Notch Pathway: A Journey from Notching Phenotypes to Cancer Immunotherapy

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Abstract

Notch is a key evolutionary conserved pathway, which has fascinated and engaged the work of investigators in an uncountable number of biological fields, from development of metazoans to immunotherapy for cancer. The study of Notch has greatly contributed to the understanding of cancer biology and a substantial effort has been spent in designing Notch-targeting therapies. Due to its broad involvement in cancer, targeting Notch would allow to virtually modulate any aspect of the disease. However, this means that Notch-based therapies must be highly specific to avoid off-target effects. This review will present the newest mechanistic and therapeutic advances in the Notch field and discuss the promises and challenges of this constantly evolving field.

Keywords

Notch · Drosophila · Cancer · Notch-targeting therapies · Endocytosis · Glycosylation · Metabolism · Immunotherapy · Synthetic biology

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The Basics of Notch Pathway

Notch discovery tracks back to more than 100 years ago when the geneticist John S. Dexter first observed "notches" in the veins of *Drosophila melanogaster* wings and Thomas Hunt Morgan identified the fly Notch mutant alleles (Dexter 1914; Morgan 1917). Spyros Artavanis-Tsakonas and Michael Young were the firsts to identify *Notch* gene, and link the notching fly wing phenotype to alterations in the *Notch* locus (Kidd et al. 1986; Wharton et al. 1985). As soon as a decade after its discovery, Notch made its entry in different fields from developmental to cancer biology. As we will discuss in this chapter, nowadays Notch is the protagonist of some of the most cutting-edge fields, including immunotherapy and synthetic biology.

Notch is a developmental signaling pathway evolutionary conserved across metazoans (Animalia kingdom) from *Drosophila* to humans. While only one Notch receptor and two ligands (Delta and Serrate) are present in flies, evolution provided humans with four Notch receptors (Notch1–4) and five canonical ligands (Delta-like ligand 1, 3, 4 [DLL1, DLL3, DLL4], Serrate-like ligand Jagged 1–2 [JAG1 and JAG2]) (Fleming 1998; Gordon et al. 2008; Aster et al. 2017). Notch receptors are single-pass transmembrane proteins and consist of different conserved functional domains (Fleming 1998; Gordon et al. 2008; Aster et al. 2017). The

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extracellular domain of Notch is composed by epidermal growth factor(EGFs)–like repeats, the number of which varies among species and different Notch receptors. Two functional domains are present in the extracellular region, the ligand-binding domain (EGF 11–12), which mediates the interaction with ligands, and the Abruptex domain (EGF 24–29), the function of which is not yet clear. The extracellular region is followed by the Negative Regulatory Region (NRR), which masks a cleavage site (S2) important for Notch activation, the heterodimerization domain (HD), and the transmembrane spanning region of the receptor. The intracellular domain of Notch (NICD) consists of the RBPJκ-associated molecule region, the Ankyrin domain and transactivation domain, which are involved in the transcriptional activation of Notch target genes. Finally, the C-terminal domain, known as proline, glutamic acid, serine, and threonine-rich (PEST) domain, ensures the stability of NICD. In mammals, Notch receptors are cleaved in S1 site in the HD domain in the Golgi and presented at the cell surface as noncovalently-linked heterodimers consisting of an extracellular and a transmembrane unit. Notch pathway activation starts with the binding of Notch receptor to its transmembrane ligands presented by neighboring cells, a process known as transactivation (Kopan and Ilagan 2009; Bray and Gomez-Lamarca 2018). This exposes the proteolytic cleavage site, S2, in the NRR, which is cleaved by ADAM metalloproteases. A subsequent cleavage, S3, mediated by γ-secretase occurs in the transmembrane region, releasing NICD. NICD translocates to the nucleus where together with the DNA-binding factor RBPJκ (also known as CSL in mammals or Suppressor of Hairless in *Drosophila*) and the coactivators Mastermind-like (MAMLs) triggers the transcription of target genes.

