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Review Article

The role of oncogenic Notch2 signaling in cancer: a novel therapeutic target

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Abstract: Deregulated Notch signaling is a key factor thought to facilitate the stem-like proliferation of cancer cells, thereby facilitating disease progression. Four subtypes of Notch receptor have been described to date, with each playing a distinct role in cancer development and progression, therefore warranting a careful and comprehensive examination of the targeting of each receptor subtype in the context of oncogenesis. Clinical efforts to translate the DAPT, which blocks Notch signaling, have been unsuccessful due to a combination of serious gastrointestinal side effects and a lack of complete blocking efficacy. There is therefore a clear need to identify better therapeutic strategies for targeting and manipulating Notch signaling. Notch2 is a Notch receptor that is commonly overexpressed in a range of cancers, and which is linked to a unique oncogenic mechanism. Successful efforts to block Notch2 signaling will depend upon doing so both efficiently and specifically in patients. As such, in the present review we will explore the role of Notch2 signaling in the development and progression of cancer, and we will assess agents and strategies with the potential to effectively disrupt Notch2 signaling and thereby yield novel cancer treatment regimens.

Keywords: Notch2, cancer, therapy

Introduction

Notch signaling is a tightly controlled and conserved pathway that is essential to the normal morphological development of multicellular organisms, governing the interactions between interacting cells in this multicellular context. In total, four Notch receptors (Notch1-4) and five Notch ligands (Delta-like 1, 3, 4 and Jagged 1-2) have been identified in mammals. In order to be activated, Notch receptors are proteolytically cleaved three times at specific sites located within certain functional domains on the receptor (**Figure 1**) [1].

Furin-like convertase catalyzes the initial cleavage of these Notch receptors, known as the S1 cleavage, in the Golgi, leading the immature transmembrane heterodimeric protein to adopt what has been termed the Notch extracellular domain-Notch transmembrane and intracellular domain (NECD-NTMIC) form. Fringe-mediated glycosylation then alters the glycosylation status of the EGF repeats present on this protein molecule, after which it is trafficked to the cell surface. On the cell surface, this immature Notch receptor can then interact with Notch ligands present on adjacent neighbor cells, and the resultant mechanical forces cause the HD-C receptor domain to undergo a conformational change revealing the S2 cleavage site [1, 2]. The proteins ADAM10 and/or ADAM17 are then able to cleave this S2 site, producing a Notch extracellular truncation (NEXT) form of the receptor, which is a membrane-bound Notch fragment. The γ-secretase complex, which is made up primarily of presenilin 1-2 and nicastrin, is then able to catalyze S3 site cleavage, causing the Notch intracellular domain (NICD) to be released within the cell. This NICD fragment then undergoes nuclear translocation and heterodimerization the CSL complex (CBF-1 (RBPJ)/suppressor of hairless/Lag1) [1]. This complex normally binds to transcription co-repressor proteins, but upon interacting with NICD it instead binds to transcription co-activators such as mastermind-like protein (MAML), leading to the transcription of a range of Notch target genes such as those of the Hes (hairy

