Chapter 3
Dual Function of Notch Signaling in Cancer: Oncogene and Tumor Suppressor

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Abstract The Notch cascade is an evolutionarily conserved cell-to-cell signaling system that regulates many aspects of embryonic development. It regulates also self-renewal and differentiation processes as well as tissue homeostasis in several adult vertebrate organs. In the last 15 years, it has become evident that deregulated Notch signaling is associated with several human disorders, including cancer. Recently, large sequencing efforts of cancer genomes have uncovered both gain- and loss-of-function mutations in different genes involved in the Notch signaling cascade, indicating that Notch can be both oncogenic and tumor suppressive. For specific tumor types, results generated from experimental mouse models predicted and also validated such relationships, whereas for others, the conclusive findings were unanticipated. The oncogenic and tumor-suppressive functions of Notch appear to be context- and tissue-specific. In this review we will discuss the context-dependent and tissue-specific oncogenic and tumor-suppressive functions of Notch.

Keywords Notch · Cancer · Oncogene · Tumor Suppressor · Mutations

3.1 A Brief Introduction to the Notch Cascade

The Notch signaling cascade consists of membrane-bound receptors and ligands that regulate multiple functions in adult vertebrate tissues including stem cell self-renewal, cell fate specification, proliferation, and apoptosis through cell-to-cell signaling [48, 50]. Mammals possess four Notch receptors (Notch1–4), four delta-like ligands (Dll1–4), and two ligands of the Jagged family (Jag1 and 2). During maturation and transport to the cell surface, Notch receptors traffic from the endoplasmic reticulum to the Golgi where they undergo proteolytic cleavage by a furin-like protease. At the cell surface, Notch receptors exist as heterodimeric receptors consisting of an extracellular subunit (N^{EC}), which is non-covalently bound to a second

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subunit harboring one part of the extracellular heterodimerization (HD) domain followed by the transmembrane domain and the cytoplasmic region of the Notch receptor (N^{TM}). The extracellular subunit of Notch receptors contains between 29 and 36 epidermal growth factor-like repeats (involved in ligand binding), 3 cysteine-rich LIN12 repeats (LNR), and a hydrophobic stretch of amino acids involved in the heterodimerization of N^{EC} and N^{TM}. The LNR together with the hydrophobic stretch of amino acids constitute the negative regulatory region (NRR), which prevents ligand-independent activation of Notch receptors. The intracellular part of Notch receptors contains multiple elements including nuclear localization signals, protein-protein interaction domains, transcriptional activation domains, and a PEST sequence involved in regulating protein stability (Fig. 3.1).

Normally, Notch signaling is initiated by ligand-receptor interaction between neighboring cells. This triggers a series of proteolytic cleavage events, the first of which is mediated by metalloproteases of the ADAM family (ADAM-10 or ADAM-17), located 12–13 amino acids external to the transmembrane domain. Subsequently, the γ-secretase multi-protein complex cleaves the remaining Notch receptor within the transmembrane domain, resulting in the release of the intracellular cytoplasmic domain of Notch (NICD). NICD subsequently translocates to the nucleus where it interacts with the DNA-binding transcription factor RBP-J (also known as CSL) in order to form a short-lived transcription activation complex. The binding of NICD to RBP-J results in the recruitment of other coactivators including mastermind-like proteins (MAML1–3), p300, and many other proteins in order to induce transcription of downstream target genes [124] (for a more comprehensive description of the molecular aspects of the Notch cascade, the reader is referred to excellent reviews [11, 50]).

3.2 Notch Functions as Oncoprotein

The first evidence demonstrating that components of the Notch cascade can function as oncoproteins resulted from the finding that the NOTCH1 locus is disrupted by t(7;9) translocations in rare cases (<1%) of human T cell acute lymphoblastic leukemia (T-ALL). This translocation results in the fusion of the 3'end of the human *NOTCH1* gene to the *TCRB* promoter/enhancer region. *TCRB*-driven transcripts of this fusion gene generate a truncated, dominant active NOTCH1 protein that lacks the NRR domain [29, 86]. The oncogenic potential of this truncated form of the NOTCH1 protein was demonstrated in murine bone marrow reconstitution experiments in which expression of the truncated NOTCH1 protein was shown to be sufficient to cause T-ALL in mice [12, 70]. However, the clinical relevance of this finding appeared to be limited due to the rare number of patients that carry such translocations. In the last decade, large sequencing efforts were performed with the aim to uncover the genomic landscape of many tumor types. In the context of T-ALL, Aster and colleagues sequenced the cancer genome of multiple human T-ALL cell lines as well as of 96 primary T-ALL samples from children and

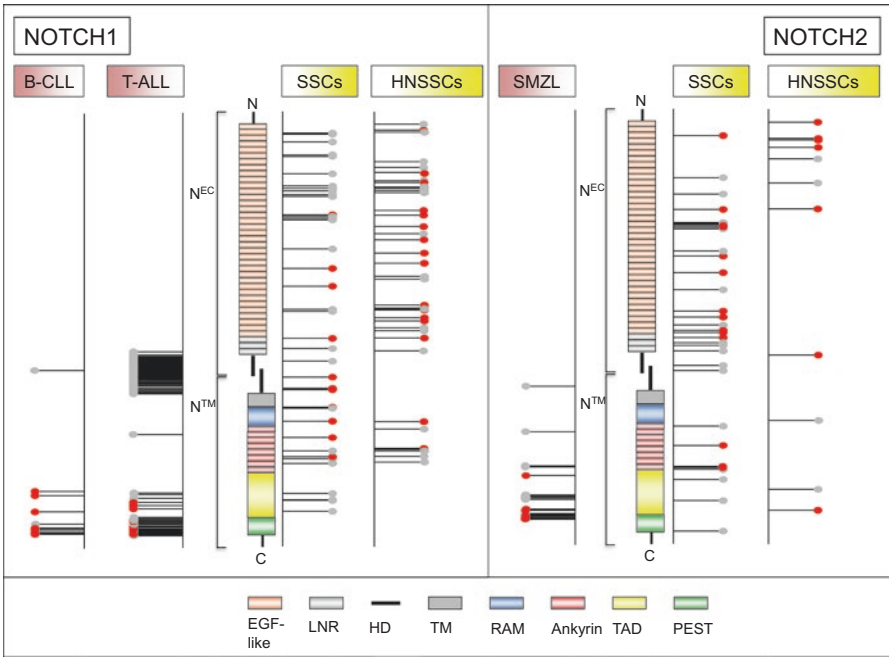

Fig. 3.1 Mutational landscape of NOTCH1 and NOTCH2 in cancers in which Notch functions as oncogene or tumor suppressor. Schematic representation of the NOTCH1 and NOTCH2 receptors with their structural distribution of missense (gray) and nonsense (red dots) mutations in indicated neoplasms. B-CLL, T-ALL, and SMZL indicated in red are neoplasms in which Notch functions as oncogene, whereas SSCS and HNSSCs indicated in yellow are cancer in which Notch exerts tumor-suppressive mutations. NOTCH1 oncogenic driver mutations in T cell acute lymphoblastic leukemia (T-ALL) [116] and B cell chronic lymphocytic leukemia (B-CLL) [24, 32, 77, 103] and Notch2 driver mutations in splenic marginal zone lymphomas (SMZL) [46, 88] [69] are displayed on the left side of the schematic Notch receptors. For NOTCH1 and T-ALL, missense mutations cluster largely to the NRR domain, while truncating mutations are mostly confined to the PEST domain. In B-CLL truncating mutations are predominant and cluster to the C-terminal PEST domain and adjacent transactivation domain. Similarly NOTCH2 mutations in SMZL are mostly confined to the PEST domain. In contrast, mutations depicted on the right side of the schematic receptors from squamous cell carcinoma (SSC) [73, 113] and head and neck squamous cell carcinoma (HNSCC) [2, 73, 106] in which Notch1 and 2 exert tumor-suppressive functions appear all over the protein, with a tendency to be more frequent in the N^{EC} domain of the receptors. Below are indicated the different protein domains of the Notch receptors. The extracellular part of the Notch receptors (N^{EC}) contains EGF-like repeats (EGF-like) followed by three cysteine-rich LIN domains (LNR) that prevent ligand-independent cleavage, the heterodimerization domain (HD) and the transmembrane domain (TM). The N^{TM} part of the receptors includes the cytoplasmic domain consisting of a RAM domain (RAM) followed by six ankyrin repeats (ankyrin) that bind to the RBP-J transcription factor, a transactivation domain (TAD) and a PEST sequence (PEST) involved in regulating protein stability