Notch is a master regulator of cell fate and tissue homeostasis and the variety of outcomes of Notch signaling in these processes is astonishing. Notch acts as an oncogene or tumor suppressor, thus either promoting proliferation or apoptosis, in different tissues or subset of cells or cellular contexts (Ntziachristos et al. 2014; Bray 2016). Notch activation has different and sometimes opposite outcomes in developmental processes depending on when and how Notch is activated (Bray 2016; Artavanis-Tsakonas et al. 1999). Given that Notch core pathway seems relatively simple, how can we explain its versatility? The interaction between different Notch receptors and ligands does change the outcome of Notch; however, this is not enough to sustain its multiple and versatile functions. It is becoming clear that Notch pathway relies on a complex regulation, which goes beyond ligand–receptor interactions from the maturation in the Golgi/ER, to the cell membrane, endosomes, and nucleus. One of the easiest explanations of Notch vary outcomes is that in cells with different chromatin states, Notch activates different sets of genes. However possible, it also seems that the kinetic, time, and interaction with enhancers of NICD might be responsible of different transcriptional outcomes (Falo-Sanjuan et al. 2019; Gomez-Lamarca et al. 2018). Further, crosstalks with other pathways within the nucleus and upstream, which have been reported also in disease contexts, might lead to a different outcome (Collu et al. 2014; Gutierrez and Look 2007). Finally, increasing evidence has shown that posttranslational modifications of Notch play important roles in the regulation of the pathway, ranging from glycosylation for the correct maturation of the protein and ligand–receptor interactions (Harvey et al. 2016; Kakuda and Haltiwanger 2017), ubiquitination-dependent regulation of Notch endocytic trafficking and degradation (Shimizu et al. 2014; Steinbuck et al. 2018), to phosphorylation regulating NICD turnover (Fryer et al. 2004; Carrieri et al. 2019). Notch versatile function and regulation have deep implication in physiological processes and diseases.

Functional Roles of Notch in Cancer and How to Target Them

The first proof of a link between Notch pathway and cancer was provided by the identification of Notch mutants in T-ALL and breast cancer (Ellisen et al. 1991; Gallahan and Callahan 1997).

Up to date, the list of cancer-associated Notch mutations has grown together with the number of functions of Notch in cancer. In the past years, studies of Notch in cancers have highlighted its role in tumor growth, cancer-stem like cells, and metastasis, and now Notch role has expanded to metabolism regulation, microenvironment, and tumor immunity. Because of its extensive involvement in cancer, Notch could be a promising target for anticancer therapies. However, its pleiotropic nature poses some challenges.

In recent years, the high-throughput sequencing of tumors has provided a lot of data about the mutational landscape of different tumors, aiming at identifying the most suitable targeting therapies for selected patients. A recent trial, named MOSCATO trial (NCT01566019), aimed to identify genetic alterations in a group of patients with advanced stage malignancies and treat these patients with targeted therapies against the altered pathways identified, including Notch. Although the beneficial outcome was observed in only 7% of the total patients screened and in 24% of patients treated with Notch-targeted therapy (Massard et al. 2017), the results were encouraging and showed that certain patients may benefit from the therapy selection based on genomic landscape. These studies might be greatly improved by a mechanistic investigation of Notch in tumors. For this purpose, understanding Notch status (whether Notch is activated or not) and role in individual tumors/patients will be key to identify tumor/patient responders to selected Notch-targeting therapies.

As we mentioned, Notch can either be an oncogene or a tumor suppressor gene depending on the cell type and tissue in which is expressed. This adds more complexity to Notch targeting in cancer because Notch will have to be either inhibited or activated depending on Notch role in the tissue in which the tumor originated and the cell type we wish to target. Usually Notch is predicted to be tumor suppressive or oncogenic, depending on the role of Notch in the tissue in which the tumor originated; however, this approach might be imprecise. Notch loss-of-function mutations and the suppressive effect of Notch ectopic expression in small cell lung cancers (SCLCs) suggest that Notch acts as a tumor suppressor in these tumors (George et al. 2015). However, an elegant work by Julien Sage's research group showed that Notch is activated and is oncogenic in a subset of tumor cells (10–50%) in SCLC mouse models and human tumors. These cells showed a non-neuroendocrine (non-NE) phenotype, were slow growing, more chemoresistant, and supported the growth of the neuroendocrine (NE) tumor cells (Lim et al. 2017). The activation of Notch in non-NE cells might result from the expression of Notch ligands on NE cells and from the tumor microenvironment (Lim et al. 2017), suggesting that the tumor itself and its microenvironment trigger the generation of non-NE oncogenic-Notch cells. These findings provide a strong rationale for the use of Notch inhibition in combination with other therapies in selected SCLCs or certain stages of the disease where non-NE are found. A similar study showed that subpopulations of cancer stem-like cells with different Notch activation status and different metabolic profiles coexist in glioblastoma (Bayin et al. 2017). These works emphasize the importance of identifying Notch status in tumors to choose the most suitable treatment. For this purpose, the use of biomarkers for Notch signaling activation might be of great help. Not only Notch target genes, but also protein/factors produced by Notch-expressing tumor cells could be used as Notch biomarkers upon identification.

