-translational regulator.

3) ***The tumorigenic role of the intracellular domain of Jagged1 in CRC (Pelullo, Nardozza, Zema et al., 2019)***

Colorectal cancer (CRC) is characterized by well-known genetic defects and about 50% of the cases harbour oncogenic *RAS* mutations. Increased expression of Notch-ligand Jagged1 occurs in several human malignancies, including CRC, and correlates with cancer progression, poor prognosis and recurrence. Herein, we demonstrate that Jagged1 is constitutively processed in CRC tumours with mutant Kras, ultimately triggering an intrinsic reverse signalling via its nuclear-targeted intracellular domain (Jag1-ICD). We provide evidence that the processing occurs when a Kras/Erk/ADAM17 signalling axis is switched on, demonstrating that Jagged1 is a novel target of Kras signalling pathway. Notably, we show that Jag1-ICD promotes tumour growth and epithelial-mesenchymal transition, enhancing CRC progression and chemoresistance both *in vitro* and *in vivo*. Our data pinpoint a novel role for Jagged1 in CRC tumour biology that may go beyond its effect on canonical Notch activation and suggest that Jag1-ICD may behave as a novel oncogenic driver, able to sustain tumour pathogenesis and to confer chemoresistance, through a non-canonical mechanism. By unveiling the Kras/Erk/ADAM17/Jagged1 signalling axis, we provide new mechanistic insights on CRC tumour biology and highlight a novel attractive target for CRC therapy.

III. Results I

1. Maml1 acts cooperatively with Gli proteins to regulate Sonic hedgehog signalling pathway (Quaranta et al., 2017)

Hedgehog (Hh) signalling has been implicated in the regulation of key events during developmental processes (Hui & Angers, 2011). The Hh pathway is controlled by extracellular ligands (Sonic, Indian and Desert hedgehog) through interaction with the receptor Patched (Ptch), thereby enhancing Smoothened (Smo) function, which activates Gli transcription factors (Ingham & McMahon, 2011). Transcriptional activation is largely derived from Gli1 and Gli2, whereas Gli3 mainly shows repressor activity in the absence of ligand. Gli1, the final and strongest transcriptional activator (Kimura et al., 2005), is both the downstream effector and a target gene of the pathway, representing a feedback loop that serves as a readout of Hh activity (Hui & Angers, 2011; Sasaki et al., 1999; Stecca & Ruiz I Altaba, 2010). Signalling through Smo causes nuclear translocation of Gli1, able to induce the expression of pro-proliferative target genes, including Cyclins D1 and D2 (Behesti & Marino, 2009; Kenney & Rowitch, 2000), which directly promote the entry into the cell cycle and DNA replication. Sonic hedgehog (Shh) pathway has a pivotal role in controlling embryonic patterning and is a master regulator of cerebellar granule cell progenitors (GCPs) development (Ruiz i Altaba et al., 2002).

Cerebellar development is a finely orchestrated process that produces an elaborate set of folia separated by fissures. The process of foliation begins during the prenatal period with the formation of four principal fissures, which divide the cerebellum into five cardinal lobes (Sillitoe & Joyner, 2007). Shh secreted by Purkinje cells (PCs) from E17.5 onward in the mouse, is a key GCPs mitogen that promotes proliferation (Dahmane & Ruiz-i-Altaba, 1999; Wallace, 1999) and sustains normal cerebellum foliation (Corrales et al., 2004; Lewis et al., 2004). Especially, it has been shown that Shh signalling spatially and temporarily correlates with fissures formation, regulating the number of folia through its influence on GCPs expansion (Corrales et al., 2004). Proliferation of granule cells and the process of cerebellar development appear to be strongly related to one another (Corrales et al., 2006; Corrales et al., 2004; Sudarov & Joyner, 2007). Indeed, a deregulated Shh signalling alters the development of GCPs making them hyperproliferative and susceptible to malignant transformation into medulloblastoma (MB), the most frequent childhood brain tumour

(Kadin et al., 1970; Ruiz i Altaba et al., 2002). The biological and pathogenic importance of Shh signalling emphasizes the need to tightly control its action.

In this study, we identify Mastermind-like 1 (Maml1) as a novel regulator of Shh signalling. In mammals, Maml1 (Kitagawa et al., 2001; Wu et al., 2000) belongs to a family of proteins, also including Maml2 and Maml3 (Lin et al., 2002; Wu et al., 2002), which act as transcriptional coactivators for Notch signalling (Petcherski & Kimble, 2000)(Borggrefe & Oswald, 2009; Talora et al., 2008). Maml1 has been recently shown to act as a coactivator in other cell signalling pathways, including p53 (Zhao et al., 2007), MEF2C (Shen et al., 2006) and β-catenin (Alves-Guerra et al., 2007), in a Notch-independent manner. These findings suggest broader roles for Maml1 protein in regulating important physiological processes.

Here, we present evidence that Maml1 enforces the Shh pathway, via a novel Notch-independent mechanism. At the molecular level, we found that Maml1 physically interacts with Gli1 and Gli2, promoting Shh-dependent transcriptional events. In addition, we show that Maml1 silencing disrupts Shh signalling with a significant reduction of Gli target genes expression. Noteworthy, in MEFs and GCPs deriving from *Maml1*$^{-/-}$ mice, the Shh pathway is strongly compromised, resulting in a decreased expression of Gli1 and Gli2, which impacts on GCPs proliferation and cerebellum development.

The experiments presented below have been conducted in collaboration with the lab of Prof. Diana Bellavia (Quaranta R, Pelullo M. and Nardozza F.).

1.1 Maml1/Gli1 protein-protein interaction reinforces the activation of Shh target genes

Based on Maml1 functions as a transcriptional coactivator in several signalling pathways (McElhinny et al., 2008) and being its expression significantly higher in cerebellum than in other tissues (Supplementary Figure S1a), we sought to examine Maml1 role in the activation of Shh pathway. To address this issue, HEK293T cells were co-transfected with 12xGli-luc (an artificial Gli reporter containing twelve copies of Gli-responsive elements) or Patched1-luc and vectors expressing Gli1 or Gli2 alone and in combination with Maml1. Figure 1a shows that Maml1 strongly cooperates with Gli proteins to potentiate both of Shh-responsive reporters. Moreover, these results confirm that Gli1 is a stronger transactivator than Gli2, also in the presence of Maml1 (Kimura et al., 2005). We examined whether Maml1 sustains the endogenous Gli1 transcriptional activity, the readout of Shh activation pathway. Therefore, we monitored Gli1 expression in NIH3T3 cells transfected with Maml1. Figure 1b shows increasing expression levels of endogenous

Gli1 in a dose-dependent manner. The cooperation of Maml1 with Gli1 or Gli2 function suggests their physical association. Fig 1c shows Gli1 and Gli2 in Maml1 immunoprecipitates, indicating the formation of Maml1/Gli complexes, confirmed also by reciprocal co-immunoprecipitation assay using anti-Gli1 antibody (Fig 1d). To demonstrate endogenous Gli1/Maml1 protein interaction, we used Maml1 immunoprecipitates from *Ptch1*$^{-/-}$ MEF cells, with a constitutively active Shh pathway (Fig 1e) (Goodrich et al., 1997). Figure 1f also reveals the endogenous Gli1/Maml1 complex in NIH3T3 cells by *in situ* proximity ligation assay (PLA), detecting single interaction pairs of native proteins by using antibodies directed against Maml1 and Gli1. Only interacting proteins pairing displays a red signal by confocal microscopy. Indeed, a high degree of Maml1/Gli1 interaction was observed both in cytoplasmic and nuclear compartment. To determine the occupancy of the Maml1/Gli complex on Shh-target genes, we performed a chromatin immunoprecipitation (ChIP) assay. Figure 1g (upper panel) shows that both Maml1 and Gli1 are recruited at the same Gli binding sites in the human *Patched1* promoter, as shown in the schematic representation of Figure 1f (lower panel). These data indicate that Maml1 physically interacts with Gli proteins and cooperatively they activate specific Shh-responsive target genes.

1.2 Maml1 C-terminal region is required to allow its cooperation with Gli1

To examine which region of Maml1 interacts with Gli, we used truncated mutant Maml1 proteins (schematic representation in Figure 2a) to perform co-immunoprecipitation assays. We focused on Gli1, the most powerful effector of the Shh pathway, which is able to enhance its own expression, auto reinforcing the signalling strength. We show in Figure 2b that Gli1 is able to bind both the N-terminal region (amino acids 1-302) and the C-terminal region (amino acids 303-1016) of Maml1 protein, independently. This observation suggests that Gli1 binds Maml1 at least in two distinct domains. To determine which region of Maml1 is important for the transcriptional activity of Gli1, we cotransfected HEK293T cells with the combination of Gli1, Maml1 mutant forms and 12xGli- or Patched1-luc reporter constructs. Figure 2c shows that Maml1 full-length (FL) and Maml1 124-1016 strongly enhance luciferase reporter gene activity in the presence of Gli1 vector either on 12xGli-promoter (upper panel) or Patched1-promoter (lower panel). Conversely, the Maml1 COOH-terminal deleted mutant (1-302) and the Mam11 303-1016 without the nuclear localization signal (NLS), have no detectable effect on Gli1 transcriptional activity. Interestingly, the Maml1 FL protein has been previously shown to drive Maml1-interacting proteins, such as Notch (Fryer et al., 2002; Wu et al., 2000), p300 (Hansson et al., 2009), MEF2C (Shen et al., 2006) and GSK3β (Saint Just Ribeiro et al., 2009) to the nucleus, in particular into nuclear bodies (Saint Just Ribeiro et al.,

2009). To this regard, Maml1 mutant proteins appeared to exert a distinct influence on Gli1 subcellular localization. In fact, Figure 2d shows that Maml1 FL is able to address Gli1 into the nucleus, particularly into nuclear bodies (panel h versus d). Instead, in cells transfected with Maml1 124-1016, Gli1 completely diffuse into the nucleus (panel p). Contrarily, Maml1 1-302 is not able to address Gli1 into the nucleus (panel l) and Maml1 303-1016, deleted of the NLS domain, sequesters Gli1 into the cytoplasm, where the two proteins preferentially interact (panel t). Subcellular distribution of transiently transfected Flag-tagged Maml1 FL and mutant forms is shown in Supplementary Figure S2. Overall the data suggest that Maml1 is required to sustain the nuclear localization of Gli1, as further supported by the nuclear and cytoplasmic fractionation assay (Supplementary Figure S3), and that Maml1 COOH-terminal region is required to reinforce the transcriptional activity of Gli1 *in vitro*.

1.3 Maml1 is required to fully activate Gli-mediated target gene transcription

Overall, our data suggest that Maml1 influences Gli1 subcellular localization and acts as a transcriptional coactivator strengthening the expression of Gli-target genes. To further support the model, we analysed whether Maml1 loss of function influences the transcriptional activity of endogenous Gli1. Importantly, the siRNA-mediated depletion of Maml1 induces a significant impairment of Gli1 protein expression (Figure 3a) and Shh target genes, as *Gli1* itself, *Ptch1*, *Cyclin D1*, *Cyclin D2* and *Hip1* (Figure 3b).

Notably, Maml1 is a well-known transcriptional coactivator of the Notch pathway and to exclude the possibility that Notch signalling impairment might be responsible of the observed effects, we investigated the expression of Hh target genes in the presence of the γ-secretase inhibitor (DAPT), that blocks the Notch pathway activation. We treated NIH3T3 cells with DAPT that affects Notch1 activation, as revealed by using the antibody against the valine 1744 (N1ICDVal1744) (Figure 3c, left panel), and down-modulates its target gene, *Hes1* (Figure 3c, right panel). In contrast, qRT-PCR assays reveal that *Gli1* itself and Shh-target gene expression levels are not significantly modified by Notch inhibition (Figure 3d), indicating that the role of Maml1 on Hh/Gli1 signalling is independent of the Notch activity. Accordingly, Maml1 potently enhances Gli1 activity upon co-transfection with 12xGli-luc (Figure 3e, left panel) or Patched1-luc (Figure 3e, right panel), independently from the presence of DAPT. Together these findings directly and functionally connect Maml1 to the Shh pathway, suggesting a Maml1-dependent reinforcement mechanism of Gli1 transcriptional activity in a Notch-independent manner.

1.4 Maml1 deletion results in an impaired Shh signalling cascade

To further validate that Maml1 functions as a coactivator of Gli transcription factors, we used *Maml1*$^{-/-}$ mouse embryo fibroblasts (MEFs) (Oyama et al., 2007) model. Interestingly, the absence of *Maml1* gene determines a significant reduction of endogenous Gli1 (Figure 4a, left panel and 4b) and Gli2 (Figure 4a, right panel and 4b) expression levels in *Maml1*$^{-/-}$ MEFs, compared to control. Moreover, the absence of Maml1 determines an impaired activation of Shh-signalling in *Maml1*$^{-/-}$ MEFs in response to treatment with the Smo agonist, SAG (Chen et al., 2002a). Figure 4c shows an important decrease of Shh-target genes expression, such as *Gli1* itself, *Ptch1* and *Cyclin D1*, in *Maml1*$^{-/-}$ versus *Maml1*$^{+/+}$ SAG-treated MEFs. Supplementary Figure S4 also shows reduced Gli1 expression levels upon SAG treatment in *Maml1*$^{-/-}$ respect to control, in a time-dependent manner. To investigate the requirement of Maml1 for Gli function, we used the *Ptch1*$^{-/-}$ MEFs, in which *Ptch1* deletion causes constitutive Gli activation. Notably, the Maml1 silencing impairs Shh signalling, by promoting the inhibition of *Gli1* and *Gli2* and specific Shh-target genes (Figure 4d), associated to a decreased proliferation rate (Figure 4e), also revealed by MTT assays (Figure 4f). These data further demonstrate that Maml1 is functionally required to sustain full activation of Shh-signalling, by acting as a crucial co-effector also when the pathway is constitutively activated.

1.5 Reduced GCPs proliferation correlates with a decreased Gli activity in Maml1$^{-/-}$ *mice*

Shh signalling is a master regulator of the development of cerebellar granule cell progenitors (GCPs) (Ruiz i Altaba et al., 2002). To investigate the relevant role of Maml1 in sustaining Shh signalling in GCP cells, we measured endogenous Shh/Gli1 target genes in primary GCPs, derived from *Maml1*$^{-/-}$ and control mice, by qRT-PCR analysis. *Maml1*$^{-/-}$ mice were studied up to E19.5, since they die at perinatal period. Figure 5a shows that the absence of Maml1 determines a remarkable reduction of Gli1 target genes in *Maml1*$^{-/-}$ GCPs when compared to *Maml1*$^{+/+}$ littermates, associated to an important decrease of Gli1 protein expression (~39%) (Fig 5b). To further investigate whether loss of Maml1 activity might result in a defective Shh signalling, we measured the expression of the direct Shh target genes *Gli1* by qRT-PCR, upon SAG stimulation. Figure 5c shows that the Gli1 activity is significantly reduced in GCPs of *Maml1*$^{-/-}$, when compared to wild-type (wt) mice.

Shh signalling supports the proliferation of GCPs during cerebellar development (Corrales et al., 2006, 2004). Thus, we examined whether the absence of Maml1 can antagonize the mitogenic effect of Shh on GCPs proliferation. For this purpose, we cultured GCPs deriving from *Maml1*$^{-/-}$

and wt littermates, after SAG-treatment and pulsed with bromodeoxyuridine (BrdU) to label proliferating cells. Intriguingly, only a small percentage of *Maml1*$^{-/-}$ GCPs incorporates BrdU (Figure 5d), with nearly 3-fold decrease in BrdU-positive cells number (Figure 5e). Moreover, Figure 5f shows a reduced endogenous *Pcna* (proliferating cell nuclear antigen) expression in *Maml1*$^{-/-}$ GCPs, which correlates to a decrease in number of total cerebella-derived GCPs in E19.5 *Maml1*$^{-/-}$ mice (Figure 5g).

To discriminate the role sustained by Shh and Notch signalling in GCPs, we performed specific pharmacological treatments in order to compare the outcomes of these signalling pathways on cerebellar progenitors proliferation. Firstly, we evaluated the expression of Notch pathway genes in GCPs. Supplementary Figure S5a shows that Notch receptors and specific target genes are similarly expressed in *Maml1*$^{-/-}$ and *Maml1*$^{+/+}$ GPCs, except for *Hes1*, which appears decreased in *Maml1*$^{-/-}$. Noteworthy, *Hes1* has been described to be also a Shh signalling target (Solecki et al., 2001). Then, we performed BrdU assay in SAG-activated *Maml1*$^{+/+}$ GCPs, treated with DAPT or KAAD/cyclopamine, specific inhibitors for Notch and Shh signalling, respectively (Chen et al., 2002b). Interestingly, Supplementary Figure S5b and c shows that KAAD/cyclopamine treatment impairs GCPs proliferation with a significant effect, when compared to DAPT, and is associated with an important down-regulation of Shh/Gli1 target genes (Fig S5d).

Furthermore, Supplementary Figure S6a shows that the proliferation of DAPT-treated *Ptch1*$^{-/-}$ MEFs, revealed by MTT analysis, is more preserved, when compared to KAAD/cyclopamine treatment, suggesting that Notch pathway plays a marginal role in controlling the proliferation on Hh-activated cells. In addition, the inhibition of Notch pathway by DAPT does not influence the expression of Shh target genes (figure S6 b and c), as instead does the KAAD treatment (Fig S6 b and d). Notably, Figure S6b confirms *Hes1* as a common target gene of Notch and Shh pathways (Solecki et al., 2001).

6.1 Cerebellar defects in Maml1$^{-/-}$ *mice*

Shh signalling plays a critical role and regulates the complexity of cerebellar foliation (Corrales et al., 2006; Dahmane & Ruiz-i-Altaba, 1999). To establish whether the decreased proliferation observed in GCPs derived from *Maml1*$^{-/-}$ mice may negatively impact on the foliation pattern, histological sections of cerebella at E18.5 and E19.5 were stained with Haematoxylin and Eosin (H&E). Figure 6a shows a defective foliation pattern in *Maml1*$^{-/-}$ cerebella at E18.5 with slight indentations corresponding to preculminate (pc) and primary (pr) cardinal fissures, furthermore the

cardinal lobes are not identifiable, with respect to control (panel B versus A). Sagittal sections of cerebella at E19.5 in Figure 6a show that in wild-type mice the four principal fissures are formed, as well as two additional fissures. In contrast, E19.5 *Maml1*$^{-/-}$ mice have only two primary fissures (pc and pr), although they are very shallow (Fig 6a, panel D versus C). Of note, the posterolateral and secondary fissures are not visible, corresponding to regions with a highest Shh signalling (Corrales et al., 2006, 2004). Then, we performed immunostaining assay with PCNA, confirming that the cells from *Maml1*$^{-/-}$ cerebellum are less mitotically active than in control (Figure 6b and c), in particular in correspondence of secondary and posterolateral fissures (Fig 6b, C versus A and D versus B). The shallow principal fissures at E18.5 and delayed lobularization of *Maml1*$^{-/-}$ cerebella are reminiscent of foliation defects observed in mice with altered Shh signalling (Corrales et al., 2006; Lewis et al., 2004). In addition, morphometric analysis reveals that the perimeter and total area of *Maml1*$^{-/-}$ cerebella are reduced, suggesting that Maml1 depletion negatively affects cerebellar size (Figure 6d). Overall, these results support our hypothesis that Maml1 protein is critical to mediate Shh signalling with an impact on cerebellum size and foliation during development in vivo.

Figures and Supplementary

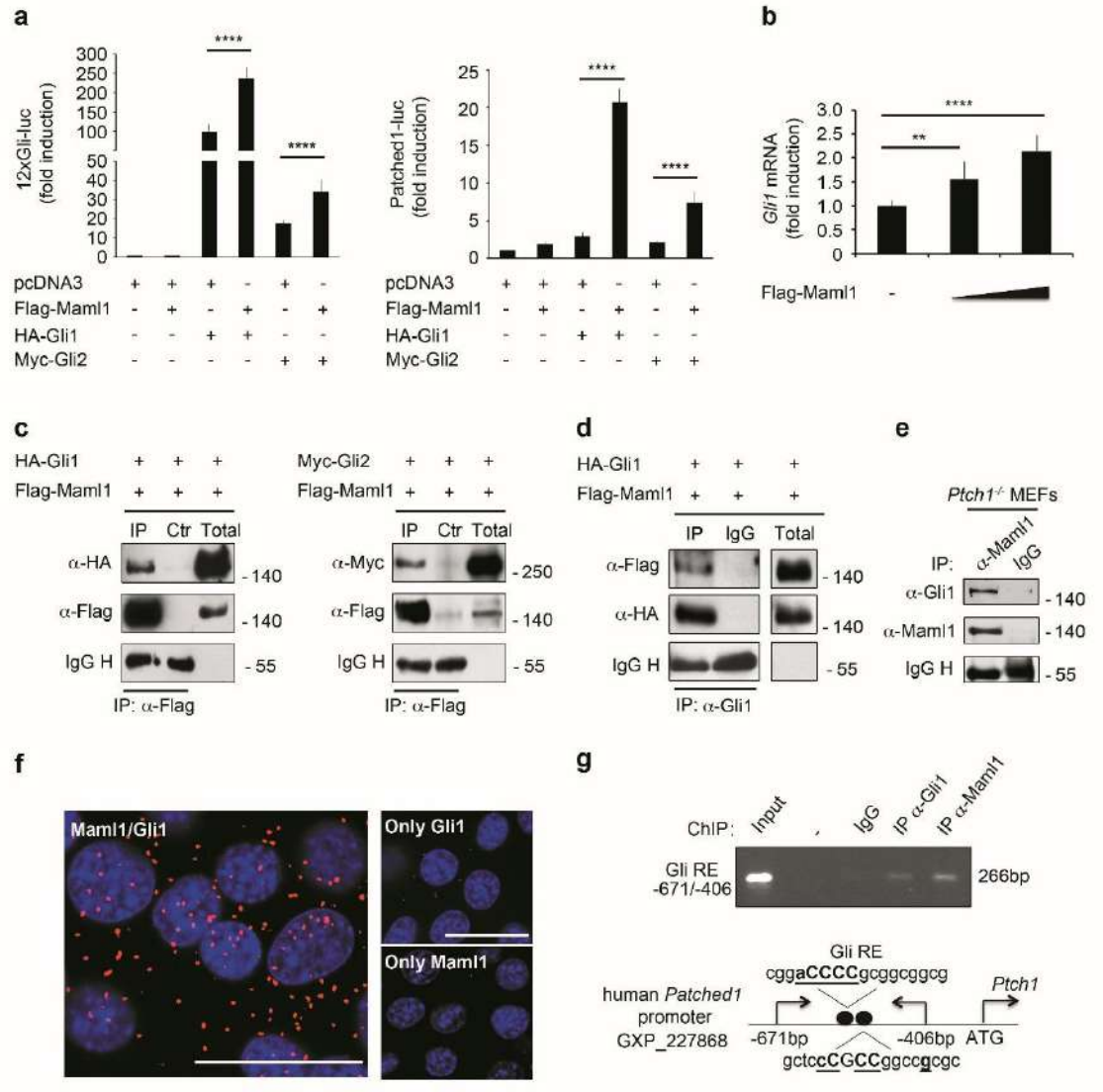


Figure 1. Maml1 interacts with Gli proteins and sustains Gli-mediated transcription. (a) Luciferase assay in HEK293T cells transfected with 12xGli-luc (left panel) or Patched1-luc (right panel) reporter and different combinations of plasmids encoding for Maml1, Gli1 and Gli2 as indicated. Luciferase activity is expressed as fold induction relative to pcDNA3 alone (empty control). (b) Endogenous *Gli1* mRNA levels evaluated by qRT-PCR in NIH3T3 cells transfected with increasing amounts of Maml1, compared to pcDNA3. (c) Coimmunoprecipitation (co-IP) experiments performed in HEK293T cells, transfected with expression vectors encoding for the indicated tagged proteins and immunoprecipitated with anti-Flag without (IP) or with (Ctr) blocking peptide. The interaction with Gli1 and Gli2 was revealed with anti-HA (left panel) or anti-Myc (right panel) respectively. (d) Reciprocal co-IP was performed by using anti-Gli1 antibody or control mouse antisera (IgG) and the interaction with Maml1 was detected with anti-Flag. Gli1 and Maml1 protein levels in total cell lysates are shown. (e) *Ptch1*$^{-/-}$ MEFs were lysed and immunoprecipitated with Maml1 antibody or control rabbit antisera (IgG) and the blot was reprobed with Gli1. (f) Representative immunofluorescence images of endogenous Gli1/Maml1 interaction in NIH3T3 cells analysed by *in situ* proximity ligation assay (PLA). Negative controls lacking one of the primary antibodies. No significant fluorescent signal was detected in NIH3T3 cells incubated with only one primary antibody (only Gli1; only Maml1). Single plane confocal images were captured using a 60x oil objective. Protein complexes were visualized in red; nuclei were DAPI-labelled (blue). Scale bar: 100μm. (g) ChIP assay in HEK293T cells. PCR was performed using primers that amplify Gli consensus binding sites on human *Ptch1* promoter (upper panel) as shown in schematic representation (lower panel). According to Genomatix, basepairs in bold and underlined are important, since they appear in a position where the matrix exhibits a high conservation profile (ci-value>60); basepairs in capital letters denote the core sequence used by MatInspector. Dark circles represent predict binding sites. Data reported as mean ± S.D. ** $p \leq 0.01$; **** $p \leq 0.0001$

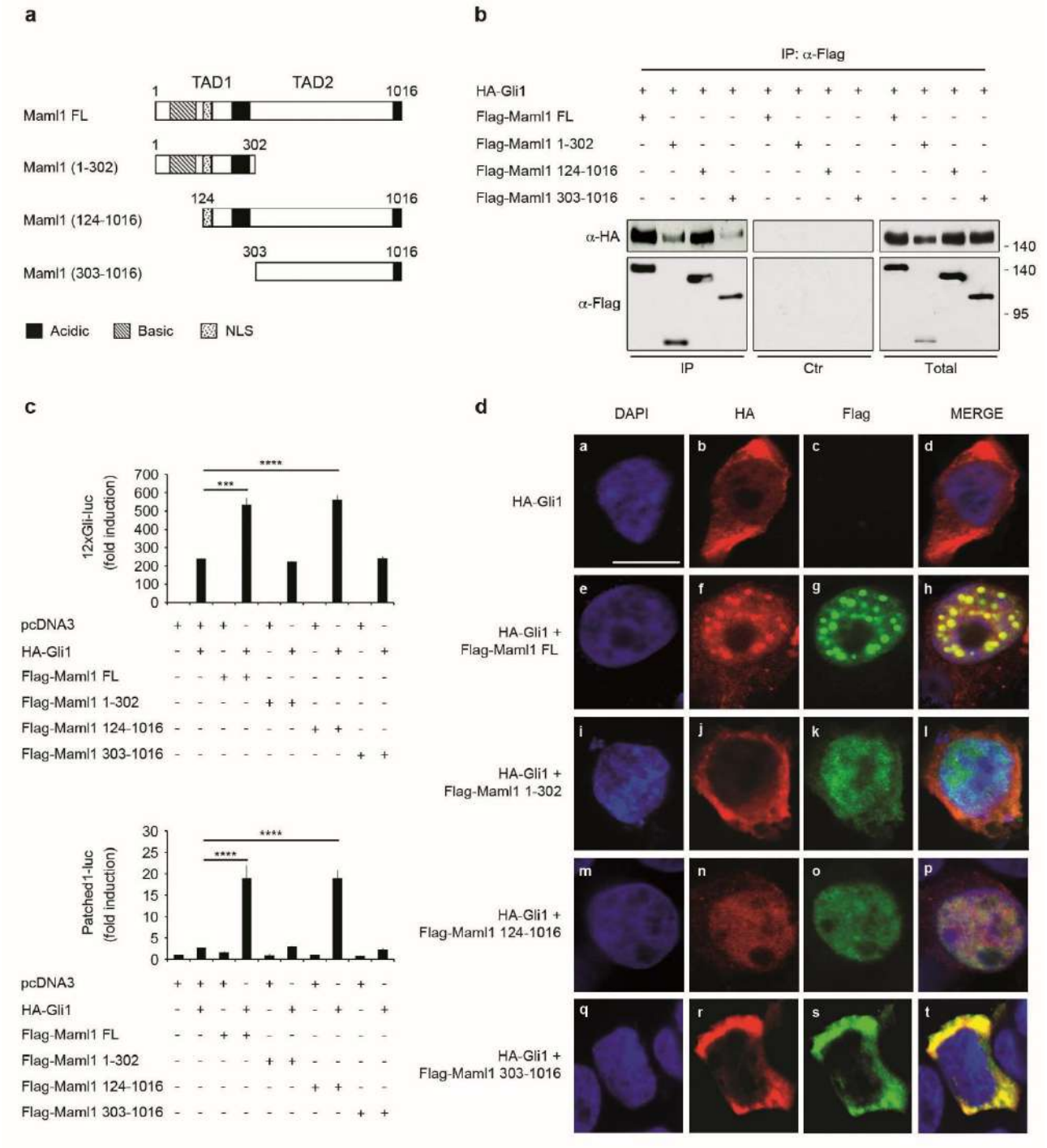


Figure 2. Maml1 COOH-terminus plays a functional role on Gli1 activity

(a) Schematic representation of full-length (FL) and truncated Maml1 constructs. Transcription activation domain (TAD): TAD1 (75-301 aa); TAD2 (303-1016 aa). (b) Whole-cell extract (WCE) from HEK293T cells co-transfected with indicated expressing vectors were immunoprecipitated with anti-Flag (IP) or anti-Flag with blocking peptide (Ctr). Immunoprecipitates and aliquots of cell lysates (Total) were immunoblotted with the indicated antibodies. (c) Luciferase assays of co-transfected HEK293T with 12xGli-luc (upper panel) or Patched1-luc (lower panel) and different combinations of expression vectors, as indicated. Luciferase activity is expressed as fold induction relative to control (pcDNA3). Data represent mean ± S.D. *** $p \leq 0.001$; **** $p \leq 0.0001$ (d) Representative single plane confocal immunofluorescence images of HEK293T cells co-transfected with indicated expression vectors. Flag- (green) or HA- (red) tags were visualized by confocal microscopy. Confocal images were captured using a 60x oil objective. Nuclei were DAPI-labelled (blue). Scale bar: 10 μm.

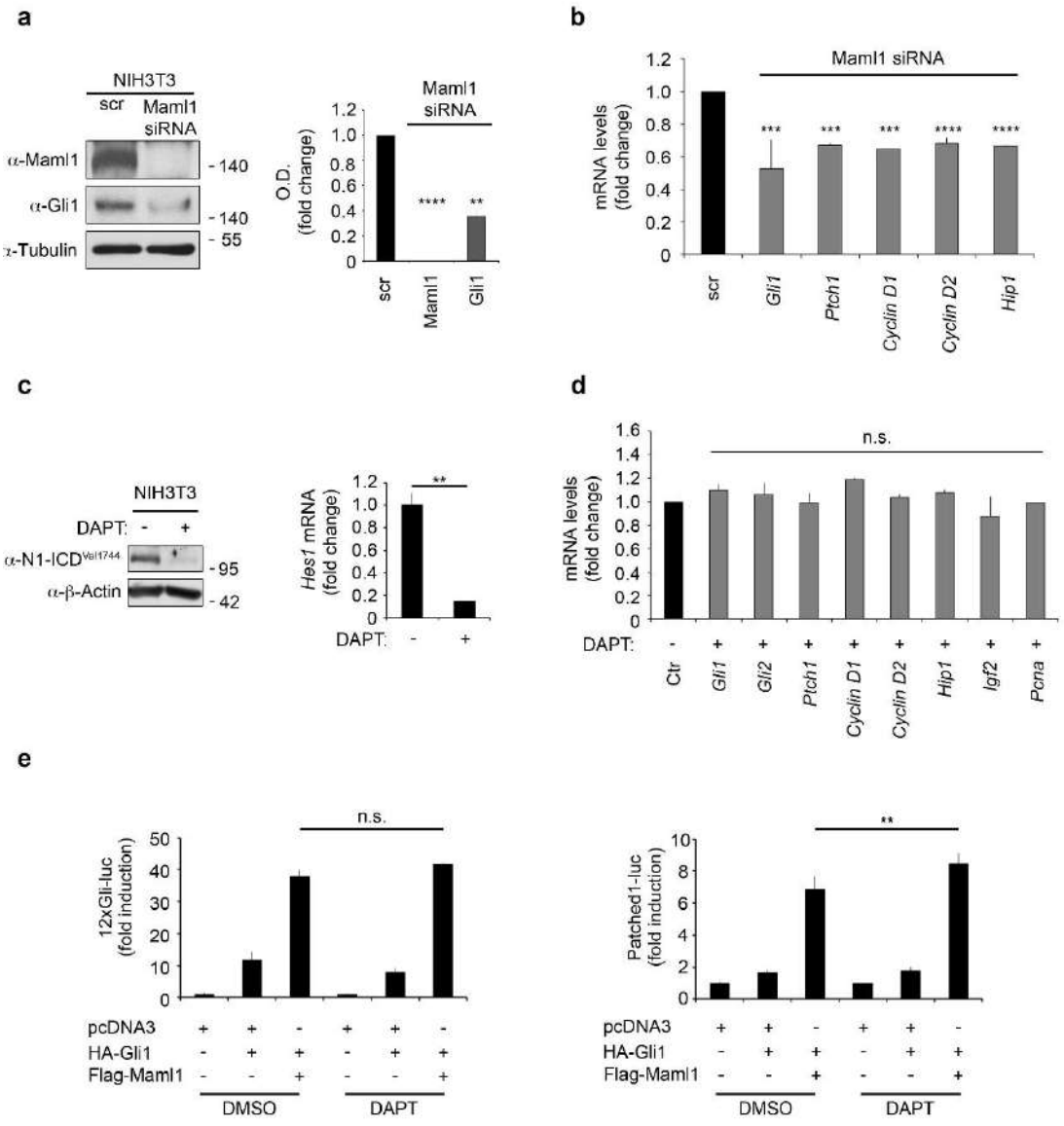


Figure 3. Maml1 cooperates with Gli1 in a Notch-independent manner

(a) Proteins expression was detected by immunoblot analysis as indicated in WCE derived from NIH3T3 cells transfected with Maml1 siRNA or control siRNA (scr). Anti-Tubulin was used as a loading control (left panel). Optical densitometry (O.D.) analysis of Maml1 and Gli1 protein expression (right panel). (b) qRT-PCR analysis of Shh target genes in NIH3T3 48h after silencing of Maml1 compared to control (scr). (c) WCE separated by SDS-page from NIH3T3 upon 72h treatment with DAPT or control vehicle (DMSO). To reveal the activated form of Notch1, an anti-Notch1 Val 1744 antibody was used (left panel). Expression level of *Hes1* mRNA was evaluated by qRT-PCR (right panel). (d) Expression levels of Shh target genes evaluated by qRT-PCR in DAPT or DMSO (Ctr) treated NIH3T3 cells. (e) Luciferase assays in HEK293T cells cotransfected with 12xGli-luc (left panel) or Patched1-luc (right panel) and different combinations of expression vectors as indicated. After DAPT or control vehicle treatment, cell lysates were harvested for luciferase assay. Data represent mean ± S.D. n.s. (not significant); ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$

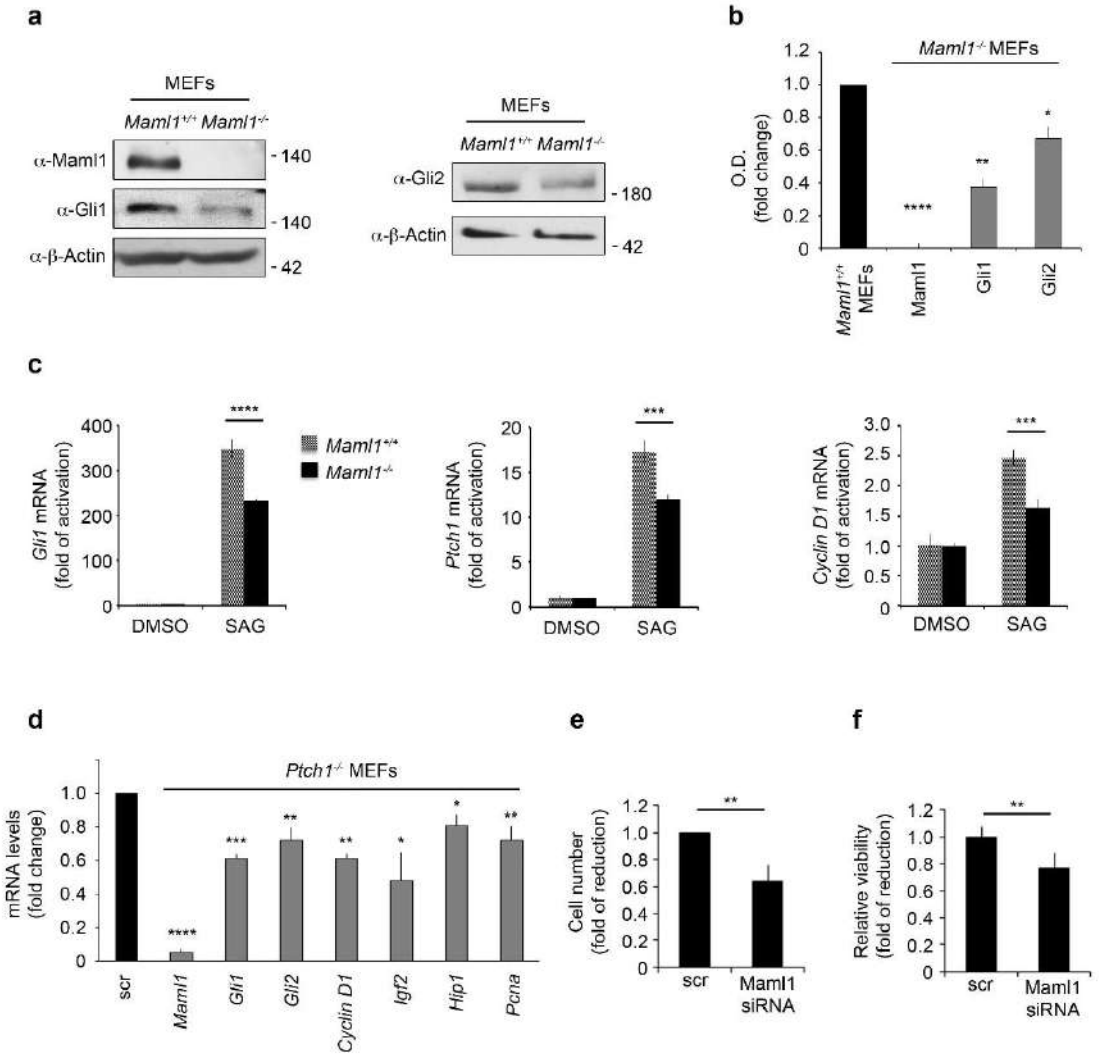


Figure 4. Maml1 loss of function determines an impaired Shh signalling pathway

(a) Immunoblot analysis of WCE from *Maml1*-deficient (*Maml1*$^{-/-}$) and control (*Maml1*$^{+/+}$) MEFs, using the indicated antibody. (b) Optical densitometry (O.D.) analysis of Maml1, Gli1 and Gli2 protein expression evaluated by immunoblotting, related to panel a. (c) Representative qRT-PCR analysis of Hh target genes in *Maml1*$^{-/-}$ MEFs compared to control upon SAG treatment for 48h. The data are presented as fold of activation respect to DMSO. (d) qRT-PCR analysis of *Maml1* mRNAs and Shh target genes in *Ptch1*$^{-/-}$ MEF cells transfected with Maml1 siRNA or control siRNA (scr) for 48 hours. (e) Trypan blue cell counting to determine the rate of proliferation and the number of viable cells in *Ptch1*$^{-/-}$ MEFs after transfection with Maml1 siRNA for 48 hours, compared to scramble (scr). (f) MTT cell proliferation assay in *Ptch1*$^{-/-}$ MEF cells Maml1-silenced compared to control. The data are presented as fold of reduction respect to scramble. Data represent mean ± S.D. ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$

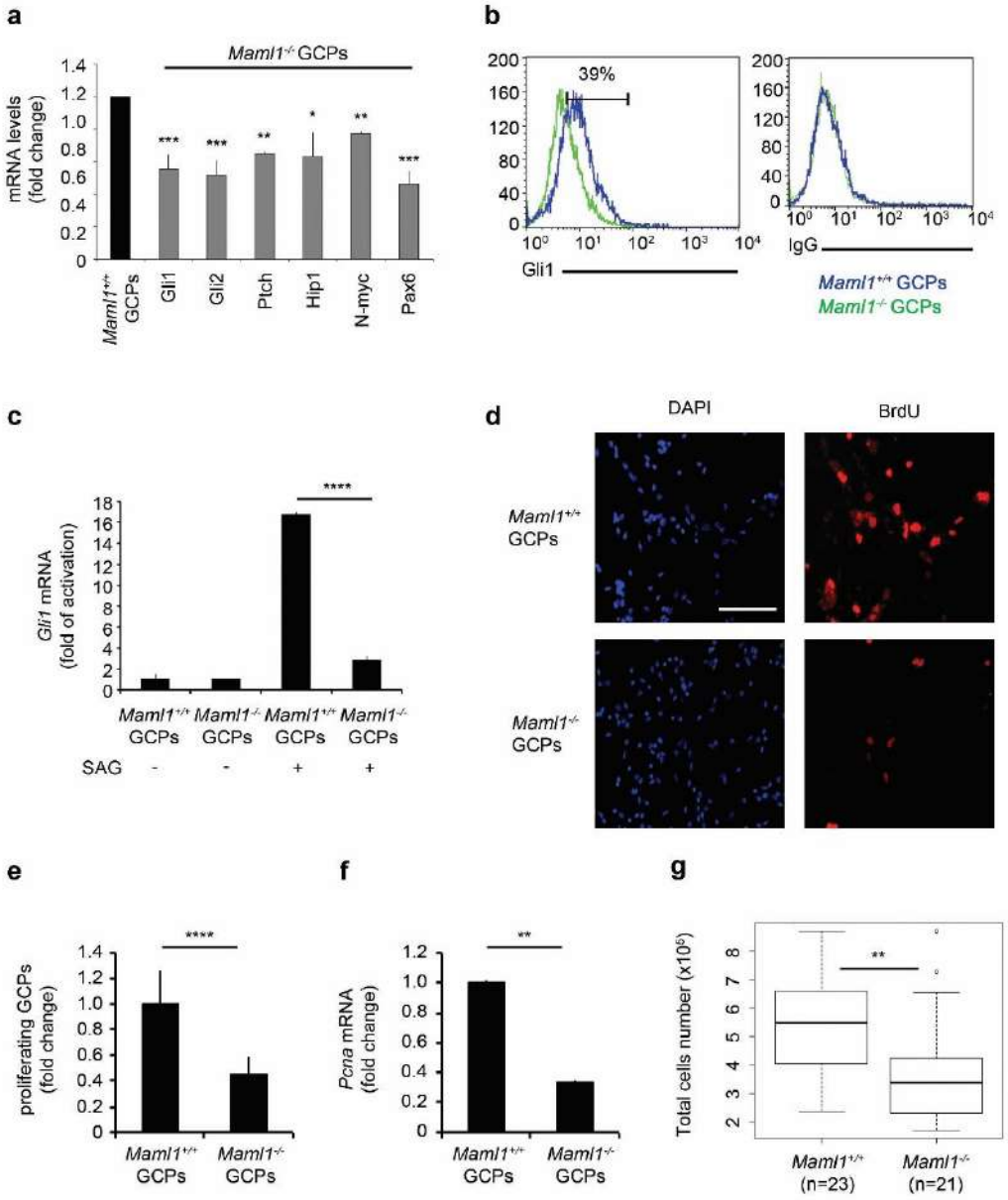


Figure 5. Reduced GCPs proliferation correlates with impaired expression of Shh target genes in *Maml1*$^{-/-}$ mice.

