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Review

Notch and cancer: a double-edged sword

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Abstract. The highly conserved Notch signaling pathway plays pleiotropic roles during embryonic development and is important for the regulation of self-renewing tissues. The physiological functions of this signaling cascade range from stem cell maintenance and influencing cell fate decisions of barely differentiated progenitor cells, to the induction of terminal differentiation processes, all of which have been found to be recapitulated in different forms of cancers. Although Notch signaling has mostly been associated with oncogenic and growth-promoting roles, depending on the tissue type it can also function as a tumor suppressor. Here we describe recent findings on Notch signaling in cancer and tumor angiogenesis, and highlight some of the therapeutic approaches that are currently being developed to interfere with tumor growth and progression.

Keywords. Notch signaling, oncogene, tumor suppressor, cancer, therapy.

The Notch signaling cascade

Notch genes encode evolutionarily conserved transmembrane bound receptors that are activated by two families of distinct but equally conserved transmembrane bound ligands [1]. Thus, Notch signaling is mediated via cell-to-cell contact. Mammals have four Notch receptors (Notch1–4) and five ligands named Delta-like (DLL) 1, 3 and 4 (homologues of Delta) and Jagged1 and 2, which are related to *Drosophila* Serrate. Notch receptors are synthesized as single precursor proteins that are cleaved (at site S1) during their transport to the cell surface by a furin-like protease [2, 3]. They are expressed at the cell surface as heterodimers comprising an extracellular subunit (N^{EC}), which is linked to a second subunit containing the extracellular heterodimerization domain followed by a transmembrane domain and the cytoplasmic region of the receptors (N^{TM}). The extracellular portions of Notch receptors contain 29–36 epidermal growth factor-like repeats implicated in ligand binding, followed by three cysteine-rich LIN12/Notch repeats that prevent ligand-independent signaling and a C-terminal hydrophobic region mediating heterodimerization between N^{EC} and N^{TM} [4]. The intracellular portion of N^{TM} mediates cell signaling, and contains multiple conserved protein domains (Fig. 1).

Ligand-receptor interactions between two neighboring cells activate Notch signaling by the induction of two successive proteolytic cleavages. The first cleavage occurs extracellularly, close to the transmembrane domain (at site S2) and is mediated by a metalloprotease of the ADAM family [5, 6]. This cleavage is most likely triggered by a physical 'tug' [7] as the ligands, together with N^{EC} are subsequently endocytosed by the ligand-expressing cell [8]. A monoubi-

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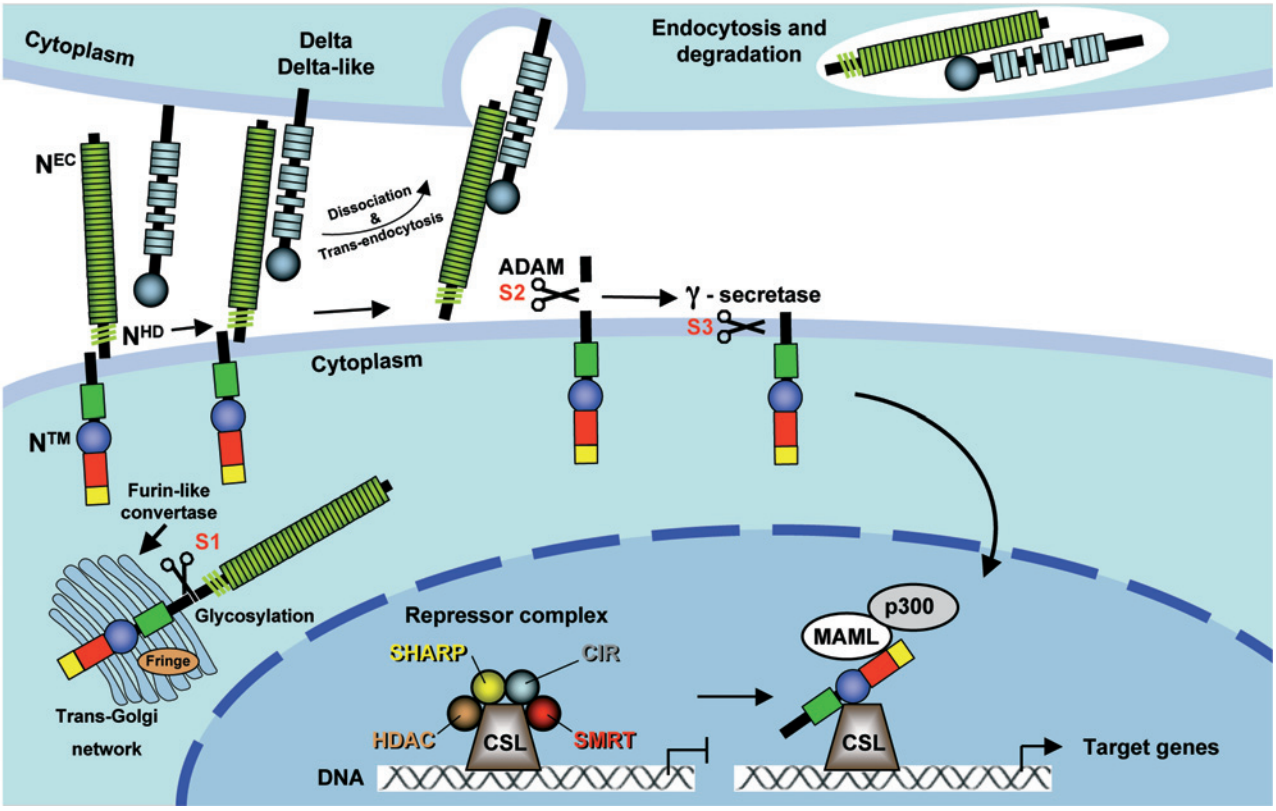