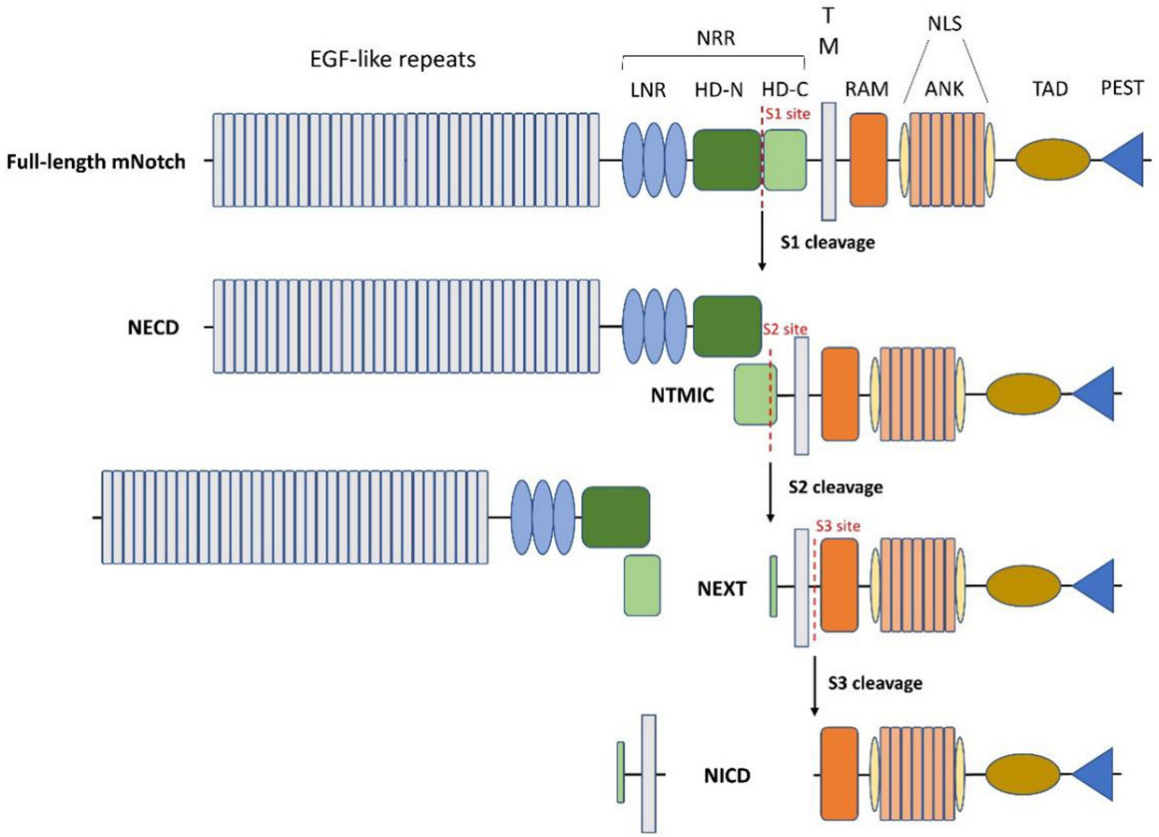

Figure 1. The structure of mammalian Notch receptors following cleavage events. The full-length Notch2 receptor consists of 29-36 epidermal growth factor (EGF) repeats, a negative regulatory region (NRR) (composed of three Lin-Notch repeats (LNRs) and a heterodimerization domain (HD)), a transmembrane domain, an RBPJ-association module (RAM) domain, seven ankyrin (ANK) repeats, two nuclear localization signals (NLS), a trans-activation domain (TAD) and a C-terminal proline (P), glutamic acid (E), serine (S), threonine (T)-rich (PEST) domain. After S1 cleavage occurs, the HD-N and the HD-C domains are separated. Upon EGF binding to a target ligand, the mechanical stability of the NRR domain is disrupted, facilitating S2 cleavage. NICD is then released by S3 cleavage, leading to its nuclear translocation, allowing it to interact via the CBF-1-binding sites located in the RAM and ANK domain in order to form the NICD-CSL complex. The TAD domain is present in Notch1 and Notch2 and plays key roles in activating downstream gene expression. In the final step, NICD undergoes ubiquitin-mediated proteasomal degradation induced by the PEST domain.

enhance of split) and Hey (Hairy/Enhancer of spit related with YRPW motif) families, c-myc, NF-κB, p21, cyclinD1, and many other targets which remain to be fully characterized [3-5].

Notch2 is encoded on chromosome 19p12 (19: 119911553-120069703), and is made up of 34 exons encoded by a total of 2471 amino acids. Notch2 is structurally similar to Notch1, but Notch2 signals less strongly than does Notch1 [6], and it also exhibits unique functional activity in the context of liver [7], kidney [8], ovary [9], smooth muscle [10], T cell [11], and B cell [12] development.

Notch2 gene stability is essential in order for an organism or a cell to develop normally. Mutations leading to overactive Notch2 activity can result in systemic problems characteristic of Alagille and Hajdu-Cheney syndromes, including cardiac defects, chronic cholestasis, polycystic kidneys, osteoporosis, skeletal malformations, and neurological difficulties [13, 14].

A wide range of cancer types have been found to overexpress Notch2 or to exhibit Notch2 gain-of-function mutations, with this enhancement in Notch2 activity playing a vital role in tumor progression. Overactive Notch2 signaling has also been linked to the dysregulation of certain miRNAs, to tumor-associated stromal cell input, and to modulation of internal and external stimulation conditions in tumor cells.

Notch2 plays a number of unique roles in the context of oncogenesis, and in some instances its effects are in opposition to those of other Notch receptors [15, 16]. In the present article, we therefore review the current state of research with respect to the role of Notch2 in different forms of cancer, with a particular focus on how frequently it is involved, how it mediates oncogenesis and disease progression, and treatment strategies targeting this signaling. In addition, we propose potential targets that may facilitate the inhibition of Notch2 signaling.

Oncogenic Notch2 signaling in cancer

Notch2 signaling in liver cancer

Unlike Notch1, Notch2 is essential for both hepatogenesis and hepatocarcinogenesis. In the context of normal liver development, bile duct formation is Notch2-dependent, with Notch2 activation driving embryonic hepatoblasts to differentiate into biliary epithelial cells (BECs), and similarly inducing mature hepatocyte trans-differentiation into BECs [7, 17, 18]. In the context of AKT/Yap-induced intrahepatic cholangiocarcinoma (ICC), Notch2 depletion leads to reduced expression of the Sox9 marker consistent with a loss of hepatocyte-derived BECs, with ICC lesions instead being replaced by hepatocellular adenomas and HCC lesions [19].

Hepatocellular carcinoma (HCC) cells develop from progenitor cells within the liver, and those HCC cells which are Notch2-positive are poorly differentiated and morphologically immature, expressing hepatoblast markers and exhibiting a high N/C ratio and nuclear density [20, 21]. In *Pten* null tumors, miRNA-21 sustains elevated Notch2 expression normally present only within hepatic progenitor cells [22]. Notch2 silencing can cause HCC cells to lose their ability to self-renew in a stem-like fashion, leading to their increased sensitivity to 5-FU [23].

YEATS4 and LEF1 are thought to drive enhanced Notch2 expression via direct binding to the Notch2 promoter in cells, with the lncRNA AKHE recruiting YEATS4, and LEF1 being believed to be activated in response to canonical Wnt signaling [24-26].

Notch2 nuclear translocation can be disrupted by the thyroid cancer 1 (TC1) protein, thereby inhibiting Notch2 signaling. HCC cell self-renewal is suppressed upon TC1 overexpression, and consistent with this it is often inactivated in the context of HCC [27]. This activity is further complicated by the fact that the TC1 homolog 1810011o10 Rik is highly expressed in $CD8^+$ T cells within the tumor, thereby inhibiting Notch2 signaling in these cells and thereby disrupting their ability to achieve anti-tumor killing activity [28].

Notch2 signaling in gastric cancer

In both humans and mice, Notch1 and Notch2 expression in morphologically normal gastric corpus epithelial tissue help to maintain normal tissue homeostasis and differentiation [29, 30]. However, when Notch is overexpressed in these cells this can result in their abnormal proliferation and dedifferentiation, ultimately leading to tumor development [31, 32]. Premalignant mucosal lesions exhibit a significant increase in Notch2 expression relative to adjacent noncancerous tissue (71.4 vs. 10.0%), suggesting that Notch2 plays a key role in the early development of tumors in this tissue [33]. Indeed, there is a significant association between elevated Notch2 expression and a poorer patient prognosis in individuals with intestinal and diffuse-type gastric cancers [34]. Human stomach adenocarcinoma cells have been found the exhibit constitutive Notch2 intracellular domain (N2ICD) activation, and this activated protein directly promotes the elevated expression of cyclooxygenase-2 (COX-2). COX-2 in turn drives prostaglandin E2 (PGE2) expression, thereby promoting the epithelial-mesenchymal transition (EMT) in tumor cells [35].