(a) qRT–PCR analysis shows mRNA expression level of Shh target genes in GCP cultures from *Maml1*$^{-/-}$ and control mice. (b) GCPs from E19.5 *Maml1*$^{+/+}$ (blue) and *Maml1*$^{-/-}$ (green) mice were analysed by flow cytometry analysis with a specific anti-Gli1 antibody or IgG, used as isotype control. (c) GCPs from E19.5 *Maml1*$^{-/-}$ and control littermates cerebella were treated with SAG or vehicle alone (DMSO) for 24h. Endogenous *Gli1* mRNA expression level was determined by qRT-PCR analysis. The results were analysed as fold of activation, compared to control (DMSO). (d) BrdU incorporation assay in SAG-treated *Maml1*$^{+/+}$ and *Maml1*$^{-/-}$ GCPs at E19.5 after a 24h BrdU pulse. Proliferating cells are visualized in red; nucleus was labelled in blue. Scale bar: 200 μm. (e) Mitotic index was calculated by number of BrdU-positive GCPs/total GCPs. The results were analysed as fold of reduction respect to control cells. (f) qRT–PCR analysis of *Pcna*, a Shh target involved in cellular proliferation, in *ex-vivo* GCP cultures from *Maml1*$^{-/-}$ and control mice. (g) Trypan blue cell counting was performed to estimate the growth rate of viable cells obtained from GCP cultures from *Maml1*$^{+/+}$ (n=23 mice) and *Maml1*$^{-/-}$ (n=21 mice) at E19.5. The box plot shows the distribution of cellular counts. Data represent mean ± S.D. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$

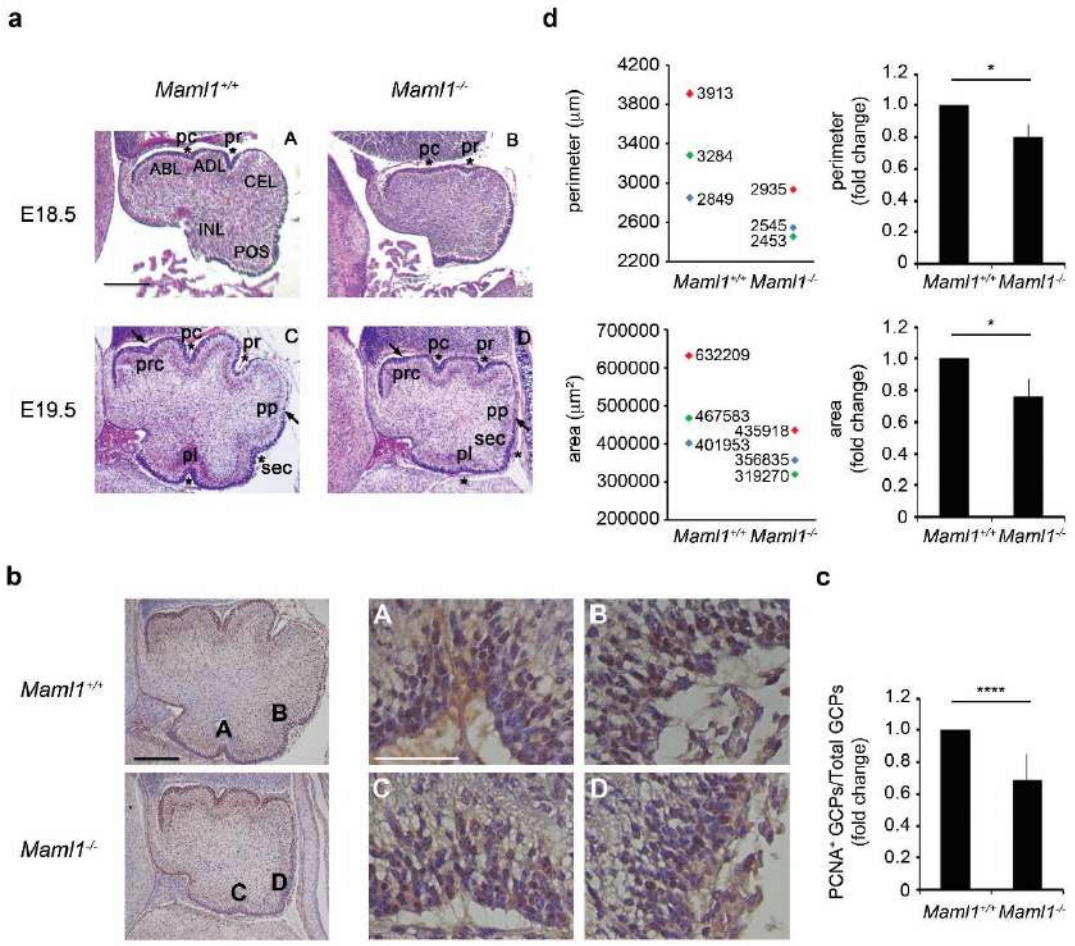
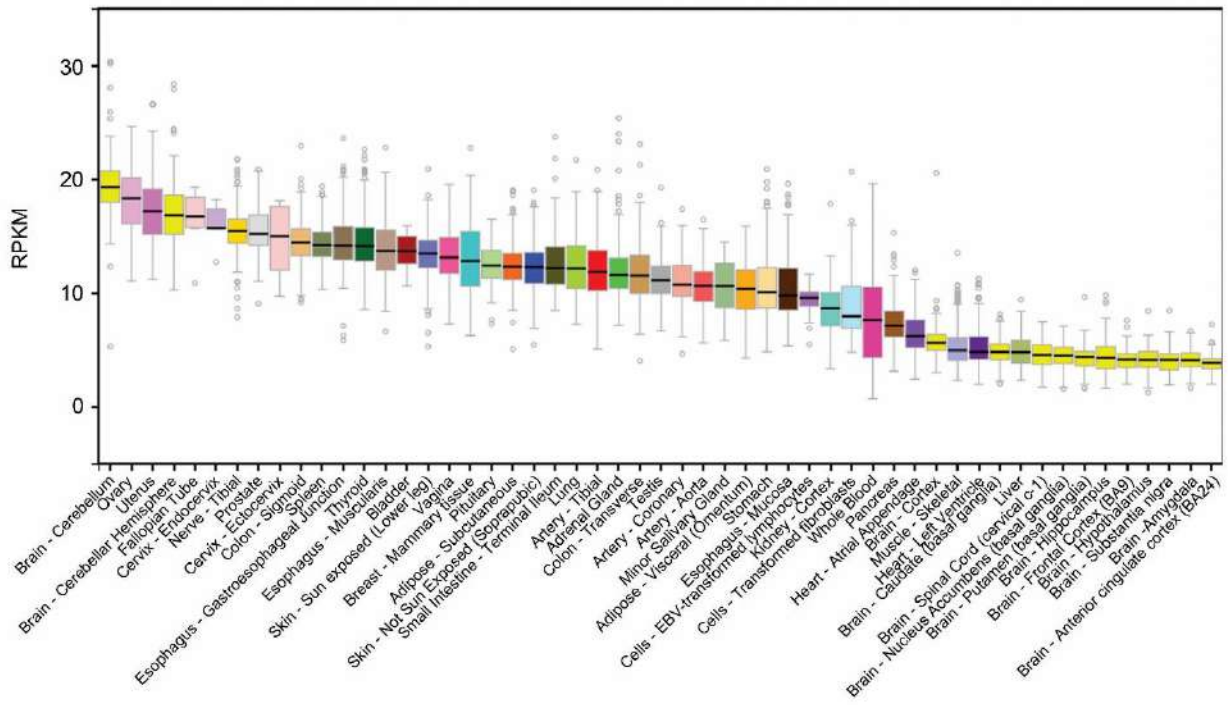


Figure 6. *Maml1*$^{-/-}$ mutant mice present foliation defects in developing cerebellum

(a) Midsagittal sections of E18.5 and E19.5 *Maml1*$^{-/-}$ and control cerebella stained with H&E. The four principal fissures (denoted by asterisks) as well as two additional fissures (indicated by the arrows) are shown in the figure. Abbreviations: prc, precentral; pc, pre-culminate; pr, primary; pp, prepyramidal; sec, secondary; pl, posterolateral fissures; ABL, anterobasal; ADL, anterodorsal; CEL, central; POS, posterior; INL, inferior lobes. Images of each panel were taken at the same magnification. Scale bar: 250 μm. (b) Immunohistochemical staining for PCNA in midsagittal cerebellar sections from *Maml1*$^{+/+}$ and *Maml1*$^{-/-}$ mice at E19.5 (left panels). Scale bar: 250μm. High magnification images of PCNA staining in the EGL, corresponding to posterolateral (A, C) and secondary (B, D) fissures (right panels). Scale bar: 50 μm (c) Graph shows the number of PCNA-positive cells, analysed as fold of reduction in comparison to *Maml1*$^{+/+}$ control mice. Five sections/mouse n=3 mice per group were analysed. **** $p \leq 0.0001$ (d) Quantitative analyses of cerebellar morphology. Values of perimeter (upper) and area (lower) of midsagittal cerebella sections from three mice of each genotype are represented in the graphs (left panels). Littermates are indicated with the symbol of the same colour. The histograms (right panels) show *Maml1*$^{-/-}$ cerebellum perimeter (upper) and area (lower), represented as fold of reduction compared to control. * $p \leq 0.05$

a

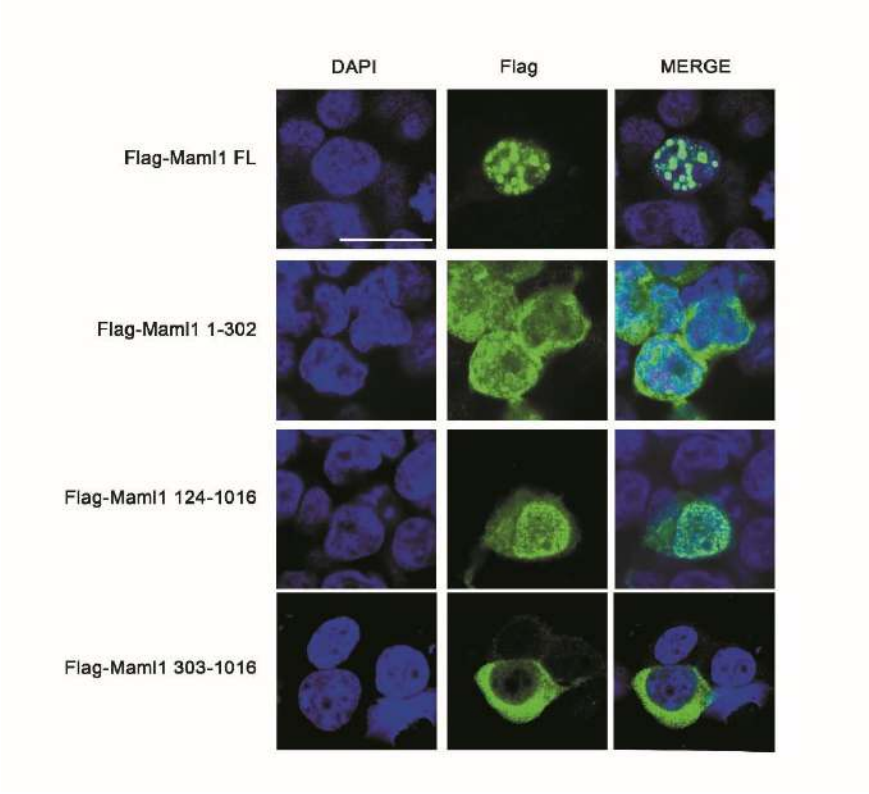


b

Gene	Design element	'pediatric high grade glioma' vs 'normal'	'adult high grade glioma' vs 'normal'	'atypical teratoid/ rhabdoid tumor' vs 'normal'	'group 3 medulloblastoma' vs 'normal'	'group 4 medulloblastoma' vs 'normal'	'pilocytic astrocytoma' vs 'normal'	'posterior fossa group A ependymoma' vs 'normal'	'posterior fossa group B ependymoma' vs 'normal'	'sonic hedgehog group medulloblastoma' vs 'normal'	'supratentorial ependymoma' vs 'normal'
maml1	202360_at				1					1.2	

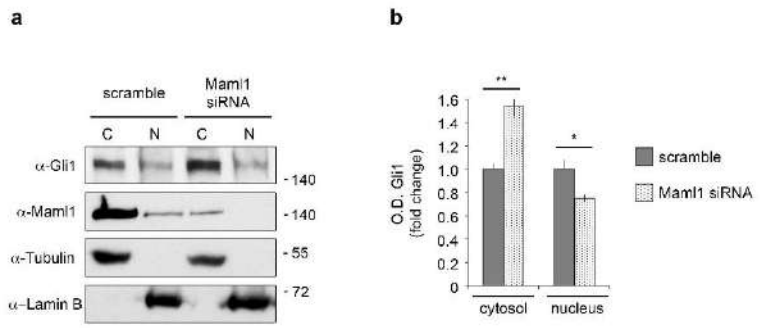
Supplementary Figure S1. Maml1 expression in silico analysis.

(a) RNA-seq of human tissue samples as indicated in Baseline Expression Atlas [http://www.ebi.ac.uk/gxa; experiment name: 53 GTEx; accession number: E-MTAB-2919; release 22.06.2016]. Maml1 transcripts expression is higher in cerebellum, as shown in the boxplot from Genotype-Tissue Expression (GTEx) Project website. RPKM=reads per kilobase per million mapped reads. (b) Expression of probe set 202360_at, indicating *Maml1* gene, from gene expression profile of paediatric and adult brain tumour types, compared to healthy brain tissue. From Differential Atlas database [array design: Affymetrix GeneChip Human Genome U133 Plus 2.0 (HG-U133-Plus_2). The red box shows *Maml1* gene up-regulation in the test condition. The colour intensity is representative of the $\log_2$ fold-change variation respect to control: the larger the $\log_2$ fold-change and the more intense is the red colour.



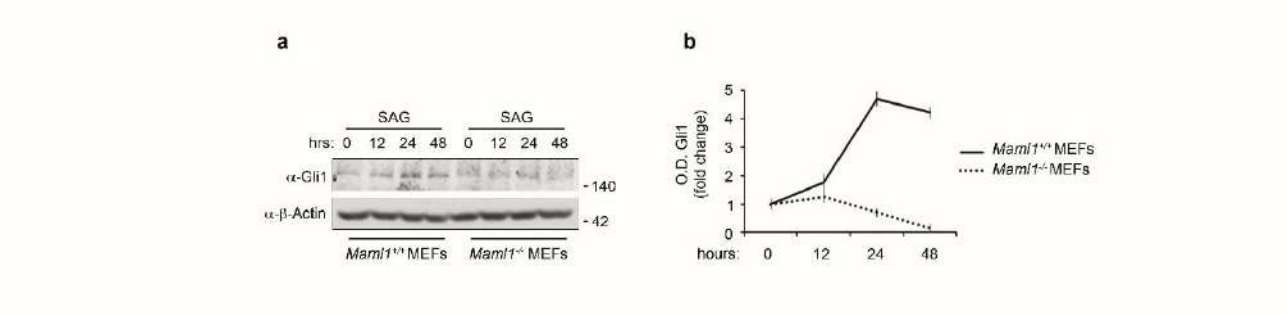
Supplementary Figure S2. Subcellular distribution of transiently expressed Flag-tagged Maml1 FL and mutant forms.

Representative single plane immunofluorescences images of HEK293T cell transfected with Flag-Maml1 full-length or mutant vectors, as indicated in Figure. Flag– (green) tags were visualised by confocal microscopy. Images were acquired as described in M&M. Figure shows that Maml1 FL is able to move preferentially into the nucleus, in particular into the nuclear bodies. On the contrary, Maml1 mutant forms present a different subcellular localization: Maml1 1-302 is localised into the nucleus and also in the cytoplasm compartment; Maml1 124-1016 truncated form is present mostly into the nucleus, in a diffuse manner; Maml1 303-1016 (containing the TAD2, but without the NLS region) is preferentially located in the cytoplasm. Nuclei were DAPI-labelled (blue). Scale bar: 15µm.



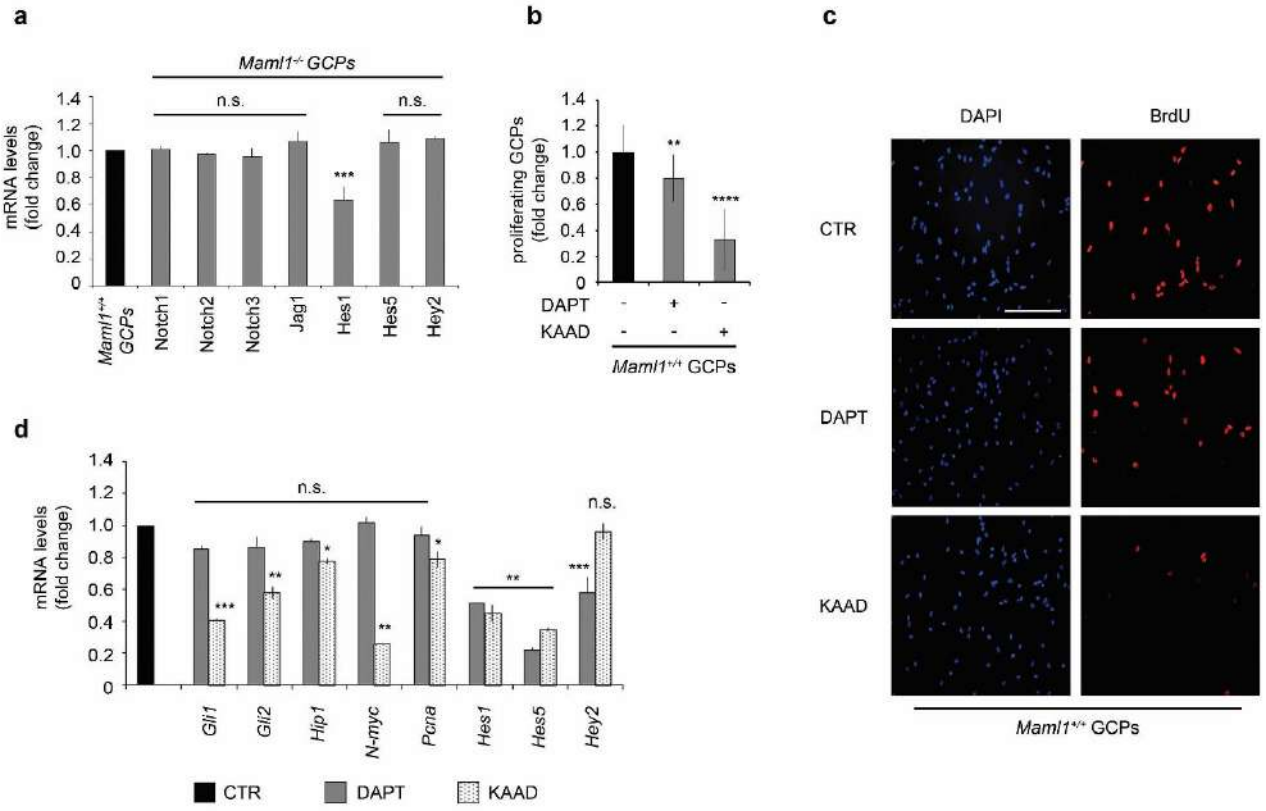
Supplementary Figure S3. Maml1 influences the subcellular localization of Gli1

(a) Immunoblot analysis of citosolyc (C) and nuclear (N) protein fractions from wild-type MEFs Maml1-silenced, compared to scramble control with the indicated antibodies. α-Lamin B and α-Tubulin were used as quality control of fractionated protein extracts and as loading control. (b) Optical densitometry (O.D.) analysis of Gli1 protein expression evaluated by immunoblotting, related to panel a. The blot presented is representative of three independent experiments.



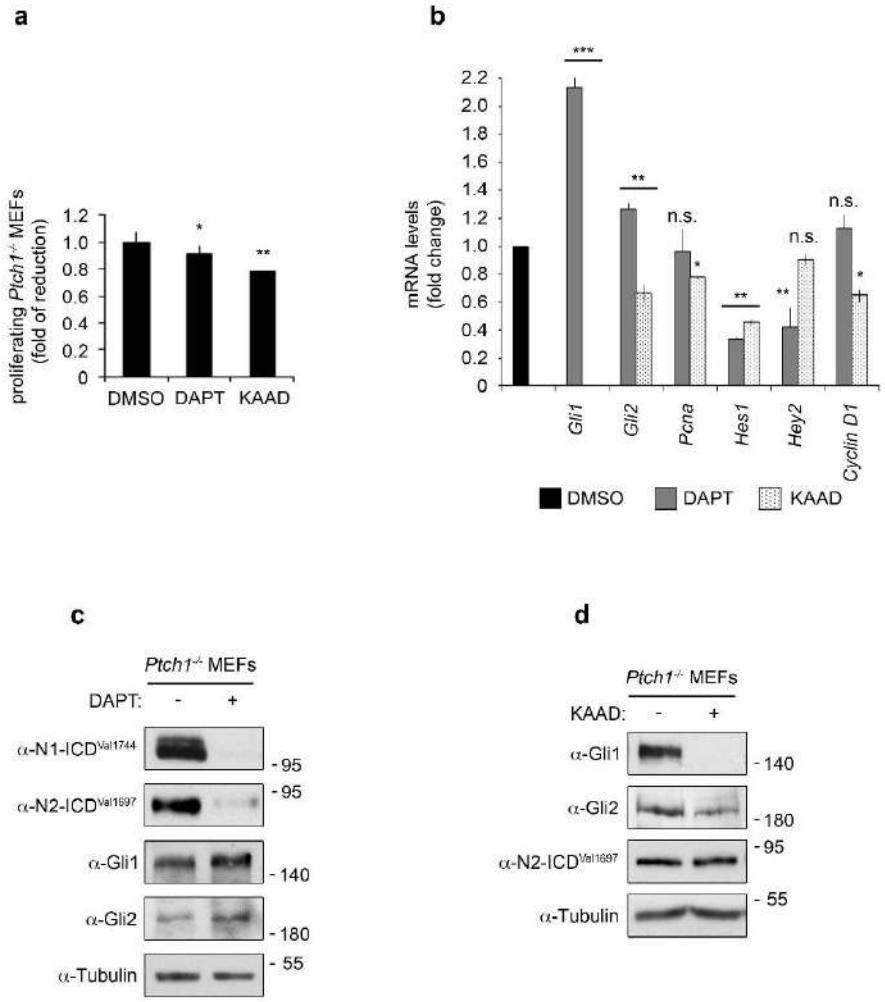
Supplementary Figure S4. Maml1 depletion determines an impaired Shh signalling activation

(a) Gli1 expression in WCE prepared from *Maml1*$^{-/-}$ and control MEFs, treated with SAG for the indicated times, was detected by immunoblot. (b) Optical densitometry (O.D.) analysis of Gli1 protein expression evaluated by immunoblotting, related to panel a.



Supplementary Figure S5. Compared effects on GCPs proliferation after Notch and Shh inhibition

(a) Basal expression of components of Notch pathway in GCPs cultures from E19.5 *Maml1*$^{-/-}$ and control mice, evaluated by qRT-PCR analysis. (b,c) BrdU incorporation assay in *Maml1*$^{+/+}$ GCPs at E19.5 treated with DAPT or KAAD and 24h BrdU pulse. Proliferating GCPs were calculated by mitotic index (BrdU-positive GCPs/total GCPs), analysed as fold of reduction compared to control (b). In red are visualized the proliferating cells; nucleus stained with DAPI blue. Scale bar: 200μm (c). (d) mRNA expression levels measured by q-RT-PCR, in *Maml1*$^{+/+}$ GCPs, upon Notch or Shh pathway pharmacological inhibition, respectively with DAPT or KAAD. Data represent mean ± S.D. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$



Supplementary Figure S6. Compared effects on Ptch-/- MEF proliferation after Notch and Shh inhibition

(a) MTT cell proliferation assay in *Ptch*$^{-/-}$ MEF upon DAPT or KAAD treatment for 48h. The data are presented as fold of reduction compared to control (DMSO). (b) Notch and Shh target genes expression evaluated by qRT-PCR analysis after DAPT or KAAD treatment. The results are analysed as fold change compared to control (DMSO). (c,d) Western blot analysis of whole cell extract from *Ptch*$^{-/-}$ and WT MEF after DAPT or KAAD treatment. Data represent mean ± S.D. n.s. (not significant); *p≤0.05; **p≤0.01; ***p≤0,001

2. **Maml1 acts as negative regulator of Itch** (Zema et al., manuscript in preparation)

Post-translation modifications are regulatory mechanisms inside the cells that modulate specific information in almost every transduction route of signal. Ubiquitylation is a highly versatile post-translational modification and a fine molecular mechanism that controls the regulation of the most important cellular functions, such as replication, transcription, proliferation and differentiation, apoptosis, immune response, endocytosis and signal transduction (Woelk et al., 2007). Initially only described as a degradative system, it is known that the ubiquitylation process is involved in several cell mechanisms (e.g. protein shuffling from different cell compartment, protein-protein interaction and modulation of protein catalytic activity) and it can be considered not only as a degradative pathway.

Ubiquitin is a polypeptide of 76 amino acids, highly conserved in evolution, and can be added to substrate proteins as poly-ubiquitin chains, multi- or mono-ubiquitylation events or hybrid or branched chains, inducing different signal inside the cells for target protein and different outcomes (Welchman et al., 2005). Ubiquitylation is a covalent and reversible post-translation modification that involves an enzymatic cascade by E1, E2 and E3 enzymes and results in the binding of ubiquitin molecules on the lysines of target proteins (Pickart, 2001). The enzymatic cascade begins with the activation of ubiquitin through a high-energy thioester bond between ubiquitin Gly76 residue and the active cysteine at the catalytic site of the enzyme E1 (ubiquitin-activating enzyme), in an ATP-dependent manner. Subsequently, the active ubiquitin molecule is transferred to the reactive cysteine of the enzyme E2 (ubiquitin- conjugating enzyme) with a transient interaction that allows the shift to an E3 ubiquitin ligase. At the end of this process, the E3 ubiquitin ligase is able to catalyse an isopeptide bond between the Gly76 on the C-terminus of ubiquitin and the lysine on the N-terminus of the target protein (Pickart, 2001; Woelk et al., 2007).

Protein poly-ubiquitylation is commonly associated to proteolytic events lead by the 26S proteasome system. Noteworthy, it is known that ubiquitination signals induce several non-proteolytic events (e.g. DNA repair, transcription, signal transduction, endocytosis and sorting) (Welchman et al., 2005). To note, Ub chains can be formed from seven different lysine residues on target proteins (i.e. K6, K11, K27, K31, K33, K48 and K63) and in this case ubiquitin molecules are linked by isopeptide bonds to the ubiquitin that was previously added to the chain (Fig. 5). Two of the most studied lysine residues are the K48 and K63; while the first one allows protein degradation

by the 26s proteasome, the K63 linkage, and also K6, K27 and K33 linkage, represent a non-degradative signal and they are suggested to regulate protein localization, complex formation, selective autophagy, DNA damage repair and innate immunity (Scialpi et al., 2008; van Wijk et al., 2019).

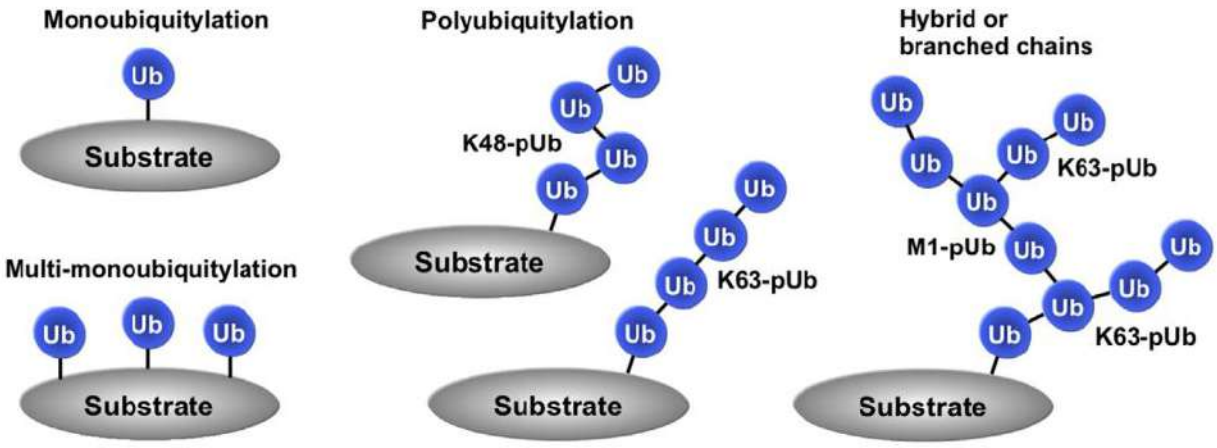


Figure 5. Ubiquitin chains and different lysine-linkage (Emmerich & Cohen, 2015)

In eukaryotes only one E1 isoform has been identified. On the contrary, several E2 isoforms have been characterized, and these interact with specific E3 ubiquitin ligases through the E3 domains. Based on the functional domain we can identify different classes of E3-ligases: The RING-finger (Really Interesting New Gene), the U-box and HECT (Homologous to the E6 associated protein Carboxyl-Terminus) E3 ligases.

The E3 ubiquitin ligase Itch, also known as AIP4 (atrophin-1 interacting protein 4) was first identified through genetic studies on the *agouti* locus in mice. The mutation a18H results from a paracentric inversion that involves two loci: *agouti* and *itchy* (*Itch*), the latter described for the first time by Perry and colleagues (1998). The mutated mice develop immune and inflammatory disease and skin inflammation and scarring due to constant itching.

Itch is a monomeric protein of 845 amino acids that belongs to Homologous to the E6 associated protein Carboxyl-Terminus E3 ligase family. Its molecular structure consists of a N-terminal Ca^{2+} lipid-interacting C2 domain, four protein-protein interaction WW domains and a C-terminal catalytic HECT domain (Bernassola et al., 2008) (Fig. 6). The four WW domains and a unique proline rich motif (PRR, between amino acids 195-294) are involved in the interaction with target proteins through several proline-rich motifs. Similarly to other HECT-E3 ligases, they also recognise phospho-serine and phospho-threonine residues followed by proline (Lu et al., 1999). The HECT domain is catalytic active and binds to E2 ubiquitin-conjugating enzyme in order to transfer the ubiquitin molecules to substrates. Consisting of 350 amino acids, the HECT domain contains a

N-terminal lobe, with the binding site for E2 enzymes, connected by an intradomain flexible hinge loop to a C-terminal lobe, characterised by the active site (Bernassola et al., 2008). The two lobes of the HECT domain undergo a conformational change to allow the trans thiolation reaction and the ubiquitin transfer from the catalytic cysteine of E2 to that of E3 (Verdecia et al., 2003). The N- and C-lobe present an inactive T-shaped conformation or a catalytically active L-shaped structure (Lorenz et al., 2013; Maspero et al., 2013; Verdecia et al., 2003).

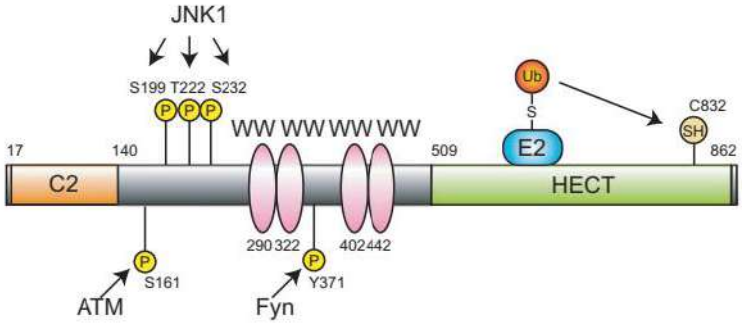


Figure 6. Itch protein structure (Aki et al., 2015)

During the binding with the E2, the hinge region allows a conformation change that results in a rapprochement between N- and C-lobes to juxtapose the reactive group of E2 and E3 and the ubiquitin transfer, suggesting that the structural plasticity of the HECT domain is essential for the catalytic activity (Lorenz et al., 2013; Zhu et al., 2017).

Generally, E3 ligases exist in an inactive steady state, in order to prevent non-specific ubiquitylation events on themselves and their target proteins, and probably other domains, beside HECT domain, play a role in maintaining this control. Besides, it is demonstrated that Itch WW domains are capable to block E2-E3 trans-thiolation and, subsequently, Itch activity (Riling et al., 2015). The auto-inhibitory loop between the WW and HECT domains can be released upon JNK phosphorylation. JNK recognizes three different residues in the PRR of Itch: serine 199 (S199); threonine 222 (T222) and serine 232 (S232). The phosphorylation mediated by JNK induces a conformational change of the WW domain, probably caused by an electrostatic repulsion, and enhances Itch catalytic activity (Gallagher et al., 2006). The JNK sites in Itch PRR are not present in other Nedd4 family E3s, suggesting an exclusive positive regulation by the upstream JNK signal on Itch. Furthermore, Zhu and colleagues demonstrated *via* biochemical and structural analysis that the WW2 and the linker region that connects WW2 and 3 domains bind a hydrophobic surface, in opposite position of the HECT domain. This linkage induces allosterically a closed conformation of HECT domain, leading to an inactive inhibitory state (Zhu et al., 2017). The binding of Ndfip1 (NEDD4 family-interacting protein 1) on the WW domains, release this inactive conformation. To

note, Itch activation by JNK depends on WW2 domain (Zhu et al., 2017). Itch activity is involved in immune responses, T-cell activation and T-helper cell differentiation (Aki et al., 2015). Upon TCR (T cell receptor) activation, Itch activity is promoted by JNK-mediated phosphorylation events, that results in ubiquitination and degradation of JunB (Gao et al., 2004). On the contrary, Src Kinase Fyn phosphorylates tyrosine 371 residue, on Itch WW domain, altering the affinity between Itch and JunB or recruiting inhibitory components, to reduce JunB degradation by Itch (Yang et al., 2006).

A further level of control on Itch activity is mediated by autocatalytic events that negatively control its protein levels, inducing proteasomal degradation. Nevertheless, the direct interaction with ubiquitin-protease FAM/USP9X prevents Itch proteasomal degradation (Mouchantaf et al., 2006). It has been reported that Itch autoubiquitylation occurs through the K63-linkage and induces a non-proteolytic regulation at different levels (i.e. catalytic activity, interaction with target proteins, cytoplasmic-nuclear shuffling) (Scialpi et al., 2008).

Among the several Itch target proteins we find Notch; in fact, Itch binds Notch via the N-terminal domain of the transcriptional factor Notch and its WW domains, to induce lysosomal degradation *via* K-29 linkage of Notch or proteasomal degradation (Chastagner et al., 2008; McGill et al., 2009; McGill & McGlade, 2003; Qiu et al., 2000; Wakamatsu et al., 1999).

The interaction between Notch and Itch is made possible by Numb, a co-adaptor, first identified in *D. melanogaster* as a negative regulator of Notch (Spana & Doe, 1996; Zhong et al., 1996). In mammals, the *Numb* gene encodes four protein isoforms by alternative splicing (i.e. Nbp65, Nbp66, Nbp71, Nbp72) (Dho et al., 1999; Verdi et al., 1999). Numb belongs to the Ndfip (NEDD4 family-interacting protein) family and is an evolutionarily conserved developmental protein involved in cell-fate determination and differentiation events (Guo et al., 1996; Rhyu et al., 1994; Verdi et al., 1996; Zilian et al., 2001). Numb acts as an adaptor protein that modulate the interaction between E3 ubiquitin ligases and the target protein to induce their degradation or endocytosis (Gulino et al., 2010). Moreover, Numb is recognized as a tumour-suppressor in several contexts, where it shows low expression levels (Pece et al., 2011).

Numb binds Itch on its WW domains by phospho-tyrosine-binding (PTB) domain to promote Notch ubiquitylation and degradation and Notch sorting to late endosome where it is degraded (McGill et al., 2009; McGill & McGlade, 2003). Numb promotes Notch ubiquitylation and degradation during myogenic differentiation, on the contrary Numb-effects are not so strong on Notch2 and Notch3 which are not polyubiquitinated by Numb (Beres et al., 2011).

Itch mediates DTX (human ortholog of deltex) lysosomal degradation via K-29 linkage (Chastagner et al., 2006). Beyond Numb also β-arrestin, a cytosolic adaptor proteins for G protein-coupled receptors (GPCRs), promotes Notch ubiquitylation and lysosomal degradation by Itch (Puca et al., 2013). These results suggest a double regulation of Itch on Notch pathway: via Notch and DTX degradation (Aki et al., 2015).

Shh pathway is negatively regulated by Numb and Itch (Di Marcotullio et al., 2011; Di Marcotullio et al., 2006). It is known that Numb interacts with Itch trough the PTB (phosphor-tyrosine-binding) domain of Numb and the WW2 domain of Itch (McGill & McGlade, 2003). This binding destabilizes the auto-inhibitory loop between Itch WW and HECT domains releasing it from its inactive state. Furthermore, the region from 174 to 421 aa of Numb recruits Gli1, to form a ternary complex with Itch. It has been shown that Itch and Numb interact to promote Gli1 degradation and to reduce its nuclear levels. In fact, Numb and Itch colocalize in the cytoplasm and their activity result in Gli1 proteasomal-dependent degradation (Di Marcotullio et al., 2006). The transcription factor Gli1 is unable to accumulate into the nucleus to promote the transcription of target genes. The overexpression of Numb, in medulloblastoma cell lines, impairs their proliferation rate, promoting differentiation of neuronal cells and on the contrary knock-down of Numb improve their colony-capacity formation. The authors suggest that the anti-proliferative and pro-differentiation effects of Numb on tumour cells are due to its suppression of functional Gli1 activity mediated by Itch-dependent degradation. Besides, Numb antagonizes Hh activity in cerebellum, inducing differentiation of GCPs, while Numb-deficient mice are characterized by an impairment of granule cells maturation and a concomitant overexpression of Gli1. Numb ΔPTB mutant, when unable to interact with Itch, does not induce Gli1 degradation, exactly as it happens with Itch C830A, thus suggesting that the maintenance of Gli1 protein level is mediated by functional cooperation between Itch and Numb (Di Marcotullio et al., 2006).

Scholars (Di Marcotullio et al., 2011) recognize a new Itch-dependent degron on Gli1 protein structure: two PPXY motifs in its C-terminal domain (775-1106aa) and a $pS^{1060}P$ motif. These three motifs are essential for Itch binding and to mediate Gli1 ubiquitylation and degradation. Gli1 triple mutant in these sites induces an increase of cell proliferation, migration and invasion (Di Marcotullio et al., 2011).

Itch acts as a negative regulator of Shh pathway inducing SuFu (Suppressor of Fused homolog, a negative regulator of Hh signalling pathway) poli-ubiquitylation on lysine 321 and 457 (Infante et al., 2018). SuFu interacts with WW1 and WW2 domains of Itch and this binding induces a poli-ubiquitylation of SuFu. Nevertheless, the poli-ubiquitylation occurs *via* K-63 linkage and mediates

non-proteolytic events (i.e. protein-protein interactions). SuFu K321/457R mutant is not ubiquitinated by Itch, resulting in an impairment of SuFu/Gli3 interaction and, subsequently, in Gli3R cleavage and repression of target genes. β-arrestin2 positively mediates SuFu/Itch binding, acting as an adaptor protein and allowing the formation of the trimeric complex SuFu/Itch/β-arrestin2. Moreover, in murine model of Medulloblastoma SuFu K321/457R mutant induces an increase in tumour volume, while, on the contrary, SuFu wild-type impairs proliferation of malignant cells (Infante et al., 2018).

Itch can be considered a negative regulator for both Shh and Notch pathways, leading to repress target genes. Different studies demonstrate that Itch is involved in tumour development (Aki et al., 2015). Itch can act as either tumour-promoting or -suppressing factor, depending on the cell context and the target proteins. Itch is involved in the formation of different types of isopeptide bond between the Gly76 on the C-terminus of ubiquitin and the lysine on the N-terminus of the target protein that can lead to non-proteolytic events. Further research on regulatory mechanisms Itch E3 ligase activity-dependent could provide a new road for therapeutic intervention targeting Itch.

2.1 Maml1 counteracts the Itch effects on Gli1 protein.

The data presented so far elucidate the role of Maml1 as transcriptional co-activators of Gli1 and Gli2, transcription factors in Shh pathway, both *in vitro* and *in vivo*. Noteworthy, Maml1 not only acts as co-factors for transcriptional events. It is known that Maml1 has a double role of coactivator to p53, stabilizing p53 protein at post-transcriptional level and promoting phosphorylation and acetylation events (Zhao et al., 2007).

To this regard, we sought to investigate whether Maml1 is able to play a role in controlling the activation of Shh signalling by triggering post-translational modifications in Gli1 proteins, regulating their activity. For this purpose, NIH3T3 cells (Figure 1a, right panel) and WT MEFs cell line (left panel) were firstly transfected with an increasing amount of Flag-Maml1. Interestingly, Figure 1a shows an increase in Gli1 protein levels in a dose-dependent manner.

To discriminate whether the increased levels of Gli1 protein are dependent non only on transcriptional activity but also on post-translational modifications we performed a cycloheximide assay in WT MEFs cells. After 6hrs of treatment, it is possible to observe that endogenous Gli1 levels are strongly reduced in absence of Maml1, on the contrary exogenous expression of Maml1 plays a significant role in stabilising Gli1 protein up to 24hrs, depending on the Maml1 expression levels (Figure 1b).

These results implicate a role for Maml1 in Gli1 post-translational regulation. It is known that Gli1-degradation signals are mediated through Numb-Itch interaction. The trimeric complex Numb-Itch-Gli1 induces Gli1 proteasomal degradation (Di Marcotullio et al., 2011; Di Marcotullio et al., 2006). To further demonstrate a regulation that goes beyond transcription, HEK293T cells were transfected with GFP-Gli1 in combination with HA-Ub and Myc-Itch to study Gli1 ubiquitylation levels. As suggested, the presence of Itch drives a sustained ubiquitylation of Gli1 (Figure 1c). Notably, the presence of Maml1 is able to protect Gli1 from Itch-mediated ubiquitylation, restoring the normal ubiquitinated levels in a dose-dependent manner (Figure 1c). Furthermore, ubiquitylation assay in WT MEFs shows similar results (Figure 1d), suggesting that the effects of Maml1 on Gli1 protein in regulating the ubiquitylation levels are independent from cellular context.

2.2 Maml1 is a new interacting factor for Itch.

Itch is an E3 ubiquitin ligases, involved in the regulation of different pathways (Aki et al., 2015). In particular, Itch, in combination with Numb, mediates Gli1 ubiquitylation and degradation, negatively modulating Shh signalling activation (Di Marcotullio et al., 2011; Di Marcotullio et al., 2006). We already demonstrated that overexpression of Maml1 induces an increase of both mRNA and protein levels of Gli1. On the contrary, Maml1 loss of function results in a decrease of Shh target genes transcription, mediated by Gli1.