Figure 1. Notch signaling pathway. Notch receptors are synthesized as single precursor proteins, which are cleaved in the Golgi apparatus by a Furin-like convertase at site S1. Cleavage at site S1 during receptor maturation generates two non-covalently associated subunits (N^{EC} and N^{TM}). Notch signaling is initiated through ligand binding to the EGF-like repeats, which induces a second cleavage at site S2 by ADAM-type metalloproteases generating a short-lived intermediate N^{TM}. This cleavage is triggered by a physical 'tug' as the ligands, together with N^{EC} are trans-endocytosed by the ligand-expressing cell. The third cleavage occurs at site S3 within the transmembrane domain and is mediated by γ-secretase, which releases NICD. NICD translocates into the nucleus and binds to the transcription factor CSL. This interaction leads to transcriptional activation by displacement of corepressors (HDAC, SHARP, CIR and SMRT) and simultaneous recruitment of coactivators including MAML and CBP/p300. EC, extracellular subunit; HD, heterodimerization domain; TM, transmembrane domain; NICD, cytoplasmic domain; HDAC, histone-deacetylase; CIR, CBF1-interacting corepressor; SMRT, silencing mediator of retinoid and thyroid receptors; SHARP, SMRT/HDAC-1-associated repressor protein; MAML, Mastermind-like proteins; CBP, CREB binding protein; CSL, **C**BF1 in humans, Suppressor of Hairless in *Drosophila* and LAG in *C. elegans*. Structure of NICD: green rectangle, RAM domain; blue circle, ANK repeats (seven iterated ankyrin-like repeats; red rectangle, TAD (transcriptional activation domain); yellow rectangle, PEST domain (proline, glutamate, serine and threonine-rich degradation sequence).

quitination process follows cleavage at the S2 site and eventually triggers the endocytosis-dependent γ-secretase-mediated cleavage within the transmembrane domain (at the S3 site) [9]. This process liberates the cytoplasmic domain (NICD), which subsequently translocates to the nucleus [10, 11]. Once in the nucleus, NICD heterodimerizes with the transcription factor CSL (CBF-1 in humans, suppressor of hairless in *Drosophila*, Lag-1 in *Caenorhabditis elegans*, also known as RBP-J in mice) [12]. In the absence of Notch signaling, CSL functions as a transcriptional repressor by binding several co-repressors [13]. NICD converts CSL into a transcriptional activator by recruiting and binding co-activators including Mastermind-like proteins (MAML) [14, 15] and CBP/p300 [16] (Fig. 1). Among the best-known Notch target genes is the family of transcription factors known as *Hairy enhancer of split* genes (*Hes*). Members of this family often negatively regulate the transcription of genes such as those of the *achete scute* gene family that are involved in neuronal differentiation [17]. The related *HERP* (Hes-related repressor protein) transcription factor family [18], the cell cycle regulator *Cdkn1a* [19], *cyclin D1* [20], the gene for *Notch-regulated ankyrin repeat protein* (*Nrarp*) [21], *Deltex1* [22], the *pre T cell receptor α* gene [23], the ubiquitin ligase *SKIP2* [24] and the proto-oncogene *c-myc* [25, 26] have all been identified as Notch target genes. This incomplete list of Notch target genes is likely to be extended, and appears to be context dependent. Although the Notch cascade is mechanistically relatively simple, the role of Notch signaling and the activation of downstream target genes in a given tissue is often unpredictable. With this outline of the Notch

signaling pathway the stage is set to discuss its crucial role in various forms of cancer.

Oncogenic functions of Notch in solid tumors

Notch and breast cancer

The first data describing the oncogenic consequences of aberrant Notch signaling in solid tumors were derived from animal studies characterizing a frequent insertion site, named int3, of the mouse mammary tumor virus (MMTV) [27]. In contrast to retroviruses that carry oncogenes, MMTV induces mammary tumors by insertion into the genome and deregulating expression of adjacent genes. MMTV-deregulated genes are therefore referred to as *"int"* (integration) genes. The int3 site was later identified as the *Notch4* locus [28]. Integration of MMTV in this locus results in the LTR-driven expression of transcripts that encode a truncated version of the Notch4 receptor lacking most of the extracellular domain [29]. Transgenic mice expressing this truncated dominant active form of the *Notch4/int3* gene under the control of mammary-specific regulatory elements confirmed a causative role of aberrant Notch signaling for the development of mammary tumors [30–32]. Microarray studies recently performed on Notch4/int3-induced tumors identified high expression levels of c-kit and PDGFR. Treating these mice with the tyrosine kinase inhibitor Gleevec (Imatinib mesylate) resulted in decreased proliferation and angiogenesis, and in the induction of apoptosis within these mammary tumors. This indicated an oncogenic role for c-kit and PDGFRα tyrosine kinases in the context of int3 signaling [33].

Whether Notch4 plays a genuine role during normal mammary development is not clear. Gene targeted mice for *Notch4* show normal mammary gland development and normal lactation, suggesting that Notch4 is either not required for normal mammary development, or that another Notch family member compensates for loss of Notch4 function [21]. The role of Notch signaling during normal mammary development was recently investigated by conditional inactivation of the *RBP-J* gene (encoding the transcriptional mediator of all Notch receptors) and the *Pofut* gene (encoding a fucosyltransferase gene, necessary for efficient ligand receptor binding). In the absence of either one of these genes a phenotype was only observed during pregnancy. The RBP-J-deficient secretory luminal cells acquired the characteristics of basal cells, and the myoepithelial cells showed excessive proliferation, resulting in disorganized alveolar structures. These results led to the interpretation that physiological Notch signaling during pregnancy maintains the luminal cell fate and prevents uncontrolled proliferation of basal cells [34].

MMTV insertions have also been found in the *Notch1* locus, albeit with a lower frequency [35]. These insertions caused the expression of a truncated Notch1 protein similar to the case of int3. Transgenic mice expressing a Notch1-derived NICD under the control of the MMTV LTR develop noninvasive lactation-dependent papillary tumors that regress upon gland involution. However, after additional pregnancies, invasive tumors start to appear, most likely through the accumulation of secondary mutations [36]. Comparative expression profile analysis using microarrays led to the identification of the proto-oncogene *c-myc* as a direct target gene of Notch1-induced mammary tumors [25].

All these results on aberrant Notch signaling and mouse mammary tumorigenesis lead to the question of how significant aberrant Notch signaling is for human breast cancer. To date we have only correlative evidence for the involvement of Notch signaling in human breast cancer. Expression studies of NOTCH1 on ductal carcinoma *in situ* tumors showed positive NOTCH1 staining in approximately two thirds of the examined cases [37]. Approximately half of the NOTCH1-positive tumors were H-ras positive, and *in vitro* data suggested that H-ras activity increases NOTCH1 signaling activity [37]. This seems to be important for the maintenance of the neoplastic phenotype in ras-transformed human cells. Interestingly, high levels of NOTCH1 and JAGGED1 expression correlate with poor survival of breast cancer patients [38]. Consistent with the murine data suggesting that c-myc is a direct downstream effector of Notch1, coexpression of NOTCH1 and c-MYC has been found in a large fraction of examined human breast cancers [39]. Additional evidence that aberrant Notch signaling might be of importance for human breast cancer is derived from studies on Numb, a negative regulator of the Notch cascade [40, 41]. Approximately 50% of the examined human mammary carcinomas, in particular the more aggressive forms, and most of the breast cancer cell lines exhibit loss of Numb expression. This finding correlated inversely with NICD expression and signaling as well with tumor grade and cell proliferation [42, 43]. The inverse expression pattern of Numb and Notch might be caused by Notch negatively regulating Numb levels as recently suggested by Chapman and colleagues [44]. Although these findings are largely correlative, they strongly suggest that aberrant Notch signaling might be of great importance in human breast cancer.