Multiple different non-coding RNAs have been found to be linked to the regulation of Notch2 in gastric cancer. For example, reversing a loss of miRNA-23b expression was able to disrupt tumor progression owing to its ability to directly bind to the Notch2 mRNA and impair its translation [36]. In an alternative regulatory mechanism, miRNA-133a is able to target and inhibit the translation of presenilin 1, thereby interfering with the activation of the γ-secretase complex, thus preventing NICD release and blocking pro-oncogenic Notch signaling [37]. The lncRNA MIR22HG has also been shown to be able to impair gastric cancer cell proliferation and invasion via ablating Notch2-dependent signaling, although the underlying molecular

mechanism has not been fully characterized [38].

Immunohistochemistry staining of gastric tumor samples in patients before and after undergoing chemotherapy has highlighted a central role for Notch2 in regulating drug resistance [39]. Those patients exhibiting disease regression following chemotherapy exhibit significantly elevated Notch2 expression at the mRNA and protein levels, and this elevated Notch2 expression appears to be induced by chemotherapy and not to be the result of chemotherapeutic enrichment for Notch2-positive cells [39]. Another Notch2-dependent drug resistance mechanism relies upon the pro-metastatic protein tetraspanin-8 (TSPAN8), which can bind Notch2 and maintain its expression, allowing Notch2 to in turn directly activate Wnt/β-catenin signaling, thereby inducing drug resistance in gastric cancer cells [40].

Notch2 signaling in brain cancer

Notch2 is normally expressed in both the hippocampus and cerebellum, and during brain development it plays a central role in governing the negative regulation of glial cell differentiation [41]. Notch2 has been found to be overexpressed in pilocytic astrocytoma (PA) (WTO grade I) tumors relative to normal tissue control samples, and this upregulation is particularly pronounced in those tumors of hypothalamo-chiasmatic origin [42]. Using an shRNA targeting CBF1 to disrupt the CSL complex was sufficient to impair PA cell growth, migration, and invasion [43]. Similarly, Notch2 overexpression has been detected in glioblastoma (GBM) (WHO grade IV), medulloblastoma (MB), and choroid plexus tumors [44-46]. Notch2 and Notch1 play opposing roles in regulating cerebellar granule cell proliferation in normal tissue, with Notch2 being responsible for promoting their proliferation [16]. The majority of MB tumors (35/47 in one study) exhibit Jagged1 overexpression, leading to increasing Notch2 activation and signaling. This facilitates MB cell survival, and disrupting Jagged1 expression can decrease this survival via the downregulation of the Notch2 target Hes1 [45].

Survival analyses of patients with GBM have revealed that a combination of elevated Notch2 expression and decreased miRNA-181a expression are associated with decreased overall survival (OS) [47]. In contrast, heterozygous gene deletions in the Notch2 locus of chromosome 1 (1p12) are highlight predictive of longer survival in GBM patients [48]. While clearly important in GBM, Notch2 expression has not been detected in oligodendroglioma (OD), with one study revealing that an L1711M loss-of-function mutation in the RAM domain of Notch2 is evident in OD tissues, thereby disrupting Notch2 signaling. This disrupted signaling in turn prevents the upregulation of Tenascin-C, which is a glycoprotein associated with Notch2-mediated GBM cell migration [44].

Notch2 signaling exhibits crosstalk with a range of other signaling pathways, including the STAT3 pathway, wherein c-MET-mediated PKCδ activation can induce the Tyr418 phosphorylation of the non-receptor tyrosine kinase SRC, in turn activating this STAT3/Notch2 pathway in GBM [49]. In breast cancer, radiation-induced IL-6 expression is able to promote STAT3 activation and subsequent Notch2 upregulation, thereby facilitating the EMT [50]. The Notch2 effector protein Hes1 can drive further activation of JAK2/STAT3 signaling, promoting glial cell differentiation and HaCaT cell EMT onset [51, 52].

Endogenous miRNAs which suppress Notch2 include miRNA-29a [53], miRNA-34a [54, 55], miRNA-107 [53, 56-58], miRNA-146a [59], miRNA-181a [47], miRNA-181c [53, 60, 61], and miRNA-326 [62], but these miRNAs are largely absent in brain tumors. When their expression is induced, this typically disrupts the tumorigenic potential of glioma cells owing to the consequent suppression of Notch2 activation. This glioma cell proliferation can be similarly inhibited via direct siRNA- or shRNA-mediated targeting of Notch2, thereby driving the induction of apoptosis in these cells [53, 63, 64]. Notch2 inhibition or silencing leads to G0/G1 cell cycle phase arrest as a consequence of p21 upregulation and MCM2, CDK2, cyclin-D1, and cyclin-E downregulation in both glioma and melanoma cell lines [63-66].

On the surface of glioma cells, the diffusible protein Netrin-1 (NTN1) is able to co-localize and interact with Notch2 and Jagged1, mediating Jagged1 endocytosis and consequent Notch2 activation and glioma cell invasiveness. One study found that a NTN1 EGF domain-containing fragment was able to maintain the

Notch2-Jagged1 complex at the cell surface, preventing its internalization and thereby counteracting this normal pro-oncogenic NTN1/Notch2 pathway [67].

Notch2 signaling in B cell malignancies

Notch2 activity has been found to be enhancing in B cell malignancies, but the underlying pathogenic mechanisms differ according to whether or not the Notch2 gene is mutated in these forms of cancer.