adolescents at time of diagnosis for *NOTCH1* mutations [116]. Fifty-six percent of the investigated tumor samples were found to carry mutations in the *NOTCH1* gene throughout all major molecular T-ALL subtypes, rendering *NOTCH1* as the most frequently mutated gene in human T-ALL. The somatic mutations cluster to two regions within the human *NOTCH1* gene (Fig. 3.1). The most common mutations are found in exon 26 and 27 coding for the HD domain, which is the region of the NRR that normally prevents activation of Notch receptor signaling in the absence of ligand binding. Mutations in the NRR facilitate ligand-independent activation of Notch receptors. The second cluster of mutations localize to the C-terminal PEST sequence encoded by exon 34 of NOTCH1. Most of these mutations consist of either nonsense or frame-shift mutations resulting in the deletion of the PEST domain which is involved in targeting NICD for degradation [16]. Mutations in the PEST domain are present in 20–30% of tumors resulting in increased Notch activity due to persistent stabilization of NICD. Ten to twenty percent of primary human T-ALLs carry mutations both the HD and the PEST domains [116]. This study identified NOTCH1 as being a major player in the pathogenesis of human T-ALL. Surprisingly, and in contrast to the truncated dominant active form of NICD, HD, PEST, and HD/PEST mutations were found to be weak inducers of Notch activity in in vitro Notch-driven reporter assays. When tested in retroviral mouse models, introduction of these mutations led to ectopic T cell development but were not sufficient to induce leukemia. However, when these constructs were studied in an oncogenic LSL-K-ras^{G12D} background that predisposes mice to T-ALL development, these relatively weak activating Notch mutations shortened disease latency and gave rise to T-ALL cell lines whose growth was dependent on Notch signaling [15]. These data suggest that frequently occurring *NOTCH1* mutations found in human T-ALL patients do not solely generate a sufficiently strong Notch signal to initiate leukemic development in mouse models. However these weakly leukemogenic *NOTCH1* mutations can complement and synergize with other already existing leukemogenic events. In agreement with this, other transgenic mouse models of T-ALL such as TAL1/SCL, OLIG2, and LMO1/1 have been found to develop spontaneous activating mutations in the Notch1 gene [4]. Taken together, these data suggest that the commonly found *NOTCH1* mutations in human T-ALL are likely to be secondary events that contribute to preexisting primary tumor initiating hits in order to accelerate tumor progression. Importantly, arising tumors remained sensitive to growth inhibition by pharmacological (γ-secretase inhibitor) Notch inhibitors, indicating that these tumors remained addicted to Notch. This warrants the rational to therapeutically block Notch signaling in T-ALL patients.

Activating mutations in the *NOTCH1* gene have also been identified in B cell chronic lymphocytic leukemia (B-CLL) [24, 32, 77, 103]. This was originally unanticipated due to early studies showing that overexpression of NICD antagonizes early B cell development in the bone marrow [78] and/or induces growth arrest and apoptosis in both murine and human B cell lines and in multiple B cell neoplasms [129]. On the other hand, Notch2-mediated signaling is essential for the development and maintenance of splenic marginal zone B cells [90]. Furthermore, Notch1 has been shown to synergize with B cell receptor and/or CD40 signaling to enhance

B cell activation and function [108]. Although high levels of Notch signaling are incompatible with early stages of B cell development, Notch signaling is important in more mature B cells. Therefore, retrospectively, the identification of activating Notch mutations in B cell neoplasms appears to be less of a surprise. B-CLL is among the most common types of leukemia in the Western world. Clinically, the progression of the disease is very heterogeneous. CLL patients can be subdivided in two major subgroups based on the immunoglobulin heavy chain variable (IGHV) gene status of CLL cells. Approximately 60% of CLLs carry immunoglobulin heavy chain variable genes exhibiting somatic hypermutations in their variable regions, while the remaining 40% of CLLs do not carry IGHV gene mutations. The subgroup carrying unmutated IGHV genes is associated with a more aggressive form of the disease [127]. *NOTCH1* mutations in CLL were first identified in 2009 in 2 out of 43 CLL cases analyzed. In both cases the mutations localized to exon 34 leading to predicted loss of the PEST domain [24]. A follow-up analysis of 133 CLL patients by the same group identified *NOTCH1* PEST domain mutations to occur with a frequency of approximately 5%. Neither HD domain nor NOTCH2 gene mutations were identified in this study, suggesting that mutations are restricted to the *NOTCH1* gene and in particular to exon 34 coding for the PEST sequence [103]. In 2011, two groups used next-generation sequencing to identify recurrent mutations in larger CLL cohorts. *NOTCH1* mutations were found to occur at a frequency of 8.3% and 12.2%, respectively [32, 77], all localized to the PEST domain. A more recent study reports that mutations can also occur within the noncoding region of *NOTCH1*, namely, the 3' UTR. These mutations generate a new splice acceptor site within the 3' UTR inducing aberrant splicing events that lead to deletions that include the final 158 coding bases of exon 34 [76]. Although the frequency of *NOTCH1* PEST mutations seems to vary between 5% and 10% at diagnosis, they are primarily found in samples of the more aggressive IGHV non-mutated CLL patient subgroup and often correlate with trisomy 12. Moreover, the mutation frequency appears to increase with disease progression reaching 31% in patients subsequently diagnosed with Richter transformation and 21% in chemorefractory CLL [32]. Taken together although *NOTCH1* mutations do not appear to be causative in CLL, they are associated with poor prognosis and worse outcome and are most likely acquired during disease progression.

NOTCH1 mutations clustering to the PEST sequence have also been identified in Mantle cell lymphoma (in 12% of clinical cases and 20% of cell lines) [51], an aggressive subtype of non-Hodgkin lymphoma.

Even though the identification of *NOTCH1* mutations in CLL and MCL was somewhat unanticipated, the identification of mutations in genes involved in the Notch pathway in splenic marginal zone lymphoma (SMZL) was less surprising, as NOTCH2 is a well-established master regulator of marginal zone B cell development and maintenance [42, 90]. Next-generation sequencing identified *NOTCH2* mutations in 20–25% of SMZL cases [46, 88] establishing *NOTCH2* as one of the most frequently mutated genes in SMZL. NOTCH2 mutations were also associated with adverse clinical outcomes including reduced treatment-free and overall survival [46, 69]. Surprisingly mutations in the *NOTCH1* gene were

also identified in approximately 5% of the investigated cases. Mutations in both *NOTCH1* and *NOTCH2* predominantly cluster to the PEST sequence and are therefore predicted to cause sustained Notch signaling due to increased protein stability. Additional mutations in Notch signaling-associated genes such as *SPEN*, *DTX1*, and *MAML2* have also been identified, though with lower frequency to *NOTCH1/Notch2* [46, 88].