Notch and medulloblastoma

Medulloblastoma is the most common malignant brain tumor in childhood [45]. These primitive neuroectodermal tumors are thought to arise from neuronal stem or precursor cells of the ventricular zone and cerebellar external germinal layer [46]. Medulloblastomas have primarily been associated with aberrant sonic hedgehog signaling (Shh), which induces expression of N-MYC [47], a protein frequently overexpressed in these type of tumors [48]. Increased activity of the Shh cascade in medulloblastomas was reported to be caused by inactivating mutations in Patched1 or Suppressor-of-Fused, two negative regulators of Shh signaling, as well as activating mutations in the *Smoothened* gene. Mutations in the *Axin* and *APC* genes causing increased activity of the Wnt pathway have also been identified in some medulloblastomas [49, 50]. Until recently the Notch pathway was mechanistically not associated with the development of these tumors. However, the primitive nature of these tumor cells and the fact that Notch signaling is involved in the maintenance of neural stem and progenitor cells [51, 52] motivated several groups to investigate the potential role of Notch in medulloblastoma. Expression studies using primary medulloblastoma tumor samples showed increased mRNA expression of NOTCH2 but not of NOTCH1. In 15 % of the examined tumors increased NOTCH2 expression levels were shown to result from *NOTCH2* gene amplification. Moreover, increased expression of the target gene *Hes1* in medulloblastomas correlated with a poor patient survival prognosis [53]. Increased expression of Notch2 and Hes5 has also been observed in a medulloblastoma mouse model based on the expression of a constitutively active form of the *Smoothened* gene in cerebellar granule neuron precursors [54]. These results suggest that Shh signaling can induce Notch signaling. Since Numb has recently been shown to function as a suppressor of hedgehog signaling [55] and Notch can negatively regulate Numb levels [44], it is tempting to speculate that Shh signaling in medulloblastoma activates the Notch cascade to escape a Numb-mediated regulatory loop, which would limit the extent and duration of hedgehog signaling.

Impediment of Notch signaling, either using γ-secretase inhibitors, soluble Delta ligands or siRNA approaches resulted in increased apoptosis and a pronounced reduction of viable cells in medulloblastoma cell lines and/or primary tumor cell cultures [53, 54]. Gain-of-function studies using a dominant active form of Notch2 promoted cell proliferation, soft agar colony formation and xenograft growth, whereas forced expression of Notch1 ICD (N1ICD) was growth inhibitory, suggesting that both Notch receptors exhibit distinct functions [53].

Accumulating evidence supports the concept that many cancer types harbor rare cell populations with stem cell-like properties, which are responsible for the propagation of tumor growth [56, 57]. Markers such as CD133 and side population activity have been used to identify rare cells in brain tumors that have the unique ability to form tumor neurospheres and xenografts [58, 59]. Interestingly, in this context a recent report shows that pharmacological inhibition of Notch signaling results in the depletion of a cancer stem cell-like population characterized by the expression of $CD133^+$ and side population activity. More importantly, loss of Notch signaling within this cell population inhibited medulloblastoma growth both *in vitro* and in *in vivo* xenografts [60]. Although the results are very promising, additional genetic loss-of-function experiments in established tumors need to be performed to confirm that Notch signaling is actually required for the maintenance of these cancer stem-like cells.

Notch and colorectal cancer

In the intestine Notch signaling has been shown to be essential for the maintenance of the crypt compartment. Post-natal gut-specific inactivation of the *CSL/RBP-J* gene results in the complete loss of proliferating transient amplifying (TA) cells followed by their conversion into mucus-secreting goblet cells [61]. In reciprocal experiments expression of NICD in the gut inhibits differentiation of crypt progenitors [62]. The intestine of these transgenic mice consists mostly of undifferentiated TA cells. These reciprocal genetic loss- and gain-of-function data established a gatekeeper function of Notch for intestinal crypt progenitor cells in mice. These conclusions have been supported by toxicology studies of γ-secretase inhibitors (GSI), which are currently developed by pharmaceutical companies to inhibit the protease (γ-secretase) activity of presenilins for treatment of Alzheimer's disease. Rodents treated with GSI display unforeseen side effects, such as loss of proliferating TA cells accompanied by goblet cell metaplasia within the crypt compartment due to inhibition of Notch signaling [63, 64]. This increase in goblet cell differentiation at the expense of enterocytes suggests an additional function for the Notch cascade in lineage specification of enterocytes, which is in agreement with the phenotype observed in gene targeted mice for the *Hes1* gene showing increased mucus-secreting and enteroendocrine cells at the expense of enterocytes [65]. A largely reciprocal phenotype, characterized by intestines only populated with enterocytes, is observed in gene-targeted mice for

Math1, which is transcriptionally repressed by Hes1 [66]. Taken together these results point to at least two physiological functions for Notch; one is the maintenance of proliferating undifferentiated crypt progenitors and the other is to control binary cell fate decisions of progenitors that will differentiate into either the adsorptive or secretory lineage.

Another signaling cascade that is also of importance for the maintenance of the crypt progenitors is the Wnt pathway. Gut-specific inhibition of Wnt signaling results in the loss of the proliferative crypt compartment [67], suggesting that both the Notch and the Wnt cascades are necessary for normal gut homeostasis.