Mutated Notch2 in B cell malignancies

B cell lymphomas commonly exhibit nonsense and frameshift mutations in Notch2, with rates of such mutations varying by tumor type from FL (1.8%) [68], to MCL (5.2-6.3%) [69, 70], DLBCL (4.3-8%) [71, 72], and MZL (5-25%) [73-78]. These mutations typically arise in the TAD and PEST domains of Notch2, yielding a truncated C-terminal fragment of the Notch2 protein that is able to disrupt PEST domain-dependent degradation of N2ICD, thereby extending its signaling half-life.

Recent studies have identified specific DLBCL subsets on the basis of clustering following gene sequencing, with the BN2/Cluster 1 subset being composed of tumors containing Notch2 mutations and BCL6 fusions/structural variants [79, 80]. These Notch2 mutations in DLBCL also commonly coincide with increased activation of MUM1, hepatitis C virus, and NF-κB signaling, and a lack of CD10 expression in these tumors [71, 72, 81]. Truncated forms of Notch2 are able to promote the proliferation of lymphoma cells and mediate NF-κB signaling cross-talk [71]. When PDTC is used to inhibit NF-κB signaling, this results in reduced Notch2 protein expression in both Notch2-mutant and wild-type DLBCL cells, thereby disrupting the aberrant proliferation of those DLBCL cells with Notch2 mutations [71].

Mutations in Notch2 are associated with poor clinical outcomes, with MCL and DLBCL patients with such mutations exhibiting shorter OS than those without [69, 81]. In MZL patients, time to treatment failure (TTF) has been suggested to be a better metric for assessing the role of Notch2 mutations in disease [73, 76, 77], indicating that there may be a malignancy-specific role for Notch2 mutations with respect to their clinical and diagnostic relevance, although further study will be needed to fully characterize such differences.

Non-mutated Notch2 in B cell malignancies

Notch2 signaling in B-CLL: In chronic B-cell lymphocytic leukemia (B-CLL), Notch2 overexpression is not the result of any mutation in the Notch2 gene but rather a consequence of microenvironmental signaling from bone-marrow mesenchymal stromal cells (BM-MSCs), resulting in improved CLL cell survival and chemoresistance [82]. Notch2 activity further promotes complement factor C1q production by BM-MSCs, and C1q in turn activates Wnt signaling within CLL cells via N-cadherin stabilization and GSK3-β inhibition [83].

IL-4 is one of the best characterized and most important microenvironmental signals responsible for activating Notch2. IL-4 signaling promotes increased activation of PKCδ within CLL cells, thereby driving ligand-independent Notch2 signaling. In addition, IL-4 favors Jagged1-Notch2 interactions on the CLL cell surface, producing a Jagged1 intracellular fragment (Jag1-IC) which in turn undergoes nuclear translocation to promote the survival of CLL cells through incompletely understood mechanisms [84]. In colorectal cancer cells, Notch2 overexpression results from miRNA-195 downregulation, leading to GATA3/IL-4 pathway activation. Subsequent IL-4 secretion by tumor cells into the local microenvironment can then promote TAM M2 polarization, thereby driving the EMT [85].

Notch2 signaling is able to promote downregulation of anti-apoptotic Mcl-1, and to additionally drive upregulation of the chemoresistance-associated protein eIF4E, thus rendering CLL cells resistant to death and therapeutic elimination [86]. The ability of Notch2 to subvert apoptotic signaling is further enhanced by the Notch2 target CD23 (FCER), which is a transmembrane glycoprotein that is associated with apoptotic resistance in CLL cells [87]. In a majority of CLL cases (23/29 in one study), the γ-secretase inhibitor DAPT failed to impair the expression of CD23 or the activation of Notch2, thus indicating that γ-secretase inhibition alone is an ineffective means of disrupting Notch2 signaling in CLL [88]. However, proteasome inhibitors (PI) have been found to promote

B-CLL apoptosis via driving CD23 and Notch2 downregulation [87]. Furthermore, gliotoxin can inhibit Notch2 transactivation via interfering with DNA-bound Notch2 complex assembly, overcoming the supportive effects of MM-MSCs and selectively impairing Notch2 expression in both CLL and solid tumor cells [89, 90].

Notch2 signaling in MM: Multiple myeloma (MM) is associated with abnormal osteoclast activation, resulting in increased osteolysis and a consequent reducing in bone matrix density. The osteoclastogenic factor RANKL promotes osteoclast differentiation from precursor cells, and Jagged/Notch2 signaling in MM cells and osteoclast progenitors drives this RANKL secretion [91]. RANKL is able to further promote the expression of Notch2 and Jagged 1 in these precursor cells, acting in concert with NF-κB signaling and the transcription factor NFATc1 to promote osteoclast generation [92]. Beyond its role in facilitating MM-associated osteoclastogenesis, Notch2 signaling can also facilitate upregulation of the cytochrome P450 enzyme CYP1A1, thereby promoting resistance to the proteasome inhibitor bortezomib in MM cells [93]. At least four different mechanisms contributing to Notch2 signaling activation in MM cells have been characterized to date:

a. Notch2 glycosylation by the secreted protein chondroitin synthase 1 (CHSY1), which has a fringe-like domain [94]. b. Interactions between Jagged and Notch2 on neighboring MM cells [91]. c. Interacitons between Notch2 on MM cells and DLL1 ligands in BM-MSCs [93]. d. High expression of the deubiquitylating enzyme USP1 [95].

Cell-cell interactions between Jagged2 and Notch2 can promote the survival both of MM and BM-MSC cells. Jagged2 upregulation on BM-MSCs further promotes miRNA-223 downregulation, leading to elevated secretion of the pro-oncogenic cytokines IL-6 and VEGF, further reducing BM-MSC osteogenic differentiation [96].

Notch2 signaling in squamous cell carcinoma

Notch2 overexpression has been detected in laryngeal squamous cell carcinoma (LSCC) tissues (83/95 in one study), primarily localizing to the cytoplasm an nucleus of cells. The tissues of LSCC patients with lymph node metastasis exhibited higher Notch2 expression levels, and knocking down Notch2 in these LSCC cells slowed their growth and we associated with p-ERK, c-Myc, and Bcl-2 downregulation as well as Bax upregulation [97].