The fact that *NOTCH* mutations within B cell neoplasms are predominantly restricted to the PEST sequence, whereas the most frequent mutations in T-ALL are localized to the HD domain (Fig. 3.1), indicates that transformed B cells do not undergo selective pressure to acquire mutations that render these cells independent of Notch ligands. Thus, transformed B cells are likely to receive their Notch ligand-mediated signals through naturally occurring ligands expressed on cells located in secondary lymphoid organs. In this scenario the prediction is that circulating transformed B cells would have little or no ongoing NOTCH signaling as they migrate out from their microenvironment(s) limiting their access to Notch ligands. The correlation between PEST mutations in *NOTCH* genes in B cell neoplasms with poor patient outcome, increased chemoresistance, and disease progression is intriguing. It suggests that the Notch mutations are likely to be acquired during disease progression. However, whether these mutations are causative, how increased Notch signaling may contribute to disease progression, and whether inhibition of Notch signaling would resensitize chemoresistant B cell neoplasms to standard of care therapies is currently poorly understood and is a matter of ongoing investigation.

The first evidence that aberrant Notch signaling has oncogenic functions in solid tumors was derived from animal studies demonstrating that integration of the mouse mammary tumor virus (MMTV) into the *Notch4* gene results in the formation of mammary tumors [34]. MMTV integration into the *Notch4* locus results in the expression of long terminal repeat (LTR)-driven transcripts encoding for truncated Notch4 mRNA species named int3 [109]. Expression of this truncated dominant active form of the *Notch4/int3* gene either under the control of MMTV LTR or the whey acidic protein (WAP) promoter in transgenic mice results in the development of mammary tumors in 100% of cases [33, 44]. Similarly, transgenic female mice carrying an MMTV-N1ICD construct encoding human *NOTCH1* cDNA also developed mammary carcinomas, but only following multiple pregnancies [47]. Increased expression of *NOTCH1* and *JAGGED1* correlates with poor overall survival in women with advanced breast cancer [84], as well as reduced disease-free survival [85]. Moreover, loss of the negative Notch regulator, Numb, is observed in approximately 50% of human mammary carcinomas [71]. A recent study identified a *NOTCH4/HES/HEY* gene signature to be predictive of poor therapeutic response and prognosis in estrogen receptor-positive (ER+) patients that often acquire de novo resistance to hormonal therapy. Mechanistically, JAGGED1-mediated NOTCH4 receptor activation increases breast cancer stem cell activity and thereby drives antiestrogen resistance in human breast tumors. Combining endocrine therapy with blockage of Notch signaling was effective in overcoming resistance in ER+ human breast cancer [98].

Additional evidence for a role of Notch signaling in breast cancer is derived from transcriptomic sequencing of breast cancer cell lines and tumors. This study identified translocations within both *NOTCH1* and *NOTCH2* genes. These genetic rearrangements lead to the generation of fusion transcripts encoding truncated versions of NOTCH1 or NOTCH2 that lack most or the entire extracellular domain of corresponding receptors including the LNR domain. These translocations have been identified in approximately 10% of investigated triple-negative breast cancer samples and have been shown to correlate with high Notch signaling activity in in vitro studies [87]. This is reminiscent to observations made on rare cases of human T-ALL that express truncated dominant forms of NOTCH1 as a consequence of t(7;9) translocations. It is worth mentioning that cancers expressing these truncated dominant active forms of Notch proteins cannot be treated therapeutically with blocking Notch antibodies and are only responsive to γ-secretase inhibitors if their S3 cleavage site of their Notch receptors remains intact. In contrast, inhibitors that block Notch signaling at the level of the transcription activation complex should be effective in such cancers.

In addition to breast cancer, increased Notch signaling is also observed in approximately one third of non-small cell lung carcinomas (NSCLCs) due to the loss of Numb expression (in 30% of cases) and due to activating mutations in the *NOTCH1* gene (in 10% of cases investigated) [118]. The oncogenic role of Notch signaling in NSCLC was confirmed in a $Kras^{G12V}$-driven experimental mouse model [8]. Genetic loss-of-function (LoF) studies on RBP-J and presenilins showed that Notch signaling is essential for the formation of NSCLC. Moreover, pharmacological inhibition of Notch signaling in mice carrying autochthonous NSCLCs prevented cancer growth, characterized by increased expression of dual-specificity phosphatase1 (DUSP1), which is directly repressed by the Notch target gene *Hes1*. DUSPs are negative regulators of MAPK/ERK signaling. Accordingly, Notch inhibition results in the upregulation of DUSP1 expression and in decreased levels of phospho-Erk correlating with the inhibited growth of murine and human NSCLCs [57].

Although activating mutations in Notch genes in other solid tumors are either rare or have yet to be reported, oncogenic roles for Notch have also been linked to other solid tumors including colorectal cancer, melanoma, pancreatic cancer, cholangiocarcinoma, and medulloblastoma [49, 80, 126].

3.3 Oncogenic Notch-Driven Signaling Events: T-ALL as Paradigm

In the past decade, oncogenic Notch signaling has been associated with multiple cancers, which leads to the important question of how Notch conveys its oncogenic potential. Identification of target genes and signaling pathways that are regulated by, or cooperate, with aberrant Notch signaling is an important field of investigation. Although the Notch signaling cascade seems surprisingly simple, it is likely that

certain Notch-driven oncogenic events can be tumor-specific, while others may be shared between different neoplasms. In this regard, T-ALL is in one of the best-understood Notch-driven cancers. Therefore, we will summarize the genes and signaling pathways controlled by NOTCH1 that are implicated in T cell transformation. *c-Myc* has been identified by several labs as being one of the most important direct Notch target genes [67, 94, 117]. ChIP-on-chip analysis demonstrated that c-Myc and NICD share common target genes involved in the regulation of growth, metabolism, and proliferation. This led to the generation of a model depicting a feed-forward loop through which Notch and c-Myc reinforce the expression of genes required for the growth of leukemic cells [67]. Although early ChIP analysis suggested that Notch regulates c-Myc expression via its binding to the c-Myc promoter, subsequent ChIP sequencing studies showed that Notch appears to preferentially regulate gene expression through its dynamic interactions with superenhancers [112]. Notch controls c-Myc expression in both murine and human T-ALL cells by binding to a distal enhancer located more than 1 megabase 3′ of the murine and human *C-MYC* gene. The Notch-bound enhancer complex loops and physically interacts with the c-Myc promoter [40, 123]. Similarly, NOTCH1 was first shown to regulate the expression of the interleukin 7 receptor α-chain (IL-7Rα, a receptor tyrosine kinase) by directly binding to the human *IL7R* gene promoter [38]. Subsequent studies extended these findings further and revealed that Notch1 in coordination with Runx1 regulates a superenhancer located 3′ of the *IL7R* gene [112]. IL-7 signaling is essential for proliferation and survival of T cell progenitors, and its importance in the context of T-ALL is highlighted by the identification of oncogenic *IL7R* gain-of-function mutations in 10% of T-ALL cases [74, 96, 125, 128]. Insulin-like growth factor receptor-1 (IGF1R) is another receptor tyrosine kinase that has been shown to be directly controlled by NOTCH1 and to be important for T-ALL cell growth and for leukemia-initiating activity in vivo [60]. Activation of receptor tyrosine kinases often correlates with activated PI3K/Akt and increased mTOR signaling. This has also been observed in the context of Notch-driven T-ALL. Protein microarray screens identified the mTOR pathway as being positively regulated by Notch in T-ALL cells. Pharmacological inhibition of Notch in T-ALL cell lines induced hypophosphorylation of multiple signaling proteins involved in the mTOR pathway. How Notch activates the mTOR pathway is not completely understood. The effects of pharmacological Notch inhibition on mTOR signaling could be rescued by c-Myc expression suggesting that mTOR is possibly activated via the direct Notch target gene, *c-Myc* [13]. Alternatively, as suggested by studies in *Drosophila* and human T-ALL cell lines, the basic helix-loop-helix Notch target gene Hes1 has been shown to negatively regulate the expression of the tumor suppressor *PTEN*. PTEN counteracts PI3K activity and, thereby, negatively regulates Akt/mTOR signaling [68]. This particular study also linked mutational loss of *PTEN* in T-ALL cells to the acquisition of therapeutic resistance to NOTCH inhibitors [68]. Experimentally induced loss of PTEN expression accelerated disease onset in murine T-ALL models, suggesting that Notch1 activation and PTEN deficiency collaborate in disease onset or progression [59].