While the causative role of aberrant Wnt signaling for the development of colorectal cancer is well established [68], it is currently less clear whether Notch signaling might have a similar oncogenic function in the gut. The fact that gene expression profiles of crypt cells and colorectal cancer cell lines appear to be very similar suggest that colorectal cancer cells represent the transformed counter part of crypt cells [69]. Since Notch is a gate keeper of crypt cells, it is likely that Notch and Wnt signaling occur simultaneously in adenomas and crypt cells. Indeed, expression of the Notch target gene *Hes1* has been observed in adenomas of APC^{Min} mice [61] as well as in primary human colorectal tumors [70]. Treatment of APC^{Min} mice with GSI partially induces goblet cell differentiation and reduces proliferation in such adenomas [61], suggesting that inhibition of Notch signaling can drive cells out of cycle and induce differentiation despite active Wnt signaling. The fact that not all of the adenoma cells can be differentiated might indicate that only early tumor stages are responsive to GSI, while later stages become resistant. Nevertheless, primary colorectal tumor samples and some colorectal cancer cell lines seem to express Hes1, suggesting that Notch target gene expression could be maintained through Notch signaling-independent mechanisms. Indeed, a recent report shows that Hes1 expression in colorectal tumor samples can be maintained through IKK activity *via* a chromatin-modifying mechanism. Importantly, pharmacological inhibition or expression of a dominant negative form of IKKα resulted in Notch target gene repression, which correlated with reduced tumor size in colorectal cancer xenografts [70]. These results indicate that maintenance of Notch target gene expression is of importance for tumor cell growth and can occur independently of Notch receptor signaling. This could explain, why only some adenoma cells are responsive to GSI. Further genetic loss- and gain-of-function approaches for Notch signaling molecules and/or target genes (such as *Hes* genes) need to be performed to show that Notch signaling is indeed important for the development or maintenance of colorectal tumors.

Notch and pancreatic cancer

An early feature of human pancreatic cancers is that they change their epithelial differentiation program. Many of the changes (including activation of the Notch cascade) documented in pathological situations are also observed during normal embryonic development of the pancreas. Notch signaling has been shown to play an important role during embryonic pancreas development maintaining an undifferentiated precursor cell type [65, 71, 72]. Notch receptors, ligands and downstream targets such as *Hes1* were found to be upregulated in pre-neoplastic lesions as well as in invasive pancreatic cancers in humans and mice [73–75], suggesting that Notch signaling in pancreatic cancers might be an early event leading to the accumulation of undifferentiated precursor cells. This notion was confirmed in explant cultures of adult mouse pancreas in which forced N1ICD expression induced a metaplastic conversion from an acinar cell-predominant epithelium to a ductal cell-predominant epithelium [73]. Transforming growth factor-α (TGF-α)-induced EGF receptor signaling is frequently found in pancreatic cancers [76]. Transgenic overexpression of TGF-α also results in acinar-ductal metaplasia, and correlated with increased Notch signaling. Interestingly, TGF-α-induced metaplasia was abolished by pharmacological blockage of Notch signaling, indicating that Notch mediates TGF-α-induced changes in epithelial differentiation during early pancreatic tumorigenesis [73]. It is likely that elevated Notch signaling levels in the pancreas are not sufficient to generate neoplastic lesions, but it appears to be sufficient to generate immature pre-neoplastic lesions susceptible to additional mutations, which eventually might lead to the development of invasive ductal carcinoma.

Notch and melanoma

Melanomas are highly aggressive tumors, which originate from melanocytes deficient in growth control signals. Melanoma development and progression can be classified into several steps: (1) acquired and congenital nevi with normal melanocytes; (2) dysplastic nevi with atypic structure; (3) non-tumorigenic primary melanoma without metastatic competence (also known as radial growth phase); (4) tumorigenic primary melanoma with competence for metastasis (also known as vertical growth phase); and (5) metastatic melanoma [77]. Once melanomas start to metastasize they become refractory to conventional cancer therapy and have a mostly fatal outcome.

Multiple aberrations due to inactivating and/or activating mutations in many signaling pathways have been found in melanoma, including loss of $p16^{INK4a}$ expression, which correlates with the invasive stage of melanoma progression [78, 79], activating mutations in B-raf [80] and N-ras [81], constitutive FGF-receptor signaling [82] and dysregulated Wnt signaling [83, 84].

Global gene expression profiling and immunohistochemistry revealing the expression of multiple Notch receptors and ligands in primary lesions of human malignant melanomas have extended the list of possible pathways involved in melanoma development [85–88]. Subsequent studies on established melanoma cell lines showed that blocking Notch signaling pharmacologically could have growth suppressive effects. However, constitutive activation of Notch promotes growth and survival and in certain experimental settings lung metastases in mice [88, 89]. Forced expression of Notch signaling in particular seems to promote phase four of melanoma, while it has little effect on already metastatic melanoma cells. The oncogenic function of Notch signaling within these cell lines was linked to increased β-catenin-mediated signaling [88], as well as to increased MAPK and AKT signaling [90]. Yet, how Notch signaling interacts with these pathways in melanoma cells is currently unclear. Although these studies suggest that aberrant Notch signaling can influence certain stages of melanoma, genetic loss-of-function experiments in established melanoma models need to be performed in the future to convincingly show that Notch is an obligate signaling cascade for melanoma development and/or tumor progression.

Nevertheless, such an essential role for Notch signaling has been shown for normal melanocyte development. Melanocyte-specific inactivation of the *RBP-J* gene, or simultaneous inactivation of *Notch1* and *Notch2* resulted in defective hair pigmentation caused by loss of melanoctye progenitor and/or stem cells due to apoptosis [91, 92]. Transgenic expression of *Hes1* in Notch signaling-deficient mice efficiently rescues melanocyte development, suggesting that Notch signaling is important for survival of melanocyte stem or progenitor cells [91].