A decrease in the expression of miRNA-1 in esophageal squamous cell cancer (ESCC) tissues and patient plasma is linked to a more advanced TNM stage, tumor invasion, and lymph node metastasis, in a mechanism that is in part Notch2-dependent [98]. MiRNA-146a, which also suppresses Notch2, can inhibit Notch2-mediated EMT induction in ESCC cells [99]. In ESCC patients, Notch2 overexpression is regarded as a prognostic indicator associated with poorer OS and PFS (progression-free survival) [100].

Potential targets to disrupt Notch2 signaling

EGF domain and S1 cleavage

Clustered loss-of-function mutations in the Notch2 N-terminal EGF-like repeats have been reported in lung squamous cell carcinoma [101, 102], and in bladder cancer [15]. Nonsense or missense mutant isoforms of Notch2 exhibit dominant negative activity, failing to respond to ligands as a consequence of disruptions in the structure of the normal ligand-binding EGF repeats 11-12 (amino acids 415-492) [103]. Recent high-throughput screening (HTS) efforts have identified a pair of small molecules (IGOR1 and IGOR2) that are able to target the Notch2 residues necessary for Notch2-Jagged2 interactions (Lys446, Gly447, Tyr448, Ile457, Glu459, Gly476, and Phe478), thereby decoupling and disrupting interactions between these two proteins [104]. Antibodies that specifically target these EGF repeats 11-12 have thus far failed to block interactions between DLL4 and mouse Notch1/Notch2 *in vitro*, suggesting that the targeting of EGF 11-12 alone is insufficient to prevent Notch signaling between adjacent cells [105]. Indeed, a truncated isoform of human Notch2 (hNotch2) lacking these EGF domains can still undergo normal S1 cleavage and mediate ligand-independent activation, indicating that such forms of the protein are not wholly inactive [106].

The necessity of S1 cleavage for Notch2 signaling remains uncertain [107]. While one study found full-length Notch2 to be unable to reach the cell surface without this cleavage event

[108], another study found that S1 cleavage only mediates slight conformational changes in Notch2, with an S1-resistant form of Notch2 exhibiting normal trafficking and ligand-dependent activation [109]. Further research will thus be needed to clarify whether furin-induced S1 cleavage represents a viable target for blocking Notch2 signaling.

NRR domain and S2 cleavage

Notch2 missense mutations in the NRR domain (amino acids 1425-1672) have been identified in 5/39 cutaneous squamous cell carcinoma cases in one study, although the resulting effects on Notch2 signaling were unclear [101]. HD domain mutations in T-ALL are linked to abnormal Notch1 signaling [101, 110]. While Notch2 is structurally similar to Notch1, four different hNotch2 HD mutant receptors mimicking this mutated Notch1 mutation (I1549E, L1566P, V1623D, and I1627N) do not undergo efficient S1 cleavage and are not as readily expressed on the cell surface. These mutant isoforms also exhibit decreases in both ligand-dependent and -independent activation, suggesting that in Notch2 these HD mutations cause a loss of normal protein function [106]. Another study of six additional hNotch2 HD mutants (F1565S, L1566P, L1573P, V1623D, I1627N, and A1647P) found these proteins to exhibit reduced NRR domain mechanical stability in a molecular dynamics stimulations, resulting in increased S2 cleavage site accessibility, potentially facilitating the activation of ligand-dependent Notch2 signaling [111].

Monoclonal antibodies targeting the NRR domain of human or mouse Notch2 are frequently employed as a means of disrupting Notch2 signaling [30, 105, 112, 113]. The fully human monoclonal antibody OMP-59R5 (tarextumab) is able to Notch2/3 activity in several tumors via binding to full-length Notch2 and Notch3 [114, 115]. In a phase I trial, OMP-59R5 was found to be well tolerated at up to 2.5 mg per week in patients with solid tumors [116].

The S2 cleavage site of Notch is found within the HD-C domain in a cleavage pocket bounded by α-helix 3, β-sheet 5, and the residue Leu1659. The conserved residue Leu1457 fills and protects the pocket and is removed directly by LNR's pulling [117]. In a study employing a MOE (Molecular Operating Environment), Dobranowski et al. found the S2 site to be biochemically different in Notch1 and Notch2. Their work suggested that small molecule drugs mimicking Leu1457 have the potential to bind a site in this cleavage pocket so as to prevent S2 exposure for occurring [118].

ADAM10 is important for mediating S2 cleavage of hNotch2, whereas ADAM17 is largely dispensable [119]. Indeed, in B cells a lack of ADAM10 renders these cells insensitive to Notch2 ligands [120]. ADAM17, meanwhile, is unable to facilitate the ligand-dependent or -independent Notch2 signaling [106, 119], indicating that only ADAM10 and not ADA17 is needed for hNotch2 signaling, making it a potential target as a means of disrupting S2 cleavage.

Notch2-related miRNAs

MicroRNAs are a class of small non-coding RNAs which are able to bind to the 3'-UTR of target mRNAs, thereby facilitating their degradation or translational suppression. A wide range of miRNAs to date have been characterized to influence cancer progression in a Notch2-dependent manner (**Table 1**). The majority of these Notch2-associated miRNAs antagonize Notch2, and as such their expression tends to be decreased in tumor tissues and to be linked to cancer progression. These miRNAs can be inactivated by a variety of mechanisms, including as a result of elevated Notch2 expression or the following:

a. Loss-of-function mutations in the miRNA transcriptional activator p53 [58]. b. Inactivation of promoter regions driving miRNA expression as a result of CTFT depletion-induced CpG methylation or EZH2-induced H3K27 trimethylation [60, 121]. c. miRNA sequestration by the lncRNAs SNHG12 or PVT1 [122, 123].