Notably, NIH3T3 cell line transfected with Maml1-small interference RNA (Maml1 siRNA) shows a significant up-regulation of Itch and Numb endogenous protein levels, compared to control (Scr) (Figure 2a). In line with these observation, cycloheximide assays show that the overexpression of Maml1 in WT MEFs induces an important reduction of Itch expression levels, respect to empty vector (pcDNA3) along a time course (Figure 2b). Altogether, these results strongly suggest that Maml1 could be involved in controlling the stability of Itch protein with important effects on the functional activity of target proteins, such as Gli1.

Overall these observations suggest a physical association between Maml1 and Itch and to address this issue we performed an immunoprecipitation assay. HEK293T cells were transfected with Maml1 mutant vectors (see Result I, Figure 2a), to reveal their protein/protein interaction and to determine the interacting domain between Flag-Maml1 and Myc-Itch. Of great interest is the observation that Maml1 is able to directly/indirectly associate to Itch and to regulate its stability and function. Moreover, we have identified at least two interaction points that involve both the N-terminal and C-terminal domains of Maml1 (Figure 2c). Moreover, Figure 2d shows the existence

of Maml1 interaction with Itch and/or Numb also at endogenous level. In order to study the biological effects of Maml1/Itch interaction, we analysed the expression levels of Gli1, a direct Itch target protein (Di Marcotullio et al., 2011). To this purpose, we co-transfected HEK293T with vectors expressing HA-Gli1, Myc-Itch ad increasing doses of Flag-Maml1 and we performed immunoprecipitation assays using specific antibodies, as shown in Figure 2e. Of note, Figure 2e displays that increasing doses of Maml1 determine a reduced interaction between Gli1 and Itch, resulting in an important increase in Gli1 expression levels. Immunoblotting assays, using the same whole cell extract (WCE) of Figure 2e, show that the up-regulation of Gli1 protein is associated to a decrease of Itch protein in a Maml1-dose dependent manner.

Overall these data demonstrate that Maml1 could act as a negative regulator for Itch protein, impinging on its binding with Gli1 target protein and indirectly regulating its activity on Shh pathway.

2.3 Maml1 induces Itch autoubiquitylation events by K63 linkage.

E3 ubiquitin ligases are characterized by a protein structure that can allow conformational change to induce the catalytic activity (Lorenz et al., 2013). The inactive form is caused trough an interaction between the HECT and WW domains of E3 ubiquitin ligases. The release of this inhibitory loop is mediated by interactions with adaptor proteins (e.g. Numb) or phosphorylation events (Di Marcotullio et al., 2011; Gallagher et al., 2006).

To further analyse the role of Maml1 on Itch activity different ubiquitylation assays were performed. HEK293T cells were co-transfected with Myc-Itch and increasing amount of Flag-Maml1. As shown in Figure 3a, Maml1 induces an increase of the ubiquitination state of Itch in a dose-dependent manner, suggesting that Maml1 is able to directly induce post-translational modifications of Itch protein with a negative impact on its activity. Moreover, in order to identify the Maml1 functional domain able to induce post-translational modification, we analysed the Itch ubiquitination state in presence of Maml1 mutant vectors. Interestingly, Figure 3b shows that only the Maml1 C-terminal domain (i.e. 303-1016) is able to mediate ubiquitination events, comparable to Maml1-FL. On the contrary, the Mam 1-302 mutant does not induce ubiquitylation processes on Itch at all.

Ub chains are constituted by seven different lysine residues (i.e. K6, K11, K27, K31, K33, K48 and K63) on target proteins. The ubiquitination signal induces several events as degradation and non-proteolytic events (Welchman et al., 2005). It is demonstrated that Itch undergoes auto-

ubiquitination processes, through the K63-linkage, to regulate its own activity (Scialpi et al., 2008). To analyse the molecular mechanism of Itch ubiquitination Maml1-mediated and to discriminate which kind of ubiquitination signal Maml1 induces, we performed ubiquitination assays using wild type (HA-Ub WT) or mutant ubiquitin vectors (HA-Ub-K48R; HA-Ub-K63R). In order to assess the different linkage involved in Itch ubiquitination Maml1-mediated, WT MEFs cells were co-transfected with Myc-Itch, Flag-Maml1 and mutant ubiquitin vectors. Interestingly, Figure 3c shows that in presence of Flag-Maml1, Myc-Itch ubiquitination levels are extremely increased with both vector HA-Ub-WT and HA-Ub-K48R. Ubiquitin molecules mutated in the Lysine 48 are unable to perform a K48 linkage, involved in proteasomal degradation (Welchman et al., 2005). These data suggest that the ubiquitination on Itch, due to Maml1 presence, is able to go beyond the mutation in lysine 48, suggesting a different linkage Maml1-mediated. On the contrary, in absence or presence of Flag-Maml1 the ubiquitination mediated by the mutant vector HA-Ub-K63R presents no modulation, suggesting that Maml1 acts on Itch ubiquitination levels through K63-linkage (Figure 3c).

Auto-ubiquitylation events require an active E3 ligase. To determine if Maml1 activity depends on Itch activation, necessary to induce its own auto-proteolytic processes, we carried out an ubiquitination assay in WT MEFs cell line, transfected with plasmid vectors encoding for Flag-Itch wild type or C830A. The mutant vector Flag-Itch-C830A is characterised by a mutation in HECT domain, which impairs its catalytic activity. The cells were co-transfected in combination with V5-Maml1-FL or pcDNA3, as control. Noteworthy, the immunoprecipitation in Figure 3d shows that Flag-Itch-WT in presence of Maml1 is more ubiquitinated, respect to control (pcDNA3). On the contrary, Maml1 is not able to restore Itch ubiquitination levels in the vector Flag-Itch-C830A, mutated in active cysteine unable to trigger ubiquitination processes of target proteins or itself. Overall, these results suggest that Maml1 activity requires an active Itch to modulate its ubiquitination state, reinforcing the idea that Maml1 induces auto-catalytic events on Itch. Indeed, even in presence of Maml1 the mutant is unable to restore the same ubiquitination levels as the wild type.

Here, we demonstrate the existence of a novel role for Maml1, able to trigger post-translational modifications that switch off/switch on the activity of Itch, controlling in this way the function of several downstream signalling pathways.

Figures

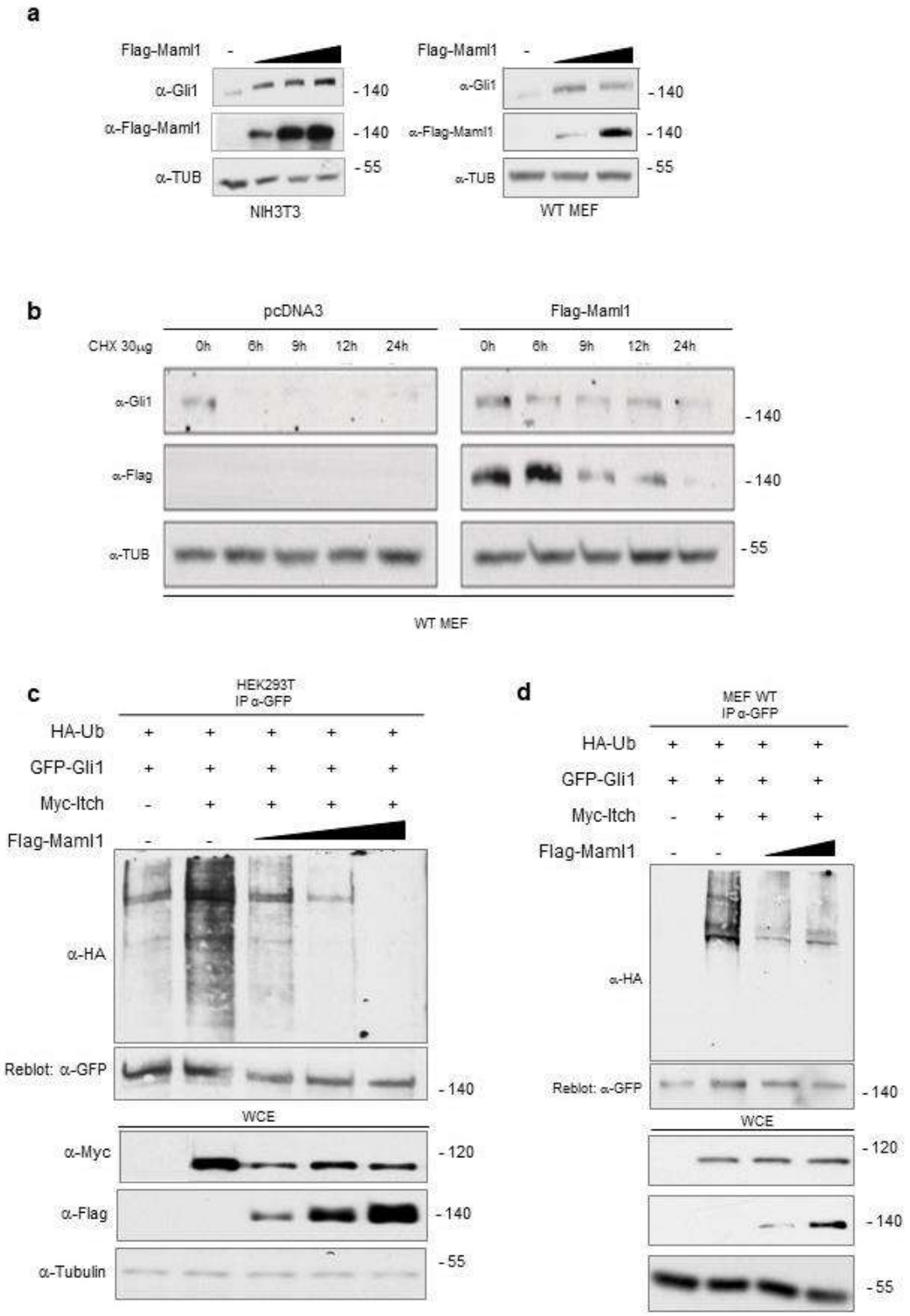


Figure 1. Maml1 enhances and stabilizes Gli1 protein levels. (a) WCE analysis of NIH3T3 and WT MEF upon 48h transfection of increasing amounts of Maml1, compared to control empty vector (pcDNA3). (b) Cycloheximide assays in WT MEF transfected with Maml1 or empty vector. After 24h of transfection the cells were treated with 30μg of CHX along a time course. (c-d) Ubiquitylation assay in HEK293T (c) and WT MEF (d). α-HA-immunoblotting of immunoprecipitated GFP-Gli1. The lower panels show western blot analysis of the respectively WCEs. Protein levels normalized relative to α -Tubulin.

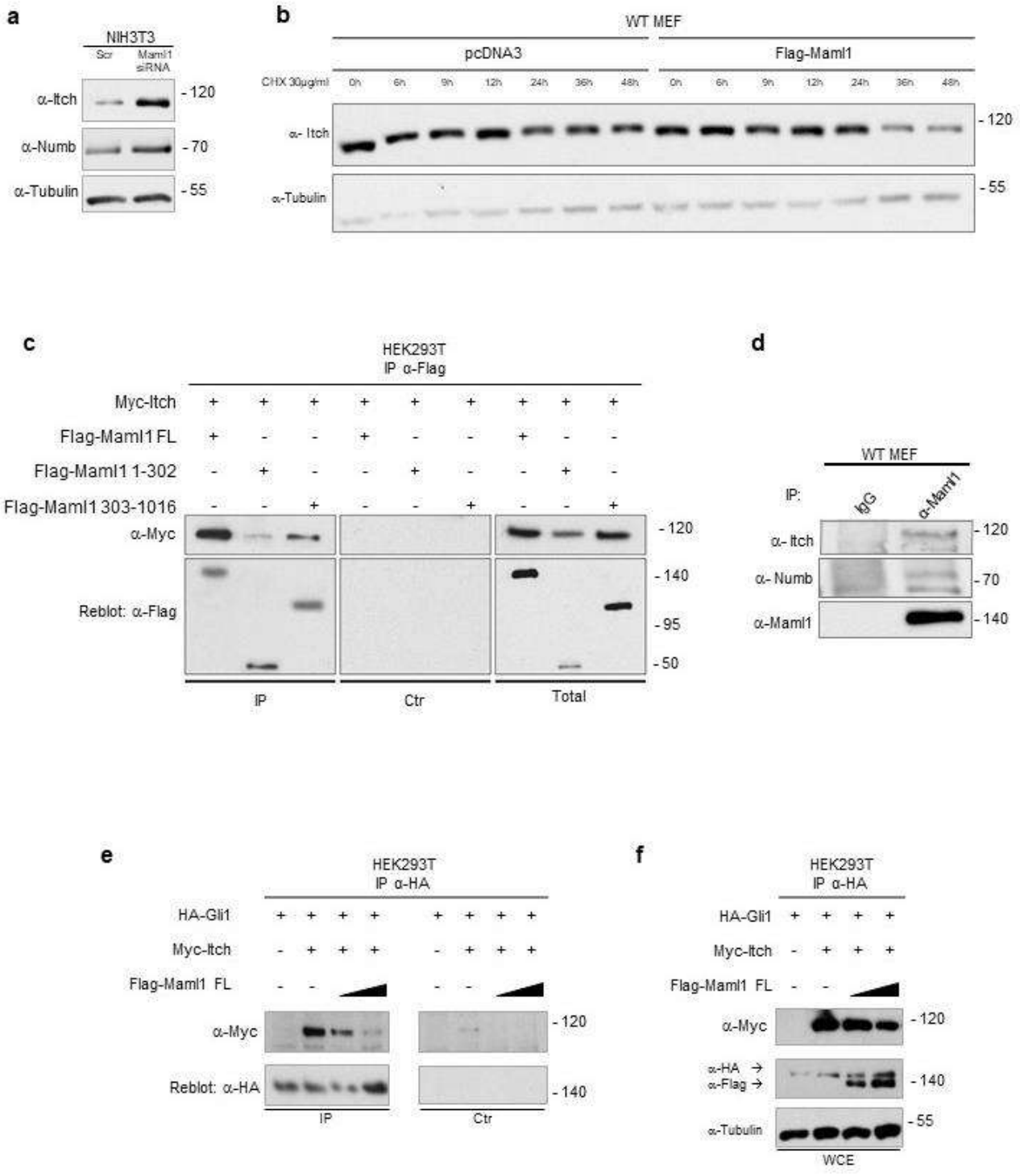


Figure 2. Maml1 is a negative regulator of Itch, impinging on Gli1/Itch interaction. (a) WCE analysis of NIH3T3 transfected with small interference RNA Maml1 (siRNA Maml1) and control (scr). (b) Western blot analysis of Itch endogenous levels upon cycloheximide assay in WT MEF after 48h of transfection of Flag-Maml1. (c) Co-IP in HEK293T transfected with Myc-Itch in combination with Maml1 FL and truncated mutants. α-Myc-immunoblotting of immunoprecipitated α-Flag. (d) Endogenous Co-IP in WT MEF. Immunoprecipitation α-Maml1 e immunoblotting α-Itch and Numb. (e) Gli1/Itch interaction analysis in HEK293T upon transfection of HA-Gli1 and Myc-Itch in combination with Maml1. IP α-HA and immunoblot α-Myc (f) Relative WCE analysis of panel (e).

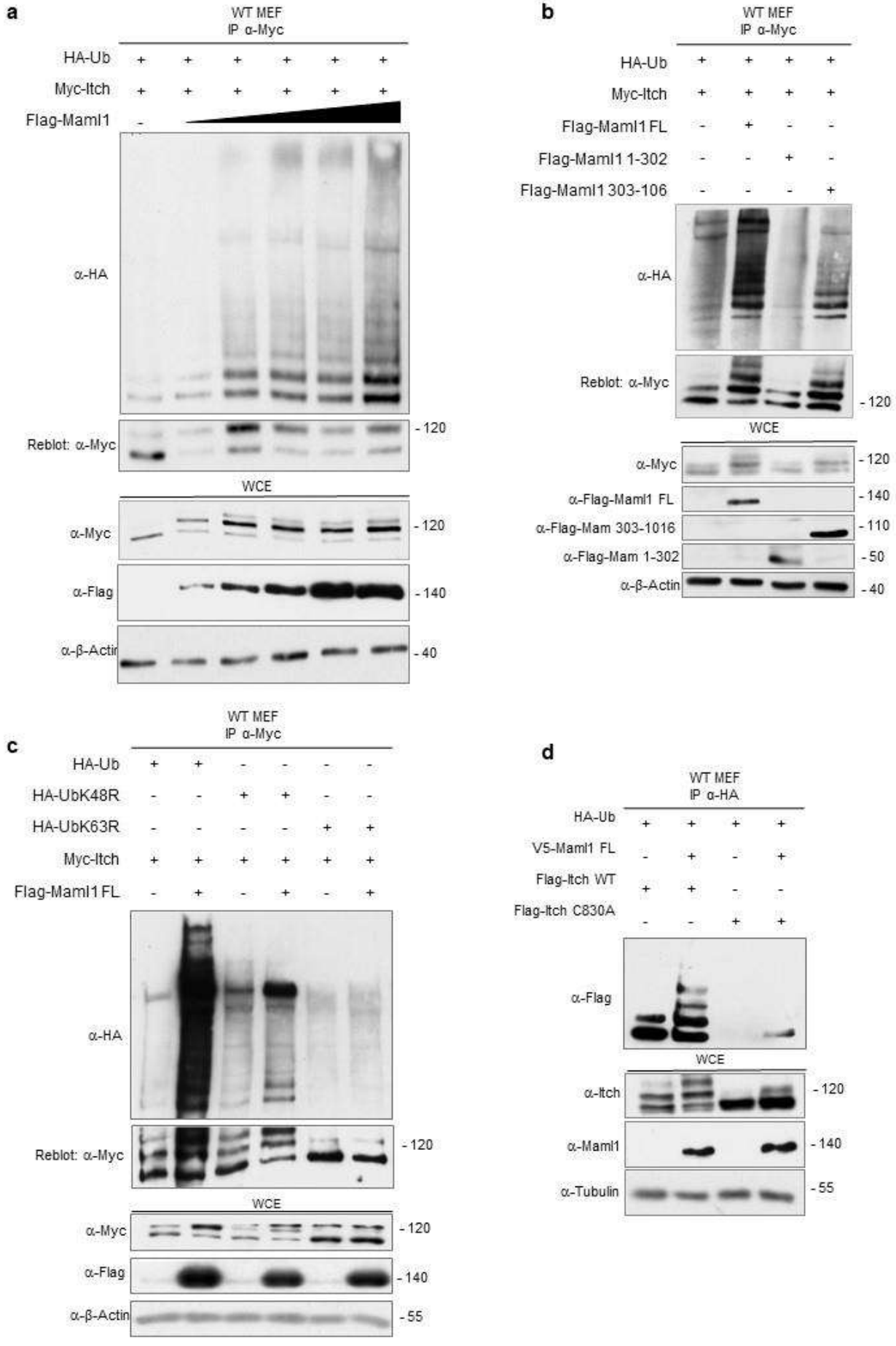

a
WT MEF
IP α-Myc
HA-Ub + + + + + +
Myc-Itch + + + + + +
Flag-Maml1 -
α-HA
Reblot: α-Myc
- 120
WCE
α-Myc
- 120
α-Flag
- 140
α-β-Actin
- 40
b
WT MEF
IP α-Myc
HA-Ub + + + +
Myc-Itch + + + +
Flag-Maml1 FL - + - -
Flag-Maml1 1-302 - - + -
Flag-Maml1 303-106 - - - +
α-HA
Reblot: α-Myc
- 120
WCE
α-Myc
- 120
α-Flag-Maml1 FL
- 140
α-Flag-Mam 303-1016
- 110
α-Flag-Mam 1-302
- 50
α-β-Actin
- 40
c
WT MEF
IP α-Myc
HA-Ub + + - - - -
HA-UbK48R - - + + - -
HA-UbK63R - - - - + +
Myc-Itch + + + + + +
Flag-Maml1 FL - + - + - +
α-HA
Reblot: α-Myc
- 120
WCE
α-Myc
- 120
α-Flag
- 140
α-β-Actin
- 55
d
WT MEF
IP α-HA
HA-Ub + + + +
V5-Maml1 FL - + - +
Flag-Itch WT + + - -
Flag-Itch C830A - - + +
α-Flag
WCE
α-Itch
- 120
α-Maml1
- 140
α-Tubulin
- 55

Figure 3. Maml1 induces Itch-ubiquitylation events by K63 linkage. (a) Ubiquitylation assay in WT MEF. The cells were transfected as indicated in the figure. IP α-Myc and Blot α-HA to observe ubiquitylation levels. The lower panel show the WCE of the same experiment. (b) Itch ubiquitylation assay in combination with Maml1 mutants. The lysates were analysed by western blot in the lower panel. (c) IP α-Myc and Blot α-HA. The panel shows the plasmids transfected for each experimental point. The lower panel indicates the western blot analysis of the relative immunoprecipitants. (d) Itch WT and inactive mutant were transfected in WT MEFs in combination with V5-Maml1, as indicated in the panel. HA-Ub was immunoprecipitated with α-HA antibody and reblot α-Flag. WCE were normalized through α-Tubulin expression levels.

3. Discussion and Conclusions

The regulation of transcription mechanisms is an event finely regulated inside the cells to control the activation or repression of specific genes in response to external or internal stimuli. Several co-factors act in cooperation with transcription factors to modulate their response and activity to signal transduction. Transcriptional co-activators are key components of transcriptional complexes and they recruit other proteins on DNA-binding complexes to promote transcriptional activity. Their dysregulation results in altered and/or pathological conditions.

Maml1 is a glutamine-rich nuclear protein, at first recognised as transcriptional co-activators for Notch signalling, an evolutionarily conserved pathway (Petcherski & Kimble, 2000; Wu et al., 2002; Wu et al., 2000). Maml1 modulate the activity of Notch receptors into the nucleus, binding with other proteins such as p300 and CDK8 to drive Notch acetylation and transcription of target genes (Fryer et al., 2004; Hansson et al., 2009; Saint Just Ribeiro et al., 2007). Noteworthy, in recent years the role of Maml1 as transcriptional co-factor for different signalling pathway has been elucidated. In fact, it is demonstrated Maml1 activity on p53 (Zhao et al., 2007), MEF2C (Shen et al., 2006), β-catenin (Alves-Guerra et al., 2007), EGR1 (Hansson et al., 2012), NF-κB (Jin et al., 2010) and Runx2 signalling pathways (Watanabe et al., 2013).

In silico analysis by Differential Atlas database revealed that Maml1 expression is higher in cerebellum in respect to other human tissues. Interestingly, Maml1 is overexpressed in cerebellar cancer medulloblastoma Shh-driven. Based on these data and literature observations, we sought to examine if Maml1 has a role in regulating Shh signalling. To note, Maml1 enhances Gli1 and Gli2 transcriptional activity. Gli1 is a stronger transcriptional factor than Gli2, which in contrast is required in the first step of Shh activation, promoting Gli1 transcription (Bai et al., 2002). It is known that Gli1 is both the principal effector of Shh pathway and a target gene, in a positive feedback loop (Dai et al., 1999; Sasaki et al., 1999). Notable, Maml1 induces an increase in Gli1 mRNA levels in a dose-dependent manner, and they co-localise in Gli1 target promoters. These results suggest a synergistic cooperation between Gli1 and Maml1, enhancing transcription of Shh target genes due to recruitment of Gli1 in the nucleus by Maml1. Remarkably, Maml1 loss of function induces a down-regulation of Gli1 and Shh target genes, with a Notch-independent mechanism that underlines the activity of Maml1 as a specific co-factor for Gli1. These events lead to a decrease of cell proliferation rates non only *in vitro* conditions but also *in vivo*. It is known that Shh pathway regulates proliferation of progenitor cells and foliation events in cerebellum (Corrales et al., 2004; Dahmane & Ruiz-i-Altaba, 1999; Lewis et al., 2004; Wallace, 1999). Notably, GCPs derived from a murine model *Maml1* depleted show a reduction of Gli1 activity and expression of

Shh target genes with a negative impact on cell proliferation. Moreover, pharmacological treatment with specific Smo agonist (i.e. SAG) is not able to restore Gli1 activity in *Maml1*$^{-/-}$ GCPs as the same level of the wild type. Overall these data demonstrate that Maml1 loss of function impairs Shh signalling cascade. Notch pharmacological inhibition on WT GCPs does not affect Shh target genes, showing a marginal role for Notch pathway on GCPs or Shh mediated proliferation and reinforcing the hypothesis of a Notch-independent mechanism. Finally, cerebella from *Maml1*$^{-/-}$ mice present an impaired foliation process, compared to control. Interestingly, the posterolateral and secondary fissures, characterised by high levels of Shh activation, are almost completely absent in *Maml1*$^{-/-}$ mice. Accordingly, analysis of PCNA expression levels revealed a down-regulation of its expression in *Maml1*$^{-/-}$ cerebellum, and morphometric analysis confirmed a reduction of perimeter and total area of Maml1 deficient mice, due to the reduced proliferation. A similar phenotype is obtained in mice with a down-regulation of Shh signalling, elucidating the strong connection between Maml1 and Gli1. Maml1 depletion affects Shh signal transduction impinging on GCPs proliferation, cerebellum foliation and size. These observations suggest an important role for Maml1 in cerebellum development, linked to Gli1 activity, providing a new integrated level of regulation in Shh/Gli pathway by identifying Maml1 as a novel coactivator that empowers Shh signalling.

It is known that Maml1 protein is able to induce post-translational modification in target proteins, as p53, NF-κB and EGR1, but the molecular mechanism has not been demonstrated yet. Here, we suggest that Maml1 is able to play a dual role: as a transcriptional coactivator and a post-translational regulator. Moreover, for the first time, we describe the molecular mechanism that underlies the stability of Gli1 target protein, which impinges on Itch functional role.

Indeed, Maml1 shows a role in Gli1 post-translational regulation preventing Itch-mediated ubiquitylation, able to restore the normal ubiquitylated levels in a dose dependent manner.

Itch is an E3 ubiquitin ligase, able to form a trimeric complex with the adaptor Numb and Gli1 to induce Gli1 ubiquitylation and proteasomal degradation (Di Marcotullio et al., 2011; Di Marcotullio et al., 2006). Of interest is the observation that NIH3T3 depleted of Maml1 show an increase in Itch and Numb protein levels, and on the contrary, Maml1 overexpression induces a decrease of Itch stability.

The endogenous interaction between Maml1, Itch and Numb suggest a direct role of Maml1 on Itch activity. It is known that Itch function is controlled through phosphorylation mechanisms, binding with adaptor proteins, and autocatalytic processes to regulate ubiquitylation events inside the cells

(Di Marcotullio et al., 2011; Gallagher et al., 2006; Melino et al., 2008; Scialpi et al., 2008). Notably, Maml1 regulates Itch ubiquitylated state in a dose-dependent manner through the C-terminal domain (TAD2). Interestingly, the TAD2 is also the domain involved in the control of Gli1 transcriptional activity. On the contrary, the N-terminal domain (TAD1) of Maml1 is recognised as the interacting domain for Notch, MEF2C and p53 signalling, suggesting a new role for Maml1 and the TAD2 domain which have not been characterised in other contexts.

In addition, Maml1 activity requires an active form of Itch and induces Itch ubiquitylation by a K63-linkage, known as a regulatory signal (van Wijk et al., 2019). The biological significance of the interaction between Maml1 and Itch, and the K63-linked ubiquitylation need further explanation. We can hypothesize that Maml1 binding could impinge on Itch/ Numb interaction and/or induce a conformational change in Itch protein folding, resulting in auto-catalytic events. On this regard, we propose to carry out further experiments to elucidate the mechanism that underlie the effects of Maml1 on Shh pathway. In the meantime, we are generating different cell lines depleted of Maml1 by CRISPR/Cas9 technology to validate our model. Furthermore, we intend to define Maml1/Itch domain of interaction and establish the X-ray structure of Itch upon Maml1 binding. Finally, we propose to study Maml1/Itch interaction on different tumoral backgrounds to observe the ubiquitylation status of different Itch target proteins in presence and absence of Maml1, to identify Maml1 as a novel factor involved in tumorigenesis.

Overall these results suggest a novel Maml1-dependent post-translation mechanism, which imping on Gli1 degradation mediated by Itch. Moreover, Maml1 and Itch are both two proteins involved in several pathway and these observations could set out a new molecular mechanism that applies to different signalling, characterised by Maml1 and Itch activity. A general mechanism of control of the Itch-mediated degradation processes by Maml1 could be used both in physiological and pathological contexts.

Overall these results suggest a novel Maml1-dependent post-translation mechanism, which imping on Gli1 degradation mediated by Itch. Maml1 and Itch are both involved in controlling the activity of several pathways. Therefore, the ability of Maml1 in controlling the activity of Itch/E3 ubiquitin ligase could have an impact in controlling the force of several signalling pathways inside the cell, as Shh and Notch, both in physiological and pathological contexts.

4. Materials and Methods

Mice

The generation and typing of *Maml1*$^{-/-}$ mouse have been described elsewhere (Oyama et al., 2007). Mice were maintained on a C57BL/6 background; they were bread and held under specific pathogen-free conditions in animal facility. The studies involving animals have been conducted following the Italian national guidelines for use and care of experimental animals, established in D.Lgs. n.26/2014, and in accordance with European Directive 2010/63/UE.

Cell culture, proliferation assay and treatments

NIH3T3, HEK293T and wild type and *Ptch1*$^{-/-}$ MEFs were maintained as described elsewhere (Mazzà et al., 2013). Primary wild-type and *Maml1*$^{-/-}$ MEFs were isolated from E13.5 littermates embryos, following the protocol from J. Xu, 2005. Primary granule cell precursor (GCPs) cells were cultured from E19.5 cerebella, according to established protocols (Argenti et al., 2005; Wechsler-Reya & Scott, 1999) and after 3 hours the medium was replaced for the starvation in serum free medium and the cells were treated with 200 nM SAG or vehicle alone (DMSO). Cell proliferation was evaluated by BrdU-Labeling and Detection Kit (cat.#11296736001, Roche, Welwyn Garden City, UK), as previously described (De Smaele et al., 2011). Nuclei were counterstained with Hoechst reagent and images were acquired with an Axio Vert.A1 microscope and analyzed with Axio Vision LE64 Software (Carl Zeiss Microscopy GmbH, Jena, Germany). At least 500 nuclei were counted in triplicate and the number of BrdU-positive nuclei respect to total cells number was evaluated to calculate the proliferation rate. To analyze the cell growth rate in *Ptch1*$^{-/-}$ MEFs, 2500 cells per well were plated onto a 96-well plate. The WST1 solution (cat.#5015944001, Roche) was added to each well according to the manufacturer's instructions. Spectrophotometric absorbance at 450 nm wavelength was determined by the plate reader GloMax-Multi Detection System (Promega, Madison, WI, USA). Cells were treated with different compounds: 10μM γ-secretase inhibitor IX (DAPT, cat.#565770, Calbiochem, Merck Millipore, Darmstadt, Germany), 200nM Smoothened agonist (SAG, cat.#ALX-270-426-M001, Enzo Life Sciences, Farmingdale, NY, USA) and 1mM KAAD/cyclopamine (Shh pathway inhibitor) (cat.#239804, Calbiochem), for the times indicated in the figures. Upon 24h of transfection the cells were treated with 30μg/ml of Cycloheximide solution (cat.#C4859, Sigma-Aldrich, St. Louis, MO, USA) along a time course as indicated in the figures. All compounds were dissolved in sterile DMSO, and DMSO was used in control treatments. Before SAG treatments, cell cultures were subjected to serum starvation.

Cell transfection, luciferase assay and plasmids

Transient transfection of HEK293T and NIH3T3 cell lines were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) or TransFectin™ Lipid Reagent (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Luciferase Assays were performed using the indicated reporter plasmids with different combinations of expression vectors, as shown in figures. pRL-TK Renilla was used as normalization control and pcDNA3 as control empty vector. Luciferase activity was assayed with a Dual Luciferase Assay system (Promega) as described previously 51. All luciferase activity data are presented as means ± S.D. of value from at least three independent experiments, each performed in triplicate. The following plasmids were described elsewhere: human Patched1 promoter (Patched1-luc) and promoter 12GLI-RETKO-luc (12xGli-luc) (Kogerman et al., 1999); pCS2-HA3-Gli1 (Di Marcotullio et al., 2011), pCS2-MT Gli2FL-Myc (Canettieri et al., 2010), GFP-Gli1 (Di Marcotullio et al., 2006), pFLAG-CMV-2 Maml1 full-length (1-1016) (Pelullo et al., 2014), pFLAG-CMV-2 Maml1 (1-302) and pFLAG-CMV-2 Maml1 (124-1016) (Wu et al., 2000), p6872 pHAGE-N-V5-MAML1-FL was a gift from Peter Howley (Addgene plasmid # 37048 ; http://n2t.net/addgene:37048 ; RRID:Addgene_37048), HA-Ub-WT, HA-Ub-K48R, HA-Ub-K63R (Di Marcotullio et al., 2006), pcDNA-Myc-Itch (Di Marcotullio et al., 2011), Flag-Itch-WT, Flag-Itch-C830A (Di Marcotullio et al., 2006). cDNA corresponding to Maml1 303-1016 was amplified by PCR from pFLAG-CMV-2 Maml1 full-length (1-1016) and cloned as SalI/NotI fragment in pFLAG-CMV-2 (#E7033 Sigma-Aldrich).

siRNA silencing

Small interference RNA (siRNA) was performed using 100nM SMART pool siRNA duplexes (cat.#L-059179-01-0005 for Maml1) or 100nM scrambled control (cat.#D-001810-10-20) purchased by Dharmacon Inc. (Lafayette, CO, USA), using Lipofectamine RNAiMAX (Life Technologies), according to the manufacturer's instructions.

RT-PCR/qRT-PCR

Total RNA extraction and reverse transcription PCR (RT-PCR) were previously described (Cialfi et al., 2013). Extraction and reverse transcription of mRNA from GCPs was achieved through the use of Cells-to-CT™ 1-Step TaqMan® Kit (Life Technologies), according to the manufacturer's instructions. Analysis of gene expression were realized by quantitative real-time PCR (qRT-PCR) using Taq-Man designed assays on demand (Invitrogen, Life Technologies) for the specific target genes, reported in Table S1, on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies), following the manufacturer's protocol for the comparative CT method. mRNA

quantification was expressed, in arbitrary units, as ratio of sample quantity to the mean value of control sample. Normalization was carried out using hypoxanthine guanine phosphoribosyl transferase (*Hprt*) as internal control gene.

Protein extract, immunoprecipitation and immunoblot analysis

Preparation of whole cell lysates, fractionation of cytoplasmic and nuclear proteins and immunoprecipitation assays were performed as described elsewhere (Checquolo et al., 2010). Briefly, for coimmunoprecipitation in transfected HEK293T cells, cell lysate were incubated with agarose conjugate Flag M2 beads (cat.#A2220, Sigma-Aldrich, St. Louis, MO, USA) or anti-HA agarose (cat.#A2095, Sigma-Aldrich) for 2 hours at 4°. In control sample the antibody was saturated with anti-Flag peptide (cat.#F4799, Sigma-Aldrich) or anti-HA peptide (cat.#I2149, Sigma-Aldrich). For reciprocal immunoprecipitation assay, after a pre-clearing step with Protein G-Agarose (cat.#sc-2002, Santa Cruz Biotechnology, Dallas, TX, USA), cell lysate were incubated with anti-Gli1 (C-1) (cat.#sc-515751, Santa Cruz Biotechnology) or anti-Myc (cat.#M4439, Sigma-Aldrich) or normal mouse IgG (cat.#sc-2025, Santa Cruz Biotechnology) as control for overnight at 4°C. The complexes were precipitated with Protein G-Agarose, then the beads were washed extensively with wash buffer, and the interaction was evaluated by Western blot analysis. Similarly, for wild type and *Ptch1*$^{-/-}$ MEFs the coimmunoprecipitation was realized with anti-Maml1 (D3E9) (cat.#11959; Cell Signalling, Danvers, MA, USA) or normal rabbit IgG (cat.#sc-2027, Santa Cruz Biotechnology) as control for overnight at 4°C; the pre-clearing step and precipitation of complexes were realized with Protein A-Agarose (cat.#sc-2001, Santa Cruz Biotechnology). For immunoblot analysis were used the following primary antibodies: anti-Gli1 (L42B10) (cat.#2643), anti-Maml1 (D3E9) and anti-Notch1 (Val1744) (D3B8) (cat.#4147) purchased from Cell Signaling; anti-Flag (cat.#F3165), anti-Myc (cat.#M4439), anti-Notch2 (Val1697) (cat.#SAB450200) and anti-β-Actin (cat.#A5441) from Sigma-Aldrich; anti-HA (cat.#sc-7392), anti-GST B-14 (cat.#sc-138), anti-α-Tubulin (cat.#sc-8035) and anti-Lamin B (M20) (cat.#sc-6217) from Santa Cruz Biotechnology; anti-Gli2 (cat.#AF3635) from R&D Systems (Minneapolis, MN, USA); anti-Numb (cat.#ab14140) from Abcam (Cambridge, UK); anti-Itch (cat.#611199) from BD Bioscience (Heidelberg, Germany). Bound antibodies were detected with enhanced chemiluminescence (ECL kit, Amersham, GE Healthcare, Lafayette, CO, USA). The intensity of protein expression was quantified using Quantity One Analysis Software (Bio-Rad). Values were normalized to housekeeping protein expression and represented as relative levels with respect to control sample.

In vivo ubiquitylation assay

In vivo ubiquitylation assay were performed as previously described (Mazzà et al., 2013). HEK293T and WT MEFs cell lines were transfected with different vectors, as indicated in the figures. Upon 24hr of transfection the cells were lysed with denaturing buffer (1% SDS, 50 mM Tris at pH 7.5, 0.5 mM EDTA, 1 mM DTT) to disrupt protein/protein interactions and then lysates were diluted 10 times with lysis buffer and subjected to immunoprecipitation with antibodies indicated in figures for 2hr (Flag M2 beads, anti-HA Agarose, anti-Myc from Sigma-Aldrich; anti-GFP from Santa Cruz Biotechnology). The immunoprecipitated proteins were then washed five times with the lysis buffer described above, resuspended in sample loading buffer, boiled for 5 min, resolved in SDS-PAGE, and then subjected to immunoblot analysis. Ubiquitylated forms were detected using anti-HA or anti-Flag antibodies. Where indicated, wild-type ubiquitin was substituted with ubiquitin mutants (K48R and K63R).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described earlier (Barisone et al., 2012; Bellavia et al., 2007). Immunoprecipitated DNA from HEK293T with Gli1 (H300) (cat.#sc-20687, Santa Cruz Biotechnology) or Maml1 (D3E9) or IgG (normal rabbit IgG) antibodies was eluted and analyzed by semiquantitative PCR, using a primer set encompassing two predicted binding sites for Gli zinc finger family (V$GLIF Matrix Family) (from -538 to -521; from -461 to -445, corresponding to dark circles in Figure 1f, low panel), on human Patched1 promoter (GXP_227868, from -891 to -87 relative to start codon). Human Patched1 promoter was identified using MatInspector (Software GmbH, Munich, Germany). The primer set used to specifically amplify Gli binding sites is the following: 5'-GAACCCAGCAGCCAGAGC-3' and 5'-CGACCCCTTCACTGCAGAA-3'.

Immunostaining and confocal imaging

Immunofluorescence staining of HEK293T cells was performed as described elsewhere (Pelullo et al., 2014). 24h after transfection, the cells were stained with primary antibody: mouse anti-HA (cat.#MMS-101P, Covance, Princeton, NJ, USA) and rabbit anti-Flag (cat.#F7425, Sigma-Aldrich). The secondary antibodies used were Alexa Fluor 594- and 488-conjugated respectively anti-mouse and anti-rabbit (Molecular Probes, Life Technologies). Nuclei were counterstained with Hoechst reagent. Single plane confocal images in the center of the cell were acquired using an inverted Olympus iX73 microscope equipped with an X-light Nipkow spinning-disk head (Crest Optics, Rome, Italy) and Lumencor Spectra X Led illumination. Images were collected using a CoolSNAP

MYO CCD camera (Photometrics, Tucson, AZ, USA) and MetaMorph Software (Molecular Device, Sunnyvale, CA, USA) with a 60x oil objective.

Proximity ligation assay (PLA)

in situ proximity ligation assay was performed in NIH3T3 cells using the Duolink® In situ-Fluorescence Technology, Olink® Bioscience (Sigma-Aldrich). All the steps were performed according to the manufacturer's protocol. Primary antibodies: anti-Gli1 (H300) and anti-Maml1 (N-20) (cat.#sc-18506) from Santa Cruz Biotechnology. Hybridization between the two PLA anti-rabbit PLUS and anti-goat MINUS probes leading the fluorescent red signal only occurs when the distance between the two antigens is less than 40 nm. In control experiment, cells were incubated with only one primary antibody and no significant binding was detected (only Gli1; only Maml1). Single plane confocal images were acquired using an inverted Olympus iX73 confocal microscope as described in immunofluorescence staining.

Immunohistochemistry

Cerebella from E18.5 and E19.5 mice were collected, and tissues were fixed in 4% formalin and paraffin embedded. Consecutive sections (2μm thick) were stained with Hematoxylin and Eosin (H&E). Immunocytochemical assay was performed using an anti-PCNA antibody (cat.#ab15497; Abcam, Cambridge, UK). Detection was carried out with Mouse-to-Mouse HRP (DAB) staining system (cat.#MTM001-IFU ScyTek Laboratories, Logan, UT, USA), according to the manufacturer's instructions. Images were acquired with a Leica DM1000 microscope equipped with a ProgRes® Speed XTcore 3 CCD camera and collected using ProgRes® CapturePro 2.8 software (Jenoptik Optical Systems GmbH, Jena, Germany). Proliferation index was deduced by the count of PCNA-positive GCPs/total GCPs in EGL of wild-type and *Maml1*$^{-/-}$ cerebella. The midsagittal area and perimeter of cerebella were measured from pictures captured using Aperio ImageScope (Aperio, Leica Biosystems, Germany) image analysis software.

Cytofluorimetric analysis

Freshly isolated GCP cells from cerebellum were stained and analyzed on a FACS-Calibur with CellQuest software (BD Biosciences, San Jose, CA, USA). For Gli1 intracellular staining, BD Fixation/Permeabilization kit was used (cat.#554714, BD Biosciences) and cells were incubated with anti-Gli1 antibody (L42B10) (cat.#2643, Cell Signaling) or normal mouse IgG (cat.#sc-2025, Santa Cruz Biotechnology), used as a negative control.

Statistical analysis

All Results were reported as the mean ± S.D. of at least three independent experiments, each performed in triplicate. Student's t test for unpaired samples was used to assess differences among groups. A p value < 0.05 was considered statistically significant (n.s. $p>0.05$; * $p<0.05$; ** $p< 0.01$; *** $p< 0.001$; **** $p< 0.0001$).

Table S1-Quantitative real time PCR oligonucleotide sequences.