Notch and leukemia

Historically, human NOTCH was identified at the chromosomal breakpoint of a subset of T cell lymphoblastic leukemias/lymphomas containing a t(7;9)(q34;q34.3) chromosomal translocation [93]. The translocation fuses the 3' portion of NOTCH1 to the T cell receptor Jβ locus. This results in a truncated NOTCH1 protein (N1ICD), which is constitutively active and aberrantly expressed [93]. However, this seminal discovery did not reveal the full oncogenic potential of the truncated version of N1ICD. A causative role of Notch1 in T cell lymphomagenesis was only shown when Pear and colleagues [94] showed in a murine system that the overexpression of N1ICD using a retroviral transduction assay of hematopoietic stem cells led to immature T cell neoplasms. These and subsequent studies [95, 96] provided the initial basis for an experimental model whereby mutations in Notch1 have been analyzed for their onocgenic potential. This was shortly followed by studies with transgenic mice expressing dominant-active forms of Notch1 [22, 97, 98], by the identification of additional Notch1 rearrangements in mice with radiation-induced thymomas [99], and Notch1 mutations in murine transgenic models of T-ALL [100]. Despite the efficiency of Notch1 in inducing T-ALL in murine models, it became soon clear that the rare t(7;9) translocation event could account for only a minor fraction of T-ALL cases [101]. Ellisen et al. [93] originally screened 40 T cell leukemia/lymphoma patient samples and found 4 with the t(7;9) translocation. Based on this, an incidence of ~10 % was estimated. However, in subsequent studies, it appears that <1 % of all human T cell leukemias or lymphomas contain this translocation [101]. However, more importantly, aberrant Notch signaling was subsequently found in several human leukemias and lymphomas that lacked genomic rearrangements [102–104], signifying that upregulated Notch signaling might have a common role in human leukemogenesis. Jundt et al. [102] found high levels of NOTCH1 protein expression in 12 T cell anaplastic large cell lymphoma (ALCL) samples as compared to B cell lymphomas, and high levels of cleaved (activated) NOTCH1 were seen in 2 human ALCL-derived cell lines, compared to normal T cells. Furthermore, NOTCH3 was consistently expressed in a sample of 30 human T cell acute leukemias, and dramatically reduced levels were seen at clinical remission [103]. Interestingly, in T-ALL, *Notch-3* is associated with the expression of its target gene, *HES1*, and of the gene encoding pTα [103]. Expression of these three genes is normally limited to thymocytes and none is usually expressed in normal mature peripheral T cells. Thus, a T-ALL signature, resulting from the combined expression of NOTCH3, pTα and HES1, characterizes the active and relapsing disease. Intriguingly, in a study designed to identify NOTCH-1 downstream targets in T-ALL cells, NOTCH3 was one of the genes up-regulated by NOTCH1 in human T-ALL cell lines [26]. This could actually suggest that a combination of N1ICD and N3ICD may be important in the develop-

ment of T cell leukemia. N3ICD could possibly function by activating NF-κB *via* its recently described effect on IKKα [105]. Although these observations do not establish a causal role for Notch in these T cell malignancies, they suggest the possibility that up-regulation of Notch may play a role in more than the small subset of lymphomas that have the t(7;9) translocation. More compelling evidence was brought about by a study published from the laboratory of Aifantis [106]. They analyzed the gene expression profile of primary hematopoietic stem and lymphocyte progenitor cells, as these are the cell populations where random *Notch1* activating mutations initially occur. The analysis revealed that a panel of significantly up-regulated genes were components of the NF-κB pathway and included target genes such as *Nfkb2*, *Relb*, *Nfkbia*, *Bcl2a1* and *Ccr7*. A direct link between the Notch and NF-κB pathway was provided using N1ICD to induce the activity of NF-κB reporters. Dominant negative forms of the *MAML1* and *IkBα* genes, respectively, could efficiently antagonize these interactions. Conclusive evidence was provided in the same study showing that *NOTCH1* human T-ALL derived mutations activate the NF-κB pathway and that T-ALL cell lines have an activated NF-κB pathway. Definitive proof for a central role of NOTCH1 in human T-ALL came from a recent study identifying somatic activating mutations in the NOTCH1 receptor independent of the t(7;9) translocation, which were detected in more than 50 % of human T-ALL cases [107]. Additionally, they are found in all previously defined T-ALL subtypes. One set of mutations destabilizes the Notch heterodimerization domain (HD) (gain-of-function mutations), probably facilitating ligand-independent pathway activation, whereas mutations that disrupt the intracellular PEST domain might function by increasing the half-life of transcriptionally active N1ICD [9]. In contrast to human T-ALL, HD mutations are rare and insertions/frameshift mutations in the PEST region of Notch1 predominate in mouse T-ALL models [100, 108–110]. However, the precise mechanism by which Notch1 induces T-ALL is not yet fully elucidated.

Several studies published recently have identified potential Notch target genes that may play a role in the oncogenic potential of Notch in the development and/or maintenance of disease. One of these target genes may be the *E2A* transcription factor. Lack of E2A predisposes mice to T-ALL. Lymphomas developing in $E2A^{-/-}$ mice were shown to be critically dependent on Notch signaling, and Notch1 promoted the survival and proliferation of these cells, in part through the induction of pTα [110]. Reschly et al. [110] demonstrated that the T cell lymphomas accumulated mutations in or near the PEST domain, which mediates degradation of the active form of Notch1. Cell cycle-related genes have been identified to be candidates for Notch target genes in T cell malignancies. Numerous observations have linked Notch signaling to the cell cycle machinery, which are likely to correlate with the ability of Notch to function as an oncogene [94] or tumor suppressor gene [111]. Yet, a direct interaction between these two molecular pathways was first implicated by Sarmento et al. [24], revealing that the F-box protein, SKP2, serves to connect Notch1 activation with $p27^{Kip1}$ and $p21^{WAF1/Cip1}$ regulation. Notch activation led to increased degradation of $p27^{Kip1}$ and $p21^{Cip1}$ and to enhanced G1-S transition. Although the work was done in non-transformed cell lines, it is possible that the capacity of Notch to induce SKP2 and to down-regulate $p27^{Kip1}$ expression may constitute the basis of its oncogenic potential in T-ALL. Sicinska and co-workers [112] revealed that mice lacking cyclin D3 are resistant to leukemogenesis induced by N1ICD. Their results indicate that cyclin D3 is required for the Notch oncogenic pathway that signals through pre-TCR, as well as for the $p56^{LCK}$ pathway that signals downstream of the pre-TCR [112]. Specifically, it was observed that mice lacking cyclin D3 are resistant to Notch-driven leukemias and show reduced susceptibility to T cell malignancies triggered by $p56^{LCK}$ but remain fully susceptible to a pre-TCR-independent oncogenic pathway. In the context of T cell leukemias, it was demonstrated that suppression of p53 by Notch is an important event in the development of lymphoma and the activation of p53 mediates regression of disease [88]. Using a tetracycline-inducible model for N1ICD activation Beverly et al. [113] showed that Notch suppresses p53 in lymphomagenesis through repression of the ARF-mdm2-p53 tumor surveillance network. Although inactivation of the N1ICD transgene leads to tumor regression, 100 % of the mice relapse within 6 weeks. Interestingly, the Notch transgene is reactivated in a majority of the relapsed tumors, indicating that there is a strong selective pressure to reacquire Notch activity. N1ICD has also been shown to directly repress p53 through an mdm2-independent pathway, by inhibiting its activating phosphorylations as well as nuclear localization. Blockage of p53 by N1ICD mainly occurred through the mammalian target of rapamycin (mTOR) using phosphatidylinositol 3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway as the mTOR inhibitor rapamycin abrogated N1ICD inhibition of p53 [114]. Another recent study also implicated several proteins in the mTOR pathway as targets of Notch signaling [74]. The mTOR pathway received activating signals from Notch and the simultaneous blockade of the mTOR and Notch pathway with small molecule