The importance of the transcriptional repressor Hes1 in T-ALL, which is one of the best-known Notch target genes, has been shown through conditional LoF experiments in murine T-ALL models as well as by gene knockdown studies in human T-ALL cell lines [93, 115]. Hes1 is required for the development and the maintenance of T-ALL in both mouse and human T-ALL cells. In addition, HES1 is known to be able repress *PTEN* expression and thereby contribute to increased Akt/mTOR signaling [68]. Furthermore, a more recent study revealed that HES1 is critically required for T-ALL tumor cell survival. Mechanistically, Hes1 appears to directly downregulate the expression of the *BCL2 binding component 3* (*BBC3)* gene in T-ALL cells, which encodes for the BH3-only pro-apoptotic factor, Puma [93]. Moreover, HES1 was also implicated in transcriptionally repressing the expression of the deubiquitinase CYLD, which negatively regulates the IKK complex, consequently leading to sustained NF-κB activation in T-ALL cells [30] [21]. CYLD functions as tumor suppressor in the skin, and LoF mutations in this gene have been identified in familial cylindromatosis [9]. The fact that (i) cylindromatosis patients do not have an increased risk of developing T-ALL and that (ii) CYLD mutations in T-ALL patients have currently not been described suggest that Notch signaling must be able to maintain strong NF-κB signaling at least in part through alternative mechanisms. Strong NF-κB signaling in Notch-driven T-ALL is characterized by nuclear localization of NF-κB, which is in part mediated by nuclear retention [95] resulting in the expression of NF-κB target genes including *Bcl-2A1*, *NF-κB2*, and *ICAM1*. Importantly, repression of NF-κB signaling in T-ALL cells inhibited cell growth both in vitro and in vivo [110].

NFAT signaling is another cascade known to be activated as a result of aberrant Notch signaling in T-ALL. High levels of Notch signaling induce the expression of calcineurin, which is a calcium-activated serine/threonine phosphatase. This phosphatase is important for the activation of NFAT transcription factors and for their translocation from the cytoplasm to the nucleus. Inhibitors of calcineurin such as cyclosporin A or FK506, which are used clinically as immunosuppressants, have been shown to induce cell death in T-ALL, leading to tumor regression and prolonged survival in murine T-ALL models.

Finally, aberrant Notch signaling has also been shown to directly regulate the expression of cell cycle proteins. Expression of G1 proteins such as cyclin D3, CDK4, and CDK6 is Notch-dependent in vitro and in vivo, and *CCND3* has been identified as a direct Notch target gene [45]. Cyclin D3-deficient mice exhibit greatly reduced susceptibility to Notch-induced leukemogenesis, and knockdown of cyclin D3 in human T-ALL cells inhibited their proliferation. The results indicate that cyclin D3 may be an essential cell cycle protein through which Notch can exert its oncogenic effects [97]. In agreement with the findings that Notch directly regulates the expression of cell cycle proteins, GSI-mediated inhibition of Notch signaling has been shown to lead to the upregulated expression of cyclin-dependent kinase inhibitors CDKN2B *($p19^{INK4d}$)* and *CDKN1B ($p27^{Kip1}$)* in T-ALL cell lines [82]. Consistently, increased Notch signaling induces the transcriptional expression of S phase kinase-associated protein 2 (SKP2), an F-box protein that functions as a component of the E3-ligase complex. The E3-ligase complex functions to degrade

$p27^{Kip1}$ and $p21^{Cip1}$, thereby promoting the G1-S transition [91] [26]. Notch was also shown to suppress p53 through the repression of the ARF-mdm2-p53 surveillance network in mice. Attenuation of Notch signaling led to increased p53 expression and to tumor regression by inducing apoptosis. Inhibition of the mdm2-p53 interaction by the small molecule nutlin resulted in the stabilization of p53, leading to induction of cell death even in the presence of sustained Notch signaling. These findings provide a proof of principle for p53 being a potential therapeutic target in the context of Notch-driven T-ALL.

Although many of the herein described potential mediators of oncogenic Notch signaling may be specific to T-ALL, it is interesting to note that a Notch-c-Myc axis has also been described in the context of Notch-driven breast cancers [47, 87]. Whether this is a general hallmark of oncogenic Notch signaling awaits further investigation.

3.4 Changing Metabolism Is Part of the Oncogenic Notch Program

In recent years, it has become clear that intrinsic alterations in metabolism occur as a direct consequence of aberrant oncogenic signaling in cancer cells, as opposed to a passive response of damaged mitochondria [114]. Metabolic reprogramming is now an established hallmark of cancer [39]. Cancer cells tend to rely on glycolysis for energy production and anabolic growth, even if sufficient oxygen is available for oxidative phosphorylation, a phenomenon known as the Warburg effect. One of the main reasons for cancer cells to do so is that cell growth is dependent on the biosynthesis of cellular building blocks derived from metabolic intermediates that are largely generated via the glycolytic and tricarboxylic acid pathway [114].

One of the first studies linking Notch signaling to anabolic growth is derived from work on T cell progenitors which demonstrated that Notch regulates cell size, glucose uptake, and glycolysis through PI3K/Akt activation [18]. Subsequently, gene expression analysis of T-ALL cells revealed that NOTCH1 promotes leukemic cell growth through direct transcriptional regulation of anabolic genes involved in protein translation, ribosome biosynthesis, and nucleotide and amino acid metabolism. Similarly and equally important is the regulation of anabolic gene expression by the Notch target *C-MYC*. As a result, a Notch-Myc feed-forward loop has been proposed in which Notch functions as the driver of cell growth and anabolism in T-ALL [67, 117]. A recent study by Ferrando and colleagues using murine models and xenografted primary human T-ALL shows that leukemic cells harboring *NOTCH1* mutations utilize glutamine as their predominant carbon source, which is used to generate all tricarboxylic acid cycle intermediates. Pharmacological or genetic Notch inhibition results in a dramatic reduction in glutamine usage and triggers autophagy as a compensatory mechanism to support leukemic cell metabolism. Inhibition of glutaminolysis and autophagy synergistically enhanced the efficiency

of therapeutic Notch inhibition in mice harboring T-ALL. Moreover, loss of PTEN resulted in increased glycolysis in targeted cells, rescuing the metabolism of leukemic cells that were treated with Notch inhibitors [41]. This study offers a possible explanation as to why the therapeutic response of T-ALL patients to pharmacological Notch inhibitors has been rather limited so far. Furthermore, it suggests that combining Notch inhibitors with drugs that target glutaminolysis and/or autophagy could be a more effective means for treatment of T-ALL. Since the Notch-MYC-AKT/mTOR axis is also conserved in other cancers, it is possible that metabolic reprogramming by Notch signaling may be a general mechanism by which Notch exerts its oncogenic properties.