Species	Gene name	Symbol	RefSeq	Assay ID
Mouse	Cyclin D1	Ccnd1	NM_007631	Mm00432359_m1
Mouse	Cyclin D2	Ccnd2	NM_009829	Mm00438070_m1
Mouse	GLI-Kruppel family member GLI1	Gli1	NM_010296	Mm00494654_m1
Mouse	GLI-Kruppel family member GLI2	Gli2	NM_001081125	Mm01293117_m1
Mouse	Hairy and enhancer of split 1	Hes1	NM_008235	Mm01342805_m1
Mouse	Hairy and enhancer of split 5	Hes5	NM_010419	Mm00439311_g1
Mouse	Hairy/enhancer-of-split related with YRPW motif 2	Hey2	NM_013904	Mm00469280_m1
Mouse	Huntingtin interacting protein 1	Hip1	NM_146001	Mm00524503_m1
Mouse	Hypoxanthine guanine phosphoribosyl transferase	Hprt	NM_013556	Mm01545399_m1 (FAM)
Mouse	Hypoxanthine guanine phosphoribosyl transferase	Hprt	NM_013556	Mm00446968_m1 (VIC)
Mouse	Insulin-like growth factor 2	Igf2	NM_001122736	Mm00439564_m1
Mouse	Jagged1	Jagged1	NM_013822	Mm00496902_m1
Mouse	Mastermind-like 1	Maml1	NM_175334	Mm00614627_m1
Mouse	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived	MycN	NM_008709	Mm00476449_m1
Mouse	Notch1	Notch1	NM_008714	Mm00435235_m1
Mouse	Notch2	Notch2	NM_010928	Mm00803077_m1
Mouse	Notch3	Notch3	NM_008716	Mm01345646_m1
Mouse	Paired box 6	Pax6	NM_001244198	Mm00443081_m1
Mouse	Proliferating cell nuclear antigen	Pcna	NM_011045	Mm00448100_g1
Mouse	Patched homolog 1	Ptch1	NM_008957	Mm00436026_m1

TaqMan Assay on Demand (Life Technologies, Applied Biosystems, Carlsbad, CA) code for the indicated genes.

IV. Results II

1. Kras/ADAM17-dependent Jag1-ICD reverse signalling sustains CRC progression and chemoresistance (Pelullo, Nardozza, Zema et al., 2019)

Sporadic colorectal cancer (CRC) development is characterized by well-known histopathological changes, resulting from specific genetic defects in selected oncogenes and tumour suppressor genes. The most of sporadic CRCs and hereditary colorectal tumours show loss of APC function, the negative regulator of Wnt signalling, ultimately leading to abnormal β-catenin-dependent gene expression (Frattini et al., 2004). In intestinal epithelial cells, constitutive activation of β-catenin/TCF leads to adenomatous polyp formation, a first step towards CRC development. Additionally, RAS driver mutations, found in about 50% of all CRCs and in advanced adenomas (Mologni et al., 2010; Pretlow & Pretlow, 2005), strongly sustain the pathogenesis of colorectal cancer, regulating tumour cell proliferation, survival, invasion, metastasis formation and drug resistance (Pretlow & Pretlow, 2005; Van Schaeybroeck et al., 2011). Constitutive activation of Kras is one of the best-characterized events in CRC development, able to trigger multiple downstream pathways, including the RAF/MEK/Erk mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)-AKT effector pathways (Mologni et al., 2012). Several observations suggest an involvement of MEK/Erk signalling in intestinal tumorigenesis (Lemieux et al., 2015), but the exact molecular mechanisms remain unclear. Of note, a growing body of evidence shows that the oncogenic Kras regulates ADAM17 activity and the shedding of several growth factors in a MEK/Erk-dependent manner (Van Schaeybroeck et al., 2014; Van Schaeybroeck et al., 2011). Kras mutations confer CRC resistance to anti-EGFR therapy and are associated with a worse prognosis (Lièvre et al., 2006). Current therapeutic options for advanced CRC have not dramatically improved clinical outcomes of patients with metastatic CRC. Therefore, a better understanding of molecular mechanisms involved in CRC development and progression is imperative for the improvement of therapeutic approaches.

Interestingly, recent studies have revealed that a sustained activation of β-catenin/TCF is responsible for transcriptional activation of Notch-ligand Jagged1, resulting in an up-regulation of Jagged1 that is required for tumorigenesis in the intestine (Rodilla et al., 2009). High expression levels of Jagged1 are associated with increased progression, metastatic potential, recurrence and

poor prognosis in several human malignancies, as prostate, renal, head and neck cancer and CRC (Lin et al., 2010; Santagata et al., 2004; Sugiyama et al., 2016; Wu et al., 2010). The commonly accepted scenario is based on the idea that Jagged1 ligand is able to contribute to tumorigenesis by activating canonical Notch signalling (Palermo et al., 2014).

Jagged1 belongs to the Delta, Serrate, Lag-2 (DSL) family of single-pass transmembrane ligands, including Delta-like (DLL1, 3 and 4) and Jagged (Jagged 1 and 2) that trans-activate the Notch receptors (Notch1-4) in signal-receiving cell (Palermo et al., 2014), through a direct contact. Receptor/ligand interaction renders Notch susceptible to proteolytic processes mediated by A-Disintegrin Metalloprotease ADAM-10 and PS/γ-secretase protein complex, which ends in the release of its intracellular domain (Notch-ICD). Notch-ICD moves into the nucleus where it binds to RBP-Jκ transcription factor and recruits co-activators to form a transcription-activating complex to activate several downstream effectors, such as hairy and enhancer of split (Hes). Aberrant activation of Notch signalling is frequently observed in many human cancers (Bellavia et al., 2007; Bellavia et al., 2018; Campese et al., 2003), including CRC (Qiao & Wong, 2009).

Emerging evidences indicate that Jagged1 is processed in a fashion similar to Notch by sequential proteolytic cleavages that involve two distinct enzymes: ADAM-17/TACE and PS/γ-secretase complex, ultimately resulting in the release of a nuclear-targeted intracellular domain (Jag1-ICD), that may play an important role in tumour development and carcinogenesis (Ascano et al., 2003; De Falco et al., 2018; LaVoie & Selkoe, 2003), possibly interacting and/or empowering the activation of other deregulated signalling pathways (Duryagina et al., 2013; Pelullo et al., 2014).

In this paper, we demonstrate that the function of Jagged1 may go beyond its effect on canonical Notch activation in colon malignancies. Indeed, we observed that in CRC cells with Kras activation, the Jagged1 ligand is not only abundantly expressed, but it undergoes a constitutive processing that ends in the aberrant generation of an intracellular fragment (Jag1-ICD), capable to move into the nucleus and to induce intrinsic reverse signalling, exerting regulatory effects on CRC tumour biology. A Kras/Erk/ADAM17 axis constitutively triggers Jag1-ICD nuclear accumulation, which favours tumour development, progression and chemoresistance through a non-canonical mechanism.

The experiments presented below have been conducted in collaboration with the lab of Prof. Diana Bellavia (Pelullo M. and Nardozza F.; Nicoletti C. for the procedures with nude mice; Besharat Z.M. for the bioinformatic analysis).

1.1 Jag1-ICD is expressed and localized into the nucleus of CRC cell lines

Based on the observation that *Jagged1* transcripts are overexpressed in a large number of human CRCs, while they are undetectable in the adjacent normal tissue (Dai et al., 2014; Guilmeau et al., 2010), we monitored the expression of *Jagged1* transcripts in several human CRC cell lines by qRT-PCR assays. Accordingly, we found a significant up-regulation of *Jagged1* mRNA in most CRC cell lines, compared to the normal colon cell line CCD-18Co (Fig. 1A), being HT29 and RKO cells the only exceptions. It is well demonstrated that the transmembrane Jag1-FL undergoes ADAM17-mediated ectodomain processing, resulting in the Jag1-ECD shedding, followed by PS/γ-secretase-dependent intramembrane proteolysis that releases an intracellular fragment (Jag1-ICD) (Ascano et al., 2003; De Falco et al., 2018; LaVoie & Selkoe, 2003; Pelullo et al., 2014). Intriguingly, here we provide the first evidence of a Jag1-FL aberrant processing in CRC cell lines, which ultimately results in the release of a remarkable amount of Jag1-ICD (Fig. 1B), able to translocate into the nucleus, as revealed by subcellular protein fractionation (Fig. 1C). Notably, as suggested by Supplementary Fig. S1, Jagged1 is strongly expressed/processed only in CRC cell lines presenting simultaneously APC-β-catenin/Kras mutations.

1.2 Jag1-ICD enhances CRC cells tumorigenicity via an intrinsic oncogenic activity

Since Jag1-ICD might have a role in tumour development and carcinogenesis (Ascano et al., 2003; Pelullo et al., 2014), we explored whether its overexpression might affect oncogenic properties of CRC cells. We found that Jag1-ICD ectopic expression in HCT15 cells (Supplementary Fig. S2A), which express low levels of endogenous Jagged1 (HCT15-V5-Jag1-ICD), determined a significant increase in cellular proliferation, as revealed by the MTT assay (Fig. 2A), induced an increased clonogenic capacity in soft agar colony formation assays (Fig. 2B) and sustained cell invasion activity *in vitro*, using transwell inserts (Fig. 2C). Interestingly, Jag1-ICD overexpression was also able to sustain CRC cells invasion/migration ability, as demonstrated by wound-healing assays (Fig. 2D). This was associated with an increased expression of invasion-related *snail* and *mmp9* genes, as revealed by qRT-PCR (Fig. 2E).

To further validate these *in vitro* results, we xenografted HCT15-V5-Jag1-ICD- or pcDNA3-Neo empty vector-transfected HCT15 cells, into nude mice. 27 days after injection, we found that Jag1-ICD expressing clones generated larger tumours when compared with control cells (Fig. 2F and G). Importantly, this was associated to an increased expression of *mmp9*, *snail1*, *snail2*, c*yclinD2* and *PCNA* transcripts in Jag1-ICD tumours, when compared with controls (Fig. 2H). In addition, Fig. 2I

shows a strong positivity for Jagged1 immunostaining in human primary colon cancer specimens and this is consistent with our preclinical data.

Overall, these data indicate that the constitutive expression of Jag1-ICD enhances the tumorigenic behaviour of CRC cells, suggesting that Jag1-ICD possesses an intrinsic oncogenic activity.

1.3 Jag1-ICD affects EMT directly controlling the expression of Snail1 and Snail2

So far, our results support an intrinsic oncogenic activity of Jag1-ICD, possibly impinging on an invasion/migration phenotype, which is typically associated with epithelial-mesenchymal transition (EMT). This is consistent with in silico analysis of a public dataset (Tsuji et al., 2012), which reveals increased Jagged1 expression in CRC metastatic patients compared to primary tumours (Fig. 3A). Since Phorbol 12-myristate 13-acetate (PMA) is known to support EMT with effects on cell migration and tumour formation in CRC cells (He et al., 2010), we assessed the potential role of Jag1-ICD in this context. Noteworthy, PMA-treated CRC cell lines readily acquired a spindle-shaped morphology consistent with mesenchymal transition (Fig. 3B), associated with a strong up-regulation of *snail1* and *snail2*, *Vimentin* and *N-cadherin* and a down-modulation of *E-cadherin* observed at the mRNA and/or protein levels (Fig. 3C and D). Interestingly, immunoblotting also revealed a time-dependent increase of cleaved Jag1-ICD in PMA-treated HCT15, SW948 and DLD1 cells (Fig. 3D). Altogether these observations support a correlation between Jag1-ICD accumulation and PMA-induced EMT in CRC cell lines. Indeed, siRNA-mediated Jag1 depletion (Supplementary Fig. S2B) significantly compromised the migratory activity of Jag1-silenced HCT15 cells both under basal (DMSO) (decreased by 40%) or PMA-induced conditions (decreased by 30%) (Fig. 3E) and significantly impaired *snail* mRNA expression (Supplementary Fig. S1C). We previously demonstrated that Jag1-ICD directly interacts with CSL/ RBP-Jκ transcription factor, sustaining its transcriptional activation (Pelullo et al., 2014). Sequence analysis of the human *snail1* and *snail2* promoters identified consensus CSL/RBP-Jκ-binding sites (Supplementary Fig. S2D). ChIP assays around these sites showed a significant recruitment of CSL/ RBP-Jκ and Jag1-ICD in PMA-treated HCT15 cells (Fig. 3F).

Overall, these findings demonstrate that nuclear accumulated Jag1-ICD directly controls the expression of EMT-related genes and the migratory activity of CRC cells, unveiling a tight link between aberrant Jagged1 processing and CRC aggressiveness.

1.4 Kras/Erk/ADAM17 signalling axis induces the constitutive activation of Jag1-ICD in CRC tumours

Jagged1 is a substrate of the catalytic activity of ADAM17 that allows the shedding of Jag1-ECD ectodomain, an obligatory step before the cleavage of Jag1-ICD by the PS/γ–secretase complex (LaVoie & Selkoe, 2003). It is known that PMA enhances ADAM17 shedding activity, by directly inducing Erk kinase phosphorylation and activation (Fan et al., 2003; Soond et al., 2005), which is an important prerequisite for ADAM17 triggering (Díaz-Rodríguez et al., 2002; Soond et al., 2005).

Firstly, to assess the phosphorylation status of ADAM17 upon PMA treatment in CRC cells, we carried out immunoprecipitation assays of endogenous proteins from DLD1 cell line. As shown in Fig. 4A, we revealed a rapid induction of Ser-phosphorylation on ADAM17 within 15 minutes of stimulation. Then, we investigated the effects of PMA on ADAM17 shedding activity, by monitoring Jagged1 cleavage in CRC cell lines. Interestingly, PMA treatment induced extensive Jagged1 processing, revealed by a significant increase of soluble Jag1-ECD and Jag1-ICD fragments in HCT15, LoVo, SW948 and DLD1 CRC cell lines with different expression levels of Jagged1, associated to an important Erk activation (Fig. 4B). Consistently, Erk inhibition via the U0126 antagonist strongly impaired Jagged1 processing, indicating that Jag1-ICD accumulation is Erk-dependent (Fig. 4C). Notably, ectopic Jag1-ICD, stably transfected in HCT15-V5-Jag1-ICD cells, is sufficient to revert the effect of U0126, as revealed by the sustained activation of EMT-linked target genes, as *snail* and *e-cadherin* (Supplementary Figure S2E and F). *In silico* analysis of a public dataset (Marisa et al., 2013), considering a large cohort of CRC patients, showed that increased expression of *Jagged1* transcripts is significantly associated to *Kras* mutation-bearing samples compared to *Kras* wt tumours (Fig. 4D). Moreover, it is reported that oncogenic *KRAS* is able to regulate ADAM17 activity in a MEK/ERK-dependent manner (Van Schaeybroeck et al., 2014). Interestingly, Jagged1 is strongly processed only in CRC cell lines bearing *Kras* mutations (Fig. 1B and Supplementary Fig. S1). These observations support the existence of a direct correlation between the aberrant activation of Kras/Erk pathway and the Jagged1 processing in CRC cells. To clarify this correlation, we investigated the status of Jagged1 protein in response to siRNA mediated Kras depletion in HCT15, SW948 and DLD1 CRC cell lines. Kras silencing resulted in a marked impairment of Jagged1 processing, revealed by a significant decrease in Jag1-ECD shedding and Jag1-ICD release, strongly suggesting a direct link between Kras activity and Jagged1 processing (Fig. 4E). Consistently, the overexpression of mutant *Kras* (pBabe Kras 12V), by retroviral infection of CCD18-Co cells (CCD18-Co-Kras cell line), causes a drastic change in cell morphology with the appearance of spindle-shaped cells, compared to empty backbone infected

cells (pBabe-Puro) (Fig. 4F). Notably, pERK was strongly induced by Kras in CCD18-Co-Kras cell line, which triggers Jag1-ICD release, compared to CCD18-Co-Puro cells (Fig. 4G, left panel). Of note, the Kras-induced Jag1-ICD processing was inhibited by TAPI-2 compound, a specific inhibitor of matrix metalloproteinase and TACE (TNF-α convertase/ADAM17/α-secretase) (Fig. 4G, right panel).

Altogether these results highlight a Kras/Erk/ADAM17/Jagged1 signalling axis in CRC cells, whereby Kras activation leads to Erk-ADAM17-dependent Jagged1 cleavage resulting in the nuclear accumulation of Jag1-ICD.

1.5 Pharmacological inhibition of Jag1-ICD activation impairs proliferation and invasiveness of Krasmut CRC cells

To explore the role of Jag1-ICD in sustaining the tumorigenic potential of CRC cells, we abrogated constitutive Jagged1 cleavage in HCT15 cells by using the TAPI-2 compound, which is able to inhibit ADAM17 activity (Fig. 5A, left panel). TAPI-2 treatment impaired HCT15 cell growth by 40%, as determined by trypan blue cell counting (Fig. 5A, right panel), associated to a G0/G1 cell cycle arrest (Fig. 5B). This was associated to the decrease of the endogenous *cyclin D2* and *PCNA* transcripts, as revealed by qRT-PCR, in TAPI-2-treated when compared to control cells (Fig. 5C). Consistent with previous results, the inhibition of Jag1-ICD release by TAPI-2 significantly decreased HCT15 invasiveness through the matrigel (56%) (Fig. 5D) and reduced the expression of invasion-related transcripts such as *mmp9*, *snail1* and *snail2* (Fig. 5E). Additionally, we carried out wound-healing assays to determine the biological effect of Jag1-ICD on the migration capability of HCT15 cells, treated with PMA alone or co-treated with TAPI-2 compound (Fig. 5F). Notably, treatment with PMA alone strongly determined a time-related increased motility (increased by 40%), when compared to control. Intriguingly, PMA effect is delayed in presence of TAPI-2 compound (decreased by 30%), which counteracts the PMA-induced Jag1-ICD shedding, indicating that Jag1-ICD release is required for CRC cell migration. Of note, TAPI-2 treatment does not have any impact on HCT15-V5-Jag1-ICD cells, expressing Jag1-ICD constitutively, revealed by sustained expression levels of EMT-target genes (Supplementary Fig. S2 G and H). To investigate the effects of Jag1-ICD on colon cancer *in vivo*, DLD1 cells were injected subcutaneously into the flanks of nude mice, which were treated with TAPI-2 or control vehicle. The results in Fig. 5G and H show that tumour volume was clearly decreased in TAPI-2-treated with respect to control mice.

Western blot results from tumour xenografted samples showed that the expression levels of Jag1-ICD were markedly decreased in samples obtained from mice treated with TAPI-2 (Fig. 5I).

Altogether these results indicate that Jag1-ICD plays a role in regulating malignant features, such as proliferation and invasion/migration ability in CRC cell lines both *in vitro* and *in vivo*.

1.6 Jag1-ICD activation confers chemoresistance in Krasmut CRC cells

Kras mutation is an important predictor of drug resistance in several cancers and is associated with a worse prognosis (Lièvre et al., 2006). Notably, it is reported that chemotherapy results in a significant increase of ADAM17 activity and growth factors shedding, which determine drug resistance in Krasmut CRC tumours (Kyula et al., 2010; Van Schaeybroeck et al., 2011). Chemoresistance is often associated to acquisition of EMT (Zhang et al., 2012), the phenotype induced in CRC cells by the Kras/Erk/ADAM17/Jag1-ICD axis that we described above. Interestingly, it is reported that high Jagged1 expression levels, combined with low E-cadherin expression, in cancer cells of CRC patients are correlated with poor prognosis, poorer survival rate and increased risk of recurrence (Sugiyama et al., 2016). Overall these observations allowed us to speculate about a direct link between an enforced Jag1-ICD shedding and the acquisition of resistance. In keeping with this hypothesis, stable Jag1-ICD overexpression in HCT15 cells was sufficient to confer resistance to 5FU and Irinotecan agents, as revealed by a sustained survival rate in CRC cells, with respect to untrasfected cells (Fig. 6A). To explore the possibility that the resistance to 5FU and/or Irinotecan may depend on Jag1-ICD, we tested the impact of both chemotherapeutic agents on Jagged1 processing. Surprisingly, treatment of HCT15 cells with 5FU (Fig. 6B) or Irinotecan (Fig. 6C) for 24 hours increased the release of Jag1-ICD in a dose-dependent manner, associated to an increased phosphorylation status of Erk and ADAM17 (Fig. 6B and C) and correlated with the modulation of the EMT-specific markers *mmp9*, *snail1*, *snail2* and *E-cadherin* (Fig. 6D). Notably, 5FU- or Irinotecan-induced Jag1-ICD processing was significantly decreased by TAPI-2 (Fig. 6E) or by U0126 (Fig. 6F) compounds. Interestingly, Supplementary Fig. S3 shows that the effects above described are also observed in DLD1, a cell line with high expression levels of endogenous Jag1-ICD. To confirm such *in vitro* results, we xenografted DLD-1 cells into nude mice, treated with 5FU or U0126 alone or in combination (5FU/U0126) and the tumour growth was measured along the time (Fig. 6G). A significant reduction of Jag1-ICD levels was observed in tumours treated with U0126 alone, associated to a drastic reduction of tumour growth (Fig. 6H and I), with respect to vehicle-treated mice. As expected, no significant difference was found in the tumour size from mice treated with 5FU alone, which further increases the release of endogenous

Jag1-ICD, when compared to control mice (Fig. 6G-I), sustaining the idea that 5FU is able to induce CRC resistance by inducing Jag1-ICD shedding. Based on the compelling *in vivo* evidences, U0126 is not able to completely counteract the 5FU-dependent Jag1-ICD increase in tumours from mice with a combined treatment, 5FU/U0126 (Fig. 6G-I).

Overall these data demonstrate that 5FU and Irinotecan are able to strongly sustain the Jagged1 processing, by triggering the Erk/ADAM17 axis, which results in the release of the Jag1-ICD oncogenic fragment, able to confer chemoresistance to CRC, both *in vitro* and *in vivo*.

Figures and Supplementary

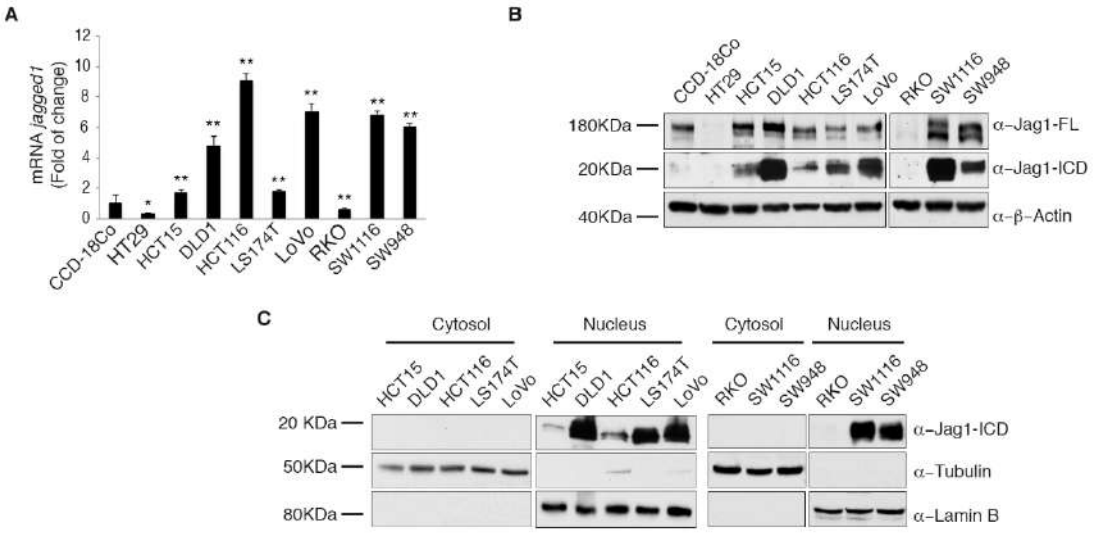


Figure 1. Jagged1 expression and constitutive processing in colorectal cancer (CRC) cell lines.
A, qRT-PCR analysis of *jagged1* gene expression in normal colon (CCD-18Co) and several CRC cell lines. Gene expression normalized relative to human *GAPDH* and depicted as fold change to CCD-18Co. Data are presented as mean ± SD. *, P<0,05; **, P<0,01 (Student's t-test). **B,** Representative immunoblots of Jag1-FL and Jag1-ICD in WCE of CRC cell lines. Protein levels normalized relative to β-Actin. **C,** Representative immunoblots of Jag1-ICD protein translocation to the nucleus. Protein levels normalized relative to Lamin B in the nuclear fraction and α-Tubulin in the cytoplasmatic fraction. All data are representative of at least three independent experiments, each in triplicate.

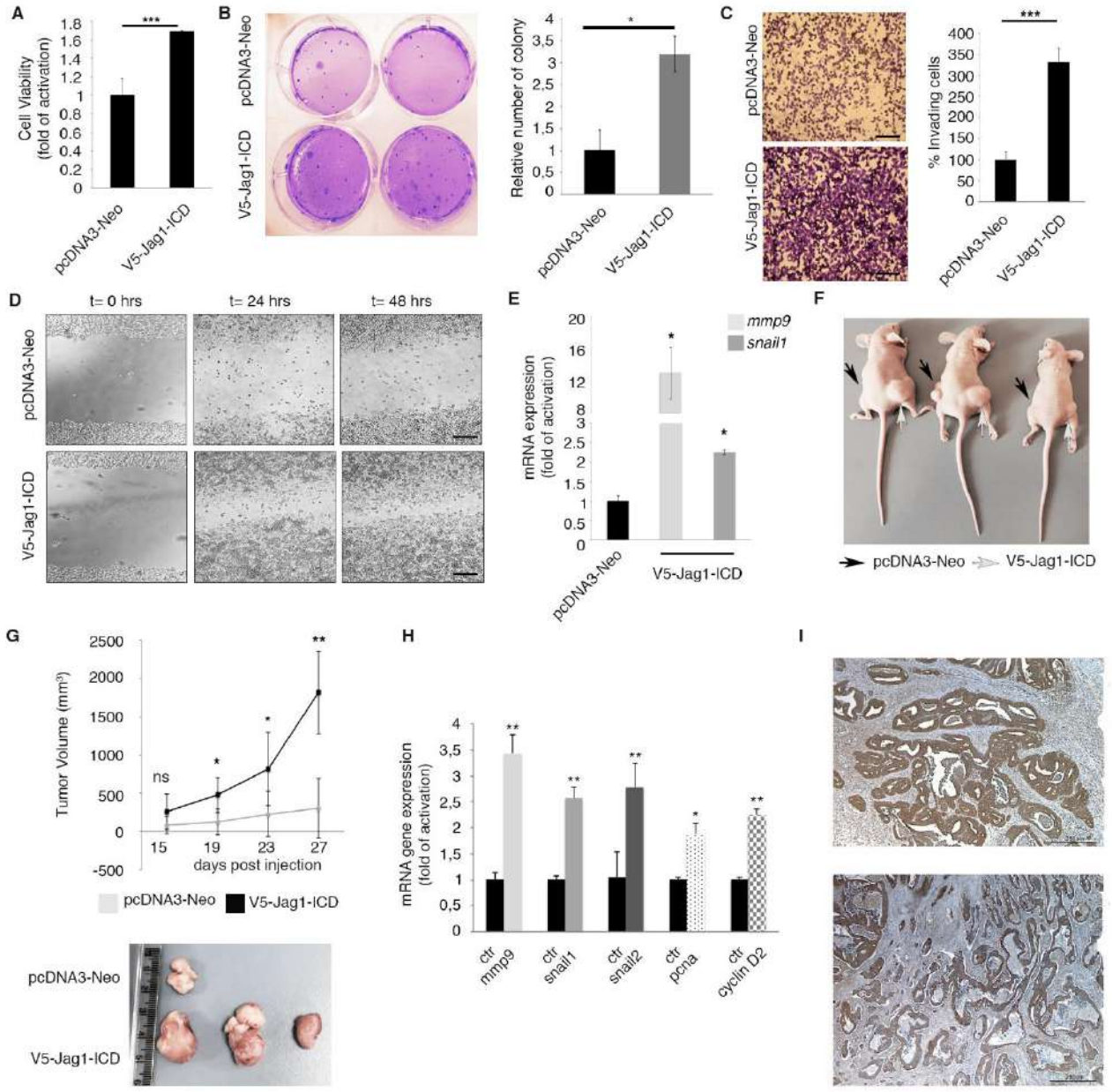


Figure 2. Jag1-ICD is required to strengthen tumorigenic behaviour of CRC.
HCT15 cells stably expressing the intracellular domain of Jag1 (V5-Jag1-ICD) or control vector (pcDNA3-Neo) were used *in vitro* and *in vivo* experiments. **A,** Cell viability of HCT15 V5-Jag1-ICD and HCT15 pcDNA3-Neo analysed by MTT assay and graphed as fold changes ± SD *versus* control. **B,** Representative image of HCT15-V5-Jag1-ICD and control, after performing soft agar assay and subsequently crystal violet staining (left panel). The number of colonies is graphed as fold of changes ± SD *versus* control (right panel). **C,** Matrigel assay for HCT15-V5-Jag1-ICD and control (left panel). The number of invading cells is graphed as percentage of total cells (right panel). Scale bar: 50 μm. **D,** Representative area for wound-healing assay of HCT15-V5-Jag1-ICD cells respect to the negative control shown after 24 and 48 hours of scratch. Scale bar: 200 μm. **E,** qRT-PCR analysis of *mmp9* and *snail1* mRNA in HCT15-V5-Jag1-ICD cells compared to control. Data are reported as fold changes ± SD after intrasample normalization to the level of *GAPDH*. **F,** Representative group of CD1/nude mice used for xenograft tumour formation deriving from subcutaneous flank injection of $1x10^7$ stably transfected HCT15 V5-Jag1-ICD or control cells, at the end point of the experiment. **G,** The volume measure of xenografted tumours derived from F is graphed (upper panel). Representative tumour masses derived F (lower panel). **H,** RNA extracted from snap-frozen xenografts from G and analysed by qRT-PCR for the expression of cell proliferation markers (*PCNA*, *cyclinD2*) and metastatic markers (*mmp9*, *snail1*, *snail2*). **I,** Representative histologic pictures of human colonic cancers showing strong positivity of immunohistochemistry for Jagged1. Data are reported as fold changes ± SD after intrasample normalization to the level of *GAPDH*. All data are representative of at least three independent experiments, each in triplicate. *, P<0,05; **, P<0,01; ***, P<0,001.

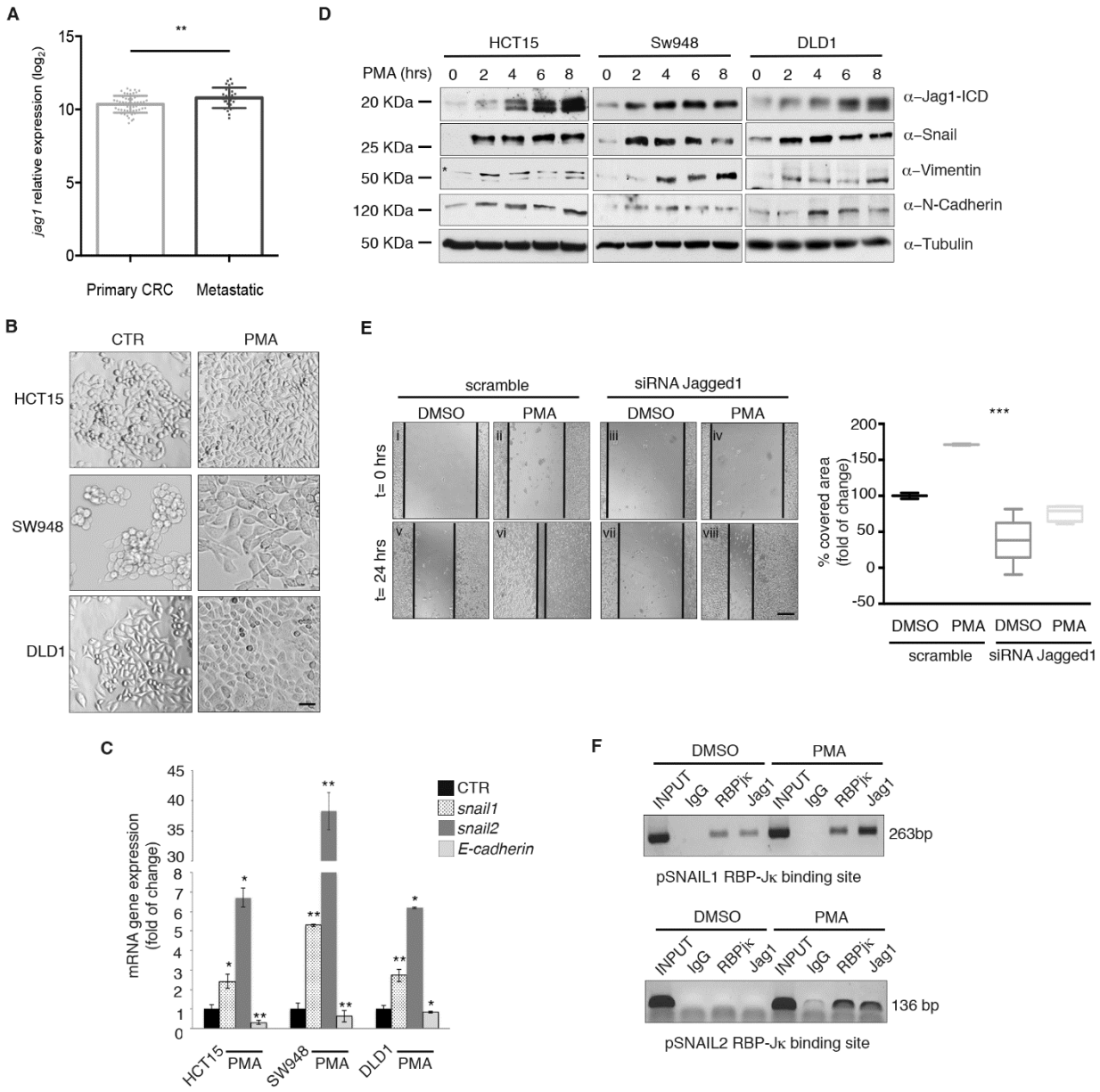


Figure 3. PMA-dependent Jagged1 activation induces EMT.
A, *jagged1* gene expression levels in primary and metastatic CRC patients by an *in silico* analysis using the probe set 209099_x_at, in a cohort of 83 CRC patients (metastasis n=27, primary n=56, GEO ID: gse28702). Data are presented as log2 scale. Each dot represents a patient. *, P<0,05; ***, P<0,001 (Student's t-test one-way ANOVA). **B,** HCT15, SW948 and DLD1 cells are treated with PMA or DMSO for 4 hours. Representative picture of plate area shows the tapered shape in PMA-treated cells respect to control. Scale bar: 20 μm **C,** qRT-PCR analysis of *snail1*, *snail2* and *E-cadherin* mRNAs expression in PMA-treated cells. Data are reported as fold changes ± SD *versus* DMSO control and normalized against the level of *GAPDH*. *, P<0,05; **, P<0,01 (Student's t-test). **D,** Representative Western blots of Jag1-ICD, Snail, Vimentin and N-Cadherin in PMA-treated cells along a time course. Protein levels normalized relative to α-Tubulin. **E,** Representative picture of plate area for wound-healing assay shown after 24 hours of scratch in HCT15 cells silenced for Jagged1 or scramble control, upon PMA treatment (left panel). The dash lines show the front. Scale bar: 200 μm. The percentage of covered scratched area was graphed as mean ± SD for each group of treatment (right panel). ***, P<0,001 (Student's t-test one-way ANOVA). **F,** Chromatin immunoprecipitation (ChIP) of endogenous Jag1-ICD and RBP-Jκ from HCT15 cells treated or not with PMA for 4 hours, followed by PCR analysis for *Snail1* promoter (pSnail1) and *Snail2* promoter (pSnail2). All data are representative of at least three independent experiments, each in triplicate.

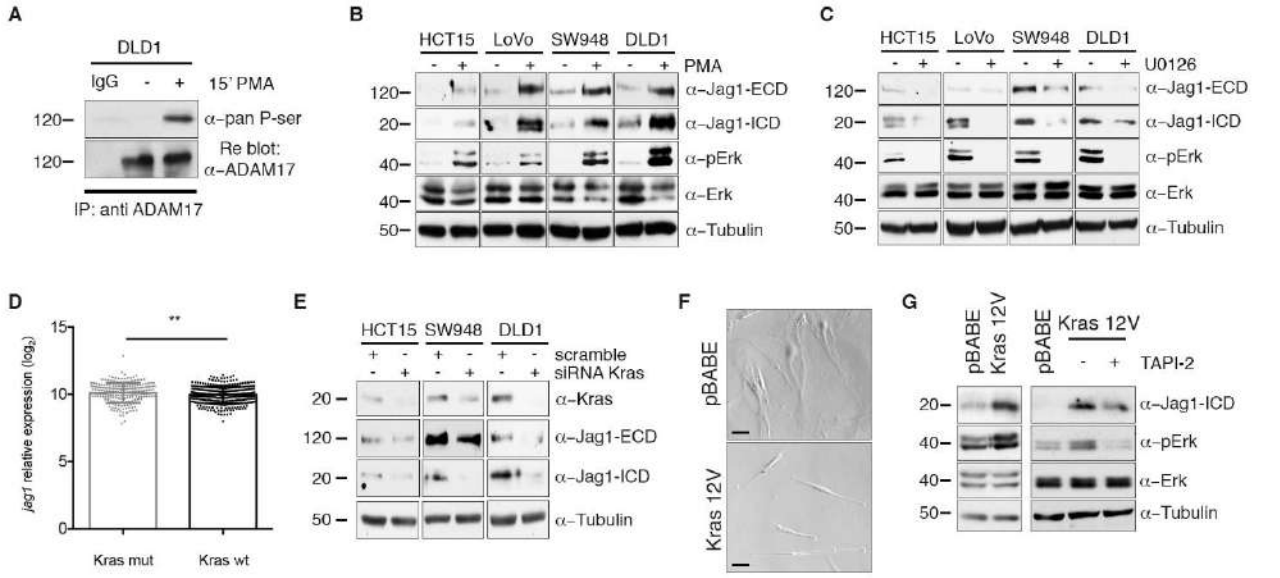


Figure 4. Kras-mediated ADAM17 activity triggers a constitutive Jagged1 processing.
A, Representative immunoblots of pan-phospho-serine for ADAM17-immunoprecipitated in DLD1 cells, treated with PMA or control, for 15'. Protein levels normalized to total ADAM17. **B and C**, Representative Western blots of Jag1-ECD, Jag1-ICD, pErk and total Erk in HCT15, LoVo, SW948 and DLD1 cells, treated with PMA (**B**), U0126 (**C**) or control for 4 hours. Protein levels normalized relative to α-Tubulin. **D,** *jag1* gene expression levels obtained by an *in silico* analysis, using the probe set 209099_x_at, in a cohort of 545 CRC patients ($Kras^{mut}$ n=217, $Kras^{wt}$ n=328, GEO ID: gse39582). Data are presented as log2 scale. Each dot represents a patient. **, P<0,01; (Student's t-test one-way ANOVA). **E,** Representative immunoblots of Kras, Jag1-ECD and Jag1-ICD in HCT15, SW948 and DLD1 cell lines transiently transfected with Kras siRNA or scramble control for 48 hours. Protein levels normalized relative to α-Tubulin. All data are representative of at least three independent experiments, each in triplicate. **F,** Representative picture of plate area shows the tapered shape in CCD18-Co infected with pBABE-KRAS 12V than negative control (pBABE). Scale bar: 10 μm **G,** Representative immunoblots of Jag1-ICD and pERK in CCD18-Co infected with pBABE-KRAS 12V or negative control (pBABE) (left panel) after 48 hours of TAPI-2 treatment (right panel). Protein levels normalized relative to α-Tubulin.

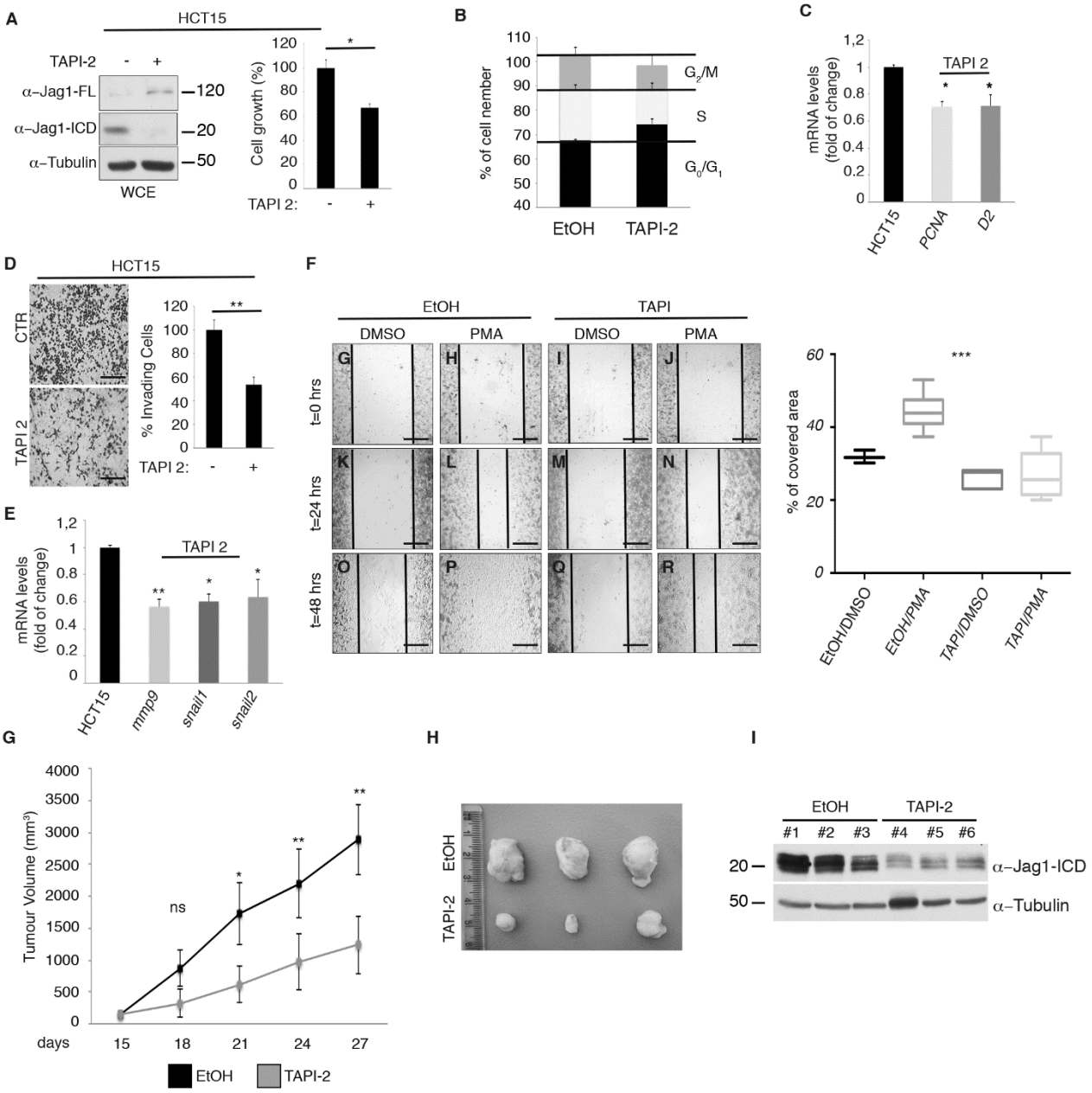


Figure 5. Jag1-ICD sustains CRC proliferation and invasion.