Efforts to restore Notch2-suppressing miRNA expression may hinge on overcoming epigenetic silencing mechanisms, interfering with the sponge-like miRNA sequestration by lncRNAs, or restoring wild-type p53 expression in tumor cells, although direct expression of miRNA mimics in tumor cells may also be a viable option. Unlike the majority of other characterized miRNAs, miRNA-21 and miRNA-1246/1248 have been found to promote Notch2 upregulation

Table 1. Multiple miRNAs affect cancer progression by regulating Notch2

MiRNAs that have tumor-suppressive functions			
miRNA	Tumor/cell type	Observations	References
miRNA-1	Esophageal squamous cell carcinoma	MiRNA-1 inhibits the proliferation, migration and invasion of ESCC cells and regulates EMT signaling by downregulating Notch2.	[98]
	Gallbladder carcinoma	MiRNA-1 inhibits the growth, migration and invasion of gallbladder carcinoma cells by downregulating Notch2.	[125]
miRNA-23b	Gastric carcinoma	MiRNA-23b inhibits the growth and lung metastasis of GC cells by downregulating Notch2.	[36]
miRNA-30a	Lymphoid malignancies	MiRNA-30a inhibits the growth and proliferation of DLBCL and T-ALL cells by suppressing Notch1/2-Myc signaling.	[126]
miRNA-34a	Gliomas	MiRNA-34a inhibits brain tumor growth by downregulating Notch1, Notch2 and c-Met.	[54, 55]
	Colorectal cancer	MiRNA-34a inhibits CRC cell growth and induces apoptosis by downregulating Notch1, Notch2 and Bcl-2.	[127]
	Pancreatic ductal adenocarcinoma	MiRNA-34a inhibits PDAC tumor growth by downregulating Notch1, Notch2 and Notch4.	[128]
	Cholangiocarcinoma	MiRNA-34a inhibits CCA cell growth by downregulating Notch1, Notch2 and Jagged1.	[121]
miRNA-133a	Gastric carcinoma	MiRNA-133a inhibits the growth, migration, and EMT of GC cells by targeting Notch1-3' Presenilin 1.	[37]
miRNA-146a	Esophageal squamous cell carcinoma	MiRNA-146a inhibits the EMT of ESCC cells by downregulating Notch2.	[99]
	Gliomas	MiRNA-146a inhibits the growth and invasion of glioma cells by downregulating Notch1 and Notch2 and the Notch1/Notch2 ratio.	[59]
miRNA-181a	Gliomas	MiRNA-181a inhibits the formation, differentiation and proliferation of glioma cells and induces apoptosis by downregulating Notch2.	[47]
miRNA-181b	Non-small cell lung cancer	MiRNA-181b inhibits NSCLC cell stemness by suppressing Notch2/Hes1 signaling.	[129]
miRNA-107	Pancreatic cancer	MiRNA-107 inhibits pancreatic cancer cell proliferation by downregulating Notch2.	[130]
	Gliomas	MiRNA-107 inhibits glioma cell invasion by suppressing Notch2/Cox-2 and Notch2/Tenascin-C/Mmp-12 signaling.	[56]
		MiRNA-107 inhibits glioma cell growth by downregulating Notch2, CD133 and Nestin.	[57]
		P53-induced miRNA-107 inhibits glioma cell growth and leads to cell cycle arrest by downregulating Notch2 and CDK6.	[58]
miRNA-29a		MiRNA-107, miRNA-181c and miRNA-29a reduce glioma cell proliferation by downregulating Notch2.	[53]
miRNA-181c		MiRNA-181c is decreased due to DNA methylation, which results in Notch2 overexpression in glioma cells.	[60]
		MiRNA-181c inhibits the proliferation, invasion, and self-renewal ability of glioma cells by downregulating Notch2.	[61]
	Endometrial cancer	Loss of miRNA-181c results in EEA recurrence via overexpression of Notch2.	[131]
miRNA-195	Colorectal cancer	MiRNA-195 inhibits stemness and 5-FU resistance of CRC cells by downregulating Notch2 and RBPJ.	[132]
		MiRNA-195 inhibits the proliferation, invasion, migration, formation, growth and EMT of CRC cells by silencing Notch2.	[85]
	Osteosarcoma	MiRNA-195 inhibits the proliferation, invasion and migration of osteosarcoma cells and induces G0/G1 cell cycle arrest by down-regulating Notch2.	[133]
miRNA-200 family	Colorectal cancer	MiRNA-200 family RNAs inhibit the growth and invasion of CRC cells and induce apoptosis by downregulating Notch1-3 and Hey1.	[134]
miRNA-205	Breast cancer	MiRNA-205 decreases tumor stem cell properties by downregulating Notch2.	[135]
miRNA-326	Gliomas	MiRNA-326 inhibits the viability, proliferation and invasion of glioma cells by downregulating Notch1 and Notch2.	[62]
miRNA-424	Cervical cancer	MiRNA-424 inhibits the proliferation and growth of cervical cancer cells and induces apoptosis by suppressing KDM5B-Notch1/2 signaling.	[136]
miRNA-488	Retinoblastoma	MiRNA-488 inhibits retinoblastoma progression by downregulating Notch2.	[123]
miRNA-598	Colorectal cancer	MiRNA-598 inhibits the migration and EMT of CRC cells by suppressing Jagged1/Notch2 signaling.	[137]
miRNA-4735	Bladder cancer	MiRNA-4735 inhibits bladder cancer cell proliferation by downregulating Notch2.	[138]
MiRNAs that have oncogenic functions			
miRNA-21	Hepatocellular carcinoma	Anti-miRNA-21 suppresses Notch2/Runx2/Opn signaling in *Pten* null liver tumors.	[22]
miRNA-1246/1248	T-ALL	MiRNA-1246/1248 promotes T-ALL cell proliferation by upregulating Notch2.	[124]

and oncogenesis, making them relevant targets for miRNA inhibition [22, 124].