3.5 Notch Functions as Tumor Suppressor

As outlined above, a highly context-dependent role of Notch signaling has been observed in a variety of blood-borne and solid tumors. Highlighted thus far were the oncogenic properties of Notch signaling and the possible molecular modes of action involved. In the subsequent sections, we will present and discuss tumor-suppressive functions of Notch signaling in various solid tumors (skin, head and neck, lung, bladder, and brain). The general aim of the following paragraphs is to highlight the most recent findings of Notch exerting its tumor-suppressive function in a variety of pathologies. We seek to highlight general paradigms and aim to put these into more a universal context of Notch acting as a tumor suppressor.

3.5.1 Notch Tumor Suppressor in the Skin: A General Paradigm

In vitro studies performed in the Dotto laboratory revealed a crucial physiological function of Notch1 in cutaneous epithelial cells [66, 81]. Here, Notch1 signaling induces growth arrest in keratinocytes as well as promotes early stages of differentiation. Therefore, Notch1 negatively regulates the proliferation of cutaneous epithelial progenitor cells and is essential for their terminal differentiation. The first conclusive evidence, however, showing that Notch acts as a tumor suppressor came from studies in the mouse skin, in which the loss of both *Notch1* alleles was shown to lead to the development of spontaneous basal cell carcinomas (BCC) in mice [63]. Additional LoF experiments unequivocally demonstrated a tumor-suppressive function of NOTCH signaling in the skin of both mice and humans [53, 75]. Although initial studies indicated that Notch1 functions as a tumor suppressor in a cell-autonomous manner, Kopan's group has highlighted a non-cell-autonomous mechanism in mice [23], supporting the notion that the carcinogenic effect is not solely dependent on cell intrinsic mechanisms driven downstream of Notch1

deficiency. In accordance with these findings, a more recent paper from Hu et al. showed that mesenchymal loss of RBP-J in the dermis was sufficient to induce inflammation and actinic keratosis, followed by the development of squamous cell carcinoma (SCC) in the overlying epidermis in aged mice [43]. Thus, defective Notch signaling in dermal fibroblasts was sufficient to induce a pro-tumorigenic microenvironment, thereby initiating carcinogenesis in the adjacent epidermis, with possible contributions by inflammatory cells recruited to tumor sites. It was further proposed that this could be due to the role of Notch in inhibiting AP1-mediated transcription of fibroblast growth factors, cytokines, proteases, and extracellular matrix proteins in dermal fibroblasts. In summary, the nonautonomous role of Notch signaling during cutaneous carcinogenesis may be coupled to its function in regulating dermal inflammation and/or barrier function of the epidermal epithelium [23, 43]. This has been shown to be a common paradigm in several epithelial malignancies creating a protumorigenic microenvironment (reviewed in Balkwill et al. [6], Balkwill and Mantovani [7], Mantovani et al. [55], and Solinas et al. [100]).

3.6 Notch Functions as a Tumor Suppressor in SCC

NOTCH1 also functions as a tumor suppressor in human non-melanoma skin cancers. Multiple components of the Notch signaling pathway, including NOTCH1, NOTCH2, and JAGGED1, are expressed at lower levels in human BCC samples compared to normal skin samples [107]. Additional evidence strengthening the role of NOTCH in human skin cancers came from the results of clinical trials employing semagacestat, a γ-secretase inhibitor, as a treatment for Alzheimer's disease. The trial revealed that treatment with this γ-secretase inhibitor was associated with an increased risk of developing skin cancer [31] (Fig. 3.2).

The tumor-suppressive role of Notch signaling is demonstrated most clearly in SCC. SCC, which causes >900,000 deaths worldwide per year, is an epidermal malignancy that occurs in tissues normally covered with stratified epithelium. SCC can arise in many different organs such as the head and neck, skin, esophagus, and lung [122]. SCCs, other than those in the skin, commonly metastasize, and survival rates have not improved in decades [20]. A better understanding of the underlying biology of SCC is only beginning to be elucidated but should be enhanced via the characterization of the molecular alterations inherent in these cells [1]. The transcriptional downregulation of Notch [53], with concomitant p53 mutations or LoF [119], is a causative event in human epithelial malignancies. In addition to the transcriptional downregulation, Notch receptor genes were also found to acquire recurrent LoF mutations. Wang et al. were the first to identify frequent somatic Notch LoF mutations in human cutaneous and lung SCCs. However, these aberrations occurred at a greater frequency in cutaneous SCCs than in lung SCCs. The detected mutations were found to cluster mainly in the extracellular EGF-like repeat region of *NOTCH1* and *NOTCH2* [113]. Utilizing cell-based assays, it was confirmed that these mutations represent LoF mutations. They cause receptor-