The HCT15 cell line was treated with 50 μM of TAPI-2 or vehicle for 48 hours. **A**, Representative immunoblots of Jag1-FL and Jag1-ICD used as control for TAPI-2 treatment. Protein levels normalized relative to α-Tubulin (left panel). Cell growth of HCT15 treated or not with TAPI-2 graphed after trypan blue staining. Quantification depicted as percentage of total cell population ± SD (error bars) of three independent experiments performed in triplicate. **B**, Histogram shows the percentage of HCT15 cells treated with TAPI-2 or EtOH, in G_0/G_1-S-G_2/M cell cycle phases. **C**, qRT-PCR analysis of *PCNA* and *cyclin D2* mRNA in HCT15 cells treated with TAPI-2 compound compared to control. Gene expression depicted as fold change to vehicle alone after intrasample normalization to the level of *GAPDH*. **D,** Matrigel assay for HCT15 treated with TAPI-2 or CTR (left panel). Scale bar: 50 μm. The number of invading cells is graphed as percentage of total cells (right panel). **E**, qRT-PCR analysis of *mmp9*, *snail1* and *snail2* mRNA showing their reduction in HCT15 cells treated with TAPI-2. Gene expression depicted as fold change to vehicle alone after intrasample normalization to the level of *GAPDH*. **F**, Representative picture of plate area for wound-healing assay shown after 24 and 48 hours of scratch in HCT15 cells treated with TAPI-2 compound, PMA or combination. The dash lines show the front. Scale bar: 200 μm. (left panel). The percentage of covered scratched area after 48hrs was graphed as mean ± SD for each group of treatment (right panel). **G,** The volume measure of xenografted tumours derived from $2x10^6$ DLD1 injected in the posterior flank of CD1/nude mice treated with vehicle control or TAPI-2 is graphed. **H,** Representative tumour masses derived from G. **I,** WCE derived from H were immunoblotted for Jag1-ICD. The amount of total extracts normalized respect to the α-Tubulin. All data are representative of at least three independent experiments, each in triplicate. *, P<0,05; **, P<0,01; ***, P<0,001

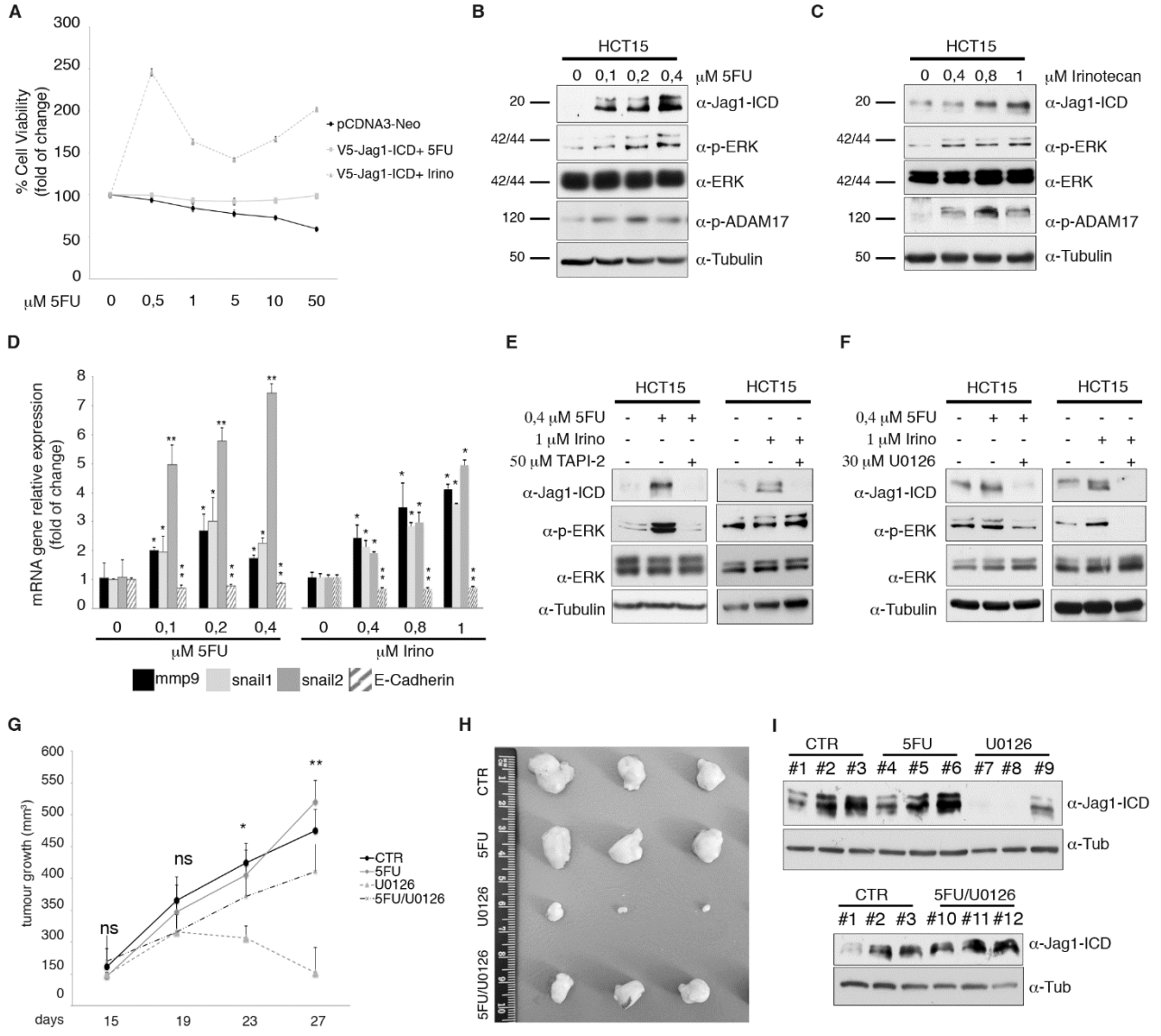
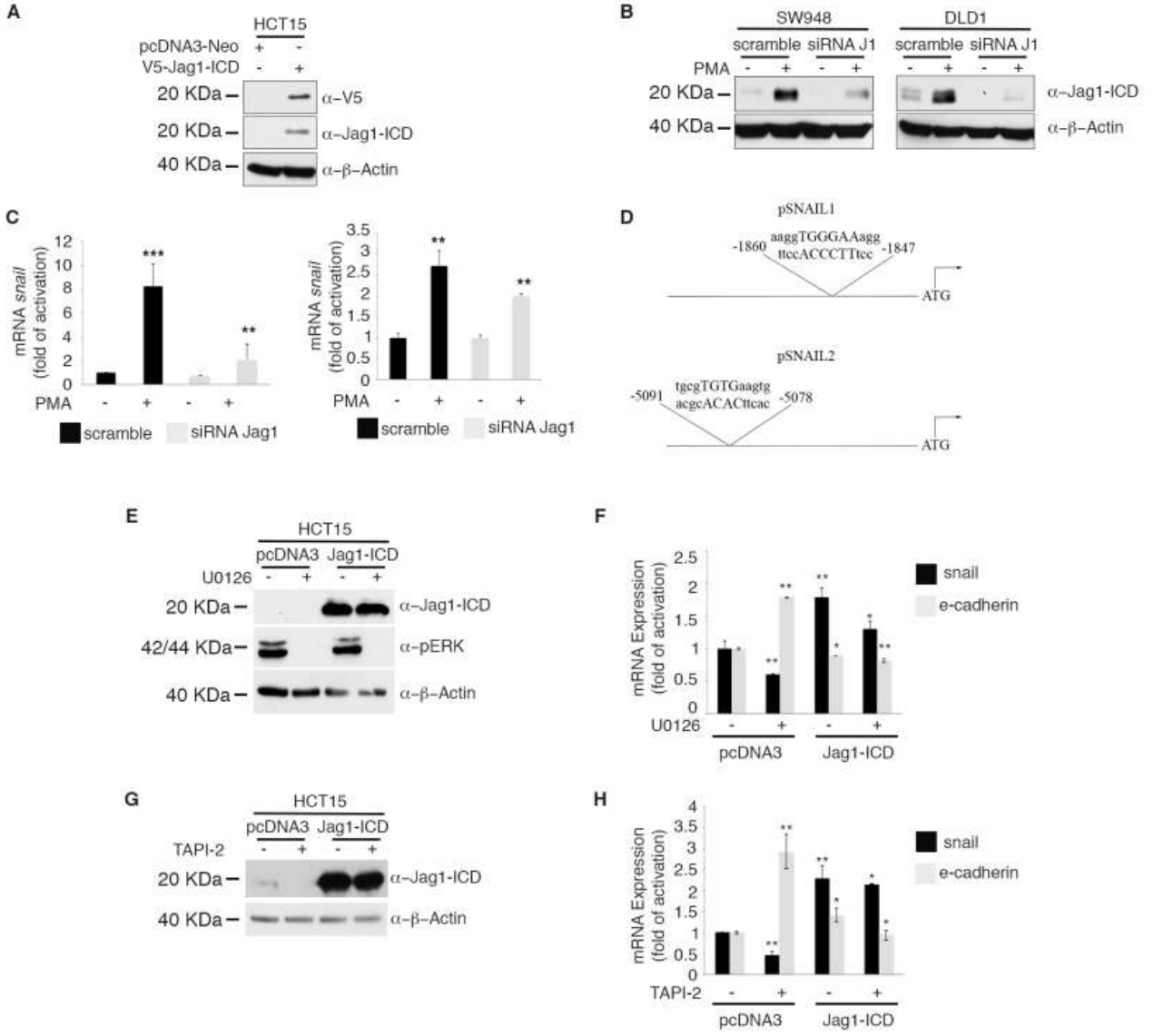


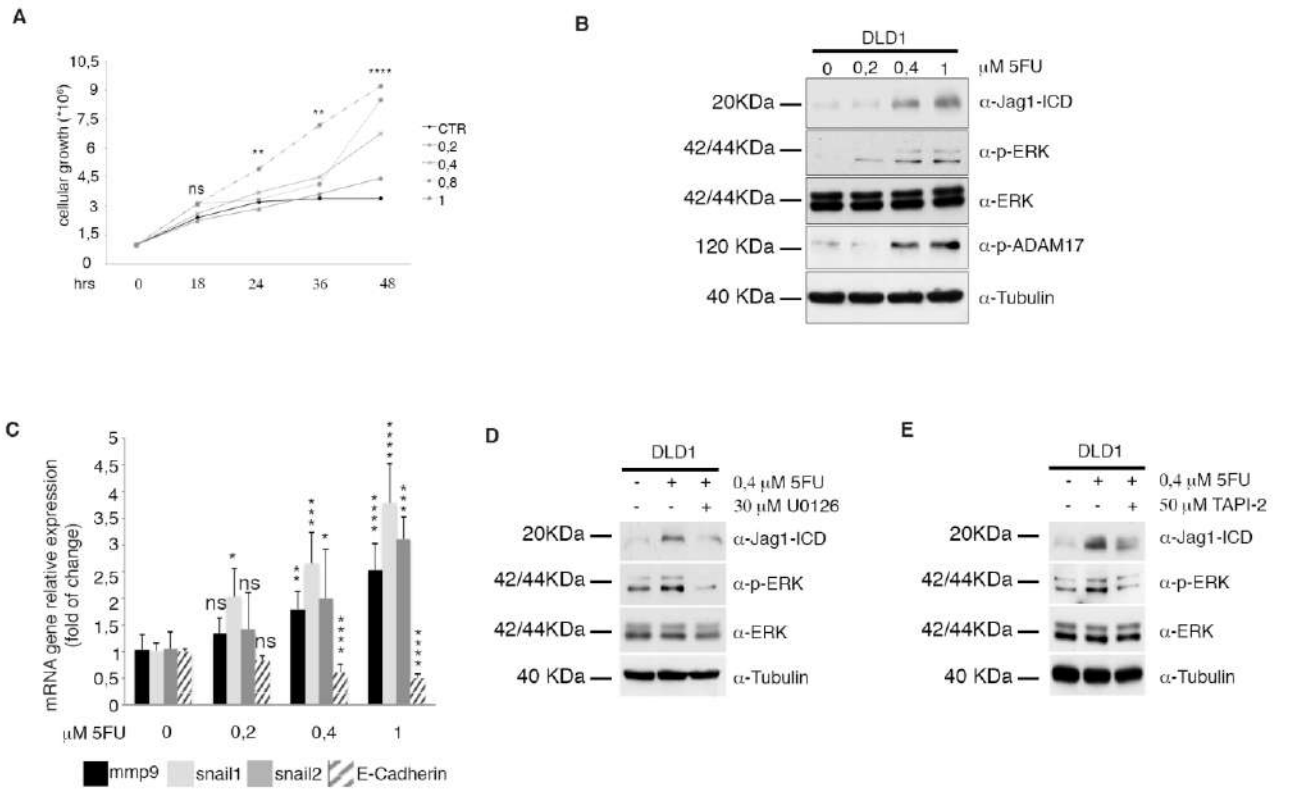
Figure 6. Jag1-ICD confers 5FU/Irinotecan resistances in CRC.
A, Proliferation rate of HCT15 cells stably expressing the intracellular domain of Jag1 (V5-Jag1-ICD) or control vector (pcDNA3-Neo) treated with an increasing amount of 5FU or Irinotecan. **B and C**, Representative immunoblot of Jag1-ICD, pERK, total ERK and pADAM17 in WCE derived from HCT15 cells treated or not with an increasing amount of 5FU (B) or Irinotecan (C) for 24 hrs. The protein levels normalized respective to α-Tubulin. **D**, qRT-PCR of HCT15 cell line derived from B and C shows the modulation of *mmp9*, *snail1*, *snail2* and *E-Cadherin* genes. Data are reported as fold changes ± SD after intrasample normalization to the level of *GAPDH*. **E and F,** Representative Western blot of Jag1-ICD, pERK and total ERK in WCE derived from HCT15 cells treated with 5FU or Irinotecan alone or in combination with TAPI-2 (E) or U0126 (F). **G**. The volume measure of xenografted tumours derived from $2x10^6$ DLD1 injected in the posterior flank of CD1/nude mice treated with vehicle control, 5FU, U0126 or combination. **H,** Representative tumour masses derived G. **I.** WCE derived from H were immunoblotted for Jag1. The amount of total extracts normalized respect to the α-Tubulin. All data are representative of at least three independent experiments, each in triplicate. *, P<0,05; **, P<0,01; ***, P<0,001.

CRC cell line	Kras	Braf	APC	CTNNB1
HT-29	wt	c. 1799T>A	c.2557G>T; c.4666_4667insA	wt
HCT15	c.38G>A	wt	c.4248delC; c.6496C>T	wt
DLD1	c.38G>A	wt	c.4248delC	wt
HCT116	c.38G>A	wt	wt	c.133_135delTCT
LS174T	c.35G>A	wt	wt	c.134C>T
LoVo	c.38G>A	wt	c.3340C>T; c.4290delC	wt
RKO	wt	c. 1799T>A	wt	wt
SW1116	c.35G>C	wt	c.790C>T; c.4287_4296delA ACCATGCCA	wt
SW948	c.182A>T	wt	c.3340C>T; c.4285C>T	wt

Supplementary Figure S1. Nature of Kras, Braf, APC and CTNNB1 mutations in CRC cell lines.



Supplementary Figure S2. Jagged1 affects EMT-related genes expression.
A, WCE derived from HCT15 cells stably transfected with V5-Jag1-ICD or negative control (pcDNA3) analysed by Western blots assay for Jagged1-ICD and V5-tag. **B,** Representative immunoblots of Jag1-ICD in WCE from SW948 (left panel) and DLD1 (right panel) cells after 48 hours of silencing for Jagged1 or scramble control. The cells were treated with PMA or vehicle alone for 4 hours. Protein levels normalized relative to β-Actin. **C**, qRT-PCR analysis of *snail* gene expression in Jagged1-silenced cells compared with scramble, upon PMA treatment. The data are presented as fold change respect to DMSO and graphed after intrasample normalization respect to the GAPDH. **, P<0,01; ***, P<0,001. **D**, Schematic representation of putative RBP-Jk binding site on *Snail1* and *Snail2* promoters ranging between 1860/-1847 bps and -5091/-5078 bps respectively upon ATG start site. All data are representative of at least three independent experiments, each in triplicate. **E and G,** Representative immunoblots of Jag1-ICD and pERK in HCT15 cells stably transfected with V5- Jag1-ICD or negative control (pcDNA3) treated with U0126 (E) or TAPI-2 (G) or vehicle alone. Protein levels normalized relative to β-Actin. **F and H,** qRT-PCR analysis of *snail* and *e-cadherin* gene expression in cells derived respectively from E and G. The data are presented as fold change respect to DMSO and graphed after intrasample normalization respect to the GAPDH. *, P<0,05, **, P<0,01.



Supplementary Figure S3. Jag1-ICD confers 5FU resistance in DLD1 cells, a CRC cell line with high levels of endogenous Jagged1.

A, Proliferation rate of DLD1 cells treated with increasing doses of 5FU, for the indicated time (hrs). **B,** Representative immunoblots of Jag1-ICD, pERK, total ERK and pADAM17 in WCE derived from DLD1 cells, treated or not with an increasing amount of 5FU. **C,** DLD1 cell line treated or not with 5FU shows the modulation of *mmp9*, *snail1*, *snail2* and *E-Cadherin* genes by qRT-PCR. Data are reported as fold changes ± SD after intrasample normalization to the level of *GAPDH*. **D and E,** Representative Western blot of Jag1-ICD, pERK and total ERK in WCE derived from DLD1 cells treated for 18 hours with 5FU alone or in combination with U0126 (D) or TAPI-2 (E). The protein levels normalized respective to α-Tubulin. All data are representative of at least three independent experiments, each in triplicate. *, P<0,05; **, P<0,01; ***, P<0,001; ****,P<0,0001.

3. Discussion

The Notch ligand Jagged1 is up-regulated in a large number of cancers, where it plays a key role in cell growth, EMT and metastatic process (Santagata et al., 2004). An increased expression of Jagged1 has been identified in about 50% of human CRC (Guilmeau et al., 2010) where it has been correlated with poor prognosis and recurrence (Sugiyama et al., 2016). To date, the most widely accepted scenario suggests that the increased expression of Jagged1 ligand identified in CRC triggers an over-activation of Notch signalling (Dai et al., 2014; Kim et al., 2013). However, Jagged1 may be processed in a fashion similar to Notch receptor, ultimately resulting in the release of the nuclear-targeted intracellular domain Jag1-ICD, thus triggering a reverse signalling (LaVoie & Selkoe, 2003; Pelullo et al., 2014). Herein, we demonstrate that Jag1-ICD is able to empower the Kras-mediated oncogenic signalling, by sustaining features of malignancies, tumour-cell invasion, migration and resistance to chemotherapy.

Previous data revealed that more than one oncogenic "driver" is deregulated in CRC tumours (Frattini et al., 2004). Mutations in the Wnt pathway cause colon cancer through constitutive activation of the β-catenin/TCF transcription complex (Bertrand et al., 2012). Recent reports have shown that β-catenin/TCF is responsible of a direct regulation of Jagged1 expression, which is required for tumorigenesis in the intestine (Rodilla et al., 2009). In addition, gain-of-function mutations in *RAS* gene are present in approximately 50% of colon cancers (Frattini et al., 2004; Mologni et al., 2012). Notably, oncogenic Kras signalling increases the β-catenin stability, by modulating its phosphorylation at serine 552 (Fang et al., 2007). Interestingly, increasing evidence suggests that the oncogenic *Kras* mutations control ADAM17 activity and growth factor shedding, *via* regulation of MEK/Erk/Adam17 signalling axis (Van Schaeybroeck et al., 2011). These results are supported by the observation that Erk activation phosphorylates and associates with ADAM17 (Díaz-Rodríguez et al., 2002; Van Schaeybroeck et al., 2014). In agreement with these data, we provide the first evidence that Krasmut CRC cells specifically show increased expression of Jagged1, which is constitutively processed by ADAM17, in a Kras-dependent manner. Of note, we show on one side that Kras-silencing attenuates significantly the Jag1-ICD release and on the other side that Kras ectopic expression directly empowers the Jagged1 cleavage, supporting the idea that Jagged1 processing is a novel substrate of Kras signalling in CRC cells. Here, we demonstrate that the constitutive Jagged1 cleavage observed in CRC cells is dependent upon Erk activation, able to phosphorylate ADAM17, as revealed by PMA stimulation or on inhibition of Erk activity with U0126 compound, both *in vitro* and *in vivo* experiments. Noteworthy, the aberrant PMA-induced Jag1-ICD release is associated to a marked increase of EMT markers Snail, Vimentin, N-cadherin

and E-cadherin. On the other side, TAPI-2-mediated ADAM17 inhibition correlates with different biological outcomes, including significant decrease of cell growth and reduction of migration and invasion phenomena, both *in vitro* and *ex viv*o, in tumour xenografts experiments. Previous observations suggest that Jag1-ICD may directly interact with RBPJ transcription factor (Pelullo et al., 2014). For the first time, we demonstrate that Jag1-ICD is able to trigger an intrinsic reverse signalling by regulating snail1 and snail2 promoter activity, via CSL/RBPJ Moreover, pre-clinical studies, performed by using HCT15-V5-Jag1-ICD xenografts experiments, sustain the idea that the persistent expression of Jag1-ICD plays an oncogenic function also in *in vivo* models.

Interestingly, *Kras* mutations are often associated with a CRC worse prognosis (Lièvre et al., 2006; Van Schaeybroeck et al., 2014; Van Schaeybroeck et al., 2011). Of note, Kras status has been correlated with Jagged1 expression in CRC patients and associated with a poorer survival rate and increased risk of recurrence, characterized by low cadherin expression and the induction of EMT, but the molecular mechanism is unknown (Sugiyama et al., 2016). In addition, it has been reported that current chemotherapy acutely activates ADAM17 that plays an important role in drug resistance in CRC tumours (Kyula et al., 2010; Van Schaeybroeck et al., 2014). Emerging evidence associates chemoresistance with the development of an EMT-like phenotype in cancer cells (Creighton et al., 2009), suggesting that EMT, metastasis and chemoresistance are closely related each other in tumour progression (Zhang et al., 2012). In accordance with these observations, we show that the 5FU or Irinotecan treatments increase the endogenous Jag1-ICD release, via Erk phosphorylation, *in vitro* or in xenografts experiments and are able to induce EMT, as revealed by modulation of endogenous specific markers. Therefore, our data indicate that the constitutive processing of Jagged1, induced by 5FU- or by Irinotecan, could be a crucial event correlated with increased risk of recurrence, poor outcome and resistance to chemotherapy of Krasmut CRC.

In conclusion, we provide evidence that Jagged1 is not only abundantly expressed but is also constitutively processed in CRC Kras molecular subtype tumours, via a Kras/Erk/ADAM17 pathway. The release of Jag1-ICD, in turn is able to empower the oncogenic Kras signalling pathway, *via* a novel mechanism, which sustains invasion and contributes to chemoresistance. Therapies targeted at this definite pathway may provide a novel method to sensitize and/or to disrupt the resistance mechanism of Kras-mutated CRC to chemotherapy, to finally improve overall tumour control and reduce tumour recurrence.

4. Materials and Methods

Animals

The six-week-old female CD1 nude mice were purchased from Charles River Laboratories Italia s.r.l. and were housed in the Institute's Animal Care Facilities.

All animal experiments were approved by local ethic authorities and conducted in accordance with Italian Governing Law (D.Lgs. n.26/2014/ Protocol Number: C1368.4) and European Directive 2010/63/UE

Cell lines and treatments

The following human colon cell lines CCD18-Co (CRL-1459TM), HT29, HCT15, DLD1, HCT116, LS174T, LoVo, RKO, SW1116 and SW948 were purchased from ATCC. Cell lines were subjected to routine cell line quality controls (e.g., morphology, Mycoplasma #G238, Abm Inc., Vancouver, CA) and authenticated by DNA profiling (short tandem repeat, STR) by the cell bank prior to shipping. The culture media were supplemented with 1% Glutammine (ECB3000D, Euroclone), 1% Antibiotics (ECB3001D, Euroclone) and 10% regular FBS (Heat-Inactivated; Life Technologies, Carlsbad, CA, USA). The media were renewal 2-3 times per week. Cells recovered from frozen aliquots were allowed one passage to reach exponential growth phase following recovery before being used. Cells at passages greater than ten were not used.

An opportune amount of cells was treated with different compounds: 50 µM TAPI-2 (# 55123-66-5; Peptides International Inc, Jefferson Town, Kentucky, USA), 200 ng/ml of Phorbol 12-myristate 13-acetate (#P8139, Sigma Aldrich, St.Louis, Missouri, USA), 30 µM di U0126 (#662005, Calbiochem, San Diego, CA), with 5-Fluorouracil (5FU) (#F6627, Sigma Aldrich, St. Louis, Missouri, USA) or Irinotecan (#134760, Sigma Aldrich, St. Louis, Missouri, USA).

Cell-cycle cytofluorimetric analysis

$1x10^6$ HCT15 cells, treated with TAPI-2 compound or vehicle alone, were fixed for 30' in EtOH 70%, washed in PBS, treated with 100µg/ml RNase A (cat. #R6513, Sigma-Aldrich, St. Louis, MO, USA) for 15' and then incubated with 10µg/ml Propidium Iodide (cat.#P4170) for 30'. The stained cells were analysed on a FACS-Calibur with CellQuest software (BD Biosciences, San Jose, CA, USA) (Antonio F Campese et al., 2009).

Plasmid construct and generation of stable cell lines

For generating cell lines stably overexpressing Jag1-ICD, murine Jag1-ICD cDNA was amplified by RT-PCR (Supplementary Table S1) and cloned into pcDNATM 3.1/V5-His TOPO TA Expression Kit (#KJ48001-01, Invitrogen by Life Technologies, Carlsbad, CA, USA) by following the manufacturer's instructions. V5-Jag1-ICD plasmid or pcDNA3-Neo was used to transfect HCT15 cell line using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. 48 hours post-transfection, the cells were cultured in selection medium containing 800ng/ml Neomycin (#A1720, Sigma-Aldrich, Saint Luis, MO, USA) for 4 weeks. pBABE-PURO (#1764) and pBABE K-RAS 12V (#12544) retroviral constructs were purchase from AddGene. Phoenix packaging cells were transfected with retroviral vectors by Lipofectamine 2000. After 48 hours of incubation at 32°C, the supernatants containing viral particles were collected and infection of CCD18-Co cells was performed, by using a 2 μgr/ml of Polybrene. Stable clones were obtained by using 1,5 μgr/ml for Puromycin for one week.

RT-PCR/q RT-PCR

Total RNA extraction and reverse transcription-PCR (RT-PCR) were previously described (S Cialfi et al., 2013; Colicchia et al., 2017). 1 μg of RNA was processed for RT-PCR using SensiFAST™ cDNA Synthesis Kit (Bioline, Taunton, MA, USA). Analysis of gene expression was realized by qPCR using Taq-Man designed assays (Supplementary Table S1) (Dharmacon Inc., Lafayette, Carlsbanb, CO, USA) on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), following the manufacturer's protocol for the comparative CT method. Data were analysed by the ΔΔCt method and *gapdh* was used for normalization (Soriani et al., 2016).

RNA interference analysis

RNA silencing was performed using 100 nM of Jagged1 (cat. # L-011060-00-0005) or Kras (cat. #L-005069-00-0005) ON-TARGET plus SMART pool small interference RNA (siRNA) or scrambled (cat. #D-001810-10-20) (Dharmacon Inc., Lafayette, CO, USA), using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

Protein extracts, subcellular fractioning, immunoprecipitation and immunoblotting

Whole cell extract (WCE) (Petroni et al., 2011), extracellular shed protein preparations (Campese et al., 2014), subcellular fractioning (Ferrandino et al., 2018) and immunoblot assay with the described antibodies (Table S2) (Vargas Romero et al., 2015) were performed as described elsewhere. Bound

antibodies were detected with enhanced chemiluminescence (ECL kit, Amersham, GE Healthcare, Lafayette, CO, USA). To perform immunoprecipitation assay (Checquolo et al., 2010), an equal amount of WCE derived from HCT15 or DLD1 cell lines, treated with the opportune dose of PMA or vehicle, were precleared with Protein A-Agarose (cat. #sc-2001; Santa Cruz Biotechnology, Dallas, TX, USA); immunoprecipitation assay was realized with ADAM17 antibody (Supplementary Table S2) or normal IgG (cat. #sc-2027; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. The complexes were precipitated with Protein A-Agarose, and the post-trasductional modifications were evaluated by using anti-phospho-Serine antibody (Table S2) (Checquolo et al., 2010).

Immunohistochemistry

Tissues were fixed in 4% formalin and paraffin embedded. Consecutive sections (2 μm thick) were stained with H&E. Immunocytochemical assay was performed using an anti-Jagged1 antibody (Abcam) (Table S2). Detection was carried out with Mouse-to-Mouse HRP (DAB) staining system (ScyTek Laboratories, Logan, UT, USA), according to the manufacturer's instructions. Images were acquired with a Leica DM1000 microscope equipped with a ProgRes Speed XTcore 3 CCD camera and collected using ProgRes CapturePro 2.8 software (Jenoptik Optical Systems GmbH, Jena, Germany) (Quaranta et al., 2017).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described earlier (Tottone et al., 2019). 1 μg of specific antibodies (Table S2), or normal IgG (cat. #sc-2027, from Santa Cruz Biotechnology, Dallas, TX, USA) was used for immunoprecipitation. *In silico* analysis using MatInspector (Genomatix Software GmbH, Munich, Germany) allowed us to identify predicted binding sites for RBP-Jκ on human *snail1* and *snail2* promoters, racing from -1860 to -1847 for snail1 and from -5091 to -5087 for snail2 (Table S1).

Cell growth and soft agar assays

HCT15 cells stably transfected with V5-Jag1-ICD expressing vector or pCDNA3-Neo control were plated in 96-well plate (5000 cells/well) and the MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was used as described elsewhere (Cialfi et al., 2014). Spectrophotometric absorbance at 570nm wavelength was determined by GloMax-Multi Detection System (Promega). Colony formation assay was performed by using a 6-well plate pre-coated with 1% of soft agar SeaKEM LE Agarose (LONZA, Allendale, NJ, USA) dissolved in medium, supplemented by 1X Glutamine, 1X Antibiotics, 20% FBS and 800ng/ml of Neomycin. 3000cells/ml were plated on the upper layer

(0,7% agarose dissolved in medium plus1X glutamine, 1X antibiotic, 20% of FBS and 800ng/ml of Neomycin). This top layer was covered by 1ml of complete medium. The cell colonies were fixed with 10% Methanol/10% Acetic Acid for 10' and then stained with a 0,005% Crystal Violet (Sigma-Aldrich).

Wound healing and invasion assays

Cell migration was analysed by wound-healing assay. Briefly, an opportune number of cells were grown in six-well plates. Wound injury was made with the tip of a sterile micropipette and cells were allowed to migrate for up to 48h. *In vitro* invasion assay was performed using a 24-well transwell insert (8 µm pore size) pre-coated with BD Matrigel matrix (BD Biosciences, San Jose, CA, USA) (Franciosa et al., 2016). The invading cells were fixed with PFA 4%, rinsed with PBS, permeabilized with EtOH 100%, stained with 1% Crystal Violet and photographed. Cells were quantified as the average number of cells found in five random microscopic fields in three independent inserts.

Animal studies

To establish xenograft tumours, $1x10^7$ HCT15 cells, stably transfected with V5-Jag1-ICD expressing vector or negative control, were respectively injected subcutaneously into right and left dorsal flank of CD1 nude mice (n=6). Conversely, $2x10^6$ DLD1 cells were injected subcutaneously into the hind leg of 6-week-old CD1 nude female (n=6). When tumour reached a mean volume of 150 mm3, the animals were randomly separated into different groups and treated respectively with 5FU at 40-50 mg/kg/2-3 days i.p. (n=4), U0126 25mol/kg/2 days i.p. (n=4) and TAPI-2 at 2mg/Kg/2 days o.g. (n=6), dissolved in 0,2 ml of saline solution. The control group received injection/oral gavage of vehicle alone. After 27 days, mice were killed, and tumours were excised. Tumour size was measured every 3/4 days with a caliper and volume was calculated according to the formula: length*width*0,5*(length+width) (Infante et al., 2015). Harvested tumour tissues were subjected to RNA and WCE extraction as described.

In silico analysis of CRC patients' deposited data

Samples from the following cohorts: 545 CRC patients (GEO ID: gse3958283) (Marisa et al., 2013) and 83 CRC patients (GEO ID: gse28702) (Tsuji et al., 2012) were selected and analysed for the *Jagged1* gene expression levels. The expression values of *Jagged1* were filtered in each analysis utilizing the expression probe set 209099_x_at. The expression value of *Jagged1* is given in log2 scale after normalizing data with rma and mas5.0 normalization. GraphPad Prism 6 (La Jolla, CA,

USA) was used for statistical analysis and p-values were calculated using Student's t-test and one-way ANOVA, where appropriate.

Statistical analysis

All results were confirmed in at least three independent experiments and all quantitative data were reported as the mean ± SD. Student's t or Anova test for unpaired samples were used to assess differences among groups. A P value < 0.05 was considered statistically significant (n.s. P>0.05, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P< 0.0001).

Table S1: List of primers utilised in this study

Gene	Taqman Ref
Jagged1	Hs01070032_m1
PCNA	Hs00427214_g1
Cyclina D2	Hs00153380_m1
MMP9	Hs00234579_m1
Snail1	Hs00195591_m1
Snail2	Hs00161904_m1
E-Cadherin	Hs01023894_m1
GAPDH	Hs02758991_g1

Gene	Sequence
Jag1-ICD-V5 (fow)	5'-ATG AGG AAG CGG CGG AAG-3'
Jag1-ICD-V5 (rev)	5' TAC GAT GTA TTC CAT CCG GTT-3'
Snail1 (fow)	5'- GAGGCTGAGCAGTTAGTGAA-3'
Snail1 (rev)	5'-CAGAGTAAAAGCCAAAGTCC-3'
Snail2 (fow)	5'-GCAAAATAAGCTACTTTGGAGGCA-3'
Snail2 (rev)	5'-AGTGCCCAACAGTGTGTGG-3'

Table S2: List of antibodies utilised in this study

Primary antibody	Source	Reference	Dilution
Jagged1	Sigma-Aldrich	HPA021555	1:1000
Jagged1	Cell Signaling	#2155	1:1000
Jagged1	Abcam	Ab192767	1:100
Kras	Abnova	H00003845-M02	1:1000
ADAM17	Abcam	Ab2251	1:1000
Total ERK	Cell Signaling	#4695	1:1000
p-ERK	Santa Cruz Biotchenology	#7383	1:1000
Phospho Serine	Abcam	AB1607	1:500
Snail	Cell Signaling	#3895	1:1000
Vimentin	Santa Cruz Biotchenology	SC-373717	1:500
N-Cadherin	Santa Cruz Biotchenology	SC-271386	1:100
Notch1 Val1774	Cell Signaling	#4147	1:1000
Notch2 Val1694	Sigma-Aldrich	SAB4502022	1:1000

Notch3	Cell Signaling	#2889	1:1000
β-actin	Sigma-Aldrich	A5441	1:20000
α-tubulin	Santa Cruz Biotchenology	SC-8035	1:500
Lamin B	Santa Cruz Biotchenology	SC-6217	1:500
RBP-J	Santa Cruz Biotchenology	SC-8213	1:1000

Secondary antibody	**Source**	**Reference**	**Dilution**
Donkey anti rabbit-HRP	Bethyl	A120-108P	1:30000
Donkey anti goat-HRP	Santa Cruz Biotchenology	SC-2020	1:3000
Goat anti mouse-HRP	Bethyl	A90-116P	1:30000

V. Conclusions

Notch signalling is an evolutionarily conserved pathway with a pleiotropic role to control several processes inside the cells, such as cell survival, proliferation, apoptosis and self-renewal events (Bray, 2006). A deregulation of the signalling is associated with increased risk of developing several malignancies. However, not always an altered expression of Notch receptors causes a deregulated signal response, but also other components of the signalling pathway can be affected.

Maml1 is known as transcriptional co-activator for Notch signalling pathways (Wu et al. 2002; Wu et al. 2000). Noteworthy, Maml1 does not always act in the shadow of Notch, in fact several signalling require Maml1 activity as co-factor to enhance the transcription of target genes (Alves-Guerra et al., 2007; Hansson et al., 2012; Jin et al., 2010; Shen et al., 2006; Watanabe et al., 2013; Zhao et al., 2007).

Interestingly, we provided experimental evidence that highlight Maml1 as a novel transcription factors for Shh signalling (Quaranta et al., 2017). *In vivo* Maml1 depletion induces a decrease in the proliferation rate of granule cell progenitors that results in cerebellum foliation defects. These data uncover the role of Maml1 in development and differentiation processes, in a Notch-independent manner. In addition, an overexpression of Maml1 is associated in medulloblastoma tumours Shh-drive, implying a possible role for Maml1 in tumorigenesis.

Moreover, we demonstrate a new Jagged1 signalling in CRC Kras molecular subtype tumours, via a Kras/Erk/ADAM17 pathway (Pelullo, Nardozza, Zema et al., 2019). The release of Jag1-ICD sustains invasion and contributes to chemoresistance, identifying a new target for personalized therapy.

Nevertheless, a deregulated signal transduction does not correlate with an altered expression of the ligands or the receptor. Post-translational modifications are involved in the aberrant response of the signalling pathway, altering the trafficking of the proteins and affecting the activation of the pathway.

Despite being preliminary data, we observed a negative regulation of Itch, E3 ubiquitin ligases mediated by Maml1. Itch activity is required to control several pathways inside the cells, and a

deregulation of its activity could cause an altered signalling in response to external or internal stimuli.

Maml1 and Itch are key components of several pathway. The interaction and negative regulation of these two components could set out a new mechanism of regulation inside the cells that can be applied to a wide range of signalling pathways, to physiological or pathological contexts.

Evidence of crosstalk between multiple signalling pathways is reported in many tumour types. 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 tumours, at the same time. Understanding the importance of these molecular interlinking networks will provide a rational basis for combined anticancer drug development (Pelullo, Zema, et al., 2019).

VI. Appendix

The following appendix presents an overview on Hh signalling and the interaction with several pathways inside in the cells, in different tumoral context. It is essential to understand the dynamics within altered contexts such as tumours, in order to design targeted drug therapies that act on a larger molecular machinery, reflecting a more realistic situation.

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REVIEW
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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

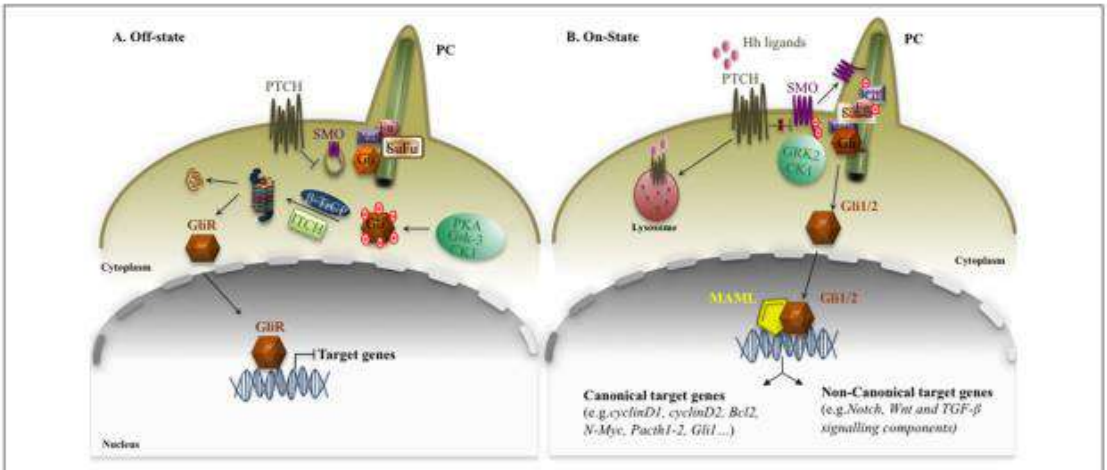

FIGURE 1 | Canonical Hh signaling. The canonical Hh signaling pathway in off (panel **A**) or on (panel **B**) state. The figure is widely discussed in the text. The red dots represent post-translational modifications: phosphorylation.