inhibitors resulted in synergistic suppression of T-ALL growth. One target gene that seems to be partially responsible for these effects is *c-MYC*, a recently described NOTCH1 transcriptional target. Enforced expression of c-myc can fully rescue mTOR effectors from Notch withdrawal in a subset of T-ALL cell lines. This finding implicates c-myc as an intermediary that connects Notch to mTOR. Although c-myc was already earlier identified as a possible Notch target in hematopoietic stem cells [115] linking Notch responsiveness to a 200-bp element lying immediately 5' of the c-myc transcriptional start site, neither a direct association of Notch with this site nor its functional importance was revealed. Recently, several groups published data placing c-MYC as a direct downstream target of NOTCH1 that maintains growth of T-ALL cells. Weng and colleagues [26] used T-ALL cell lines that are susceptible to GSI and probed for genes regulated by NOTCH1 using gene expression profiling. Among the potential target genes identified were many known Notch targets such as *Hes-1, Hey1* and *Deltex*. However, *c-MYC* was additionally identified. *c-MYC* expression was directly correlated to N1ICD levels and ChIP analysis revealed *c-MYC* to be a direct target of NOTCH1. In a similar approach Palomero et al. [116] also identified NOTCH1 as a direct regulator of cell growth in human T-ALL cell lines and placed *c-MYC* as an immediate target gene regulated by NOTCH1 in T-ALL, highlighting the importance of this interaction in the pathogenesis of human cancer. They were able to elegantly show that the interaction of NOTCH1 and c-MYC is composed of a feed-forward-loop regulatory motif controlling leukemic cell growth. To specifically identify N1ICD target genes in mouse T cell leukemia, doxycycline-regulated N1ICD T-ALL cell lines were developed [117]. Several known Notch1 target genes and signaling pathways were induced, and consistent with the above studies *c-myc* was again identified as a direct Notch1 target gene in mouse leukemic cells. These data provide compelling evidence that c-myc is also a critical downstream effector of Notch in the development of T cell leukemia. The existence of a direct link between Notch and c-myc in T-ALL cell lines and normal thymocytes has therapeutic as well as basic implications. Most mutated Notch1 receptors found in T-ALL cells depend on γ-secretase to transmit signals [107, 118], and the withdrawal of *c-myc* transgene expression cures ~50 % of mice with T-ALL [119]. That the primary effect of Notch1 signals in T-ALL cells appears to be on proliferation and metabolism, rather than differentiation or survival, suggests the use of combinatorial therapies of Notch pathway inhibitors and other therapeutic agents. This could take the form of agents that also disrupt protein synthesis through independent mechanisms, or drugs that target parallel pathways, such as those that regulate cell survival directly.

The flip side of the coin: Notch as a tumor suppressor

Up to this point, we have exclusively described growth promoting or oncogenic roles of the Notch signaling pathway. In tissues in which Notch exhibits growth promoting functions, its physiological role is mostly associated with immature progenitor stages during development or tissue homeostasis. To allow terminal differentiation of progenitor cells Notch signaling often has to be down-regulated. However, instead of maintaining progenitor cells in an undifferentiated state, or influencing their cell fate decisions, in some tissues Notch can also induce differentiation, which is associated with growth suppression. The best-studied example is the role of Notch in the skin. In the murine epidermis Notch receptors and ligand expression is mostly confined to the suprabasal layers and not found in the less differentiated stem or TA cells [19]. *In vitro* data from both human and mouse keratinocytes suggest that Notch signaling induces differentiation, which is accompanied by cell cycle arrest [19, 120, 121]. In mouse keratinocytes, but not human keratinocytes, cell cycle arrest is induced by Notch1-mediated expression of the cell cycle regulator $p21^{WAF1/Cip1}$ [19, 122]. Another property of Notch1 activation is the induction of early differentiation markers including Keratin1/10 and involucrin, and down-modulation of integrin expression [19]. Conditional inactivation of signaling components of the Notch cascade, including Notch1, RBP-J and Presenilin1 and 2 in mouse skin results in hyperproliferation of the skin, hair loss and epidermal cyst formation within less than 1 month [111, 123–125]. Over time, Notch1-deficient animals develop spontaneous, highly vascularized basal cell carcinoma-like tumors. This tumor type in mouse and man is frequently associated with deregulated Shh signaling, and Notch1 deficiency in the mouse skin leads to increased Gli2 expression, which is a downstream component of the Shh pathway [111]. Another pathway that seems to be deregulated as a consequence of loss of Notch1 signaling in the skin is the Wnt cascade. Notch1 deficiency results in increased β-catenin-mediated signaling in hyperproliferative skin and primary tumor lesions, suggesting that Notch might suppress Wnt signaling in the skin [111]. Suppression of Wnt signaling by Notch seems to be mediated indirectly by increasing $p21^{WAF1/Cip1}$ protein levels that subsequently bind to the Wnt4