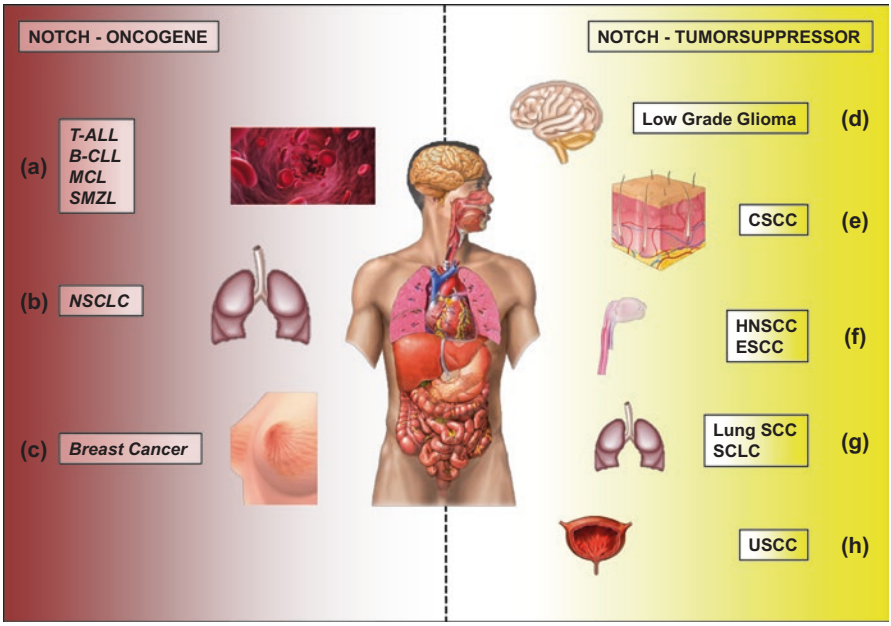

Fig. 3.2 Schematic representation of oncogenic or tumor-suppressive roles of NOTCH signaling associated with human cancers. The left side of the panel represents the major human tissues in which an *oncogenic* role for NOTCH has been described. (**a**) Weng et al. were the first to show that more than 50% of human pediatric T cell acute lymphoblastic leukemia (T-ALL) patient samples carry activating mutations in the *NOTCH1* gene [116]. Several studies [24, 32, 76, 77, 103] revealed also activating mutations in the *NOTCH1* gene in B cell chronic lymphocytic leukemia (B-CLL). *NOTCH1* mutations clustering in the PEST domain were associated with overall poor survival in mantle cell lymphoma (MCL) [51]. In splenic marginal zone lymphomas (SMZLs), next-generation sequencing identified mutations in *NOTCH2* to be more frequent than *NOTCH1*, and in both receptors, mutations clustered also mainly to the PEST domain [46, 69, 88]. (**b**) An additional oncogenic function of Notch signaling was identified in non-small cell lung carcinomas (NSCLCs) [57, 118]. (**c**) However, the first evidence that Notch signaling has an oncogenic function in solid tumors was found in breast cancer. Increased expression of NOTCH1 and JAGGED1 correlates with poor overall survival in women with advanced breast cancer, as well as reduced disease-free survival [84, 85]. On the other hand, lately several potentially *tumor-suppressive* functions of Notch signaling have been discovered in human malignancies. These are schematically highlighted on the right-side panel. (**d**) *NOTCH1* mutations have been identified in low-grade human gliomas [10], and Giachino et al. underscored the tumor-suppressive role of Notch in a mouse model [37]. (**e–g**) Notch signaling has a tumor suppressor in the skin, and its tumor-suppressive role is demonstrated most clearly in squamous cell carcinoma (SCC), and highlighted here are cutaneous (CSCC) [52, 54, 73, 102, 113], head and neck (HNSCC) [2, 106], esophageal (ESCC) [3, 35, 101], as well as lung SCC [113]. Most of these studies find potentially inactivating mutations in the *NOTCH1* and *NOTCH2* receptors, which are located predominantly in the EGF repeats (see text for details). (**g**) George et al. found, analyzing 110 human small cell lung carcinomas (SCLCs), that all four NOTCH receptors were affected with potentially inactivating mutations also clustering preferentially in the extracellular domains [36]. (**h**) NOTCH acting as a tumor suppressor in urothelial SCC (USCC), as has been shown by the groups of Real and Serrano [56] as well as by the Klinakis team [79], both using exome sequencing approaches followed by functional assays

ligand interactions to be distorted or result in truncated receptors. Truncations inducing stop codons, such as Q610 in NOTCH1 or W330 in NOTCH2, result in the loss of EGF repeats 11–13 which are essential for ligand binding [121] and will most likely prevent signaling. Other mutations resulting in secreted or membrane-tethered peptides, however, that enable Notch receptors to retain ligand-binding capacity, would create a dominant negative activity [83].

An elegant study published by the Blanpain group assessed the mutational landscape of SCCs in the frequently used DMBA-induced TPA skin SCC mouse model and its relevance to human SCCs [62]. In this model it was shown that LoF of Notch could efficiently substitute for TPA. These findings therefore established that Notch deficiency acts as a tumor-promoting event [23]. The comprehensive analysis using whole genome sequencing revealed that the genetic abnormalities in premalignant and fully malignant tumors, as well as their metastases highly resembled aberrations found in human SCCs. With respect to the *Notch1* gene, which is mutated with a frequency of 30% in mouse skin SCC – of which most mutations cluster to regions encoding the EGF repeats – the human *NOTCH1* counterpart was also found to be recurrently mutated in SCCs isolated from different human tissues. Fifty to Seventy five percent of human cutaneous SCCs were found to carry *NOTCH1* mutations, 14–19% in head and neck SCCs (HNSCCs); 8–16% in oral, esophageal, and lung SCCs; and at a low frequency (2%) in nasopharyngeal SCC. *Notch2* was found to be mutated at a low frequency of 7% in murine cutaneous SCC, whereas 31–63% of human skin SCC carried *NOTCH2* mutations. However, *NOTCH2* was mutated at much lower frequencies in HNSCC (5–9%), 6% in oral SCC, 2–4% in esophageal, and only 1% in nasopharyngeal and was absent in lung SCC. The frequently recurrent mutations detected in this study strongly correlated with previously identified NOTCH aberrations. Taken together, these findings not only validate the tumor-suppressive function of Notch in the murine skin [63] but also highlight the robust genomics approaches that will be essential to employ in order to define the mechanisms by which identified recurrently mutated genes in human cancers promote tumor initiation and/or progression.

Substantiating the study by the Cho laboratory [113], several groups recently confirmed that mutations affecting NOTCH signaling are frequently found in cutaneous SCC [52, 54, 73, 102]. A report published by South and colleagues involving whole-exome sequencing of 20 sporadic cutaneous SCC revealed an overall mean somatic mutation rate of 50 per megabase pair. The majority were C>T transitions, consistent with genetic changes found in UVR-induced DNA damage. Their findings confirm that mutations in *NOTCH1* and *NOTCH2* (frequency 82% combined) and *TP53* (63%) dominate this genetic landscape, with smaller contributions from *CDKN2A* (28%) and *RAS* family mutations (11%). Finally, the study showed that NOTCH1 expression levels were reduced in samples with Notch mutations, which is consistent with a LoF phenotype. This reduced expression pattern was even apparent in adjacent normal-appearing skin [102]. The authors concluded that NOTCH1 acts as a gatekeeper in human cutaneous SCC. The sample set was derived from a heterogeneous patient population including immunosuppressed individuals and included poorly differentiated tumors. Thus, NOTCH signaling was confirmed

to be a major tumor-suppressive mechanism in this cancer, and its disruption is likely to be an early event occurring during the development of cutaneous SCC.

In addition, Pickering et al. [73] characterized somatic mutations in aggressive metastasizing cutaneous SCC and performed whole-exome sequencing on DNA from 39 patients. Six of the top mutated genes, detected in aggressive cutaneous SCC (*TP53*, *CDKN2A*, *NOTCH1*, *NOTCH2*, *HRAS*, and *FAT1)* in this study, were also reported by South and colleagues [102] in a cohort of 20 cutaneous SCCs (see above). Although two groups had previously reported high frequencies of both *NOTCH1* and *NOTCH2* mutations in cutaneous SCC [28, 102], Pickering et al. were able to demonstrate for the first time that both genes are significantly mutated in metastasizing cutaneous SCC [73]. Since conditional *Notch1* deficiency in the mouse skin predisposes animals to skin tumors [63], it is likely that Notch1 may play a similar role in cutaneous SCC. In contrast to Notch1, skin-specific deletion of *Notch2* in mice does not predispose to tumor development or any other apparent phenotype [27], suggesting that Notch2-mediated signaling is not predominant in murine skin. Loss of *Notch2* is compensated by other redundant Notch receptors in murine skin. However, the combined inactivation of *Notch1* and *Notch2* is known to lead to more severe defects in the differentiation of skin than loss of *Notch1* alone [23, 25, 27]. Thus, *NOTCH1* and *NOTCH2* signaling may both function as a barrier against carcinogenesis in some systems (human), whereas *Notch1* may be the primary barrier for other systems (mouse).

Recently, the Khavari laboratory added a study to the growing body of work on genome-level sequencing of cutaneous SCCs [52]. The authors confirmed findings that the *TP53* and *NOTCH1* genes are each mutated in approximately half of cutaneous SCCs and that *CDKN2A*, *NOTCH2*, and *HRAS* are also commonly mutated genes albeit at slightly lower frequencies. In addition, it is interesting to note that recurrent LoF mutations in NOTCH1/2 leading to a loss of tumor suppressor function occurred in the early stages of SCC carcinogenesis, in particular during actinic keratosis (precancerous stage of cutaneous SCC) and subsequently in invasive cutaneous SCC. Overall, these data sets attest to the tumor-suppressive functions of Notch signaling in normal human skin and that perturbation of the major signaling pathways in this tissue can lead to rapid expansion of preexisting tumor-initiating clonal populations, highlighting *NOTCH1* as a gatekeeper in squamous carcinogenesis of the skin.