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),

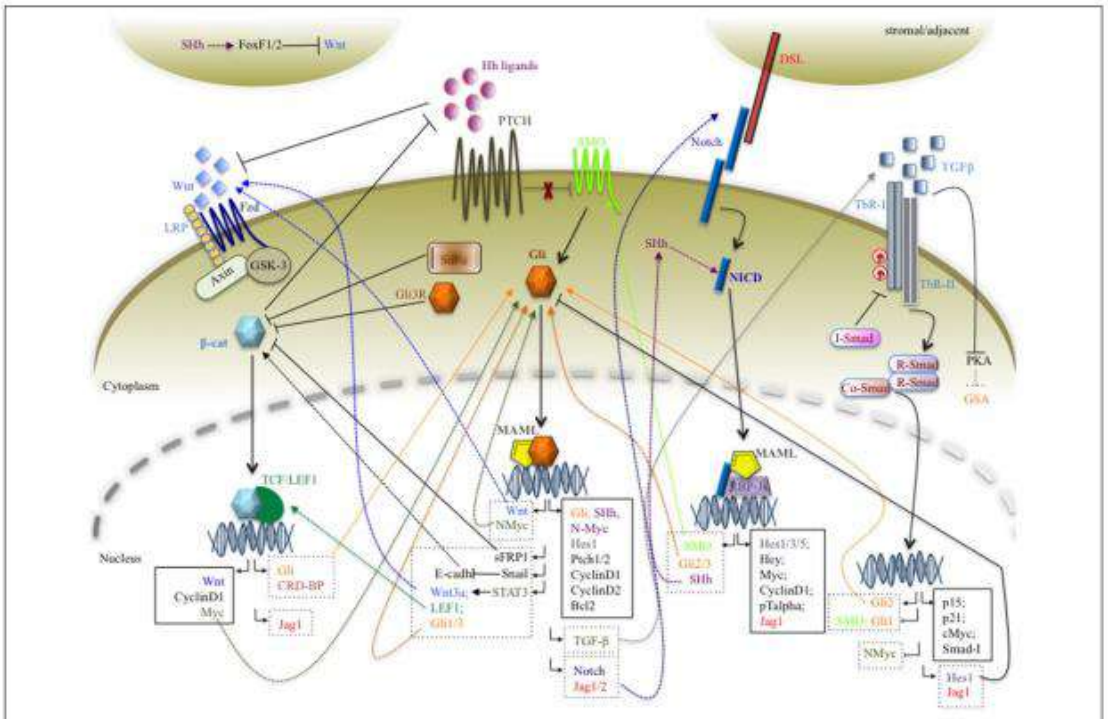

FIGURE 2 | A schematic picture of the interlinking networks between Hh, Wnt/β-catenin, Notch, and TGF-β pathways inside a cancer cell. In the picture are represented the signals deriving from major signaling pathways that converge on the Hh signaling and *vice versa*. The figure is largely discussed 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
	Ptch2	1p34.1	Rhabdomyosarcoma	Germline	Missense	Taeubner et al., 2018
			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
	Gli1	12q13.3	Leukemia	Somatic	Missense	Dagklis et al., 2015; Burns et al., 2018
			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
	Gli2	2q14.2	Medulloblastoma	Somatic	Amplification	Northcott et al., 2009; Rusert et al., 2014; Kool et al., 2014
			Leukemia	Somatic	Missense	Burns et al., 2018;
			Medulloblastoma	Somatic	Missense	Reifenberger et al., 1998; Rusert et al., 2014; Kool et al., 2014
	SMO	7q32.1	Basal cell carcinoma	Somatic	Missense	Reifenberger et al., 1998; Reifenberger et al., 2005
			Leukemia	Somatic	Frameshift	Dagklis et al., 2015
			Gastric–intestinal cancer	NA	Missense Insertion	Wang et al., 2013
			Ameloblastoma	Somatic	Missense	Sweeney et al., 2014
	SHH	7q36.3	Medulloblastoma	NA	Amplification	Reifenberger et al., 1998; Kool et al., 2014
			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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REFERENCES

Aavikko, M., Li, S. P., Saarinen, S., Alhopuro, P., Kaasinen, E., Morgunova, E., et al. (2012). Loss of SUFU function in familial multiple meningioma. *Am. J. Hum. Genet.* 91, 520–526. doi: 10.1016/j.ajhg.2012.07.015

Aberger, F., Hutterer, E., Sternberg, C., Del Burgo, P. J., and Hartmann, T. N. (2017). Acute myeloid leukemia—strategies and challenges for targeting oncogenic Hedgehog/GLI signaling. *Cell Commun. Signal* 15, 8. doi: 10.1186/s12964-017-0163-4

Akiyoshi, T., Nakamura, M., Koga, K., Nakashima, H., Yao, T., Tsuneyoshi, M., et al. (2006). Gli1, downregulated in colorectal cancers, inhibits proliferation of colon cancer cells involving Wnt signalling activation. *Gut* 55, 991–999. doi: 10.1136/gut.2005.080333

Allman, D., Karnell, F. G., Punt, J. A., Bakkour, S., Xu, L., Myung, P., et al. (2001). Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. *J. Exp. Med.* 194, 99–106. doi: 10.1084/jem.194.1.99

Alvarez-Rodriguez, R., Barzi, M., Berenguer, J., and Pons, S. (2007). Bone morphogenetic protein 2 opposes Shh-mediated proliferation in cerebellar granule cells through a TIEG-1-based regulation of Nmyc. *J. Biol. Chem.* 282, 37170–37180. doi: 10.1074/jbc.M705414200

Androutsellis-Theotokis, A., Leker, R. R., Soldner, F., Hoeppner, D. J., Ravin, R., Poser, S. W., et al. (2006). Notch signalling regulates stem cell numbers *in vitro* and *in vivo*. *Nature* 442, 823–826. doi: 10.1038/nature04940

Arruga, F., Gizdic, B., Serra, S., Vaisitti, T., Ciardullo, C., Coscia, M., et al. (2014). Functional impact of NOTCH1 mutations in chronic lymphocytic leukemia. *Leukemia* 28, 1060–1070. doi: 10.1038/leu.2013.319

Bai, C. B., Stephen, D., and Joyner, A. L. (2004). All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. *Dev. Cell* 6, 103–115. doi: 10.1016/S1534-5807(03)00394-0

Bakshi, A., Chaudhary, S. C., Rana, M., Elmets, C. A., and Athar, M. (2017). Basal cell carcinoma pathogenesis and therapy involving hedgehog signaling and beyond. *Mol. Carcinog.* 56, 2543–2557. doi: 10.1002/mc.22690

Bellavia, D., Campese, A. F., Alesse, E., Vacca, A., Felli, M. P., Balestri, A., et al. (2000). Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. *EMBO J.* 19, 3337–3348. doi: 10.1093/emboj/19.13.3337

Bellavia, D., Mecarozzi, M., Campese, A. F., Grazioli, P., Gulino, A., and Screpanti, I. (2007a). Notch and Ikaros: not only converging players in T cell leukemia. *Cell Cycle* 6, 2730–2734. doi: 10.4161/cc.6.22.4894

Bellavia, D., Mecarozzi, M., Campese, A. F., Grazioli, P., Talora, C., Frati, L., et al., (2007b). Notch3 and the Notch3-upregulated RNA-binding protein HuD regulate Ikaros alternative splicing. *EMBO J.* 26, 1670–1680. doi: 10.1038/sj.emboj.7601626

Bellavia, D., Palermo, R., Felli, M. P., Screpanti, I., and Checquolo, S. (2018). Notch signaling as a therapeutic target for acute lymphoblastic leukemia. *Expert Opin. Ther. Targets* 22, 331–342. doi: 10.1080/14728222.2018.1451840

Beverly, L. J., and Capobianco, A. J. (2003). Perturbation of Ikaros isoform selection by MLV integration is a cooperative event in Notch(IC)-induced T cell leukemogenesis. *Cancer cell* 3, 551–564. doi: 10.1016/S1535-6108(03)00137-5

Bian, Y. H., Huang, S. H., Yang, L., Ma, X. L., Xie, J. W., and Zhang, H. W. (2007). Sonic hedgehog-Gli1 pathway in colorectal adenocarcinomas. *World J. Gastroenterol.* 13, 1659–1665. doi: 10.3748/wjg.v13.i11.1659

Bigelow, R. L., Chari, N. S., Unden, A. B., Spurgers, K. B., Lee, S., Roop, D. R., et al. (2004). Transcriptional regulation of bcl-2 mediated by the sonic hedgehog signaling pathway through gli-1. *J. Biol. Chem.* 279, 1197–1205. doi: 10.1074/jbc.M310589200

Blotta, S., Jakubikova, J., Calimeri, T., Roccaro, A. M., Amodio, N., Azab, A. K., et al. (2012). Canonical and noncanonical Hedgehog pathway in the pathogenesis of multiple myeloma. *Blood* 120, 5002–5013. doi: 10.1182/blood-2011-07-368142

Bonifas, J. M., Pennypacker, S., Chuang, P. T., McMahon, A. P., Williams, M., Rosenthal, A., et al. (2001). Activation of expression of hedgehog target genes in basal cell carcinomas. *J. Investig. Dermatol.* 116, 739–742. doi: 10.1046/j.1523-1747.2001.01315.x

Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T., and Kimelman, D. (1997). A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in *Xenopus*. *Genes Dev.* 11, 2359–2370. doi: 10.1101/gad.11.18.2359

Bridge, J. A., Liu, J., Weibolt, V., Baker, K. S., Perry, D., Kruger, R., et al. (2000). Novel genomic imbalances in embryonal rhabdomyosarcoma revealed by comparative genomic hybridization and fluorescence *in situ* hybridization: an intergroup rhabdomyosarcoma study. *Genes Chromosomes Cancer* 27, 337–344. doi: 10.1002/(SICI)1098-2264(200004)27:4<337::AID-GCC1>3.0.CO;2-1

Burns, M. A., Liao, Z. W., Yamagata, N., Pouliot, G. P., Stevenson, K. E., Neuberg, D. S., et al. (2018). Hedgehog pathway mutations drive oncogenic transformation in high-risk T-cell acute lymphoblastic leukemia. *Leukemia* 32, 2126–2137. doi: 10.1038/s41375-018-0097-x

Campbell, V., and Copland, M. (2015). Hedgehog signaling in cancer stem cells: a focus on hematological cancers. *Stem Cells Cloning* 8, 27–38. doi: 10.2147/SCCAA.S58613

Campese, A. F., Bellavia, D., Gulino, A., and Screpanti, I. (2003). Notch signalling at the crossroads of T cell development and leukemogenesis. *Semin. Cell Dev. Biol.* 14, 151–157. doi: 10.1016/S1084-9521(02)00184-2

Campese, A. F., Grazioli, P., Colantoni, S., Anastasi, E., Mecarozzi, M., Checquolo, S., et al. (2009). Notch3 and pTalpha/pre-TCR sustain the *in vivo* function of naturally occurring regulatory T cells. *Int. Immunol.* 21, 727–743. doi: 10.1093/intimm/dxp042

Canettieri, G., Di Marcotullio, L., Greco, A., Coni, S., Antonucci, L., Infante, P., et al. (2010). Histone deacetylase and Cullin3–REN(KCTD11) ubiquitin ligase interplay regulates Hedgehog signalling through Gli acetylation. *Nat. Cell Biol.* 12, 132–142. doi: 10.1038/ncb2013

Carballo, G. B., Honorato, J. R., de Lopes, G. P. F., and Spohr, T. (2018). A highlight on Sonic hedgehog pathway. *Cell Commun. Signal* 16, 11. doi: 10.1186/s12964-018-0220-7

Catanzaro, G., Sabato, C., Russo, M., Rosa, A., Abballe, L., Besharat, Z. M., et al. (2017). Loss of miR-107, miR-181c and miR-29a-3p promote activation of Notch2 signaling in pediatric high-grade gliomas (pHGGs). *Int. J. Mol. Sci.* 18, 2742–2754. doi: 10.3390/ijms18122742

Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., et al. (1998). *Drosophila* Tcf and Groucho interact to repress Wingless signalling activity. *Nature* 395, 604–608. doi: 10.1038/26982

Ceccarelli, S., Megiorni, F., Bellavia, D., Marchese, C., Screpanti, I., and Checquolo, S. (2019). Notch3 targeting: a novel weapon against ovarian cancer stem cells. *Stem Cells Int.* 2019, 6264931. doi: 10.1155/2019/6264931

Checquolo, S., Palermo, R., Cialfi, S., Ferrara, G., Oliviero, C., Talora, C., et al. (2010). Differential subcellular localization regulates c-Cbl E3 ligase activity upon Notch3 protein in T-cell leukemia. *Oncogene* 29, 1463–1474. doi: 10.1038/onc.2009.446

Chen, C. R., Kang, Y., Siegel, P. M., and Massague, J. (2002). E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. *Cell* 110, 19–32. doi: 10.1016/S0092-8674(02)00801-2

Chen, Y., Sasai, N., Ma, G., Yue, T., Jia, J., Briscoe, J., et al. (2011). Sonic Hedgehog dependent phosphorylation by CK1alpha and GRK2 is required for ciliary accumulation and activation of smoothened. *PLoS Biol.* 9, e1001083. doi: 10.1371/journal.pbio.1001083

Choi, J. H., Park, J. T., Davidson, B., Morin, P. J., Shih Ie, M., and Wang, T. L. (2008). Jagged-1 and Notch3 juxtacrine loop regulates ovarian tumor growth and adhesion. *Cancer Res.* 68, 5716–5723. doi: 10.1158/0008-5472.CAN-08-0001

Cialfi, S., Palermo, R., Manca, S., Checquolo, S., Bellavia, D., Pelullo, M., et al. (2013). Glucocorticoid sensitivity of T-cell lymphoblastic leukemia/lymphoma is associated with glucocorticoid receptor-mediated inhibition of Notch1 expression. *Leukemia* 27, 485–488. doi: 10.1038/leu.2012.192

Cialfi, S., Palermo, R., Manca, S., De Blasio, C., Vargas Romero, P., Checquolo, S., et al. (2014). Loss of Notch1-dependent p21(Waf1/Cip1) expression influences the Notch1 outcome in tumorigenesis. *Cell cycle* 13, 2046–2055. doi: 10.4161/cc.29079

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. *Cell* 127, 469–480. doi: 10.1016/j.cell.2006.10.018

Cohen, B., Shimizu, M., Izrailit, J., Ng, N. F., Buchman, Y., Pan, J. G., et al. (2010). Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. *Breast Cancer Res. Treat.* 123, 113–124. doi: 10.1007/s10549-009-0621-9

Coni, S., Antonucci, L., D'Amico, D., Di Magno, L., Infante, P., De Smaele, E., et al. (2013). Gli2 acetylation at lysine 757 regulates hedgehog-dependent transcriptional output by preventing its promoter occupancy. *PloS One* 8, e65718. doi: 10.1371/journal.pone.0065718

Couve-Privat, S., Le Bret, M., Traiffort, E., Queille, S., Coulombe, J., Bouadjar, B., et al. (2004). Functional analysis of novel sonic hedgehog gene mutations identified in basal cell carcinomas from xeroderma pigmentosum patients. *Cancer Res.* 64, 3559–3565. doi: 10.1158/0008-5472.CAN-03-4040

Crompton, T., Outram, S. V., and Hager-Theodorides, A. L. (2007). Sonic hedgehog signalling in T-cell development and activation. *Nat. Rev. Immunol.* 7, 726–735. doi: 10.1038/nri2151

Dagklis, A., Demeyer, S., De Bie, J., Radaelli, E., Pauwels, D., Degryse, S., et al. (2016). Hedgehog pathway activation in T-cell acute lymphoblastic leukemia predicts response to SMO and GLI1 inhibitors. *Blood* 128, 2642–2654. doi: 10.1182/blood-2016-03-703454

Dagklis, A., Pauwels, D., Lahortiga, I., Geerdens, E., Bittoun, E., Cauwelier, B., et al. (2015). Hedgehog pathway mutations in T-cell acute lymphoblastic leukemia. *Haematologica* 100, e102–e105. doi: 10.3324/haematol.2014.119248

Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M., and Ishii, S. (1999). Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J. Biol. Chem.* 274, 8143–8152. doi: 10.1074/jbc.274.12.8143

Datto, M. B., Frederick, J. P., Pan, L., Borton, A. J., Zhuang, Y., and Wang, X. F. (1999). Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Mol. Cell. Biol.* 19, 2495–2504. doi: 10.1128/MCB.19.4.2495

De Falco, F., Del Papa, B., Baldoni, S., Sabatini, R., Falzetti, F., Di Ianni, M., et al. (2018). IL-4-dependent Jagged1 expression/processing is associated with survival of chronic lymphocytic leukemia cells but not with Notch activation. *Cell Death Dis* 9, 1160. doi: 10.1038/s41419-018-1185-6

De Falco, F., Sabatini, R., Falzetti, F., Di Ianni, M., Sportoletti, P., Baldoni, S., et al. (2015). Constitutive phosphorylation of the active Notch1 intracellular domain in chronic lymphocytic leukemia cells with NOTCH1 mutation. *Leukemia* 29, 994–998. doi: 10.1038/leu.2014.329

Decker, S., Zirlik, K., Djebatchie, L., Hartmann, D., Ihorst, G., Schmitt-Graeff, A., et al. (2012). Trisomy 12 and elevated GLI1 and PTCH1 transcript levels are biomarkers for Hedgehog-inhibitor responsiveness in CLL. *Blood* 119, 997–1007. doi: 10.1182/blood-2011-06-359075

DeCoteau, J. F., Knaus, P. I., Yankelev, H., Reis, M. D., Lowsky, R., Lodish, H. F., et al. (1997). Loss of functional cell surface transforming growth factor beta (TGF-beta) type I receptor correlates with insensitivity to TGF-beta in chronic lymphocytic leukemia. *Proc. Natl Acad. Sci. U. S. A.* 94, 5877–5881. doi: 10.1073/pnas.94.11.5877

Denef, N., Neubuser, D., Perez, L., and Cohen, S. M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell* 102, 521–531. doi: 10.1016/S0092-8674(00)00056-8

Dennler, S., Andre, J., Alexaki, I., Li, A., Magnaldo, T., ten Dijke, P., et al. (2007). Induction of sonic hedgehog mediators by transforming growth factor-beta: Smad3-dependent activation of Gli2 and Gli1 expression *in vitro* and *in vivo*. *Cancer Res.* 67, 6981–6986. doi: 10.1158/0008-5472.CAN-07-0491

Di Marcotullio, L., Ferretti, E., Greco, A., De Smaele, E., Po, A., Sico, M. A., et al. (2006). Numb is a suppressor of Hedgehog signalling and targets Gli1 for itch-dependent ubiquitination. *Nat. Cell Biol.* 8, 1415–1423. doi: 10.1038/ncb1510

Di Marcotullio, L., Ferretti, E., Greco, A., De Smaele, E., Screpanti, I., and Gulino, A. (2007). Multiple ubiquitin-dependent processing pathways regulate hedgehog/gli signaling: implications for cell development and tumorigenesis. *Cell Cycle* 6, 390–393. doi: 10.4161/cc.6.4.3809

Di Marcotullio, L., Greco, A., Mazza, D., Canettieri, G., Pietrosanti, L., Infante, P., et al. (2011). Numb activates the E3 ligase Itch to control Gli1 function through a novel degradation signal. *Oncogene* 30, 65–76. doi: 10.1038/onc.2010.394

Dierks, C., Grbic, J., Zirlik, K., Beigi, R., Englund, N. P., Guo, G. R., et al. (2007). Essential role of stromally induced hedgehog signaling in B-cell malignancies. *Nat. Med.* 13, 944–951. doi: 10.1038/nm1614

Diluvio, G., Del Gaudio, F., Giuli, M. V., Franciosa, G., Giuliani, E., Palermo, R., et al. (2018). NOTCH3 inactivation increases triple negative breast cancer sensitivity to gefitinib by promoting EGFR tyrosine dephosphorylation and its intracellular arrest. *Oncogenesis* 7, 42. doi: 10.1038/s41389-018-0051-9

Ding, Q., Fukami, Si, Meng, X., Nishizaki, Y., Zhang, X., Sasaki, H., et al. (1999). Mouse suppressor of fused is a negative regulator of sonic hedgehog signaling and alters the subcellular distribution of Gli1. *Curr. Biol.* 9, 1119–1122. doi: 10.1016/S0960-9822(99)80482-5

Domingo-Domenech, J., Vidal, S. J., Rodriguez-Bravo, V., Castillo-Martin, M., Quinn, S. A., Rodriguez-Barrueco, R., et al. (2012). Suppression of acquired docetaxel resistance in prostate cancer through depletion of notch- and hedgehog-dependent tumor-initiating cells. *Cancer Cell* 22, 373–388. doi: 10.1016/j.ccr.2012.07.016

Drakopoulou, E., Outram, S. V., Rowbotham, N. J., Ross, S. E., Furmanski, A. L., Saldana, J. I., et al. (2010). Non-redundant role for the transcription factor Gli1 at multiple stages of thymocyte development. *Cell Cycle* 9, 4144–4152. doi: 10.4161/cc.9.20.13453

Dunaeva, M., Michelson, P., Kogerman, P., and Toftgard, R. (2003). Characterization of the physical interaction of Gli proteins with SUFU proteins. *J. Biol. Chem.* 278, 5116–5122. doi: 10.1074/jbc.M209492200

Ezratty, E. J., Stokes, N., Chai, S., Shah, A. S., Williams, S. E., and Fuchs, E. (2011). A role for the primary cilium in Notch signaling and epidermal differentiation during skin development. *Cell* 145, 1129–1141. doi: 10.1016/j.cell.2011.05.030

Fan, Q., He, M., Sheng, T., Zhang, X., Sinha, M., Luxon, B., et al. (2010). Requirement of TGFbeta signaling for SMO-mediated carcinogenesis. *J. Biol. Chem.* 285, 36570–36576. doi: 10.1074/jbc.C110.164442

Fan, X., Mikolaenko, I., Elhassan, I., Ni, X., Wang, Y., Ball, D., et al. (2004). Notch1 and notch2 have opposite effects on embryonal brain tumor growth. *Cancer Res.* 64, 7787–7793. doi: 10.1158/0008-5472.CAN-04-1446

Fan, X., and Eberhart, C. G. (2008). Medulloblastoma stem cells. *J. Clin. Oncol.* 26 (17), 2821–2827. doi: 10.1200/JCO.2007.15.2264

Felli, M. P., Maroder, M., Mitsiadis, T. A., Campese, A. F., Bellavia, D., Vacca, A., et al. (1999). Expression pattern of Notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand-receptor interactions in intrathymic T cell development. *Int. Immunol.* 11, 1017–1025. doi: 10.1093/intimm/11.7.1017

Ferrandino, F., Bernardini, G., Tsaouli, G., Grazioli, P., Campese, A. F., Noce, C., et al. (2018a). Intrathymic Notch3 and CXCR4 combinatorial interplay facilitates T-cell leukemia propagation. *Oncogene* 37, 6285–6298. doi: 10.1038/s41388-018-0401-2

Ferrandino, F., Grazioli, P., Bellavia, D., Campese, A. F., Screpanti, I., and Felli, M. P. (2018b). Notch and NF-kappaB: coach and players of regulatory T-cell response in cancer. *Front. Immunol.* 9, 2165. doi: 10.3389/fimmu.2018.02165

Fortini, M. E. (2009). Notch signaling: the core pathway and its posttranslational regulation. *Dev. Cell* 16, 633–647. doi: 10.1016/j.devcel.2009.03.010

Fortunel, N. O., Hatzfeld, A., and Hatzfeld, J. A. (2000). Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis. *Blood* 96, 2022–2036.

Fortunel, N. O., Hatzfeld, J. A., Monier, M. N., and Hatzfeld, A. (2003). Control of hematopoietic stem/progenitor cell fate by transforming growth factor-beta. *Oncol. Res.* 13, 445–453. doi: 10.3727/096504003108748483

Franciosa, G., Diluvio, G., Gaudio, F. D., Giuli, M. V., Palermo, R., Grazioli, P., et al. (2016). Prolyl-isomerase Pin1 controls Notch3 protein expression and regulates T-ALL progression. *Oncogene* 35, 4741–4751. doi: 10.1038/onc.2016.5

Fu, X., Shi, L., Zhang, W., Zhang, X., Peng, Y., Chen, X., et al. (2014). Expression of Indian hedgehog is negatively correlated with APC gene mutation in colorectal tumors. *Int. J. Clin. Exp. Med.* 7, 2150–2155.

Fujii, K., Ohashi, H., Suzuki, M., Hatsuse, H., Shiohama, T., Uchikawa, H., et al. (2013). Frameshift mutation in the PTCH2 gene can cause nevoid basal cell carcinoma syndrome. *Fam. Cancer* 12, 611–614 doi: 10.1007/s10689-013-9623-1

Gailani, M. R., Stahle-Backdahl, M., Leffell, D. J., Glynn, M., Zaphiropoulos, P. G., Pressman, C., et al. (1996). The role of the human homologue of *Drosophila* patched in sporadic basal cell carcinomas. *Nat. Genet.* 14, 78–81. doi: 10.1038/ng0996-78

Gao, J., Graves, S., Koch, U., Liu, S., Jankovic, V., Buonamici, S., et al. (2009). Hedgehog signaling is dispensable for adult hematopoietic stem cell function. *Cell Stem Cell* 4, 548–558. doi: 10.1016/j.stem.2009.03.015

Geissler, K., and Zach, O. (2012). Pathways involved in *Drosophila* and human cancer development: the Notch, Hedgehog, Wingless, Runt, and Trithorax pathway. *Ann. Hematol* 91, 645–669. doi: 10.1007/s00277-012-1435-0

Goetz, S. C., and Anderson, K. V. (2010). The primary cilium: a signalling centre during vertebrate development. *Nat. Rev. Genet.* 11, 331–344. doi: 10.1038/nrg2774

Guleng, B., Tateishi, K., Ohta, M., Asaoka, Y., Jazag, A., Lin, L. J., et al. (2006). Smoothened gene mutations found in digestive cancer have no aberrant Hedgehog signaling activity. *J. Gastroenterol.* 41, 1238–1239. doi: 10.1007/s00535-006-1955-2

Guo, S., Liu, M., and Gonzalez-Perez, R. R. (2011). Role of Notch and its oncogenic signaling crosstalk in breast cancer. *Biochim. Biophys. Acta* 1815, 197–213. doi: 10.1016/j.bbcan.2010.12.002

Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., et al. (1996). Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome. *Cell* 85, 841–851. doi: 10.1016/S0092-8674(00)81268-4

Harris, P. J., Speranza, G., and Dansky Ullmann, C. (2012). Targeting embryonic signaling pathways in cancer therapy. *Expert Opin. Ther. Targets* 16, 131–145. doi: 10.1517/14728222.2011.645808

He, J., Sheng, T., Stelter, A. A., Li, C., Zhang, X., Sinha, M., et al. (2006). Suppressing Wnt signaling by the hedgehog pathway through sFRP-1. *J. Biol. Chem.* 281, 35598–35602. doi: 10.1074/jbc.C600200200

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., et al. (1998). Identification of c-MYC as a target of the APC pathway. *Science* 281, 1509–1512. doi: 10.1126/science.281.5382.1509

He, X., Semenov, M., Tamai, K., and Zeng, X. (2004). LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development* 131, 1663–1677. doi: 10.1242/dev.01117

Hou, X., Chen, X., Zhang, P., Fan, Y., Ma, A., Pang, T., et al. (2014). Inhibition of hedgehog signaling by GANT58 induces apoptosis and shows synergistic antitumor activity with AKT inhibitor in acute T cell leukemia cells. *Biochimie* 101, 50–59. doi: 10.1016/j.biochi.2013.12.019

Huang, P., Xiong, F., Megason, S. G., and Schier, A. F. (2012). Attenuation of Notch and Hedgehog signaling is required for fate specification in the spinal cord. *PLoS Genet.* 8, e1002762. doi: 10.1371/journal.pgen.1002762

Humke, E. W., Dorn, K. V., Milenkovic, L., Scott, M. P., and Rohatgi, R. (2010). The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. *Genes Dev.* 24, 670–682. doi: 10.1101/gad.1902910

Huttner, A. (2012). Overview of primary brain tumors: pathologic classification, epidemiology, molecular biology, and prognostic markers. *Hematol. Oncol. Clin. North Am.* 26, 715–732. doi: 10.1016/j.hoc.2012.05.004

Imai, Y., Kurokawa, M., Izutsu, K., Hangaishi, A., Maki, K., Ogawa, S., et al. (2001). Mutations of the Smad4 gene in acute myelogenous leukemia and their functional implications in leukemogenesis. *Oncogene* 20, 88–96. doi: 10.1038/sj.onc.1204057

Infante, P., Alfonsi, R., Ingallina, C., Quaglio, D., Ghirga, F., D'Acquarica, I., et al. (2016). Inhibition of Hedgehog-dependent tumors and cancer stem cells by a newly identified naturally occurring chemotype. *Cell Death Dis.* 7, e2376. doi: 10.1038/cddis.2016.195

Infante, P., Faedda, R., Bernardi, F., Bufalieri, F., Lospinoso Severini, L., Alfonsi, R., et al. (2018). Itch/beta-arrestin2-dependent non-proteolytic ubiquitylation of SuFu controls Hedgehog signalling and medulloblastoma tumorigenesis. *Nat. Commun.* 9, 976. doi: 10.1038/s41467-018-03339-0

Ingram, W. J., McCue, K. I., Tran, T. H., Hallahan, A. R., and Wainwright, B. J. (2008). Sonic Hedgehog regulates Hes1 through a novel mechanism that is independent of canonical Notch pathway signalling. *Oncogene* 27, 1489–1500. doi: 10.1038/sj.onc.1210767

Jakubowiak, A., Pouponnot, C., Berguido, F., Frank, R., Mao, S., Massague, J., et al. (2000). Inhibition of the transforming growth factor beta 1 signaling pathway by the AML1/ETO leukemia-associated fusion protein. *J. Biol. Chem.* 275, 40282–40287. doi: 10.1074/jbc.C000485200

Ji, Z., Mei, F. C., Johnson, B. H., Thompson, E. B., and Cheng, X. (2007). Protein kinase A, not Epac, suppresses hedgehog activity and regulates glucocorticoid sensitivity in acute lymphoblastic leukemia cells. *J. Biol. Chem.* 282, 37370–37377. doi: 10.1074/jbc.M703697200

Jiao, X., Wood, L. D., Lindman, M., Jones, S., Buckhaults, P., Polyak, K., et al. (2012). Somatic mutations in the Notch, NF-KB, PIK3CA, and Hedgehog pathways in human breast cancers. *Genes Chromosomes Cancer* 51, 480–489. doi: 10.1002/gcc.21935

Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., et al. (1996). Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* 272, 1668–1671. doi: 10.1126/science.272.5268.1668

Jones, E. A., Sajid, M. I., Shenton, A., and Evans, D. G. (2011). Basal cell carcinomas in Gorlin syndrome: a review of 202 patients. *J. Skin Cancer* 2011, 217378. doi: 10.1155/2011/217378

Jones, S., Zhang, X., Parsons, D. W., Lin, J. C., Leary, R. J., Angenendt, P., et al. (2008). Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 321, 1801–1806. doi: 10.1126/science.1164368

Joshi, I., Minter, L. M., Telfer, J., Demarest, R. M., Capobianco, A. J., Aster, J. C., et al. (2009). Notch signaling mediates G1/S cell-cycle progression in T cells *via* cyclin D3 and its dependent kinases. *Blood* 113, 1689–1698. doi: 10.1182/blood-2008-03-147967

Joutel, A., Dodick, D. D., Parisi, J. E., Cecillon, M., Tournier-Lasserve, E., and Bousser, M. G. (2000). De novo mutation in the Notch3 gene causing CADASIL. *Ann. Neurol* 47, 388–391. doi: 10.1002/1531-8249(200003)47:3<388::AID-ANA19>3.0.CO;2-Q

Katoh, Y., and Katoh, M. (2009). Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. *Curr. Mol. Med.* 9, 873–886. doi: 10.2174/156652409789105570

Kawahara, T., Kawaguchi-Ihara, N., Okuhashi, Y., Itoh, M., Nara, N., and Tohda, S. (2009). Cyclopamine and quercetin suppress the growth of leukemia and lymphoma cells. *Anticancer Res.* 29, 4629–4632.

Kenney, A. M., Cole, M. D., and Rowitch, D. H. (2003). Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. *Development* 130, 15–28. doi: 10.1242/dev.00182

Kim, T. H., Kim, B. M., Mao, J., Rowan, S., and Shivdasani, R. A. (2011). Endodermal Hedgehog signals modulate Notch pathway activity in the developing digestive tract mesenchyme. *Development* 138, 3225–3233. doi: 10.1242/dev.066233

Kodach, L. L., Wiercinska, E., de Miranda, N. F., Bleuming, S. A., Musler, A. R., Peppelenbosch, M. P., et al. (2008). The bone morphogenetic protein pathway is inactivated in the majority of sporadic colorectal cancers. *Gastroenterology* 134, 1332–1341. doi: 10.1053/j.gastro.2008.02.059

Kong, J. H., Yang, L., Dessaud, E., Chuang, K., Moore, D. M., Rohatgi, R., et al. (2015). Notch activity modulates the responsiveness of neural progenitors to sonic hedgehog signaling. *Dev. Cell* 33, 373–387. doi: 10.1016/j.devcel.2015.03.005

Kool, M., Korshunov, A., Remke, M., Jones, D. T., Schlanstein, M., Northcott, P. A., et al. (2012). Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas. *Acta Neuropathol.* 123, 473–484. doi: 10.1007/s00401-012-0958-8

Kool, M., Koster, J., Bunt, J., Hasselt, N. E., Lakeman, A., van Sluis, P., et al. (2008). Integrated genomics identifies five medulloblastoma subtypes with distinct genetic profiles, pathway signatures and clinicopathological features. *PloS One* 3, e3088. doi: 10.1371/journal.pone.0003088

Kool, M., Jones, D. T., Jager, N., Northcott, P. A., Pugh, T. J., Hovestadt, V., et al. (2014). Genome sequencing of SHH medulloblastoma predicts genotype-related response to smoothened inhibition. *Cancer Cell* 25 (3), 393–405. doi: 10.1016/j.ccr.2014.02.004

Kopan, R., and Ilagan, M. X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* 137, 216–233. doi: 10.1016/j.cell.2009.03.045

Kosinski, C., Li, V. S., Chan, A. S., Zhang, J., Ho, C., Tsui, W. Y., et al. (2007). Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. *Proc. Natl Acad. Sci. U. S. A.* 104, 15418–15423. doi: 10.1073/pnas.0707210104

Krebs, L. T., Iwai, N., Nonaka, S., Welsh, I. C., Lan, Y., Jiang, R., et al. (2003). Notch signaling regulates left-right asymmetry determination by inducing Nodal expression. *Genes Dev.* 17, 1207–1212. doi: 10.1101/gad.1084703

Kuang, S. Q., Fang, Z., Zweidler-McKay, P. A., Yang, H., Wei, Y., Gonzalez-Cervantes, E. A., et al. (2013). Epigenetic inactivation of Notch-Hes pathway in human B-cell acute lymphoblastic leukemia. *PloS One* 8, e61807. doi: 10.1371/journal.pone.0061807

Kumar, V., Palermo, R., Talora, C., Campese, A. F., Checquolo, S., Bellavia, D., et al. (2014). Notch and NF-kB signaling pathways regulate miR-223/FBXW7 axis in T-cell acute lymphoblastic leukemia. *Leukemia* 28, 2324–2335. doi: 10.1038/leu.2014.133

Larsson, J., Blank, U., Helgadottir, H., Bjornsson, J. M., Ehinger, M., Goumans, M. J., et al. (2003). TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability *in vivo* despite increased proliferative capacity *in vitro*. *Blood* 102, 3129–3135. doi: 10.1182/blood-2003-04-1300

Le Bousse-Kerdiles, M. C., Chevillard, S., Charpentier, A., Romquin, N., Clay, D., Smadja-Joffe, F., et al. (1996). Differential expression of transforming growth factor-beta, basic fibroblast growth factor, and their receptors in CD34+ hematopoietic progenitor cells from patients with myelofibrosis and myeloid metaplasia. *Blood* 88, 4534–4546.

Lee, J., Platt, K. A., Censullo, P., and Ruiz i Altaba, A. (1997). Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. *Development* 124, 2537–2552.

Lee, J. H., Song, S. Y., Kim, M. S., Yoo, N. J., and Lee, S. H. (2018). Intratumoral heterogeneity of frameshift mutations of GLI1 encoding a Hedgehog signaling protein in colorectal cancers. *Pathol. Oncol. Res.* 24, 477–481. doi: 10.1007/s12253-017-0272-9

Li, X., Deng, W., Lobo-Ruppert, S. M., and Ruppert, J. M. (2007). Gli1 acts through Snail and E-cadherin to promote nuclear signaling by beta-catenin. *Oncogene* 26, 4489–4498. doi: 10.1038/sj.onc.1210241

Li, Y., Hibbs, M. A., Gard, A. L., Shylo, N. A., and Yun, K. (2012). Genome-wide analysis of N1ICD/RBPJ targets *in vivo* reveals direct transcriptional regulation of Wnt, SHH, and hippo pathway effectors by Notch1. *Stem Cells* 30, 741–752. doi: 10.1002/stem.1030

Lindemann, R. K. (2008). Stroma-initiated hedgehog signaling takes center stage in B-cell lymphoma. *Cancer Res.* 68, 961–964. doi: 10.1158/0008-5472.CAN-07-5500

Lindstrom, E., Shimokawa, T., Toftgard, R., and Zaphiropoulos, P. G. (2006). PTCH mutations: distribution and analyses. *Human Mutat.* 27, 215–219. doi: 10.1002/humu.20296

Liu, S., Dontu, G., Mantle, I. D., Patel, S., Ahn, N. S., Jackson, K. W., et al. (2006). Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res.* 66, 6063–6071. doi: 10.1158/0008-5472.CAN-06-0054

Logan, C. Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* 20, 781–810. doi: 10.1146/annurev.cellbio.20.010403.113126

Lopes, S. S., Lourenco, R., Pacheco, L., Moreno, N., Kreiling, J., and Saude, L. (2010). Notch signalling regulates left-right asymmetry through ciliary length control. *Development* 137, 3625–3632. doi: 10.1242/dev.054452

MacDonald, B. T., Tamai, K., and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* 17, 9–26. doi: 10.1016/j.devcel.2009.06.016

Maeda, O., Kondo, M., Fujita, T., Usami, N., Fukui, T., Shimokata, K., et al. (2006). Enhancement of GLI1-transcriptional activity by beta-catenin in human cancer cells. *Oncol. Rep.* 16, 91–96. doi: 10.3892/or.16.1.91

Mar, B. G., Amakye, D., Aifantis, I., and Buonamici, S. (2011). The controversial role of the Hedgehog pathway in normal and malignant hematopoiesis. *Leukemia* 25, 1665–1673. doi: 10.1038/leu.2011.143

Mastronardi, F. G., Dimitroulakos, J., Kamel-Reid, S., and Manoukian, A. S. (2000). Co-localization of patched and activated sonic hedgehog to lysosomes in neurons. *Neuroreport* 11, 581–585. doi: 10.1097/00001756-200002280-00030

McAuliffe, S. M., Morgan, S. L., Wyant, G. A., Tran, L. T., Muto, K. W., Chen, Y. S., et al. (2012). Targeting Notch, a key pathway for ovarian cancer stem cells, sensitizes tumors to platinum therapy. *Proc. Natl Acad. Sci. U. S. A.* 109, E2939–E2948. doi: 10.1073/pnas.1206400109

Merchant, A. A., and Matsui, W. (2010). Targeting Hedgehog—a cancer stem cell pathway. *Clin. Cancer Res.* 16, 3130–3140. doi: 10.1158/1078-0432.CCR-09-2846

Micci, F., Panagopoulos, I., Tjonnfjord, G. E., Kolstad, A., Delabie, J., Beiske, K., and Heim, S. (2007). Molecular cytogenetic characterization of t(14;19)(q32;p13), a new recurrent translocation in B cell malignancies. *Virchows Arch.* 450, 559–565. doi: 10.1007/s00428-007-0407-6

Michaud, E. J., and Yoder, B. K. (2006). The primary cilium in cell signaling and cancer. *Cancer Res.* 66, 6463–6467. doi: 10.1158/0008-5472.CAN-06-0462

Moustakas, A., Souchelnytskyi, S., and Heldin, C. H. (2001). Smad regulation in TGF-beta signal transduction. *J. Cell Sci.* 114, 4359–4369.

Mullor, J. L., Dahmane, N., Sun, T., and Ruiz i Altaba, A. (2001). Wnt signals are targets and mediators of Gli function. *Curr. Biol.* 11, 769–773. doi: 10.1016/S0960-9822(01)00229-9

Mumm, J. S., and Kopan, R. (2000). Notch signaling: from the outside in. *Dev. Biol.* 228, 151–165. doi: 10.1006/dbio.2000.9960

Napolitano, M., Marfia, G. A., Vacca, A., Centonze, D., Bellavia, D., Di Marcotullio, L., et al. (1999). Modulation of gene expression following long-term synaptic depression in the striatum. *Brain Res. Mol. Brain Res.* 72, 89–96. doi: 10.1016/S0169-328X(99)00213-2

Naylor, T. L., Greshock, J., Wang, Y., Colligon, T., Yu, Q. C., Clemmer, V., et al. (2005). High resolution genomic analysis of sporadic breast cancer using array-based comparative genomic hybridization. *Breast Cancer Res.* 7, R1186–R1198. doi: 10.1186/bcr1356

Nessling, M., Richter, K., Schwaenen, C., Roerig, P., Wrobel, G., Wessendorf, S., et al. (2005). Candidate genes in breast cancer revealed by microarray-based comparative genomic hybridization of archived tissue. *Cancer Res.* 65, 439–447.

Nilsson, M., Unden, A. B., Krause, D., Malmqwist, U., Raza, K., Zaphiropoulos, P. G., and Toftgard, R. (2000). Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. *Proc. Natl Acad. Sci. U. S. A.* 97, 3438–3443. doi: 10.1073/pnas.97.7.3438

Nitzki, F., Zibat, A., Frommhold, A., Schneider, A., Schulz-Schaeffer, W., Braun, and T., Hahn, H. (2011). Uncommitted precursor cells might contribute to increased incidence of embryonal rhabdomyosarcoma in heterozygous Patched1-mutant mice. *Oncogene* 30, 4428–4436. doi: 10.1038/onc.2011.157

Northcott, P. A., Nakahara, Y., Wu, X., Feuk, L., Ellison, D. W., Croul, S., et al. (2009). Multiple recurrent genetic events converge on control of histone lysine methylation in medulloblastoma. *Nat. Genet.* 41, 465–472. doi: 10.1038/ng.336

Noubissi, F. K., Goswami, S., Sanek, N. A., Kawakami, K., Minamoto, T., Moser, A., et al. (2009). Wnt signaling stimulates transcriptional outcome of the Hedgehog pathway by stabilizing GLI1 mRNA. *Cancer Res.* 69, 8572–8578. doi: 10.1158/0008-5472.CAN-09-1500

Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287, 795–801. doi: 10.1038/287795a0

Okuyama, R., Tagami, H., and Aiba, S. (2008). Notch signaling: its role in epidermal homeostasis and in the pathogenesis of skin diseases. *J. Dermatol. Sci.* 49, 187–194. doi: 10.1016/j.jdermsci.2007.05.017

Oliver, T. G., Grasfeder, L. L., Carroll, A. L., Kaiser, C., Gillingham, C. L., Lin, S. M., et al. (2003). Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. *Proc. Natl Acad. Sci. U. S. A.* 100, 7331–7336. doi: 10.1073/pnas.0832317100

Ormestad, M., Astorga, J., Landgren, H., Wang, T., Johansson, B. R., Miura, N., and Carlsson, P. (2006). Foxf1 and Foxf2 control murine gut development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production. *Development* 133, 833–843. doi: 10.1242/dev.02252

Palermo, R., Checquolo, S., Bellavia, D., Talora, C., and Screpanti, I. (2014). The molecular basis of notch signaling regulation: a complex simplicity. *Curr. Mol. Med.* 14, 34–44. doi: 10.2174/1566524013666131118105216

Palomero, T., Lim, W. K., Odom, D. T., Sulis, M. L., Real, P. J., Margolin, A., et al. (2006). NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc. Natl Acad. Sci. U. S. A.* 103, 18261–18266. doi: 10.1073/pnas.0606108103

Pardali, K., Kurisaki, A., Moren, A., ten Dijke, P., Kardassis, D., and Moustakas, A. (2000). Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J. Biol. Chem.* 275, 29244–29256. doi: 10.1074/jbc.M909467199

Pearse, R. V., I.S, Collier, MP, Scott, and Tabin, C. J. (1999). Vertebrate homologs of *Drosophila* suppressor of fused interact with the gli family of transcriptional regulators. *Dev. Biol.* 212, 323–336. doi: 10.1006/dbio.1999.9335

Pelullo, M., Quaranta, R., Talora, C., Checquolo, S., Cialfi, S., Felli, M. P., et al. (2014). Notch3/Jagged1 circuitry reinforces notch signaling and sustains T-ALL. *Neoplasia* 16, 1007–1017. doi: 10.1016/j.neo.2014.10.004

Petrova, R., and Joyner, A. L. (2014). Roles for Hedgehog signaling in adult organ homeostasis and repair. *Development* 141, 3445–3457. doi: 10.1242/dev.083691

Pierrat, M. J., Marsaud, V., Mauviel, A., and Javelaud, D. (2012). Expression of microphthalmia-associated transcription factor (MITF), which is critical for melanoma progression, is inhibited by both transcription factor GLI2 and transforming growth factor-beta. *J. Biol. Chem.* 287, 17996–18004. doi: 10.1074/jbc.M112.358341

Poschl, J., Bartels, M., Ohli, J., Bianchi, E., Kuteykin-Teplyakov, K., Grammel, D., et al. (2014). Wnt/beta-catenin signaling inhibits the Shh pathway and impairs tumor growth in Shh-dependent medulloblastoma. *Acta Neuropathol.* 127, 605–607. doi: 10.1007/s00401-014-1258-2

Price, M. A., and Kalderon, D. (2002). Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by glycogen synthase kinase 3 and casein kinase 1. *Cell* 108, 823–835. doi: 10.1016/S0092-8674(02)00664-5

Qualtrough, D., Rees, P., Speight, B., Williams, A. C., and Paraskeva, C. (2015). The Hedgehog inhibitor cyclopamine reduces beta-catenin-Tcf transcriptional activity, induces E-cadherin expression, and reduces invasion in colorectal cancer cells. *Cancers* 7, 1885–1899. doi: 10.3390/cancers7030867

Quaranta, R., Pelullo, M., Zema, S., Nardozza, F., Checquolo, S., Lauer, D. M., et al. (2017). Maml1 acts cooperatively with Gli proteins to regulate sonic hedgehog signaling pathway. *Cell Death Dis.* 8, e2942. doi: 10.1038/cddis.2017.326

Radojcic, V., and Maillard, I. (2014). A jagged road to lymphoma aggressiveness. *Cancer cell* 25, 261–263. doi: 10.1016/j.ccr.2014.03.001

Raffel, C., Jenkins, R. B., Frederick, L., Hebrink, D., Alderete, B., Fults, D. W. (1997). Sporadic medulloblastomas contain PTCH mutations. *Cancer Res.* 57 (5), 842–845.

Ramalho-Santos, M., Melton, D. A., and McMahon, A. P. (2000). Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* 127, 2763–2772.