Notch2 activators

Endogenous and exogenous Notch2 activators can activate Notch2 either via binding to the NOTCH2 promoter region at the pre-transcriptional level [24-26], or via directly activating the Notch2 receptor at the post-translational level [52, 84, 139].

PKCδ is an endogenous activator of Notch2 found in tumor cells that can be activated by external compounds, leading to STAT3 phosphorylation and consequent Notch2 transcription, as well as to ligand-independent Notch2 signaling. When PKCδ is depleted, this leads to a suppression of Notch2 activity and a reduction in the viability, migration, and invasion of tumor cells [49, 84, 88].

Midkine (MK) is cytokine produced by tumor cells which is able to bind to Notch2 via its N-terminal ligand-binding EGF repeats, activating Notch2 signaling. This MK-Notch2 signaling axis has been found to play a role in mediating tumorigenesis and drug resistance, and inhibiting MK activity can disrupt these oncogenic pathways [52, 139, 140].

Notch2 degradation

The ubiquitin-proteasome pathway

Notch2 undergoes ubiquitin-mediated proteasomal degradation in a mechanism that is in part initiated by the poly (ADP-ribose) polymerase (PARP) tankyrase 1, which binds to and PARylates a RREPVG motif in the N2ICD RAM domain. This modification enables RNF146, an E3 ubiquitin ligase, to ubiquitylate N2ICD, thereby targeting it for proteasomal degradation [141]. Tankyrase 1 is also required for the generation of the N2ICD-like γ-secretase. Inhibiting or eliminating tankyrase results in an increase in the levels of uncleaved membrane-bound Notch2 and a reduction in cleaved N2ICD in human cells, thereby impairing Notch2 target transcription in affected cells [141].

In CLL cells, the PIs bortezomib and MG132 have been found to reduce both DNA-bound Notch2 complexes and cell viability, but this finding is not generalizable to all malignancies. For example, BL cells transfected with N2ICD or N2ICDΔPEST exhibited reduced PI-sensitivity, indicating that these drugs fail to effectively impair the activation of overactive isoforms of Notch2, particularly in B cell malignancies associated with Notch2 gain-of-function PEST mutations [87].

While bortezomib and DAPT individually are unable to inhibit Notch2 in MM cells, they are able to do so effectively in combination [93]. In MM the targeting of the deubiquitylating enzyme USP1 has instead been found to more effectively reduce tumor cell viability and bortezomib resistance. Upon USP1 inhibition using SJB, both Notch1 and Notch2 protein levels were decreased in targeted cells [95].

The autophagy-lysosome pathway

Following their endocytosis, Notch receptors can undergo trafficking to the lysosome for ubiquitylation-dependent degradation [142]. In one study, the antioxidant N-acetylcysteine (NAC) was found to mediate an antioxidant-independent reduction in Notch2 protein levels via inducing Notch2 lysosomal degradation in a mechanism dependent upon the E3 ubiquitin ligase Itch, resulting in reduced proliferation, migration, and growth of glioma cells [66].

Conclusions

Persistent Notch2 signaling facilitates the stem-like ability of tumor cells to avoid apoptotic cell death while undergoing self-renewal and promoting the EMT, and increased Notch2 expression is linked to poor clinical prognosis in patients. Notch2 further increases the chemo- and radio-resistance of tumor cells, thereby rendering these cancers less sensitive to therapeutic treatment [23, 39, 40, 50, 82, 86, 93, 129, 132, 143].

A wide range of Notch2 mutations have been identified in the context of cancer, but how these mutations correspond to differences in Notch2 signaling has not been comprehensively characterized to date. Based on the available research, we propose a range of candidate targets for the blocking of Notch2 signaling (**Figure 2**). At the pre-transcriptional level, direct Notch2 silencing is highly effective in cell-based experiments, although it has the potential for significant off-target effects making the reduction of NOTCH2 transcriptional activators a potentially more appealing strategy.