Notch mutations found in cancer are assumed to be loss or gain of function depending on whether Notch is tumor suppressor or oncogenic, respectively, in the tissue in which the tumor originated. However, the outcome of Notch mutations should be validated in order to choose whether the therapeutic intervention should inhibit or favor Notch activation. For example, Notch-targeting therapies could be applied to head and neck squamous cell carcinomas (HNSCCs), since a remarkable number of mutations in Notch genes have been found in HNSCCs in both the Caucasian (10–15%) and Asian populations (50%) (Izumchenko et al. 2014; Song et al. 2014; Agrawal et al. 2011; Stransky et al. 2011). However, it is not clear whether these mutations are gain or loss of function, thus whether Notch should be inhibited

or promoted. In the Caucasian population, the majority of Notch mutations were predicted to be loss of function, whereas in the Asian population mainly gain of function. This classification was ruled out depending on the position of the mutations in the Notch receptor: The Caucasian mutations were mainly clustering around the ligand-binding domain of Notch or causing the truncation of the Ankyrin domain, which is critical for transcription of target genes (Agrawal et al. 2011; Stransky et al. 2011); the Asian mutations were mainly in the Abruptex domain and in the NRR (Izumchenko et al. 2014; Song et al. 2014). There are contradictory evidences on whether Notch is tumor suppressor or oncogenic in HNCCs, and thus a careful functional validation will be needed to determine which of these mutants upregulate and which ones downregulate Notch. Also, these studies point out that the role of Notch might vary not only in different tumor types, but also in tumors of the same class and in different ethnicities. Therefore, a patient-based mechanistic-based use of Notch-targeting therapies is much need.

It is clear that, due to the pleiotropic nature of Notch pathway, the role and status of Notch signaling will have to be evaluated on an individual tumor and patient basis. In particular, this could be achieved with functional/mechanistic studies and identification of biomarkers. This approach will require a considerable amount of effort, but it should pay off in improving the use of Notch-targeting therapies. In the next section, we will discuss current and future Notch-targeting therapies, their mechanistic implications and rationale.

Gamma-Secretase Inhibitors: Learning from Failure

In the past years, γ-secretase inhibitors (GSIs) represented a major class of Notch-targeting agents. GSIs prevent Notch activation by hampering the γ-secretase-mediated cleavage of Notch and the release of NICD. Given the important role of Notch in cancer, GSIs held a lot of expectations in their potential to target cancer cells and especially cancer stem-like cells. Despite the fact that GSIs are the first Notch-targeting agents that saw transition to the clinic, their early-stage clinical development as single agents was challenged by low antitumor effects and severe side effects, due to Notch inhibition in healthy tissues. These included gastrointestinal toxicity, skin rushes, and immunosuppression. Several clinical trials using GSI were terminated or withdrawn before completion. Few trials reported a moderate success. For example, GSI PF-03084014 showed high tolerability, long-term control of the disease, and partial response in 71.4% of patients with desmoid tumors in a phase II clinical trial (Kummar et al. 2017; Villalobos et al. 2018). LY3039478 recently entered clinical trials after proving significant single-agent activity and manageable toxicity in preclinical models (Bender et al. 2013). This agent showed limited therapeutic success in a phase I trial, but with manageable toxicity (Massard et al. 2018). This study, as others with GSIs, was carried out in a heterogeneous cohort of patients with different types of tumors. It is likely that GSIs with improved toxicity, like LY3039478, might be more effective in a selected and validated group of patients. The identification of GSI-responder patients should be one of the major focuses to improve GSI clinical development. An interesting study from O'Rourke and colleagues has identified and validated a transcriptional signature which can predict GSI responders among patients affected by cholangiocarcinomas (CCAs) (O'Rourke et al. 2020). In this study, they first identified an increase in *Notch1*, *Notch3*, and Notch ligands in CCA patients using genomic data, thus rationalizing the potential therapeutic use of GSIs for CCA. Second, they identified a transcriptome signature by treating different CCA cell lines with GSIs, transplanting them in mice and evaluate their sensitivity or resistance to GSI treatment. This signature was then validated in a CCA mouse model and in an independent cohort of CCA patients, in which it identified 48.7% as predicted GSI-responder patients. A similar method could be applied to other cancer types to predict the subgroup of GSI-responder patients and could be very advantageous for the design of clinical trials involving GSIs. The identification