NOTCH has also been recently attributed a tumor suppressor function in HNSCC. Agrawal et al., and Stransky et al. published two landmark studies on human HNSCC revealing inactivating mutations in *NOTCH1* and provided novel insights into the genetic basis underlying HNSCC [2, 106]. Both groups performed next-generation sequencing of the exons of all known human genes on tumor-derived DNA isolated from two distinct patient cohorts. The two groups analyzed a total of 32 [2] and 74 [106] tumor samples including tumors being either positive or negative for the human papillomavirus. Both groups independently identified *NOTCH1* mutations among other genetic aberrations previously identified as key players (*TP53, CDK2A, PIK3CA, PTEN, HRAS*) in HNSCC. In accordance with the findings from Nassar et al. [62], both studies reported inactivating mutations in

NOTCH1 in 10–15% of head and neck tumors, and it was the second most frequently mutated gene after *TP53* (mutated in 50–80% of tumors). In several tumors, both alleles harbored inactivating *NOTCH1* mutations. This finding in correlation with the observation that mice with a disrupted *Notch1* gene in the skin show a malignant phenotype [63, 75] provides strong evidence that *NOTCH1* has an important tumor suppressor function in HNSCC. Stransky et al. [106] also found mutations in other cell differentiation-related genes, such as *NOTCH2, NOTCH3*, and *TP63*, suggesting that deregulation of the terminal differentiation program of mucosal keratinocytes is critical for SCC development. *Oral* SCC is the most common subtype of HNSCC, and thus it is not surprising that mutations in *TP53, FAT1, HRAS, CASP8,* and *NOTCH1* are shared with other HNSCCs [19]. In oral SCC inactivating mutations of *NOTCH1* are also found in about 10–15% of the tumor samples analyzed (Caucasian patients) thus reflecting an equal mutational frequency found in all HNSCCs.

Whole-exome sequencing studies performed on patient cohorts from different ethnic backgrounds (Chinese and Caucasian) using *esophageal* SCC samples found *NOTCH1*, *NOTCH2*, or *NOTCH3* mutations to occur at a similar frequency in Chinese individuals (16–22%) [35, 101] as reported in US cases (28%) [3]. Thus, different ethnic populations reveal similar mutational frequencies in *NOTCH* receptor family genes in esophageal SCC.

In summary, all these observations would indicate that loss of Notch signaling activity is possibly a crucial event for the growth of tumor cells with epithelial squamous differentiation characteristics. Although high-throughput sequencing approaches can reveal many mutations in a large number of genes, this does not necessarily imply that all identified genes carry "driver mutations" causally related to the malignant transformation process. It is noteworthy though to mention here a study published by Martincorena et al. [58] that has led to a paradigm shift in the understanding of cutaneous SCC. Ultra-deep genome sequencing of normal eyelid skin was used to identify clones of cells carrying genes with attributed tumor suppressor activity. Martincorena et al. assessed 74 cancer genes in 234 biopsies isolated from the normal eyelid skin of 4 individuals. They confirmed a remarkably high frequency of somatic mutations in key genes, including *TP53*, *NOTCH1–3*, *FGFR3*, *FAT1*, and *RBM10*, and demonstrated tolerance to cancer-causing mutations in normal skin. Mutations in *NOTCH1* were especially frequent and found in up to 25% of normal keratinocytes and often occurred in conjunction with loss of heterozygosity, resulting in biallelic NOTCH1 inactivation. In 1 cm^2 of normal skin, the authors identified six clones each containing up to six driver mutations within a given cell. These findings could raise many questions about the mechanisms/gatekeepers that block progression to actinic keratosis and to invasive cutaneous SCC. It is widely assumed that driver mutations occur infrequently in long-lived lineages of rare subclones of cells [64] and that most arise in cancerous tissue that is too small to be clinically detectable. Therefore, the report by Martincorena et al. [58] overrode these assumptions and revealed that sun-exposed normal skin is already composed of a polyclonal mixture of driver mutations including NOTCH. Tumors have to be viewed as genetically unstable and acquiring many mutations including "pas-

senger mutations" [99, 120] that occur during the progression, rather than in the initiation of the disease. Therefore scrutinizing functional studies in animal models are required to elucidate the exact role of the NOTCH receptors and other genes mutated in SCC. In conclusion, all of the abovementioned studies confirm the assumption by Nassar et al. [62] that extensive genomics studies in mice can provide a valuable resource to define genetic heterogeneity found in human cancers. However, it will be inevitable to define mechanisms by which certain genes such as *NOTCH1* and *NOTCH2* promote tumor initiation and progression. These mechanisms may be investigated in light of the Notch signaling cascade being highly context- and tissue-specific.

3.6.1 Dual Function of Notch in Lung Cancer

There are two major types of lung cancers: non-small cell lung cancers (*NSCLCs*) and small cell lung cancer (*SCLC*). *NSCLCs* account for about 85% of lung cancers, are strongly correlated with tobacco smoke, and can be further subdivided into the following three subtypes: (i) lung adenocarcinoma accounts for approximately 50% of all NSCLCs and is the most common form of lung cancer in the United States and arises predominantly in distal airways, (ii) SCC makes up 40% of NSCLCs and develops within the lining of the bronchial tubes (proximal airways), and (iii) large cell carcinomas referred to NSCLCs that are neither adenocarcinomas nor SCC [1, 22]. *SCLC* is an extremely aggressive malignancy and accounts for the remaining 15% of lung cancers in the United States. SCLC (i) occurs due to smoking at a higher frequency, (ii) grows more rapidly, and (iii) metastasizes earlier than NSCLC. However, it is also more responsive to chemotherapy. The role of the Notch signaling cascade in lung cancer is pleiotropic in terms of *tumor-suppressive* or *oncogenic* properties. Lung adenocarcinoma is the most frequent occurring NSCLC subtype, and Notch signaling has been accredited with tumor-promoting effects in this malignancy although Notch-related alterations are rare (reviewed in Ntziachristos et al. [65]). The second major type of NSCLC is SCC, and the tumor-suppressive role of Notch in this malignancy has been outlined above. SCLC is a neuroendocrine subtype of lung cancer, and although it only accounts for a smaller fraction of all lung cancers (approximately 15%), it is the most malignant type of cancer. Although earlier studies have failed to identify recurrent mutations in genes of the Notch signaling cascade [72, 89], a report published by George and colleagues recently identified recurrent somatic mutations in NOTCH1, NOTCH2, NOTCH3, and NOTCH4 in tumor specimens of patients diagnosed with stage I–IV SCLC [36]. Although, previous studies already implicated a tumor-suppressive role for Notch activity in SCLC. There, it was shown that hyperactivation of Notch signaling blocks cell cycle progression of SCLC cell lines [104, 105]. It was however not until the comprehensive genomic sequencing of 110 human SCLC and additional murine samples conducted by George et al. that Notch mutations were unequivocally identified. Mutations affected Notch receptor family genes in both

human (NOTCH1–4) and mouse (NOTCH3 only) SCLC, with NOTCH1 mutations occurring at a frequency of 20% in human patient tissue [36]. The mutations however did not cluster significantly in any specific domain, but a higher frequency of missense and nonsense mutations occurred in the extracellular domain. Overall, NOTCH family genes were genetically altered in 25% of human SCLC with NOTCH1 itself being most frequently affected. A functional role of Notch signaling in lung cancer was elegantly shown in a mouse model [61, 92] of lung cancer. Notch signaling may inhibit the expansion and/or differentiation of neuroendocrine cells and thereby counteracts the expansion of SCLC tumors [104, 111]. Constitutive overexpression of either Notch1 or Notch2 in a lung cancer mouse model [61, 92] led to a significant reduction in lung tumors, overall increased survival rate and seemed to block malignant progression in early tumor initiation phase. In addition, ectopic expression of N1ICD in murine and human SCLC cell lines was shown to lead to growth arrest. These studies provide the first functional analysis to identify and validate the role of Notch as a tumor suppressor in SCLC. Its function could be postulated as a key regulator of neuroendocrine differentiation.