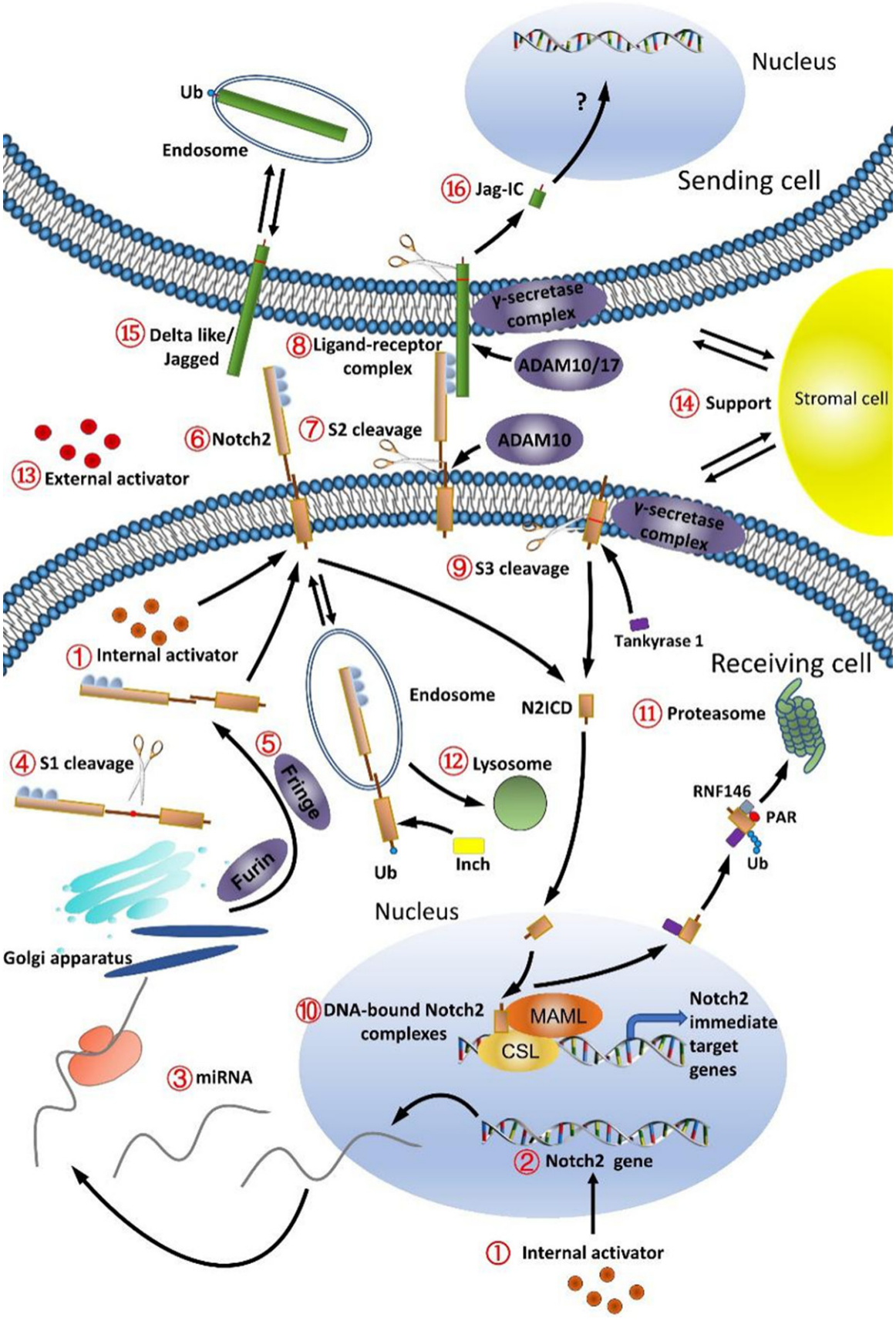
Nucleus
?
Ub
Endosome
Sending cell
16 Jag-IC
γ-secretase complex
15 Delta like/ Jagged
8 Ligand-receptor complex
ADAM10/17
14 Support
Stromal cell
6 Notch2
7 S2 cleavage
ADAM10
13 External activator
γ-secretase complex
9 S3 cleavage
Tankyrase 1
Receiving cell
1 Internal activator
Endosome
N2ICD
11 Proteasome
4 S1 cleavage
5 Fringe
12 Lysosome
RNF146
PAR
Ub
Inch
Ub
Furin
Nucleus
Golgi apparatus
10 DNA-bound Notch2 complexes
MAML
CSL
Notch2 immediate target genes
3 miRNA
2 Notch2 gene
1 Internal activator

Figure 2. Oncogenic Notch2 signaling and potential therapeutic targets. ① Eliminating internal Notch2 activators ② Silencing the NOTCH2 gene via siRNA or shRNA ③ Regulating Notch2-related miRNAs ④ Preventing S1 cleavage ⑤ Inhibiting the glycosylation of Notch2 using fringe inhibitors ⑥ Antibody-mediated blocking of the Notch2 receptor ⑦ Preventing S2 cleavage by S2 site blockers or ADAM10 inhibitors ⑧ Uncoupling the ligand-receptor complex or maintaining the ligand-receptor complex at the cell surface ⑨ Preventing S3 cleavage using γ-secretase or tankyrase 1 inhibitors ⑩ Preventing the formation of DNA-bound Notch2 complexes or silencing N2ICD, CSL, or MAML ⑪ Interfering with proteasome-dependent Notch2 degradation using proteasome-, tankyrase 1- or USP1 inhibitors ⑫ Promoting Inch-induced lysosome-dependent Notch2 degradation ⑬ Eliminating external Notch2 activators ⑭ Preventing interactions between tumor cells and stromal cells ⑮ Antibody-mediated blocking of delta-like/Jagged ligands ⑯ Targeting Notch2-induced Jag-IC activity (not fully understood yet).

At the posttranscriptional level, interfering with the cleavage-mediated maturation of Notch2 represents one potential therapeutic strategy. The S1 and S2 cleavage steps of hNotch2 differ from those of Notch1 and from those of murine Notch2, although the specifics of these differences are incompletely characterized [106]. Further efforts are therefore needed to fully understand whether these two cleavage steps represent viable targets for pharmacological inhibition. The γ-secretase inhibitor DAPT is able to block S3 cleavage, but it indiscriminately interferes with the cleavage of all Notch subtypes, and Notch2 can in some cases exhibit DAPT resistance [88, 90]. As such, tankyrase 1 represents a potentially superior target for disrupting Notch2 S3 cleavage and for promoting its proteasomal degradation [141]. Like Notch receptors, Delta-like and Jagged ligands also undergo ADAM10/17 and γ-secretase-mediated proteolysis following receptor interactions after binding to receptors [144, 145]. Using an antibody to block Jagged1 has been found to reduce Jag1-IC activation, thereby reducing cell viability [84].

Modulating or disrupting the formation, function, and degradation of Notch2, N2ICD, and the Notch2-ligand complex all have potential as viable means of targeting and interfering with Notch2 activity. NRR but not EGF domain-blocking antibodies have been found to inhibit Notch2 activity. Disrupting the interactions between supportive stromal cells and Notch2 is also an important and viable strategy to remove the microenvironmental signals that support tumor growth and survival.

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Disclosure of conflict of interest

None.

Abbreviations

YEATS4, YEATS domain containing 4; LEF1, lymphoid enhancer-binding factor 1; EMT, epithelial-mesenchymal transition; MCM2, mini-chromosome maintenance protein 2; CDK2, cyclin-dependent kinases 2; FL, follicular lymphoma; MCL, mantle cell lymphoma; DLBCL, diffuse large B cell lymphoma; MZL, marginal zone lymphoma; MUM1, multiple myeloma 1; RANKL, Receptor Activator of Nuclear Factor-κB Ligand; T-ALL, T-cell acute lymphoblastic leukemia.

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