promoter together with the E2F-1 transcription factor to down-regulate Wnt4 expression [126].
Moreover, classical chemically induced carcinogenesis experiments showed that Notch1-deficient skin is more susceptible to developing skin cancers. As the carcinogen-induced mutation predominantly affects the *HA-ras* gene, it is possible that loss of Notch1 signaling results in cooperative oncogenic effects with activated ras. This possibility has been confirmed by showing that Notch1-deficient keratinocytes forced to express an activated *ras* gene, form aggressive squamous cell carcinomas (SCC) when injected subcutaneously into nude mice, while wild-type cells do not [111]. A more recent study showed that transgenic mice expressing a dominant negative form of MAML1, which inhibits Notch signaling mediated by all Notch receptors, develop SCC that are associated with the accumulation of nuclear β-catenin and cyclin D1 in tumor cells [127]. Since the mouse skin expresses Notch1 and Notch2, it is conceivable that the squamous cell phenotype of the tumors is the result of a complete block of Notch signaling, while basal cell carcinomas may occur when only Notch1 signaling is abolished. Future investigations will be necessary to clarify this issue. Taken together, the genetic mouse studies strongly suggest that Notch signaling exhibits tumor suppressive functions in the skin. This leads to the question of whether these studies are relevant, or indicative for human skin cancers. Consistent with the genetic data from gene-manipulated mice is the finding that human basal cell carcinomas show reduced expression of NOTCH1, NOTCH2 and JAGGED1 [128]. A more recent report shows reduced expression of NOTCH1, NOTCH2 and HES1 in a panel of human oral and skin SCC cell lines, as well as in surgically excised SCCs from patients. Furthermore, suppression of Notch signaling in primary human keratinocytes that express an activated form of the *ras* gene is sufficient to cause aggressive SCC in xenograft models [129], similar to the above-mentioned studies with mouse keratinocytes [111]. Mechanistically, *Notch1* seems to be a p53 target gene, which negatively regulates Rho GTPase effector genes that have previously been linked to tumorigenesis [130–132]. Additional work with human keratinocytes and primary tumor samples needs to be performed to further clarify the role of Notch signaling in the human epidermis and skin cancer lesions. Nevertheless, it seems very likely that Notch also functions in human skin as tumor a suppressor.
Other tissues or forms of cancer where Notch signaling is associated with growth suppressive functions are the prostatic epithelium [121], hepatocellular carcinoma [133], and small cell lung cancer [134, 135]. However, the growth inhibitory role of Notch has mainly (with the exception of the prostate) been suggested on the basis of activated Notch1 overexpression studies. Thus, further experiments are clearly needed in these tissues or cancer types to clarify whether Notch indeed has tumor suppressive functions.

Notch and tumor angiogenesis

As discussed above, Notch has been associated with both oncosuppressive and oncogenic roles, and Notch signaling has been shown to play an important part in various carcinomas. In adults, blood vessels in most organs are quiescent – except notably, during the growth of solid tumors, when otherwise specific embryonic signaling pathways direct new blood vessels to grow around and into the tumor. One important player in this process is the vascular endothelial growth factor (VEGF), which is a potent inducer of angiogenesis both in embryos and in tumors [136, 137]. The other key player involved in the regulation of embryonic and tumor vessel development is the family of Notch signaling components. Several genetic studies show the importance of Notch signaling in angiogenesis as a result of gain- or loss-of-function of Notch signaling components by either promoting or inhibiting angiogenesis. Mice deficient for a variety of these components, including Notch1, Notch1/Notch4, Jagged1, Dll1, Dll4, Hey1/Hey2, and Presenilins (PS1 and PS2) resulted in embryonic lethality with vascular remodeling defects [138, 139]. Haploinsufficiency of Dll4 also resulted in embryonic lethality from severe vascular defects in mice [140]. Remarkably, endothelial cell-specific N4ICD and knockouts of either Notch1 or Notch1/Notch4 produced similar phenotypes [141]. This would imply that either excessive up- or down-regulation of Notch signaling is detrimental to vascular development, and thus a narrow range of optimal expression seems to be essential. There is ample evidence that the relative expression levels of Notch on adjacent developing cells influence cell fate decisions, originally shown by a landmarking report of Heitzler and Simpson [142] in the developing nervous system of *Drosophila.* In their studies, wild-type cells adopted the epidermal fate (the secondary fate) when neighboring cells expressed lower Notch levels, but adopted the neural fate (the primary fate) when neighboring cells expressed higher Notch levels. Presumably, a feedback loop, such as that observed in *Caenorhabditis elegans* development [143], amplifies initial differences in the expression of Notch on neighboring cells. In the mammalian system elegant studies revealed that Notch haploinsufficiency result-

ed in defined cell fate decisions during T cell development [144, 145]. Observations by various other groups revealed haploinsufficiency of Notch receptors and ligands in a plethora of developing systems [21, 140, 146–149]. However, the key point in terms of vascular development is that the various Delta/Jagged-Notch pathway members are absolutely required at early stages of vascular development [149, 150]. Although Notch signaling has been shown to be indispensable during embryonic development [150, 151], recently several groups have identified the importance of Notch signaling in tumor angiogenesis. It has been reported that DLL4 mRNA is up-regulated in the vasculature of a xenografted human breast carcinoma cell line, in endogenous human tumors and following hypoxia [152]. However, the expression of DLL4 is restricted to certain areas of microvessels suggesting that there is finely tuned regulation of Notch signaling during angiogenesis. Patel et al. [153] demonstrated that DLL4 expression is up-regulated in clear cell renal cell carcinoma and is correlated with VEGF expression. Reduction of basal Dll4 levels in endothelial cells by siRNA led to the inhibition of multiple endothelial functions *in vitro* including proliferation, migration, and network formation, implying the potential role of this pathway in cancer. In a different study, VEGF induced the expression of NOTCH1 and DLL4 through the PI3K–Akt pathway in human arterial endothelial cells [154]. These studies suggest that pro-angiogenic factors activate Notch signaling to promote angiogenesis. Wang and colleagues [155] showed that tumor-associated growth factors stimulate the direct interaction between tumor cells and endothelial cells via mitogen-activating protein kinase (MAPK) and Notch signaling pathways, promoting tumor neovascularization and tumor growth *in vivo*. They nicely demonstrated that JAGGED1 is highly expressed in head and neck squamous cell carcinoma and is induced through the MAPK pathway. The elevated JAGGED1 expressions levels on tumor cells triggered Notch activation in neighboring endothelial cells and promoted network formation. The effect was abolished through GSI, soluble JAGGED1 treatment or dominant negative RBP-Jκ expression within the endothelial cells. These studies are inspiring as they provide the first causal link between Notch signaling and tumor angiogenesis.

Preliminary results suggest that Notch signaling may also play a role in breast cancer angiogenesis. NOTCH3 is highly expressed in the neovasculature of human breast tumors, suggesting a possible role for this receptor in blood vessel maintenance [156]. Estrogen-up-regulated JAGGED1 and NOTCH1 expression in both MCF7 and endothelial cells promoted sprouting in endothelial cells [157]. The same study also showed that NOTCH1-expressing breast cancer cells induce hypoxia inducible factor-1α (HIF-1α). Other recent data suggest that HIF-1α binds and stabilizes activated Notch1, leading to enhanced Notch signaling and that Notch signaling was required to maintain an undifferentiated state in stem cells under hypoxic conditions [158]. It would be interesting to examine the relevance of these findings in tumor cells and determine if exposing tumor cells or endothelial cells to hypoxic conditions affects N1ICD transcriptional activity and tumor angiogenesis.