of gene signatures that confirm Notch pathway inhibition would also help establishing and monitoring the therapeutic window of GSIs. A study examined the transcriptome in hair follicle and blood of healthy human and nonhuman primates subministered with GSIs and identified a signature, which correlates with GSI kinetics (Tanis et al. 2016).

It is now clear that GSIs are pharmacologically distinct. GSIs like AL101 (formerly known as BMS-906024) show equal potency in inhibiting all four Notch receptors (Gavai et al. 2015). However, other compounds are more selective toward one or more Notch receptors; PF-3084014 have a stronger effect on Notch2 and, interestingly, a higher potency for Notch3 inhibition at low concentrations (Ran et al. 2017); LY3039478 is a highly potent inhibitor of Notch1 γ-secretase-mediated cleavage (Bender et al. 2013). Given that the roles of the four Notch receptors vary considerably in different cancers, choosing the right inhibitor and dosage to target Notch might improve the therapeutic effect of GSIs. It is now been evaluated the use of GSIs which are selective toward γ-secretase subunits. MRK-560 mainly targets presenilin-1 subunit (PSEN1) in the γ-secretase complex (Borgegård et al. 2012). This compound showed antitumor activity in T-ALL cell lines and patient-derived xenografts and did not cause any major effect in normal T-cells or in the gastrointestinal tract (Habets et al. 2019). This is likely because PSEN1 is highly expressed in T-ALL cells compared to normal T-cells and the lack of PSEN1 might be compensated by the other γ-secretase subunits, thus maintaining tissue homeostasis in the gastrointestinal tract (Habets et al. 2019). The use of more selective GSIs toward Notch or γ-secretase subunits in rationally selected tumors might greatly impact on lowering the toxicity and boosting GSIs anti-tumor activity.

Because γ-secretase have many substrates, GSIs have multiple targets apart from Notch (Haapasalo and Kovacs 2011). On one side, these "off-targets" might contribute to the toxicity associated with these agents, but on the other side, these might also contribute to GSIs antitumor activity. For example, among γ-secretase substrates we find CD44, a cancer stem cell marker, and E-cadherin, which are both associated with tumor progression and invasion (McAuliffe et al. 2012; Marambaud et al. 2002). E-cadherin processing by γ-secretase also increases the amount of free cytosolic β-catenin which is an important mediator of WNT signaling (Marambaud et al. 2002). It is possible that the antitumor effects of PF-03084014 observed in desmoid tumors, which are WNT pathway dependent, might result not only from the inhibition of Notch, but also from the "off-target" effect on WNT-pathway. On the same line, Morgan and colleagues showed that AL101 can enhance the effect of chemotherapy in preclinical models of non-small cell lung cancers (NSCLC), which did not harbor mutations in Notch nor in its negative regulators (Morgan et al. 2017), suggesting GSI antitumor effect does not only depend on Notch inhibition in these tumors. Therefore, upon identification and validation, the broad spectrum of GSIs might be useful to reach multiple targets, including Notch, that drive selected tumors.

As we discussed, GSIs showed a limited activity as single agents in early clinical trials; however, more clinical trials are ongoing with GSIs in combination with chemotherapy or targeted agents. A number of preclinical studies have showed GSIs to enhance the antitumor effect of other anticancer therapies (Morgan et al. 2017; Pikman et al. 2017; Zhao et al. 2016a; Qiu et al. 2013; Schott et al. 2013). Therefore, it is possible that some of these combinatorial treatments might show positive results and allow the use of lower doses of GSIs, thus reducing their side effects.