Reedijk, M., Odorcic, S., Zhang, H., Chetty, R., Tennert, C., et al. (2008). Activation of Notch signaling in human colon adenocarcinoma. *Int. J. Oncol.* 33, 1223–1229.

Regl, G., Neill, G. W., Eichberger, T., Kasper, M., Ikram, M. S., Koller, J., et al. (2002). Human GLI2 and GLI1 are part of a positive feedback mechanism in Basal Cell Carcinoma. *Oncogene* 21, 5529–5539. doi: 10.1038/sj.onc.1205748

Reifenberger, J., Wolter, M., Knobbe, C. B., Kohler, B., Schonicke, A., Scharwachter, C., et al. (2005). Somatic mutations in the PTCH, SMOH, SUFUH and TP53 genes in sporadic basal cell carcinomas. *Br. J. Dermatol.* 152, 43–51. doi: 10.1111/j.1365-2133.2005.06353.x

Reifenberger, J., Wolter, M., Weber, R. G., Megahed, M., Ruzicka, T., Lichter, P., et al. (1998). Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res.* 58, 1798–1803.

Rimkus, T. K., Carpenter, R. L., Qasem, S., Chan, M., and Lo, H. W. (2016). Targeting the sonic hedgehog signaling pathway: review of smoothened and GLI inhibitors. *Cancers* 8, 22–45. doi: 10.3390/cancers8020022

Roberts, W. M., Douglass, E. C., Peiper, S. C., Houghton, P. J., and Look, A. T. (1989). Amplification of the gli gene in childhood sarcomas. *Cancer Res.* 49, 5407–5413.

Rodilla, V., Villanueva, A., Obrador-Hevia, A., Robert-Moreno, A., Fernandez-Majada, V., Grilli, A., et al. (2009). Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. *Proc. Natl Acad. Sci. U. S. A.* 106, 6315–6320. doi: 10.1073/pnas.0813221106

Rohatgi, R., Milenkovic, L., and Scott, M. P. (2007). Patched1 regulates hedgehog signaling at the primary cilium. *Science* 317, 372–376. doi: 10.1126/science.1139740

Rosati, E., Sabatini, R., Rampino, G., Tabilio, A., Di Ianni, M., Fettucciari, K., et al. (2009). Constitutively activated Notch signaling is involved in survival and apoptosis resistance of B-CLL cells. *Blood* 113, 856–865. doi: 10.1182/blood-2008-02-139725

Ross, D. A., and Kadesch, T. (2001). The notch intracellular domain can function as a coactivator for LEF-1. *Mol. Cell. Biol.* 21, 7537–7544. doi: 10.1128/MCB.21.22.7537-7544.2001

Rowbotham, N. J., Hager-Theodorides, A. L., Furmanski, A. L., and Crompton, T. (2007). A novel role for Hedgehog in T-cell receptor signaling: implications for development and immunity. *Cell cycle* 6, 2138–2142. doi: 10.4161/cc.6.17.4644

Rusert, J. M., Wu, X., Eberhart, C. G., Taylor, M. D., and Wechsler-Reya, R. J. (2014). SnapShot: medulloblastoma. *Cancer cell* 26940-40, e1. doi: 10.1016/j.ccell.2014.11.015

Rustighi, A., Zannini, A., Tiberi, L., Sommaggio, R., Piazza, S., Sorrentino, G., et al. (2014). Prolyl-isomerase Pin1 controls normal and cancer stem cells of the breast. *EMBO Mol. Med.* 6, 99–119. doi: 10.1002/emmm.201302909

Ryan, K. E., and Chiang, C. (2012). Hedgehog secretion and signal transduction in vertebrates. *J. Biol. Chem.* 287, 17905–17913. doi: 10.1074/jbc.R112.356006

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., and Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* 126, 3915–3924.

Schreck, K. C., Taylor, P., Marchionni, L., Gopalakrishnan, V., Bar, E. E., Gaiano, N., and Eberhart, C. G. (2010). The Notch target Hes1 directly modulates Gli1 expression and Hedgehog signaling: a potential mechanism of therapeutic resistance. *Clin. Cancer Res.* 16, 6060–6070. doi: 10.1158/1078-0432.CCR-10-1624

Sengupta, A., Banerjee, D., Chandra, S., Banerji, S. K., Ghosh, R., Roy, R., et al. (2007). Deregulation and cross talk among Sonic hedgehog, Wnt, Hox and Notch signaling in chronic myeloid leukemia progression. *Leukemia* 21, 949–955. doi: 10.1038/sj.leu.2404657

Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M., and Massague, J. (2001). TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat. Cell Biol.* 3, 400–408. doi: 10.1038/35070086

Sheng, T., Li, C., Zhang, X., Chi, S., He, N., Chen, K., et al. (2004). Activation of the hedgehog pathway in advanced prostate cancer. *Mol. Cancer* 3, 29. doi: 10.1186/1476-4598-3-29

Shin, H. M., Minter, L. M., Cho, O. H., Gottipati, S., Fauq, A. H., Golde, T. E., et al. (2006). Notch1 augments NF-kappaB activity by facilitating its nuclear retention. *EMBO J.* 25, 129–138. doi: 10.1038/sj.emboj.7600902

Siegel, P. M., and Massague, J. (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat. Rev. Cancer* 3, 807–821. doi: 10.1038/nrc1208

Sikandar, S. S., Pate, K. T., Anderson, S., Dizon, D., Edwards, R. A., Waterman, M. L., et al. (2010). NOTCH signaling is required for formation and self-renewal of tumor-initiating cells and for repression of secretory cell differentiation in colon cancer. *Cancer Res.* 70, 1469–1478. doi: 10.1158/0008-5472.CAN-09-2557

Singh, R. R., Kim, J. E., Davuluri, Y., Drakos, E., Cho-Vega, J. H., Amin, H. M., et al. (2010). Hedgehog signaling pathway is activated in diffuse large B-cell lymphoma and contributes to tumor cell survival and proliferation. *Leukemia* 24, 1025–1036. doi: 10.1038/leu.2010.35

Sinha, S., Singh, R. K., Alam, N., Roy, A., Roychoudhury, S., and Panda, C. K. (2008). Frequent alterations of hMLH1 and RBSP3/HYA22 at chromosomal 3p22.3 region in early and late-onset breast carcinoma: clinical and prognostic significance. *Cancer Sci.* 99, 1984–1991. doi: 10.1111/j.1349-7006.2008.00952.x

Sisson, B. E., Ziegenhorn, S. L., and Holmgren, R. A. (2006). Regulation of Ci and Su(fu) nuclear import in *Drosophila*. *Dev. Biol.* 294, 258–270. doi: 10.1016/j.ydbio.2006.02.050

Smith, M. J., Beetz, C., Williams, S. G., Bhaskar, S. S., O'Sullivan, J., Anderson, B., et al. (2014). Germline mutations in SUFU cause Gorlin syndrome–associated childhood medulloblastoma and redefine the risk associated with PTCH1 mutations. *J. Clin. Oncol.* 32, 4155–4161. doi: 10.1200/JCO.2014.58.2569

Staal, F. J., Famili, F., Garcia Perez, L., and Pike-Overzet, K. (2016). Aberrant Wnt signaling in leukemia. *Cancers* 8, 78–93. doi: 10.3390/cancers8090078

Stasiulewicz, M., Gray, S. D., Mastromina, I., Silva, J. C., Bjorklund, M., Seymour, P. A., et al. (2015). A conserved role for Notch signaling in priming the cellular response to Shh through ciliary localisation of the key Shh transducer Smo. *Development* 142, 2291–2303. doi: 10.1242/dev.125237

Stecca, B., and Ruiz i Altaba, A. (2009). A GLI1–p53 inhibitory loop controls neural stem cell and tumour cell numbers. *EMBO J.* 28, 663–676. doi: 10.1038/emboj.2009.16

Steg, A. D., Katre, A. A., Goodman, B., Han, H. D., Nick, A. M., Stone, R. L., et al. (2011). Targeting the notch ligand JAGGED1 in both tumor cells and stroma in ovarian cancer. *Clin. Cancer Res.* 17, 5674–5685. doi: 10.1158/1078-0432.CCR-11-0432

Stone, D. M., Murone, M., Luoh, S., Ye, W., Armanini, M. P., Gurney, A., et al. (1999). Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli. *J. Cell Sci.* 112 (Pt 23), 4437–4448.

Swartling, F. J., Grimmer, M. R., Hackett, C. S., Northcott, P. A., Fan, Q. W., Goldenberg, D. D., et al. (2010). Pleiotropic role for MYCN in medulloblastoma. *Genes Dev.* 24, 1059–1072. doi: 10.1101/gad.1907510

Sweeney, R. T., McClary, A. C., Myers, B. R., Biscocho, J., Neahring, L., Kwei, K. A., et al. (2014). Identification of recurrent SMO and BRAF mutations in ameloblastomas. *Nat. Genet.* 46, 722–725. doi: 10.1038/ng.2986

Syed, V. (2016). TGF-beta signaling in cancer. *J. Cell. Biochem.* 117, 1279–1287. doi: 10.1002/jcb.25496

Taeubner, J., Brozou, T., Qin, N., Bartl, J., Ginzel, S., Schaper, J., et al. (2018). Congenital embryonal rhabdomyosarcoma caused by heterozygous concomitant PTCH1 and PTCH2 germline mutations. *Eur. J. Human Genet.* 26, 137–142. doi: 10.1038/s41431-017-0048-4

Taipale, J., Cooper, M. K., Maiti, T., and Beachy, P. A. (2002). Patched acts catalytically to suppress the activity of Smoothened. *Nature* 418, 892–897. doi: 10.1038/nature00989

Takebe, N., Harris, P. J., Warren, R. Q., and Ivy, S. P. (2011). Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. *Nat. Rev. Clin. Oncol.* 8, 97–106. doi: 10.1038/nrclinonc.2010.196

Talora, C., Campese, A. F., Bellavia, D., Felli, M. P., Vacca, A., Gulino, A., et al. (2008). Notch signaling and diseases: an evolutionary journey from a simple beginning to complex outcomes. *Biochim. Biophys. Acta* 1782, 489–497. doi: 10.1016/j.bbadis.2008.06.008

Talora, C., Cialfi, S., Oliviero, C., Palermo, R., Pascucci, M., Frati, L., et al. (2006). Cross talk among Notch3, pre-TCR, and Tal1 in T-cell development and leukemogenesis. *Blood* 107, 3313–3320. doi: 10.1182/blood-2005-07-2823

Taylor, M. D., Liu, L., Raffel, C., Hui, C. C., Mainprize, T. G., Zhang, X., et al. (2002). Mutations in SUFU predispose to medulloblastoma. *Nat. Genet.* 31, 306–310. doi: 10.1038/ng916

Taylor, M. D., Zhang, X., Liu, L., Hui, C. C., Mainprize, T. G., Scherer, S. W., et al. (2004). Failure of a medulloblastoma-derived mutant of SUFU to suppress WNT signaling. *Oncogene* 23, 4577–4583. doi: 10.1038/sj.onc.1207605

Teglund, S., and Toftgard, R. (2010). Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim. Biophys. Acta* 1805, 181–208. doi: 10.1016/j.bbcan.2010.01.003

Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 398, 422–426. doi: 10.1038/18884

Thayer, S. P., di Magliano, M. P., Heiser, P. W., Nielsen, C. M., Roberts, D. J., Lauwers, G. Y., et al. (2003). Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* 425, 851–856. doi: 10.1038/nature02009

Thomas, W. D., Chen, J., Gao, Y. R., Cheung, B., Koach, J., Sekyere, E., et al. (2009). Patched1 deletion increases N-Myc protein stability as a mechanism of medulloblastoma initiation and progression. *Oncogene* 28, 1605–1615. doi: 10.1038/onc.2009.3

Thompson, M. C., Fuller, C., Hogg, T. L., Dalton, J., Finkelstein, D., Lau, C. C., et al. (2006). Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. *J. Clin. Oncol.* 24, 1924–1931. doi: 10.1200/JCO.2005.04.4974

Tottone, L., Zhdanovskaya, N., Carmona Pestana, A., Zampieri, M., Simeoni, F., Lazzari, S., et al. (2019). Histone modifications drive aberrant Notch3 expression/activity and growth in T-ALL. *Front. Oncol.* 9, 198. doi: 10.3389/fonc.2019.00198

Ulloa, F., Itasaki, N., and Briscoe, J. (2007). Inhibitory Gli3 activity negatively regulates Wnt/beta-catenin signaling. *Curr. Biol.* 17, 545–550. doi: 10.1016/j.cub.2007.01.062

Vacca, A., Felli, M. P., Palermo, R., Di Mario, G., Calce, A., Di Giovine, M., et al. (2006). Notch3 and pre-TCR interaction unveils distinct NF-kappaB pathways in T-cell development and leukemia. *EMBO J.* 25, 1000–1008. doi: 10.1038/sj.emboj.7600996

van den Brink, G. R., Bleuming, S. A., Hardwick, J. C., Schepman, B. L., Offerhaus, G. J., Keller, J. J., et al. (2004). Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. *Nat. Genet.* 36, 277–282. doi: 10.1038/ng1304

Vargas Romero, P., Cialfi, S., Palermo, R., De Blasio, C., Checquolo, S., Bellavia, D., et al. (2015). The deregulated expression of miR-125b in acute myeloid leukemia is dependent on the transcription factor C/EBPalpha. *Leukemia* 29, 2442–2445. doi: 10.1038/leu.2015.117

Varjosalo, M., and Taipale, J. (2008). Hedgehog: functions and mechanisms. *Genes Dev.* 22, 2454–2472. doi: 10.1101/gad.1693608

Varnat, F., Duquet, A., Malerba, M., Zbinden, M., Mas, C., Gervaz, P. and Ruiz i Altaba, A., et al. (2009). Human colon cancer epithelial cells harbour active HEDGEHOG-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. *EMBO Mol. Med.* 1, 338–351. doi: 10.1002/emmm.200900039

Varnat, F., Siegl-Cachedenier, I., Malerba, M., Gervaz, P., and Ruiz i Altaba, A. (2010). Loss of WNT-TCF addiction and enhancement of HH-GLI1 signalling define the metastatic transition of human colon carcinomas. *EMBO Mol. Med.* 2, 440–457. doi: 10.1002/emmm.201000098

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., et al. (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat. Med.* 6, 1278–1281. doi: 10.1038/81390

Verrecchia, F., and Mauviel, A. (2007). Transforming growth factor-beta and fibrosis. *World J. Gastroenterol.* 13, 3056–3062. doi: 10.3748/wjg.v13.i22.3056

Vied, C., and Kalderon, D. (2009). Hedgehog-stimulated stem cells depend on non-canonical activity of the Notch co-activator Mastermind. *Development* 136, 2177–2186. doi: 10.1242/dev.035329

Vokes, S. A., Ji, H., McCuine, S., Tenzen, T., Giles, S., Zhong, S., et al. (2007). Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. *Development* 134, 1977–1989. doi: 10.1242/dev.001966

Wahl, S. M. (1994). Transforming growth factor beta: the good, the bad, and the ugly. *J. Exp. Med.* 180, 1587–1590. doi: 10.1084/jem.180.5.1587

Wall, D. S., Mears, A. J., McNeill, B., Mazerolle, C., Thurig, S., Wang, Y., et al. (2009). Progenitor cell proliferation in the retina is dependent on Notch-independent Sonic hedgehog/Hes1 activity. *J. Cell Biol.* 184, 101–112. doi: 10.1083/jcb.200805155

Wang, X. D., Inzunza, H., Chang, H., Qi, Z., Hu, B., Malone, D., Cogswell, J. (2013). Mutations in the hedgehog pathway genes SMO and PTCH1 in human gastric tumors. *PloS One* 8, e54415. doi: 10.1371/journal.pone.0054415

Weng, A. P., Millholland, J. M., Yashiro-Ohtani, Y., Arcangeli, M. L., Lau, A., Wai, C., et al. (2006). c-Myc is an important direct target of Notch1 in T-cell

acute lymphoblastic leukemia/lymphoma. *Genes Dev.* 20, 2096–2109. doi: 10.1101/gad.1450406

Wiemels, J., Wrensch, M., and Claus, E. B. (2010). Epidemiology and etiology of meningioma. *J. Neuro-oncol.* 99, 307–314. doi: 10.1007/s11060-010-0386-3

Wils, L. J., and Bijlsma, M. F. (2018). Epigenetic regulation of the Hedgehog and Wnt pathways in cancer. *Crit. Rev. Oncol. Hematol.* 121, 23–44. doi: 10.1016/j.critrevonc.2017.11.013

Wolfraim, L. A., Fernandez, T. M., Mamura, M., Fuller, W. L., Kumar, R., Cole, D. E., et al. (2004). Loss of Smad3 in acute T-cell lymphoblastic leukemia. *N. Engl. J. Med.* 351, 552–559. doi: 10.1056/NEJMoa031197

Wong, S. Y., and Reiter, J. F. (2008). The primary cilium at the crossroads of mammalian hedgehog signaling. *Curr. Top. Dev. Biol.* 85, 225–260. doi: 10.1016/S0070-2153(08)00809-0

Wood, L. D., Parsons, D. W., Jones, S., Lin, J., Sjoblom, T., Leary, R. J., et al. (2007). The genomic landscapes of human breast and colorectal cancers. *Science* 318, 1108–1113. doi: 10.1126/science.1145720

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J. D. (2000). MAML1, a human homologue of *Drosophila* mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat. Genet.* 26, 484–489. doi: 10.1038/82644

Xie, G., Karaca, G., Swiderska-Syn, M., Michelotti, G. A., Kruger, L., Chen, Y., et al. (2013). Cross-talk between Notch and Hedgehog regulates hepatic stellate cell fate in mice. *Hepatology* 58, 1801–1813. doi: 10.1002/hep.26511

Xie, J., Murone, M., Luoh, S. M., Ryan, A., Gu, Q., Zhang, C., et al. (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* 391, 90–92. doi: 10.1038/34201

Yang, X., Letterio, J. J., Lechleider, R. J., Chen, L., Hayman, R., Gu, H., et al. (1999). Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J.* 18, 1280–1291. doi: 10.1093/emboj/18.5.1280

Yavropoulou, M. P., Maladaki, A., and Yovos, J. G. (2015). The role of Notch and Hedgehog signaling pathways in pituitary development and pathogenesis of pituitary adenomas. *Hormones* 14, 5–18. doi: 10.1007/BF03401377

Yoshikawa, K., Shimada, M., Miyamoto, H., Higashijima, J., Miyatani, T., Nishioka, M., et al. (2009). Sonic hedgehog relates to colorectal carcinogenesis. *J. Gastroenterol.* 44, 1113–1117. doi: 10.1007/s00535-009-0110-2

Yoshimoto, A. N., Bernardazzi, C., Carneiro, A. J., Elia, C. C., Martinusso, C. A., Ventura, G. M., et al. (2012). Hedgehog pathway signaling regulates human colon carcinoma HT-29 epithelial cell line apoptosis and cytokine secretion. *PloS One* 7, e45332. doi: 10.1371/journal.pone.0045332

Zavadil, J., and Bottinger, E. P. (2005). TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* 24, 5764–5774. doi: 10.1038/sj.onc.1208927

Zavadil, J., Cermak, L., Soto-Nieves, N., and Bottinger, E. P. (2004). Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. *EMBO J.* 23, 1155–1165. doi: 10.1038/sj.emboj.7600069

Zi, Z., Chapnick, D. A., and Liu, X. (2012). Dynamics of TGF-beta/Smad signaling. *FEBS Lett.* 586, 1921–1928. doi: 10.1016/j.febslet.2012.03.063

Zinke, J., Schneider, F. T., Harter, P. N., Thom, S., Ziegler, N., Toftgard, R., et al. (2015). Beta-catenin-Gli1 interaction regulates proliferation and tumor growth in medulloblastoma. *Mol. Cancer* 14, 17. doi: 10.1186/s12943-015-0294-4

Zurawel, R. H., Allen, C., Chiappa, S., Cato, W., Biegel, J., Cogen, P., et al. (2000). Analysis of PTCH/SMO/SHH pathway genes in medulloblastoma. *Genes Chromosomes Cancer* 27, 44–51. doi: 10.1002/(SICI)1098-2264(200001)27:1<44::AID-GCC6>3.0.CO;2-V

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VII. References

Agrawal, N., Frederick, M. J., Pickering, C. R., Bettegowda, C., Chang, K., Li, R. J., … Myers, J. N. (2011). Exome Sequencing of Head and Neck Squamous Cell Carcinoma Reveals Inactivating Mutations in NOTCH1 *Science*, *333*(6046), 1154 LP – 1157. https://doi.org/10.1126/science.1206923

AITHAL, M. G. S., & RAJESWARI, N. (2013). Role of Notch signalling pathway in cancer and its association with DNA methylation. *Journal of Genetics*, *92*(3), 667–675. https://doi.org/10.1007/s12041-013-0284-5

Aki, D., Zhang, W., & Liu, Y.-C. (2015). The E3 ligase Itch in immune regulation and beyond. *Immunological Reviews*, *266*(1), 6–26. https://doi.org/10.1111/imr.12301

Alves-Guerra, M.-C., Ronchini, C., & Capobianco, A. J. (2007). Mastermind-like 1 Is a Specific Coactivator of β-Catenin Transcription Activation and Is Essential for Colon Carcinoma Cell Survival. *Cancer Research*, *67*(18), 8690 LP – 8698. https://doi.org/10.1158/0008-5472.CAN-07-1720

Argenti, B., Gallo, R., Di Marcotullio, L., Ferretti, E., Napolitano, M., Canterini, S., … Gulino, A. (2005). Hedgehog antagonist REN(KCTD11) regulates proliferation and apoptosis of developing granule cell progenitors. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(36), 8338–8346. https://doi.org/10.1523/JNEUROSCI.2438-05.2005

Artavanis-Tsakonas, S., & Muskavitch, M. A. T. (2010). Notch: The Past, the Present, and the Future. *Current Topics in Developmental Biology*, *92*, 1–29. https://doi.org/10.1016/S0070-2153(10)92001-2

Artavanis-Tsakonas, S., Rand, M. D., & Lake, R. J. (1999, April 30). Notch signaling: Cell fate control and signal integration in development. *Science*, Vol. 284, pp. 770–776. https://doi.org/10.1126/science.284.5415.770

Ascano, J. M., Beverly, L. J., & Capobianco, A. J. (2003). The C-terminal PDZ-ligand of JAGGED1 is essential for cellular transformation. *The Journal of Biological Chemistry*, *278*(10), 8771–8779. https://doi.org/10.1074/jbc.M211427200

Aster, J. C., Pear, W. S., & Blacklow, S. C. (2008). Notch signaling in leukemia. *Annual Review of Pathology*, *3*, 587–613. https://doi.org/10.1146/annurev.pathmechdis.3.121806.154300

Bai, C. B., Auerbach, W., Lee, J. S., Stephen, D., & Joyner, A. L. (2002). Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. *Development*, *129*(20), 4753 LP – 4761. Retrieved from http://dev.biologists.org/content/129/20/4753.abstract

Barisone, G. A., Ngo, T., Tran, M., Cortes, D., Shahi, M. H., Nguyen, T.-V., … Díaz, E. (2012). Role of MXD3 in proliferation of DAOY human medulloblastoma cells. *PloS One*, *7*(7), e38508–e38508. https://doi.org/10.1371/journal.pone.0038508

Behesti, H., & Marino, S. (2009). Cerebellar granule cells: Insights into proliferation, differentiation, and role in medulloblastoma pathogenesis. *The International Journal of Biochemistry & Cell Biology*, *41*(3), 435–445. https://doi.org/10.1016/J.BIOCEL.2008.06.017

Bellavia, D, Campese, A. F., Alesse, E., Vacca, A., Felli, M. P., Balestri, A., … Screpanti, I. (2000). Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. *The EMBO Journal*, *19*(13), 3337–3348. https://doi.org/10.1093/emboj/19.13.3337

Bellavia, Diana, Mecarozzi, M., Campese, A. F., Grazioli, P., Gulino, A., & Screpanti, I. (2007). Notch and Ikaros: Not Only Converging Players in T Cell Leukemia. *Cell Cycle*, *6*(22), 2730–2734. https://doi.org/10.4161/cc.6.22.4894

Bellavia, Diana, Mecarozzi, M., Campese, A. F., Grazioli, P., Talora, C., Frati, L., … Screpanti, I. (2007). Notch3 and the Notch3-upregulated RNA-binding protein HuD regulate Ikaros alternative splicing. *The EMBO Journal*, *26*(6), 1670–1680. https://doi.org/10.1038/sj.emboj.7601626

Bellavia, Diana, Palermo, R., Felli, M. P., Screpanti, I., & Checquolo, S. (2018). Notch signaling as a therapeutic target for acute lymphoblastic leukemia. *Expert Opinion on Therapeutic Targets*, *22*(4), 331–342. https://doi.org/10.1080/14728222.2018.1451840

Beres, B. J., George, R., Lougher, E. J., Barton, M., Verrelli, B. C., McGlade, C. J., … Wilson-Rawls, J. (2011). Numb regulates Notch1, but not Notch3, during myogenesis. *Mechanisms of Development*, *128*(5–6), 247–257. https://doi.org/10.1016/J.MOD.2011.02.002

Bernasconi-Elias, P., Hu, T., Jenkins, D., Firestone, B., Gans, S., Kurth, E., … Fryer, C. J. (2016). Characterization of activating mutations of NOTCH3 in T-cell acute lymphoblastic leukemia and anti-leukemic activity of NOTCH3 inhibitory antibodies. *Oncogene*, *35*(47), 6077–6086. https://doi.org/10.1038/onc.2016.133

Bernassola, F., Karin, M., Ciechanover, A., & Melino, G. (2008). The HECT family of E3 ubiquitin ligases: multiple players in cancer development. *Cancer Cell*, *14*(1), 10–21. https://doi.org/10.1016/j.ccr.2008.06.001

Bertrand, F. E., Angus, C. W., Partis, W. J., & Sigounas, G. (2012). Developmental pathways in colon cancer: crosstalk between WNT, BMP, Hedgehog and Notch. *Cell Cycle (Georgetown, Tex.)*, *11*(23), 4344–4351. https://doi.org/10.4161/cc.22134

Borggrefe, T., Lauth, M., Zwijsen, A., Huylebroeck, D., Oswald, F., & Giaimo, B. D. (2016). The Notch intracellular domain integrates signals from Wnt, Hedgehog, TGFβ/BMP and hypoxia pathways. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1863*(2), 303–313. https://doi.org/10.1016/J.BBAMCR.2015.11.020

Borggrefe, T., & Oswald, F. (2009). The Notch signaling pathway: Transcriptional regulation at Notch target genes. *Cell. Mol. Life Sci.*, (66), 1631. https://doi.org/https://doi.org/10.1007/s00018-009-8668-7

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. *Nature Reviews Molecular Cell Biology*, *7*(9), 678–689. https://doi.org/10.1038/nrm2009

Brimer, N., Lyons, C., Wallberg, A. E., & Vande Pol, S. B. (2012). Cutaneous papillomavirus E6 oncoproteins associate with MAML1 to repress transactivation and NOTCH signaling. *Oncogene*, *31*(43), 4639–4646. https://doi.org/10.1038/onc.2011.589

Campese, Antonio F., Bellavia, D., Gulino, A., & Screpanti, I. (2003). Notch signalling at the crossroads of T cell development and leukemogenesis. *Seminars in Cell & Developmental Biology*, *14*(2), 151–157. https://doi.org/10.1016/S1084-9521(02)00184-2

Campese, Antonio F, Grazioli, P., Colantoni, S., Anastasi, E., Mecarozzi, M., Checquolo, S., … Screpanti, I. (2009). Notch3 and pTα/pre-TCR sustain the in vivo function of naturally occurring regulatory T cells. *International Immunology*, *21*(6), 727–743. https://doi.org/10.1093/intimm/dxp042

Campese, Antonio Francesco, Grazioli, P., Cesaris, P. de, Riccioli, A., Bellavia, D., Pelullo, M., … Starace, D. (2014). Mouse Sertoli Cells Sustain De Novo Generation of Regulatory T Cells by Triggering the Notch Pathway Through Soluble JAGGED1. *Biology of Reproduction*, *90*(3). Retrieved from https://doi.org/10.1095/biolreprod.113.113803

Canettieri, G., Di Marcotullio, L., Greco, A., Coni, S., Antonucci, L., Infante, P., … Gulino, A. (2010). Histone deacetylase and Cullin3–RENKCTD11 ubiquitin ligase interplay regulates Hedgehog signalling through Gli acetylation. *Nature Cell Biology*, *12*, 132. Retrieved from https://doi.org/10.1038/ncb2013

Chastagner, P., Israël, A., & Brou, C. (2006). Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. *EMBO Reports*, *7*(11), 1147–1153. https://doi.org/10.1038/sj.embor.7400822

Chastagner, P., Israël, A., & Brou, C. (2008). AIP4/Itch Regulates Notch Receptor Degradation in the Absence of Ligand. *PLOS ONE*, *3*(7), e2735. Retrieved from https://doi.org/10.1371/journal.pone.0002735

Checquolo, S., Palermo, R., Cialfi, S., Ferrara, G., Oliviero, C., Talora, C., … Screpanti, I. (2010). Differential subcellular localization regulates c-Cbl E3 ligase activity upon Notch3 protein in T-cell leukemia. *Oncogene*, *29*(10), 1463–1474. https://doi.org/10.1038/onc.2009.446

Chen, C., Wei, Y., Hummel, M., Hoffmann, T. K., Gross, M., Kaufmann, A. M., & Albers, A. E. (2011). Evidence for epithelial-mesenchymal transition in cancer stem cells of head and neck squamous cell carcinoma. *PloS One*, *6*(1), e16466–e16466. https://doi.org/10.1371/journal.pone.0016466

Chen, J. J., Hong, Y., Rustamzadeh, E., Baleja, J. D., & Androphy, E. J. (1998). Identification of an α Helical Motif Sufficient for Association with Papillomavirus E6. *Journal of Biological Chemistry*, *273*(22), 13537–13544. https://doi.org/10.1074/JBC.273.22.13537

Chen, J. K., Taipale, J., Cooper, M. K., & Beachy, P. A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes & Development*, *16*(21), 2743–2748. https://doi.org/10.1101/gad.1025302

Chen, J. K., Taipale, J., Young, K. E., Maiti, T., & Beachy, P. A. (2002). Small molecule modulation of Smoothened activity. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(22), 14071–14076. https://doi.org/10.1073/pnas.182542899

Chen, Xiangrong, Chen, L., Zhang, R., Yi, Y., Ma, Y., Yan, K., … Wang, X. (2013). ADAM17 regulates self-renewal and differentiation of U87 glioblastoma stem cells. *Neuroscience Letters*, *537*, 44–49. https://doi.org/10.1016/J.NEULET.2013.01.021

Chen, Xu, Stoeck, A., Lee, S. J., Shih, I.-M., Wang, M. M., & Wang, T.-L. (2010). Jagged1 expression regulated by Notch3 and Wnt/β-catenin signaling pathways in ovarian cancer. *Oncotarget*, *1*(3), 210–218. https://doi.org/10.18632/oncotarget.127

Cheng, H., Chen, L., Hu, X., Qiu, H., Xu, X., Gao, L., … Huang, C. (2019). Knockdown of MAML1 inhibits proliferation and

induces apoptosis of T-cell acute lymphoblastic leukemia cells through SP1-dependent inactivation of TRIM59. *Journal of Cellular Physiology*, *234*(4), 5186–5195. https://doi.org/10.1002/jcp.27323

Choi, J.-H., Park, J. T., Davidson, B., Morin, P. J., Shih, I.-M., & Wang, T.-L. (2008). Jagged-1 and Notch3 juxtacrine loop regulates ovarian tumor growth and adhesion. *Cancer Research*, *68*(14), 5716–5723. https://doi.org/10.1158/0008-5472.CAN-08-0001

Choi, S. H., Wales, T. E., Nam, Y., O'Donovan, D. J., Sliz, P., Engen, J. R., & Blacklow, S. C. (2012). Conformational Locking upon Cooperative Assembly of Notch Transcription Complexes. *Structure*, *20*(2), 340–349. https://doi.org/10.1016/J.STR.2011.12.011

Cialfi, S, Palermo, R., Manca, S., Checquolo, S., Bellavia, D., Pelullo, M., … Talora, C. (2013). Glucocorticoid sensitivity of T-cell lymphoblastic leukemia/lymphoma is associated with glucocorticoid receptor-mediated inhibition of Notch1 expression. *Leukemia*, *27*(2), 485–488. https://doi.org/10.1038/leu.2012.192

Cialfi, Samantha, Palermo, R., Manca, S., De Blasio, C., Vargas Romero, P., Checquolo, S., … Talora, C. (2014). Loss of Notch1-dependent p21(Waf1/Cip1) expression influences the Notch1 outcome in tumorigenesis. *Cell Cycle (Georgetown, Tex.)*, *13*(13), 2046–2055. https://doi.org/10.4161/cc.29079

Cohen, B., Shimizu, M., Izrailit, J., Ng, N. F. L., Buchman, Y., Pan, J. G., … Reedijk, M. (2010). Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. *Breast Cancer Research and Treatment*, *123*(1), 113–124. https://doi.org/10.1007/s10549-009-0621-9

Colicchia, V., Petroni, M., Guarguaglini, G., Sardina, F., Sahún-Roncero, M., Carbonari, M., … Giannini, G. (2017). PARP inhibitors enhance replication stress and cause mitotic catastrophe in MYCN-dependent neuroblastoma. *Oncogene*, *36*(33), 4682–4691. https://doi.org/10.1038/onc.2017.40

Cordle, J., Redfieldz, C., Stacey, M., van der Merwe, P. A., Willis, A. C., Champion, B. R., … Handford, P. A. (2008). Localization of the delta-like-1-binding site in human Notch-1 and its modulation by calcium affinity. *The Journal of Biological Chemistry*, *283*(17), 11785–11793. https://doi.org/10.1074/jbc.M708424200

Corrales, J. D., Blaess, S., Mahoney, E. M., & Joyner, A. L. (2006). The level of sonic hedgehog signaling regulates the complexity of cerebellar foliation. *Development*, *133*(9), 1811 LP – 1821. https://doi.org/10.1242/dev.02351

Corrales, J. D., Rocco, G. L., Blaess, S., Guo, Q., & Joyner, A. L. (2004). Spatial pattern of sonic hedgehog signaling through Gli genesduring cerebellum development. *Development*, *131*(22), 5581–5590. https://doi.org/10.1242/DEV.01438

Creighton, C. J., Li, X., Landis, M., Dixon, J. M., Neumeister, V. M., Sjolund, A., … Chang, J. C. (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(33), 13820–13825. https://doi.org/10.1073/pnas.0905718106

D'Souza, B., Miyamoto, A., & Weinmaster, G. (2008). The many facets of Notch ligands. *Oncogene*, *27*(38), 5148–5167. https://doi.org/10.1038/onc.2008.229

Dahmane, N., & Ruiz-i-Altaba, A. (1999). Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development*, *126*(14), 3089 LP – 3100. Retrieved from http://dev.biologists.org/content/126/14/3089.abstract

Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M., & Ishii, S. (1999). Sonic hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *Journal of Biological Chemistry*, *274*(12), 8143–8152. https://doi.org/10.1074/jbc.274.12.8143

Dai, Y., Wilson, G., Huang, B., Peng, M., Teng, G., Zhang, D., … Qiao, L. (2014). Silencing of Jagged1 inhibits cell growth and invasion in colorectal cancer. *Cell Death & Disease*, *5*(4), e1170–e1170. https://doi.org/10.1038/cddis.2014.137

De Falco, F., Del Papa, B., Baldoni, S., Sabatini, R., Falzetti, F., Di Ianni, M., … Rosati, E. (2018). IL-4-dependent Jagged1 expression/processing is associated with survival of chronic lymphocytic leukemia cells but not with Notch activation. *Cell Death & Disease*, *9*(12), 1160. https://doi.org/10.1038/s41419-018-1185-6

De Smaele, E., Di Marcotullio, L., Moretti, M., Pelloni, M., Occhione, M. A., Infante, P., … Gulino, A. (2011). Identification and characterization of KCASH2 and KCASH3, 2 novel Cullin3 adaptors suppressing histone deacetylase and Hedgehog activity in medulloblastoma. *Neoplasia (New York, N.Y.)*, *13*(4), 374–385. https://doi.org/10.1593/neo.101630

Dho, S. E., French, M. B., Woods, S. A., & McGlade, C. J. (1999). Characterization of Four Mammalian Numb Protein Isoforms. *Journal of Biological Chemistry*, *274*(46), 33097–33104. https://doi.org/10.1074/JBC.274.46.33097

Di Marcotullio, L., Greco, A., Mazzà, D., Canettieri, G., Pietrosanti, L., Infante, P., … Gulino, A. (2011). Numb activates the E3 ligase Itch to control Gli1 function through a novel degradation signal. *Oncogene*, *30*(1), 65–76. https://doi.org/10.1038/onc.2010.394

Di Marcotullio, Lucia, Ferretti, E., Greco, A., De Smaele, E., Po, A., Sico, M. A., … Gulino, A. (2006). Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. *Nature Cell Biology*, *8*(12), 1415–1423. https://doi.org/10.1038/ncb1510

Díaz-Rodríguez, E., Montero, J. C., Esparís-Ogando, A., Yuste, L., & Pandiella, A. (2002). Extracellular signal-regulated kinase

phosphorylates tumor necrosis factor alpha-converting enzyme at threonine 735: a potential role in regulated shedding. *Molecular Biology of the Cell*, *13*(6), 2031–2044. https://doi.org/10.1091/mbc.01-11-0561

Dickson, B. C., Mulligan, A. M., Zhang, H., Lockwood, G., O'Malley, F. P., Egan, S. E., & Reedijk, M. (2007). High-level JAG1 mRNA and protein predict poor outcome in breast cancer. *Modern Pathology*, *20*, 685. Retrieved from https://doi.org/10.1038/modpathol.3800785

Diluvio, G., Del Gaudio, F., Giuli, M. V., Franciosa, G., Giuliani, E., Palermo, R., … Checquolo, S. (2018). NOTCH3 inactivation increases triple negative breast cancer sensitivity to gefitinib by promoting EGFR tyrosine dephosphorylation and its intracellular arrest. *Oncogenesis*, *7*(5), 42. https://doi.org/10.1038/s41389-018-0051-9

Duryagina, R., Thieme, S., Anastassiadis, K., Werner, C., Schneider, S., Wobus, M., … Bornhäuser, M. (2013). Overexpression of Jagged-1 and Its Intracellular Domain in Human Mesenchymal Stromal Cells Differentially Affect the Interaction with Hematopoietic Stem and Progenitor Cells. *Stem Cells and Development*, *22*(20), 2736–2750. https://doi.org/10.1089/scd.2012.0638

Emmerich, C. H., & Cohen, P. (2015). Optimising methods for the preservation, capture and identification of ubiquitin chains and ubiquitylated proteins by immunoblotting. *Biochemical and Biophysical Research Communications*, *466*(1), 1–14. https://doi.org/10.1016/j.bbrc.2015.08.109

Fan, H., Turck, C. W., & Derynck, R. (2003). Characterization of growth factor-induced serine phosphorylation of tumor necrosis factor-alpha converting enzyme and of an alternatively translated polypeptide. *The Journal of Biological Chemistry*, *278*(20), 18617–18627. https://doi.org/10.1074/jbc.M300331200

Fang, D., Hawke, D., Zheng, Y., Xia, Y., Meisenhelder, J., Nika, H., … Lu, Z. (2007). Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. *The Journal of Biological Chemistry*, *282*(15), 11221–11229. https://doi.org/10.1074/jbc.M611871200

Ferrandino, F., Bernardini, G., Tsaouli, G., Grazioli, P., Campese, A. F., Noce, C., … Felli, M. P. (2018). Intrathymic Notch3 and CXCR4 combinatorial interplay facilitates T-cell leukemia propagation. *Oncogene*, *37*(49), 6285–6298. https://doi.org/10.1038/s41388-018-0401-2

Ferrando, A. A. (2009). The role of NOTCH1 signaling in T-ALL. *Hematology. American Society of Hematology. Education Program*, 353–361. https://doi.org/10.1182/asheducation-2009.1.353

Fiúza, U.-M., & Arias, A. M. (2007). Cell and molecular biology of Notch. *Journal of Endocrinology*, *194*(3), 459–474. https://doi.org/10.1677/JOE-07-0242

Franciosa, G., Diluvio, G., Gaudio, F. Del, Giuli, M. V, Palermo, R., Grazioli, P., … Checquolo, S. (2016). Prolyl-isomerase Pin1 controls Notch3 protein expression and regulates T-ALL progression. *Oncogene*, *35*(36), 4741–4751. https://doi.org/10.1038/onc.2016.5

Frattini, M., Balestra, D., Suardi, S., Oggionni, M., Alberici, P., Radice, P., … Pierotti, M. A. (2004). Different Genetic Features Associated with Colon and Rectal Carcinogenesis. *Clinical Cancer Research*, *10*(12), 4015 LP – 4021. https://doi.org/10.1158/1078-0432.CCR-04-0031

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., & Artavanis-Tsakonas, S. (2005). Notch signals control the fate of immature progenitor cells in the intestine. *Nature*, *435*(7044), 964–968. https://doi.org/10.1038/nature03589

Fryer, C. J., Lamar, E., Turbachova, I., Kintner, C., & Jones, K. A. (2002). Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. *Genes & Development*, *16*(11), 1397–1411. https://doi.org/10.1101/gad.991602

Fryer, C. J., White, J. B., & Jones, K. A. (2004). Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Molecular Cell*, *16*(4), 509–520. https://doi.org/10.1016/j.molcel.2004.10.014

Gallagher, E., Gao, M., Liu, Y.-C., & Karin, M. (2006). Activation of the E3 ubiquitin ligase Itch through a phosphorylation-induced conformational change. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(6), 1717–1722. https://doi.org/10.1073/pnas.0510664103

Gao, J., Liu, J., Fan, D., Xu, H., Xiong, Y., Wang, Y., … Zheng, G. (2011). Up-regulated expression of Notch1 and Jagged1 in human colon adenocarcinoma. *Pathologie Biologie*, *59*(6), 298–302. https://doi.org/10.1016/J.PATBIO.2010.11.001

Gao, M., Labuda, T., Xia, Y., Gallagher, E., Fang, D., Liu, Y.-C., & Karin, M. (2004). Jun Turnover Is Controlled Through JNK-Dependent Phosphorylation of the E3 Ligase Itch. *Science*, *306*(5694), 271 LP – 275. https://doi.org/10.1126/science.1099414

Goodrich, L. V, Milenković, L., Higgins, K. M., & Scott, M. P. (1997). Altered Neural Cell Fates and Medulloblastoma in Mouse patched Mutants. *Science*, *277*(5329), 1109 LP – 1113. https://doi.org/10.1126/science.277.5329.1109

Grochowski, C. M., Loomes, K. M., & Spinner, N. B. (2016). Jagged1 (JAG1): Structure, expression, and disease associations. *Gene*, *576*(1 Pt 3), 381–384. https://doi.org/10.1016/j.gene.2015.10.065

Guilmeau, S., Flandez, M., Mariadason, J. M., & Augenlicht, L. H. (2010). Heterogeneity of Jagged1 expression in human and

mouse intestinal tumors: implications for targeting Notch signaling. *Oncogene*, *29*(7), 992–1002. https://doi.org/10.1038/onc.2009.393

Gulino, A., Di Marcotullio, L., & Screpanti, I. (2010). The multiple functions of Numb. *Experimental Cell Research*, *316*(6), 900–906. https://doi.org/10.1016/J.YEXCR.2009.11.017

Guo, M., Jan, L. Y., & Jan, Y. N. (1996). Control of Daughter Cell Fates during Asymmetric Division: Interaction of Numb and Notch. *Neuron*, *17*(1), 27–41. https://doi.org/10.1016/S0896-6273(00)80278-0

Haines, N., & Irvine, K. D. (2003). Glycosylation regulates Notch signalling. *Nature Reviews Molecular Cell Biology*, *4*(10), 786–797. https://doi.org/10.1038/nrm1228

Hansson, M. L., Behmer, S., Ceder, R., Mohammadi, S., Preta, G., Grafström, R. C., … Wallberg, A. E. (2012). MAML1 acts cooperatively with EGR1 to activate EGR1-regulated promoters: implications for nephrogenesis and the development of renal cancer. *PloS One*, *7*(9), e46001–e46001. https://doi.org/10.1371/journal.pone.0046001

Hansson, M. L., Popko-Scibor, A. E., Saint Just Ribeiro, M., Dancy, B. M., Lindberg, M. J., Cole, P. A., & Wallberg, A. E. (2009). The transcriptional coactivator MAML1 regulates p300 autoacetylation and HAT activity. *Nucleic Acids Research*, *37*(9), 2996–3006. https://doi.org/10.1093/nar/gkp163

He, H., Davidson, A. J., Wu, D., Marshall, F. F., Chung, L. W. K., Zhau, H. E., … Wang, R. (2010). Phorbol ester phorbol-12-myristate-13-acetate induces epithelial to mesenchymal transition in human prostate cancer ARCaPE cells. *The Prostate*, *70*(10), 1119–1126. https://doi.org/10.1002/pros.21146

Hori, K., Fostier, M., Ito, M., Fuwa, T. J., Go, M. J., Okano, H., … Matsuno, K. (2004). Drosophila deltex mediates suppressor of hairless-independent and late-endosomal activation of notch signaling. *Development*, *131*(22), 5527–5537. https://doi.org/10.1242/dev.01448

Hori, K., Sen, A., & Artavanis-Tsakonas, S. (2013). Notch signaling at a glance. *Journal of Cell Science*, *126*(10), 2135 LP – 2140. https://doi.org/10.1242/jcs.127308

Hui, C., & Angers, S. (2011). Gli Proteins in Development and Disease. *Annual Review of Cell and Developmental Biology*, *27*(1), 513–537. https://doi.org/10.1146/annurev-cellbio-092910-154048

Infante, P., Faedda, R., Bernardi, F., Bufalieri, F., Lospinoso Severini, L., Alfonsi, R., … Di Marcotullio, L. (2018). Itch/β-arrestin2-dependent non-proteolytic ubiquitylation of SuFu controls Hedgehog signalling and medulloblastoma tumorigenesis. *Nature Communications*, *9*(1), 976. https://doi.org/10.1038/s41467-018-03339-0

Infante, P., Mori, M., Alfonsi, R., Ghirga, F., Aiello, F., Toscano, S., … Di Marcotullio, L. (2015). Gli1/DNA interaction is a druggable target for Hedgehog-dependent tumors. *The EMBO Journal*, *34*(2), 200–217. https://doi.org/10.15252/embj.201489213

Ingham, P., & McMahon, A. (2011). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev*, (15), 3059–3087.