A series of recent landmark papers have yielded a better insight into Notch function during the formation of blood vessels in both embryos and tumors [159–165]. These new studies [164, 165] identify a novel role for Notch/Dll4 signaling during vascular development and reveal the mechanism responsible for the vascular defects that result from reduced Notch signaling. Of particular interest is the common finding that inhibition of Notch signaling led to increased sprouting and branching of blood vessels. However, the experiments performed by Noguera-Troise et al. [159] as well as Rigway et al. [160] revealed that the administration of either neutralizing antibodies against Dll4, a recombinant form of the Dll4 protein that had been generated to block Dll4/Notch signaling or adenoviruses engineered to express Dll4-Fc inhibited the growth of several different solid tumors in mice. Although blocking of Dll4 increased the sprouting and branching of blood vessels, and led to a marked elevation in blood-vessel density in the tumors, assessment of the vascular network in these tumors revealed that the new vessels functioned inefficiently and were not connected functionally to the vascular network of the tumors. This led inadvertently to an overall inhibition of tumor growth. These promising data have unveiled a new drug target for disrupting tumor angiogenesis (see below for further discussion).

Therapeutic approaches

This review has highlighted an important role of the Notch signaling pathway in cancer development. Although the causative role of activated Notch in human carcinogenesis has only been demonstrated explicitly for human NOTCH1 in several cases of T-ALL, Notch receptors and ligands are often aberrantly expressed in a wide range of cancers and in tumor-derived cell lines (see above). One approach that seems suitable is to target components of the Notch signaling pathway as a relevant option for cancer treatment. Thus, protein components of the Notch pathway – including receptor/ligand binding, release

of NICD, interaction of NICD and specific downstream targets, as well as NICD protein stability – may provide suitable drug targets; some of these are already available and others are theoretically possible (Fig. 2).

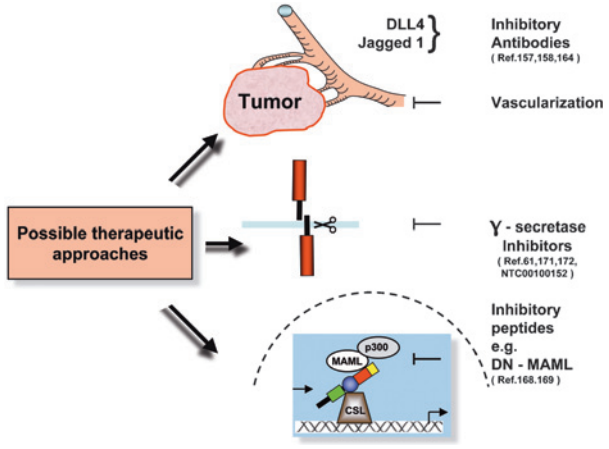

Figure 2. Therapeutic targets in Notch signaling. The Notch signaling pathway in various cancers can be targeted at various levels including receptor ligand binding, release of NICD as well as the coactivator complex. A promising strategy to block receptor ligand binding employs inhibitory antibodies directed against Jagged1 or DLL4 [155, 159, 160]. Blocking DLL4 led to dysfunctional neovascularization and inhibition of tumor growth. The most promising results have been achieved using small-molecule inhibitors of the γ-secretase complex (GSI), preventing the release of NICD [61, 172, 173]. A phase I clinical trial using the GSI MK0752 inhibitor was initiated in 2005 (http://www.clinicaltrials.gov/ct/show/NCT00100152). The third protein component of the Notch signaling that may possibly provide a suitable drug target is the coactivator complex consisting of CSL, MAML and CBP/p300. Small inhibitory peptides acting as dominant negative forms of MAML or CLS decrease the transcriptional activation of target genes [169, 170].

Various strategies that have been used to inhibit Notch signaling aim at receptor ligand interactions. Studies targeting blood vessel formation employing blocking agents to Dll4 [159, 160] revealed substantial tumor growth reduction. Although, anti-Dll4 treatment inhibited tumor growth better when combined with anti-VEGF treatment than when administered alone, the treatment with the Dll4 blockers was still effective against tumors that did not respond to anti-VEGF therapies. Thus, anti-Dll4 treatment may provide a good option for alternative or combinatorial therapy for solid tumors that are resistant to anti-VEGF. The findings of Wang and colleagues [155] highlight that tumor cells express Notch ligands that are able to stimulate tumor angiogenesis and tumor growth. Specific interruption of JAGGED1 signaling within human tumors may provide a potential novel anti-angiogenic therapy.

Although various other strategies have been used to inhibit Notch signaling, including antisense Notch [166], RNA interference [53, 167], soluble receptor decoys that act by sequestering Notch ligands [166, 168] and dominant negative forms of MAML or CSL that decrease the transcriptional activation of target genes [169, 170], they are still far from actual therapeutic application. In practice, small-molecule inhibitors of the γ-secretase complex (GSI), which prevent the release of NICD represent the most immediately promising therapeutic approach in view of the current capabilities for the delivery of anti-cancer drugs. These agents simultaneously target all Notch receptors [171]. Promising results have been shown *in vivo*. One such study involved mice carrying a mutation in the APC tumor suppressor gene. These mice spontaneously develop intestinal adenomas overexpressing Notch target genes such as *Hes1*. Treatment with GSI down-regulated Hes1 and turned proliferative adenoma cells into goblet cells [61]. Kaposi sarcoma samples and cell lines markedly overexpress activated forms of NOTCH1, 2, 4 as well as Hey1 and Hey2 compared with normal endothelial cells. GSI treatment reduced Notch signaling and induced apoptosis in Kaposi sarcoma cells *in vitro*. In xenograft models of Kaposi sarcoma tumors, intra-tumoral injection of GSI inhibited tumor growth by decreasing proliferation and increasing apoptosis [172]. Several different GSI have been shown to reduce endothelial cell proliferation, tube formation and microvessel outgrowths *in vitro*. In mouse models of human glioblastomas and lung adenocarcinomas, both highly vascularized tumors, the GSI DAPT potently reduced tumor growth and vascularization [173]. However, most promising is a phase I clinical trial launched for relapsed or refractory T-ALL patients and advanced breast cancers (http://www.clinicaltrials.gov/ct/show/NCT00100152).

Although, there seems to be quite a broad range of targeting Notch signaling in cancer therapeutics using GSI, a major challenge is the untoward side effects associated with these inhibitors, in particular the cytotoxicity in the gastrointestinal tract [174], which can be exacerbated by conventional chemotherapeutic drugs. Therefore, balancing efficacy and toxicity of GSI must be considered in future clinical applications.

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