3.7 Novel Tumor-Suppressive Roles for Notch Signaling Activity in Urothelial Cancers and Glioblastoma

As discussed above Notch signaling has a dual function acting either as an oncogene or a tumor suppressor in a highly context- and tissue-specific manner. The role of Notch signaling in other solid tumors, other than epithelial-derived, is less clear, and we would like to highlight only two other examples in which an undiscovered tumor-suppressive role was attributed to NOTCH – in *bladder cancer* and in *glioblastoma*.

Notch acts as a tumor suppressor in bladder cancer. Until recently, the role of Notch signaling in urothelial cancer (UC) remained unclear. However, lately studies were published, although with different emphasis and some divergence on the molecular and mechanistic details, and together revealed that the Notch signaling cascade is frequently inactivated in bladder cancer and exerts a tumor-suppressive role in this tissue [5, 56, 79].

The groups of Real and Serrano [56, 79], as well as the Klinakis team [79], employed exome sequencing approaches to identify somatic LoF mutations in NOTCH pathway components; in particular, *NOTCH1 and NOTCH2* genes were found to be frequently mutated. Rampias et al. [79] showed that tumors harboring NOTCH-inactivating mutations, either exclusively or in combination with *FGFR3* or *RAS* mutations, exhibited increased phosphorylation of ERK1/2, suggesting that NOTCH negatively regulates ERK1/2 activation. Consistent with this idea, activation of NOTCH signaling inhibited the proliferation of human bladder transitional carcinoma cell lines and led to reduced ERK1/2 phosphorylation via transcriptional induction of several DUSP, responsible for the dephosphorylation of ERK1/2.

Rampias et al. demonstrated that the expression of several members of the DUSP family is induced by active Notch signaling in UC cell lines [79]. Inactivation of Notch signaling results in diminished DUSP activity, thus leading to high levels of phospho-MAPK. Genetic inactivation of Notch signaling in mice promoted the development of high-grade invasive UCs characterized by high ERK1/2 phosphorylation and expression of basal cell markers, similar to the aggressive basal subtype of bladder cancer observed in humans. In contrast, the overexpression of activated NOTCH1 in UC cells reversed the cancer phenotype. Similarly, urothelium-specific loss of Notch signaling also resulted in the formation of bladder tumors exhibiting basal characteristics. These findings implicate loss of NOTCH signaling as a driver event in UC in mice and men. Although Maraver et al. [56] also concluded that the loss of tumor-suppressive activity of Notch via genetic inactivation occurs predominantly in UC with squamous features, they highlighted another consequence of LoF Notch function in UC. In a genetic LoF approach (using either tissue-specific conditional RBP-JKO or PsenKO animals), loss of Notch signaling accelerated UC tumorigenesis and promoted the formation of SCC with mesenchymal features. Notch signaling promoted the expression of the transcription factor Hes1, which prevents epithelial-mesenchymal transition (EMT). Moreover, evaluation of human bladder cancers revealed that tumors with low levels of HES1 exhibited greater EMT and invasive potential. In this context, it is interesting to note that HES1, being a transcriptional target of NOTCH, is responsible for the derepression of the EMT program in UC cells [56]. Taken together, their results also indicate that NOTCH serves as a tumor suppressor in the bladder. Therefore, inactivation of this pathway is likely to promote EMT in squamous bladder cancer cells.

A final exemplary discussion will be on the novel discovery for a role of *Notch signaling acting as a tumor suppressor in forebrain tumor subtypes* [37]. Gliomas are the most common malignancy in adult brain and have a very poor prognosis. Although Notch signaling, which is integral for neuronal stem cell (NSC) maintenance, has been suggested to play an oncogenic role in some brain tumors [14, 17], the role however of this pathway in glioma remains unclear. Giachino and colleagues [37] investigated the role of Notch signaling using mouse models of glioma. The tumor model was driven by platelet-derived growth factor (PDGF) expression and loss of *Trp53* (PDGF^{+}/*Trp53*$^{-/-}$) combined with a Hes5Cre^{ERT} mouse crossed to a Cre-inducible reporter line as a readout of activated Notch signaling. In early-stage PDGF^{+}/*Trp53*$^{-/-}$ gliomas, Hes5 was expressed in only a subpopulation of glioma cells. Targeted expression of PDGF and deletion of *Trp53* in Hes5^{+} Notch signaling cells resulted in glioma initiation. Surprisingly, loss of RBP-Jκ accelerated the growth of PDGF^{+}/*Trp53*$^{-/-}$ gliomas, which were mostly composed of HES5^{-} proliferating cells. Late-stage PDGF^{+}/*Trp53*$^{-/-}$/*Rbp-jκ*$^{-/-}$ gliomas exhibited features of poorly differentiated supratentorial primitive neuroectodermal tumors (sPNETs) and harbored proneural/mesenchymal glioblastoma gene signatures. In addition, co-deletion of *Rbp-j* and *Trp53* in quiescent NSCs induced the development of premalignant NSCs and highly penetrant sPNET-like tumors. In patients with proneural glioblastoma, *HES5* expression was inversely correlated with sur-

vival, and the expression of *HES5*, *RBP-j*, and the Notch-induced transcription factors, *HEY1* and *HEY2,* was associated with improved prognosis in grade II and III astrocytomas. In summary, these results strongly suggest that Notch signaling has a tumor suppressor function in grade II–III astrocytomas, proneural glioblastomas, and sPNETs.

It is noteworthy that NOTCH1 mutations, most likely inactivating, have been identified in low-grade human gliomas by exome sequencing [10]. Thus, the data provided by Giachino et al. [37] highlight the tumor-suppressive role of Notch in a cell-autonomous fashion in a genetic animal model and point to the feasibility of using Notch targets as biomarkers for patient prognosis and as potential treatment options.

3.8 Conclusions

Research of the last decade has emphasized the dual function of Notch in cancer. It can function as oncogene as well as tumor suppressor. The oncogenic function of Notch signaling is best understood in T-ALL, in which it is the most frequently mutated oncogene and where the availability of suitable mouse models has enabled us to gain deep insight in the molecular mechanisms underlying this disease. Next-generation sequencing has uncovered gain-of-function mutations of Notch genes in other malignancies including CLL, SMZL, Mantle cell lymphoma, and non-small cell lung cancer. Notch mutations in these malignancies often correlate with poor prognosis and occur more frequently in cases of acquired resistance to chemotherapy. This correlation infers that increased Notch signaling is involved in tumor progression or in escape to therapy. In many cases, whether these gain-of-function mutations are indeed causative and how they mechanistically contribute to tumor progression and/or to therapeutic resistance remains unknown and requires further investigation. Conversely, loss-of-function mutations have frequently been observed in cutaneous SSC, followed by head and neck SSCs, lung cancer, bladder cancer, and others indicating that Notch signaling has a tumor-suppressive function in these tissues. In agreement with this, Alzheimer patients that were treated in a phase III trial for a prolonged period of time with γ-secretase inhibitor in order to inhibit cleavage of the amyloid precursor protein and thereby the generation of the pathogenic peptides exhibited an increased incidence of skin cancer. Thus the dual function of Notch in cancer complicates the attempts to treat patients with blocking antibodies or drugs that suppress Notch signaling. It will be important to carefully select the patients as well as the indications in which blocking Notch therapeutics will be used. However, with increasing knowledge about mechanisms and the tissues in which Notch functions as an oncogene or as a tumor suppressor, combined with appropriate biomarkers, it should be possible to safely select the appropriate patients that would benefit from therapeutic Notch inhibition.

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