Ishimoto, T., Nagano, O., Yae, T., Tamada, M., Motohara, T., Oshima, H., … Saya, H. (2011). CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(-) and thereby promotes tumor growth. *Cancer Cell*, *19*(3), 387–400. https://doi.org/10.1016/j.ccr.2011.01.038

Itoh, M., Kim, C.-H., Palardy, G., Oda, T., Jiang, Y.-J., Maust, D., … Chitnis, A. B. (2003). Mind Bomb Is a Ubiquitin Ligase that Is Essential for Efficient Activation of Notch Signaling by Delta. *Developmental Cell*, *4*(1), 67–82. https://doi.org/10.1016/S1534-5807(02)00409-4

Jin, B., Shen, H., Lin, S., Li, J.-L., Chen, Z., Griffin, J. D., & Wu, L. (2010). The mastermind-like 1 (MAML1) co-activator regulates constitutive NF-kappaB signaling and cell survival. *The Journal of Biological Chemistry*, *285*(19), 14356–14365. https://doi.org/10.1074/jbc.M109.078865

Jin, K., Zhou, W., Han, X., Wang, Z., Li, B., Jeffries, S., … Capobianco, A. J. (2017). Acetylation of mastermind-like 1 by p300 drives the recruitment of NACK to initiate Notch-dependent transcription. *Cancer Research*, *77*(16), 4228–4237. https://doi.org/10.1158/0008-5472.CAN-16-3156

Kadin, M. E., Rubinstein, L. J., & Nelson, J. S. (1970). Neonatal Cerebellar Medulloblastoma Originating from the Fetal External Granular Layer. *Journal of Neuropathology & Experimental Neurology*, *29*(4), 583–600. https://doi.org/10.1097/00005072-197010000-00005

Kamarajan, P., Shin, J. M., Qian, X., Matte, B., Zhu, J. Y., & Kapila, Y. L. (2013). ADAM17-mediated CD44 cleavage promotes orasphere formation or stemness and tumorigenesis in HNSCC. *Cancer Medicine*, *2*(6), 793–802. https://doi.org/10.1002/cam4.147

Kenney, A. M., & Rowitch, D. H. (2000). Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Molecular and Cellular Biology*, *20*(23), 9055–9067. https://doi.org/10.1128/mcb.20.23.9055-9067.2000

Kim, M.-H., Kim, H.-B., Yoon, S. P., Lim, S.-C., Cha, M. J., Jeon, Y. J., … You, H. J. (2013). Colon cancer progression is driven by APEX1-mediated upregulation of Jagged. *The Journal of Clinical Investigation*, *123*(8), 3211–3230. https://doi.org/10.1172/JCI65521

Kimura, H., Stephen, D., Joyner, A., & Curran, T. (2005). Gli1 is important for medulloblastoma formation in Ptc1+/− mice. *Oncogene*, *24*(25), 4026–4036. https://doi.org/10.1038/sj.onc.1208567

Kitagawa, M, Oyama, T., Kawashima, T., Yedvobnick, B., Kumar, A., Matsuno, K., & Harigaya, K. (2001). A human protein with sequence similarity to Drosophila mastermind coordinates the nuclear form of notch and a CSL protein to build a transcriptional activator complex on target promoters. *Molecular and Cellular Biology*, *21*(13), 4337–4346. https://doi.org/10.1128/MCB.21.13.4337-4346.2001

Kitagawa, Motoo. (2015, August 13). Notch signalling in the nucleus: Roles of Mastermind-like (MAML) transcriptional coactivators. *Journal of Biochemistry*, Vol. 159, pp. 287–294. https://doi.org/10.1093/jb/mvv123

Klinakis, A., Lobry, C., Abdel-Wahab, O., Oh, P., Haeno, H., Buonamici, S., … Aifantis, I. (2011). A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. *Nature*, *473*, 230. Retrieved from https://doi.org/10.1038/nature09999

Kogerman, P., Grimm, T., Kogerman, L., Krause, D., Undén, A. B., Sandstedt, B., … Zaphiropoulos, P. G. (1999). Mammalian Suppressor-of-Fused modulates nuclear–cytoplasmic shuttling of GLI-1. *Nature Cell Biology*, *1*(5), 312–319. https://doi.org/10.1038/13031

Kopan, R., & Ilagan, M. X. G. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*, *137*(2), 216–233. https://doi.org/10.1016/j.cell.2009.03.045

Kovall, R. A., & Hendrickson, W. A. (2004). Crystal structure of the nuclear effector of Notch signaling, CSL, bound to DNA. *The EMBO Journal*, *23*(17), 3441–3451. https://doi.org/10.1038/sj.emboj.7600349

Kyula, J. N., Van Schaeybroeck, S., Doherty, J., Fenning, C. S., Longley, D. B., & Johnston, P. G. (2010). Chemotherapy-induced activation of ADAM-17: a novel mechanism of drug resistance in colorectal cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *16*(13), 3378–3389. https://doi.org/10.1158/1078-0432.CCR-10-0014

Lacroix, M., Toillon, R.-A., & Leclercq, G. (2006). p53 and breast cancer, an update. *Endocrine-Related Cancer Endocr Relat Cancer*, *13*(2), 293–325. https://doi.org/10.1677/erc.1.01172

LaFoya, B., Munroe, J. A., Mia, M. M., Detweiler, M. A., Crow, J. J., Wood, T., … Albig, A. R. (2016). Notch: A multi-functional integrating system of microenvironmental signals. *Developmental Biology*, *418*(2), 227–241. https://doi.org/10.1016/j.ydbio.2016.08.023

Lai, E. C. (2002). Protein Degradation: Four E3s For The Notch Pathway. *Current Biology*, *12*(2), R74–R78. https://doi.org/10.1016/S0960-9822(01)00679-0

Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development*, *131*(5), 965 LP – 973. https://doi.org/10.1242/dev.01074

LaVoie, M. J., & Selkoe, D. J. (2003). The Notch Ligands, Jagged and Delta, Are Sequentially Processed by α-Secretase and Presenilin/γ-Secretase and Release Signaling Fragments. *Journal of Biological Chemistry*, *278*(36), 34427–34437. https://doi.org/10.1074/JBC.M302659200

Lehmann, R., Jiménez, F., Dietrich, U., & Campos-Ortega, J. A. (1983). On the phenotype and development of mutants of early neurogenesis inDrosophila melanogaster. *Wilhelm Roux's Archives of Developmental Biology*, *192*(2), 62–74. https://doi.org/10.1007/BF00848482

Lemieux, E., Cagnol, S., Beaudry, K., Carrier, J., & Rivard, N. (2015). Oncogenic KRAS signalling promotes the Wnt/β-catenin pathway through LRP6 in colorectal cancer. *Oncogene*, *34*(38), 4914–4927. https://doi.org/10.1038/onc.2014.416

Lewis, P. M., Gritli-Linde, A., Smeyne, R., Kottmann, A., & McMahon, A. P. (2004). Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. *Developmental Biology*, *270*(2), 393–410. https://doi.org/10.1016/J.YDBIO.2004.03.007

Lièvre, A., Bachet, J.-B., Le Corre, D., Boige, V., Landi, B., Emile, J.-F., … Laurent-Puig, P. (2006). KRAS Mutation Status Is Predictive of Response to Cetuximab Therapy in Colorectal Cancer. *Cancer Research*, *66*(8), 3992 LP – 3995. https://doi.org/10.1158/0008-5472.CAN-06-0191

Lin, J.-T., Chen, M.-K., Yeh, K.-T., Chang, C.-S., Chang, T.-H., Lin, C.-Y., … Chang, P.-J. (2010). Association of High Levels of Jagged-1 and Notch-1 Expression with Poor Prognosis in Head and Neck Cancer. *Annals of Surgical Oncology*, *17*(11), 2976–2983. https://doi.org/10.1245/s10434-010-1118-9

Lin, S.-E., Oyama, T., Nagase, T., Harigaya, K., & Kitagawa, M. (2002). Identification of new human mastermind proteins defines a family that consists of positive regulators for notch signaling. *The Journal of Biological Chemistry*, *277*(52), 50612–50620. https://doi.org/10.1074/jbc.M209529200

Lorenz, S., Cantor, A. J., Rape, M., & Kuriyan, J. (2013). Macromolecular juggling by ubiquitylation enzymes. *BMC Biology*, *11*, 65. https://doi.org/10.1186/1741-7007-11-65

Louvi, A., & Artavanis-Tsakonas, S. (2012). Notch and disease: a growing field. *Seminars in Cell & Developmental Biology*, *23*(4), 473–480. https://doi.org/10.1016/j.semcdb.2012.02.005

Lu, P.-J., Zhou, X. Z., Shen, M., & Lu, K. P. (1999). Function of WW Domains as Phosphoserine- or Phosphothreonine-Binding Modules. *Science*, *283*(5406), 1325 LP – 1328. https://doi.org/10.1126/science.283.5406.1325

Marcotullio, Lucia Di, Ferretti, E., Greco, A., De Smaele, E., Po, A., Sico, M. A., … Gulino, A. (2006). Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. *Nature Cell Biology*, *8*(12), 1415–1423. https://doi.org/10.1038/ncb1510

Marisa, L., de Reyniès, A., Duval, A., Selves, J., Gaub, M. P., Vescovo, L., … Boige, V. (2013). Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. *PLoS Medicine*, *10*(5), e1001453–e1001453. https://doi.org/10.1371/journal.pmed.1001453

Maspero, E., Valentini, E., Mari, S., Cecatiello, V., Soffientini, P., Pasqualato, S., & Polo, S. (2013). Structure of a ubiquitin-loaded HECT ligase reveals the molecular basis for catalytic priming. *Nature Structural &Amp; Molecular Biology*, *20*, 696. Retrieved from https://doi.org/10.1038/nsmb.2566

Matsuno, K, Diederich, R. J., Go, M. J., Blaumueller, C. M., & Artavanis-Tsakonas, S. (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development*, *121*(8), 2633 LP – 2644. Retrieved from http://dev.biologists.org/content/121/8/2633.abstract

Matsuno, Kenji, Eastman, D., Mitsiades, T., Quinn, A. M., Carcanciu, M. L., Ordentlich, P., … Artavanis-Tsakonas, S. (1998). Human deltex is a conserved regulator of Notch signalling. *Nature Genetics*, *19*(1), 74–78. https://doi.org/10.1038/ng0598-74

Mazzà, D., Infante, P., Colicchia, V., Greco, A., Alfonsi, R., Siler, M., … Di Marcotullio, L. (2013). PCAF ubiquitin ligase activity inhibits Hedgehog/Gli1 signaling in p53-dependent response to genotoxic stress. *Cell Death and Differentiation*, *20*(12), 1688–1697. https://doi.org/10.1038/cdd.2013.120

McElhinny, A. S., Li, J.-L., & Wu, L. (2008). Mastermind-like transcriptional co-activators: emerging roles in regulating cross talk among multiple signaling pathways. *Oncogene*, *27*, 5138. Retrieved from https://doi.org/10.1038/onc.2008.228

McGill, M. A., Dho, S. E., Weinmaster, G., & McGlade, C. J. (2009). Numb regulates post-endocytic trafficking and degradation of Notch1. *The Journal of Biological Chemistry*, *284*(39), 26427–26438. https://doi.org/10.1074/jbc.M109.014845

McGill, M. A., & McGlade, C. J. (2003). Mammalian Numb Proteins Promote Notch1 Receptor Ubiquitination and Degradation of the Notch1 Intracellular Domain. *Journal of Biological Chemistry*, *278*(25), 23196–23203. https://doi.org/10.1074/JBC.M302827200

Melino, G., Gallagher, E., Aqeilan, R. I., Knight, R., Peschiaroli, A., Rossi, M., … Bernassola, F. (2008). Itch: a HECT-type E3 ligase regulating immunity, skin and cancer. *Cell Death And Differentiation*, *15*, 1103. Retrieved from https://doi.org/10.1038/cdd.2008.60

Moghbeli, M., Mosannen Mozaffari, H., Memar, B., Forghanifard, M. M., Gholamin, M., & Abbaszadegan, M. R. (2019). Role of MAML1 in targeted therapy against the esophageal cancer stem cells. *Journal of Translational Medicine*, *17*(1), 126. https://doi.org/10.1186/s12967-019-1876-5

Mologni, L., Brussolo, S., Ceccon, M., & Gambacorti-Passerini, C. (2012). Synergistic effects of combined Wnt/KRAS inhibition in colorectal cancer cells. *PloS One*, *7*(12), e51449–e51449. https://doi.org/10.1371/journal.pone.0051449

Mologni, L., Dekhil, H., Ceccon, M., Purgante, S., Lan, C., Cleris, L., … Gambacorti-Passerini, C. B. (2010). Colorectal Tumors Are Effectively Eradicated by Combined Inhibition of β-Catenin, KRAS, and the Oncogenic Transcription Factor ITF2. *Cancer Research*, *70*(18), 7253 LP – 7263. https://doi.org/10.1158/0008-5472.CAN-10-1108

Mouchantaf, R., Azakir, B. A., McPherson, P. S., Millard, S. M., Wood, S. A., & Angers, A. (2006). The Ubiquitin Ligase Itch Is Auto-ubiquitylated in Vivo and in Vitro but Is Protected from Degradation by Interacting with the Deubiquitylating Enzyme FAM/USP9X. *Journal of Biological Chemistry*, *281*(50), 38738–38747. https://doi.org/10.1074/JBC.M605959200

Mukherjee, A., Veraksa, A., Bauer, A., Rosse, C., Camonis, J., & Artavanis-Tsakonas, S. (2005). Regulation of Notch signalling by non-visual β-arrestin. *Nature Cell Biology*, *7*(12), 1191–1201. https://doi.org/10.1038/ncb1327

Nam, Y., Sliz, P., Song, L., Aster, J. C., & Blacklow, S. C. (2006). Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. *Cell*, *124*(5), 973–983. https://doi.org/10.1016/j.cell.2005.12.037

Ntziachristos, P., Lim, J. S., Sage, J., & Aifantis, I. (2014). From fly wings to targeted cancer therapies: a centennial for notch signaling. *Cancer Cell*, *25*(3), 318–334. https://doi.org/10.1016/j.ccr.2014.02.018

Nüsslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature*, *287*(5785), 795–801. https://doi.org/10.1038/287795a0

Orth, G. (2006). Genetics of epidermodysplasia verruciformis: Insights into host defense against papillomaviruses. *Seminars in Immunology*, *18*(6), 362–374. https://doi.org/10.1016/J.SMIM.2006.07.008

Oyama, T., Harigaya, K., Muradil, A., Hozumi, K., Habu, S., Oguro, H., … Kitagawa, M. (2007). Mastermind-1 is required for Notch signal-dependent steps in lymphocyte development in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(23), 9764–9769. https://doi.org/10.1073/pnas.0700240104

Ozato, K., Shin, D.-M., Chang, T.-H., & Morse 3rd, H. C. (2008). TRIM family proteins and their emerging roles in innate immunity. *Nature Reviews. Immunology*, *8*(11), 849–860. https://doi.org/10.1038/nri2413

Palermo, R., Checquolo, S., Bellavia, D., & Screpanti, C. T. and I. (2014). The Molecular Basis of Notch Signaling Regulation: A Complex Simplicity. *Current Molecular Medicine*, Vol. 14, pp. 34–44. https://doi.org/http://dx.doi.org/10.2174/1566524013666131118105216

Palomero, T., McKenna, K., O-Neil, J., Galinsky, I., Stone, R., Suzukawa, K., … Ferrando, A. A. (2006). Activating mutations in NOTCH1 in acute myeloid leukemia and lineage switch leukemias. *Leukemia*, *20*(11), 1963–1966. https://doi.org/10.1038/sj.leu.2404409

Panin, V. M., Shao, L., Lei, L., Moloney, D. J., Irvine, K. D., & Haltiwanger, R. S. (2002). Notch ligands are substrates for protein O-fucosyltransferase-1 and Fringe. *The Journal of Biological Chemistry*, *277*(33), 29945–29952. https://doi.org/10.1074/jbc.M204445200

Pannequin, J., Bonnans, C., Delaunay, N., Ryan, J., Bourgaux, J.-F., Joubert, D., & Hollande, F. (2009). The Wnt Target Jagged-1 Mediates the Activation of Notch Signaling by Progastrin in Human Colorectal Cancer Cells. *Cancer Research*, *69*(15), 6065 LP – 6073. https://doi.org/10.1158/0008-5472.CAN-08-2409

Pece, S., Confalonieri, S., R. Romano, P., & Di Fiore, P. P. (2011). NUMB-ing down cancer by more than just a NOTCH. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, *1815*(1), 26–43. https://doi.org/10.1016/J.BBCAN.2010.10.001

Pelullo, M., Nardozza, F., Zema, S., Quaranta, R., Nicoletti, C., Besharat, Z. M., … Bellavia, D. (2019). Kras/ADAM17-dependent Jag1-ICD reverse signalling sustains colorectal cancer progression and chemoresistance. *Cancer Research*, canres.0145.2019. https://doi.org/10.1158/0008-5472.CAN-19-0145

Pelullo, M., Quaranta, R., Talora, C., Checquolo, S., Cialfi, S., Felli, M. P., … Bellavia, D. (2014). Notch3/Jagged1 circuitry reinforces notch signaling and sustains T-ALL. *Neoplasia (New York, N.Y.)*, *16*(12), 1007–1017. https://doi.org/10.1016/j.neo.2014.10.004

Pelullo, M., Zema, S., Nardozza, F., Checquolo, S., Screpanti, I., & Bellavia, D. (2019). Wnt, Notch, and TGF-β Pathways Impinge on Hedgehog Signaling Complexity: An Open Window on Cancer . *Frontiers in Genetics* , Vol. 10, p. 711. Retrieved from https://www.frontiersin.org/article/10.3389/fgene.2019.00711

Perry, W. L., Hustad, C. M., Swing, D. A., O'Sullivan, T. N., Jenkins, N. A., & Copeland, N. G. (1998). The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. *Nature Genetics*, *18*(2), 143–146. https://doi.org/10.1038/ng0298-143

Petcherski, A. G., & Kimble, J. (2000). Mastermind is a putative activator for Notch. *Current Biology*, *10*(13), R471–R473. https://doi.org/10.1016/S0960-9822(00)00577-7

Petroni, M., Veschi, V., Prodosmo, A., Rinaldo, C., Massimi, I., Carbonari, M., … Giannini, G. (2011). MYCN Sensitizes Human Neuroblastoma to Apoptosis by HIPK2 Activation through a DNA Damage Response. *Molecular Cancer Research*, *9*(1), 67 LP – 77. https://doi.org/10.1158/1541-7786.MCR-10-0227

Pickart, C. M. (2001). Mechanisms Underlying Ubiquitination. *Annual Review of Biochemistry*, *70*(1), 503–533. https://doi.org/10.1146/annurev.biochem.70.1.503

Pretlow, T. P., & Pretlow, T. G. (2005). Mutant KRAS in aberrant crypt foci (ACF): Initiation of colorectal cancer? *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, *1756*(2), 83–96. https://doi.org/10.1016/J.BBCAN.2005.06.002

Puca, L., Chastagner, P., Meas-Yedid, V., Israël, A., & Brou, C. (2013). α-arrestin 1 (ARRDC1) and β-arrestins cooperate to mediate Notch degradation in mammals. *Journal of Cell Science*, *126*(19), 4457 LP – 4468. https://doi.org/10.1242/jcs.130500

Puente, X. S., Pinyol, M., Quesada, V., Conde, L., Ordóñez, G. R., Villamor, N., … Campo, E. (2011). Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature*, *475*(7354), 101–105. https://doi.org/10.1038/nature10113

Qiao, L., & Wong, B. C. Y. (2009). Role of Notch signaling in colorectal cancer . *Carcinogenesis*, *30*(12), 1979–1986. https://doi.org/10.1093/carcin/bgp236

Qiu, L., Joazeiro, C., Fang, N., Wang, H. Y., Elly, C., Altman, Y., … Liu, Y. C. (2000). Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. *The Journal of Biological Chemistry*, *275*(46), 35734–35737. https://doi.org/10.1074/jbc.M007300200

Quaranta, R., Pelullo, M., Zema, S., Nardozza, F., Checquolo, S., Lauer, D. M., … Bellavia, D. (2017). Maml1 acts cooperatively with Gli proteins to regulate sonic hedgehog signaling pathway. *Cell Death & Disease*, *8*(7), e2942–e2942. https://doi.org/10.1038/cddis.2017.326

Ranganathan, P., Weaver, K. L., & Capobianco, A. J. (2011). Notch signalling in solid tumours: a little bit of everything but not all the time. *Nature Reviews Cancer*, *11*, 338. Retrieved from https://doi.org/10.1038/nrc3035

Rangarajan, A., Syal, R., Selvarajah, S., Chakrabarti, O., Sarin, A., & Krishna, S. (2001). Activated Notch1 Signaling Cooperates with Papillomavirus Oncogenes in Transformation and Generates Resistance to Apoptosis on Matrix Withdrawal through PKB/Akt. *Virology*, *286*(1), 23–30. https://doi.org/10.1006/VIRO.2001.0867

Reedijk, M., Odorcic, S., Chang, L., Zhang, H., Miller, N., McCready, D. R., … Egan, S. E. (2005). High-level Coexpression of JAG1 and NOTCH1 Is Observed in Human Breast Cancer and Is Associated with Poor Overall Survival. *Cancer Research*, *65*(18), 8530 LP – 8537. https://doi.org/10.1158/0008-5472.CAN-05-1069

Rhyu, M. S., Jan, L. Y., & Jan, Y. N. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell*, *76*(3), 477–491. https://doi.org/10.1016/0092-8674(94)90112-0

Riling, C., Kamadurai, H., Kumar, S., O'Leary, C. E., Wu, K.-P., Manion, E. E., … Oliver, P. M. (2015). Itch WW Domains Inhibit Its E3 Ubiquitin Ligase Activity by Blocking E2-E3 Ligase Trans-thiolation. *The Journal of Biological Chemistry*, *290*(39), 23875–23887. https://doi.org/10.1074/jbc.M115.649269

Rodilla, V., Villanueva, A., Obrador-Hevia, A., Robert-Moreno, A., Fernández-Majada, V., Grilli, A., … Espinosa, L. (2009). Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(15), 6315–6320. https://doi.org/10.1073/pnas.0813221106

Ruiz i Altaba, A., Sánchez, P., & Dahmane, N. (2002). Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nature Reviews Cancer*, *2*(5), 361–372. https://doi.org/10.1038/nrc796

Saint Just Ribeiro, M., Hansson, M. L., Lindberg, M. J., Popko-Ścibor, A. E., & Wallberg, A. E. (2009). GSK3β is a negative regulator of the transcriptional coactivator MAML1. *Nucleic Acids Research*, *37*(20), 6691–6700. https://doi.org/10.1093/nar/gkp724

Saint Just Ribeiro, M., Hansson, M. L., & Wallberg, A. E. (2007). A proline repeat domain in the Notch co-activator MAML1 is important for the p300-mediated acetylation of MAML1. *The Biochemical Journal*, *404*(2), 289–298. https://doi.org/10.1042/BJ20061900

Santagata, S., Demichelis, F., Riva, A., Varambally, S., Hofer, M. D., Kutok, J. L., … Aster, J. C. (2004). JAGGED1 Expression Is Associated with Prostate Cancer Metastasis and Recurrence. *Cancer Research*, *64*(19), 6854 LP – 6857. https://doi.org/10.1158/0008-5472.CAN-04-2500

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., & Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development*, *126*(17), 3915 LP – 3924. Retrieved from http://dev.biologists.org/content/126/17/3915.abstract

Scialpi, F., Malatesta, M., Peschiaroli, A., Rossi, M., Melino, G., & Bernassola, F. (2008). Itch self-polyubiquitylation occurs through lysine-63 linkages. *Biochemical Pharmacology*, *76*(11), 1515–1521. https://doi.org/10.1016/J.BCP.2008.07.028

Šestan, N., Artavanis-Tsakonas, S., & Rakic, P. (1999). Contact-Dependent Inhibition of Cortical Neurite Growth Mediated by Notch Signaling. *Science*, *286*(5440), 741 LP – 746. https://doi.org/10.1126/science.286.5440.741

Shariat Razavi, S. M., Forghanifard, M. M., Kordi-Tamandani, D. M., & Abbaszadegan, M. R. (2019). MAML1 regulates EMT markers expression through NOTCH-independent pathway in breast cancer cell line MCF7. *Biochemical and Biophysical Research Communications*, *510*(3), 376–382. https://doi.org/10.1016/J.BBRC.2019.01.101

Shen, H., McElhinny, A. S., Cao, Y., Gao, P., Liu, J., Bronson, R., … Wu, L. (2006). The Notch coactivator, MAML1, functions as a novel coactivator for MEF2C-mediated transcription and is required for normal myogenesis. *Genes & Development*, *20*(6), 675–688. https://doi.org/10.1101/gad.1383706

Sillitoe, R. V, & Joyner, A. L. (2007). Morphology, Molecular Codes, and Circuitry Produce the Three-Dimensional Complexity of the Cerebellum. *Annual Review of Cell and Developmental Biology*, *23*(1), 549–577. https://doi.org/10.1146/annurev.cellbio.23.090506.123237

Small, D., Kovalenko, D., Kacer, D., Liaw, L., Landriscina, M., Serio, C. Di, … Maciag, T. (2001). Soluble Jagged 1 Represses the Function of Its Transmembrane Form to Induce the Formation of the Src-dependent Chord-like Phenotype. *Journal of Biological Chemistry*, *276*(34), 32022–32030. https://doi.org/10.1074/JBC.M100933200

Solecki, D. J., Liu, X. L., Tomoda, T., Fang, Y., & Hatten, M. E. (2001). Activated Notch2 signaling inhibits differentiation of cerebellar granule neuron precursors by maintaining proliferation. *Neuron*, *31*(4), 557–568. https://doi.org/10.1016/s0896-6273(01)00395-6

Soond, S. M., Everson, B., Riches, D. W. H., & Murphy, G. (2005). ERK-mediated phosphorylation of Thr735 in TNFα-converting

enzyme and its potential role in TACE protein trafficking. *Journal of Cell Science*, *118*(11), 2371 LP – 2380. https://doi.org/10.1242/jcs.02357

Soriani, A., Borrelli, C., Ricci, B., Molfetta, R., Zingoni, A., Fionda, C., … Santoni, A. (2016). p38 MAPK differentially controls NK activating ligands at transcriptional and post-transcriptional level on multiple myeloma cells. *Oncoimmunology*, *6*(1), e1264564–e1264564. https://doi.org/10.1080/2162402X.2016.1264564

South, A. P., Cho, R. J., & Aster, J. C. (2012). The double-edged sword of Notch signaling in cancer. *Seminars in Cell & Developmental Biology*, *23*(4), 458–464. https://doi.org/10.1016/j.semcdb.2012.01.017

Spana, E. P., & Doe, C. Q. (1996). Numb Antagonizes Notch Signaling to Specify Sibling Neuron Cell Fates. *Neuron*, *17*(1), 21–26. https://doi.org/10.1016/S0896-6273(00)80277-9

Stecca, B., & Ruiz I Altaba, A. (2010). Context-dependent regulation of the GLI code in cancer by HEDGEHOG and non-HEDGEHOG signals. *Journal of Molecular Cell Biology*, *2*(2), 84–95. https://doi.org/10.1093/jmcb/mjp052

Stransky, N., Egloff, A. M., Tward, A. D., Kostic, A. D., Cibulskis, K., Sivachenko, A., … Grandis, J. R. (2011). The Mutational Landscape of Head and Neck Squamous Cell Carcinoma. *Science*, *333*(6046), 1157 LP – 1160. https://doi.org/10.1126/science.1208130

Sudarov, A., & Joyner, A. L. (2007). Cerebellum morphogenesis: the foliation pattern is orchestrated by multi-cellular anchoring centers. *Neural Development*, *2*, 26. https://doi.org/10.1186/1749-8104-2-26

Sugiyama, M., Oki, E., Nakaji, Y., Tsutsumi, S., Ono, N., Nakanishi, R., … Maehara, Y. (2016). High expression of the Notch ligand Jagged-1 is associated with poor prognosis after surgery for colorectal cancer. *Cancer Science*, *107*(11), 1705–1716. https://doi.org/10.1111/cas.13075

Talora, C., Campese, A. F., Bellavia, D., Felli, M. P., Vacca, A., Gulino, A., & Screpanti, I. (2008). Notch signaling and diseases: An evolutionary journey from a simple beginning to complex outcomes. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1782*(9), 489–497. https://doi.org/10.1016/J.BBADIS.2008.06.008

Tan, M. J. A., White, E. A., Sowa, M. E., Harper, J. W., Aster, J. C., & Howley, P. M. (2012). Cutaneous -human papillomavirus E6 proteins bind Mastermind-like coactivators and repress Notch signaling. *Proceedings of the National Academy of Sciences*, *109*(23), E1473–E1480. https://doi.org/10.1073/pnas.1205991109

Tottone, L., Zhdanovskaya, N., Carmona Pestaña, Á., Zampieri, M., Simeoni, F., Lazzari, S., … Palermo, R. (2019). Histone Modifications Drive Aberrant Notch3 Expression/Activity and Growth in T-ALL. *Frontiers in Oncology*, *9*, 198. https://doi.org/10.3389/fonc.2019.00198

Tsuji, S., Midorikawa, Y., Takahashi, T., Yagi, K., Takayama, T., Yoshida, K., … Aburatani, H. (2012). Potential responders to FOLFOX therapy for colorectal cancer by Random Forests analysis. *British Journal of Cancer*, *106*(1), 126–132. https://doi.org/10.1038/bjc.2011.505

Van Schaeybroeck, S., Kalimutho, M., Dunne, P. D., Carson, R., Allen, W., Jithesh, P. V., … Johnston, P. G. (2014). ADAM17-Dependent c-MET-STAT3 Signaling Mediates Resistance to MEK Inhibitors in KRAS Mutant Colorectal Cancer. *Cell Reports*, *7*(6), 1940–1955. https://doi.org/10.1016/J.CELREP.2014.05.032

Van Schaeybroeck, S., Kyula, J. N., Fenton, A., Fenning, C. S., Sasazuki, T., Shirasawa, S., … Johnston, P. G. (2011). Oncogenic Kras promotes chemotherapy-induced growth factor shedding via ADAM17. *Cancer Research*, *71*(3), 1071–1080. https://doi.org/10.1158/0008-5472.CAN-10-0714

van Wijk, S. J. L., Fulda, S., Dikic, I., & Heilemann, M. (2019). Visualizing ubiquitination in mammalian cells. *EMBO Reports*, *20*(2), e46520. https://doi.org/10.15252/embr.201846520

Vargas Romero, P., Cialfi, S., Palermo, R., De Blasio, C., Checquolo, S., Bellavia, D., … Screpanti, I. (2015). The deregulated expression of miR-125b in acute myeloid leukemia is dependent on the transcription factor C/EBPα. *Leukemia*, *29*(12), 2442–2445. https://doi.org/10.1038/leu.2015.117

Veenendaal, L. M., Kranenburg, O., Smakman, N., Klomp, A., Borel Rinkes, I. H. M., & van Diest, P. J. (2008). Differential Notch and TGFbeta signaling in primary colorectal tumors and their corresponding metastases. *Cellular Oncology : The Official Journal of the International Society for Cellular Oncology*, *30*(1), 1–11. https://doi.org/10.1155/2008/839076

Veeraraghavalu, K., Subbaiah, V. K., Srivastava, S., Chakrabarti, O., Syal, R., & Krishna, S. (2005). Complementation of human papillomavirus type 16 E6 and E7 by Jagged1-specific Notch1-phosphatidylinositol 3-kinase signaling involves pleiotropic oncogenic functions independent of CBF1;Su(H);Lag-1 activation. *Journal of Virology*, *79*(12), 7889–7898. https://doi.org/10.1128/JVI.79.12.7889-7898.2005

Verdecia, M. A., Joazeiro, C. A. P., Wells, N. J., Ferrer, J.-L., Bowman, M. E., Hunter, T., & Noel, J. P. (2003). Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. *Molecular Cell*, *11*(1), 249–259. https://doi.org/10.1016/S1097-2765(02)00774-8

Verdi, J. M., Bashirullah, A., Goldhawk, D. E., Kubu, C. J., Jamali, M., Meakin, S. O., & Lipshitz, H. D. (1999). Distinct human

NUMB isoforms regulate differentiation vs. proliferation in the neuronal lineage. *Proceedings of the National Academy of Sciences*, *96*(18), 10472–10476. https://doi.org/10.1073/PNAS.96.18.10472

Verdi, J. M., Schmandt, R., Bashirullah, A., Jacob, S., Salvino, R., Craig, C. G., … McGlade, C. J. (1996). Mammalian NUMB is an evolutionarily conserved signaling adapter protein that specifies cell fate. *Current Biology*, *6*(9), 1134–1145. https://doi.org/10.1016/S0960-9822(02)70680-5

Vitt, U. A., Hsu, S. Y., & Hsueh, A. J. W. (2001). Evolution and Classification of Cystine Knot-Containing Hormones and Related Extracellular Signaling Molecules. *Molecular Endocrinology*, *15*(5), 681–694. https://doi.org/10.1210/mend.15.5.0639

Wakamatsu, Y., Maynard, T. M., Jones, S. U., & Weston, J. A. (1999). NUMB Localizes in the Basal Cortex of Mitotic Avian Neuroepithelial Cells and Modulates Neuronal Differentiation by Binding to NOTCH-1. *Neuron*, *23*(1), 71–81. https://doi.org/10.1016/S0896-6273(00)80754-0

Wallace, V. A. (1999). Purkinje-cell-derived Sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum. *Current Biology : CB*, *9*(8), 445–448. https://doi.org/10.1016/s0960-9822(99)80195-x

Wang, R., Ye, X., Bhattacharya, R., Boulbes, D. R., Fan, F., Xia, L., & Ellis, L. M. (2016). A Disintegrin and Metalloproteinase Domain 17 Regulates Colorectal Cancer Stem Cells and Chemosensitivity Via Notch1 Signaling. *Stem Cells Translational Medicine*, *5*(3), 331–338. https://doi.org/10.5966/sctm.2015-0168

Watanabe, T., Oyama, T., Asada, M., Harada, D., Ito, Y., Inagawa, M., … Asahara, H. (2013). MAML1 enhances the transcriptional activity of Runx2 and plays a role in bone development. *PLoS Genetics*, *9*(1), e1003132–e1003132. https://doi.org/10.1371/journal.pgen.1003132

Wechsler-Reya, R. J., & Scott, M. P. (1999). Control of Neuronal Precursor Proliferation in the Cerebellum by Sonic Hedgehog. *Neuron*, *22*(1), 103–114. https://doi.org/10.1016/S0896-6273(00)80682-0

Welchman, R. L., Gordon, C., & Mayer, R. J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nature Reviews Molecular Cell Biology*, *6*(8), 599–609. https://doi.org/10.1038/nrm1700

Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P., Silverman, L. B., Sanchez-Irizarry, C., … Aster, J. C. (2004). Activating Mutations of NOTCH1 in Human T Cell Acute Lymphoblastic Leukemia. *Science*, *306*(5694), 269 LP – 271. https://doi.org/10.1126/science.1102160

Wilson, J. J., & Kovall, R. A. (2006). Crystal structure of the CSL-Notch-Mastermind ternary complex bound to DNA. *Cell*, *124*(5), 985–996. https://doi.org/10.1016/j.cell.2006.01.035

Woelk, T., Sigismund, S., Penengo, L., & Polo, S. (2007). The ubiquitination code: a signalling problem. *Cell Division*, *2*(1), 11. https://doi.org/10.1186/1747-1028-2-11

Wu, K., Xu, L., Zhang, L., Lin, Z., & Hou, J. (2010). High Jagged1 Expression Predicts Poor Outcome in Clear Cell Renal Cell Carcinoma. *Japanese Journal of Clinical Oncology*, *41*(3), 411–416. https://doi.org/10.1093/jjco/hyq205

Wu, L., Sun, T., Kobayashi, K., Gao, P., & Griffin, J. D. (2002). Identification of a Family of Mastermind-Like Transcriptional Coactivators for Mammalian Notch Receptors. *Molecular and Cellular Biology*, *22*(21), 7688–7700. https://doi.org/10.1128/mcb.22.21.7688-7700.2002

Wu, Lizi, Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., & Griffin, J. D. (2000). MAML1, a human homologue of Drosophila Mastermind, is a transcriptional co-activator for NOTCH receptors. *Nature Genetics*, *26*(4), 484–489. https://doi.org/10.1038/82644

Wu, Lizi, & Griffin, J. D. (2004). Modulation of Notch signaling by mastermind-like (MAML) transcriptional co-activators and their involvement in tumorigenesis. *Seminars in Cancer Biology*, *14*(5), 348–356. https://doi.org/10.1016/j.semcancer.2004.04.014

Xu, J. (2005). Preparation, Culture, and Immortalization of Mouse Embryonic Fibroblasts. *Current Protocols in Molecular Biology*, *70*(1), 28.1.1-28.1.8. https://doi.org/10.1002/0471142727.mb2801s70

Xu, K., Usary, J., Kousis, P. C., Prat, A., Wang, D.-Y., Adams, J. R., … Egan, S. E. (2012). Lunatic fringe deficiency cooperates with the Met/Caveolin gene amplicon to induce basal-like breast cancer. *Cancer Cell*, *21*(5), 626–641. https://doi.org/10.1016/j.ccr.2012.03.041

Yae, T., Tsuchihashi, K., Ishimoto, T., Motohara, T., Yoshikawa, M., Yoshida, G. J., … Nagano, O. (2012). Alternative splicing of CD44 mRNA by ESRP1 enhances lung colonization of metastatic cancer cell. *Nature Communications*, *3*, 883. Retrieved from https://doi.org/10.1038/ncomms1892

Yang, C., Zhou, W., Jeon, M.-S., Demydenko, D., Harada, Y., Zhou, H., & Liu, Y.-C. (2006). Negative regulation of the E3 ubiquitin ligase itch via Fyn-mediated tyrosine phosphorylation. *Molecular Cell*, *21*(1), 135–141. https://doi.org/10.1016/j.molcel.2005.11.014

Yun, J., Espinoza, I., Pannuti, A., Romero, D., Martinez, L., Caskey, M., … Miele, L. (2015). p53 Modulates Notch Signaling in MCF-7 Breast Cancer Cells by Associating With the Notch Transcriptional Complex Via MAML1. *Journal of Cellular*

Physiology, *230*(12), 3115–3127. https://doi.org/10.1002/jcp.25052

Zagouras, P., Stifani, S., Blaumueller, C. M., Carcangiu, M. L., & Artavanis-Tsakonas, S. (1995). Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proceedings of the National Academy of Sciences*, *92*(14), 6414 LP – 6418. https://doi.org/10.1073/pnas.92.14.6414

Zhang, W., Feng, M., Zheng, G., Chen, Y., Wang, X., Pen, B., … He, Z. (2012). Chemoresistance to 5-fluorouracil induces epithelial–mesenchymal transition via up-regulation of Snail in MCF7 human breast cancer cells. *Biochemical and Biophysical Research Communications*, *417*(2), 679–685. https://doi.org/10.1016/J.BBRC.2011.11.142

Zhao, Y., Katzman, R. B., Delmolino, L. M., Bhat, I., Zhang, Y., Gurumurthy, C. B., … Band, V. (2007). The notch regulator MAML1 interacts with p53 and functions as a coactivator. *The Journal of Biological Chemistry*, *282*(16), 11969–11981. https://doi.org/10.1074/jbc.M608974200

Zhong, W., Feder, J. N., Jiang, M.-M., Jan, L. Y., & Jan, Y. N. (1996). Asymmetric Localization of a Mammalian Numb Homolog during Mouse Cortical Neurogenesis. *Neuron*, *17*(1), 43–53. https://doi.org/10.1016/S0896-6273(00)80279-2

Zhu, K., Shan, Z., Chen, X., Cai, Y., Cui, L., Yao, W., … Wen, W. (2017). Allosteric auto-inhibition and activation of the Nedd4 family E3 ligase Itch. *EMBO Reports*, *18*(9), 1618–1630. https://doi.org/10.15252/embr.201744454

Zilian, O., Saner, C., Hagedorn, L., Lee, H.-Y., Säuberli, E., Suter, U., … Aguet, M. (2001). Multiple roles of mouse Numb in tuning developmental cell fates. *Current Biology*, *11*(7), 494–501. https://doi.org/10.1016/S0960-9822(01)00149-X