In summary, even though the clinical development of GSIs has been challenging, the accumulated knowledge about these compounds provides chances for improvements. GSIs are more effective in selected tumors, like desmoid tumors, and it might be possible to identify GSI-responder patients depending on molecular signatures detected in tumors. Also, GSIs that are more selective toward certain Notch receptors might be used to target Notch receptors that are prevalently altered in selected tumors. The "off targets" of GSIs might be exploited to enhance their antitumor effect. GSIs that target specific subunits of

γ-secretase are now available and might reduce the side effects of the classical GSIs. Finally, combinatorial treatments might benefit the antitumor effect of GSIs. Therefore, a more rational use of GSIs should take into account the tumor type, patient responders, Notch alterations, "off targets," and potential combinatorial treatments.

Therapeutic Antibodies Against Notch and Ligands

The use of antibodies to target Notch have advantages compared to pan-Notch inhibition, including higher specificity toward the target. However, preclinical and clinical studies on antibodies targeting Notch and ligands raised some concerns about their use because of toxicity and low antitumor activity. Tarextumab, an antagonistic antibody against Notch2/3, showed promising results in preclinical studies and in a phase I clinical trial in SCLC (Yen et al. 2015), but did not show any benefit in phase II (NCT01859741). The same was for a Notch1 antagonistic antibody (NCT01778439), which also showed severe adverse effects. Anti-DLL4 antibodies were designed to disrupt DLL4–Notch1 interaction and showed to inhibit tumor angiogenesis and growth in preclinical studies (Ridgway et al. 2006; Yan 2011). However, in a phase I clinical trial anti-DLL4 showed dose-limiting adverse toxicities (Chiorean et al. 2015). Nevertheless, anti-Notch2/3 and anti-Notch1 showed a better response in patients with higher expression of Notch3 and Notch1, respectively, and anti-DLL4 showed a partial response in a subgroup of non-small cell lung cancers harboring a β-catenin mutation and in ovarian cancers in which DLL4 is overexpressed (Yen et al. 2015; Chiorean et al. 2015), suggesting that they might be more responsive in a selected patient cohort. The toxicity associated with these antibodies might result from their long half-life in the body, which can result in chronic inhibition of Notch (Yan 2011). Coach and colleagues designed an anti-DLL4, which is rapidly cleared from the body and showed that intermittent inhibition of DLL4/Notch1 mitigates the toxicities associated with continuous inhibition (Couch et al. 2016). As for GSIs, combinatorial use of these antibodies and other therapeutic agents might allow the use of a lower dose, which might decrease toxicity. On the same line, bispecific antibodies able to target multiple hits were recently designed and these might increase the antitumor effect derived from targeting of multiple pathways and also allow the use of lower doses. In a recent study, bispecific antibodies which are able to target both EGFR and Notch2/3 demonstrated anti-tumor effect decreased the number of cancer stem-like cells and presented no major toxicity in cell lines and xenografts models of triple negative breast cancer (Fu et al. 2019). Bispecific antibodies were also designed against DLL4/VEGF and demonstrated inhibition of tumor progression and angiogenesis in xenografts models of lung, breast, and gastric cancers (Lee et al. 2016; Zhou et al. 2019). These antibodies are now moving on to the clinic and showed manageable toxicity and antitumor activity in different previously treated tumors, and especially in ovarian cancer, in a phase I trial (Jimeno et al. 2019). Future clinical investigation should focus on antibody kinetic, multitargeting, and mechanism-based selection of patients to improve anti-Notch pathway antibodies.

Targeting the Sweet Side of Notch

Notch receptors' affinity for different ligands varies and has important regulatory implications on Notch function. Increasing evidence has shown that discrimination and specificity of Notch binding to different ligands rely on differences in glycosylation, binding forces and surfaces, and lipid-binding. Glycosylation is important for Notch–ligand interaction and proper transport of Notch to the cell membrane. Glycosylation in different EGF repeats of Notch receptors has been found to modify the ability of Notch to bind its ligands in *Drosophila* and mammals and is mediated by addition of O-fucose, O-GlcNAc, or O-glucose by the glycotransferases Pofut1, Fringe (Fringe in *Drosophila* and Lunatic, Maniac and Radical Fringe in mammals) and Poglut1/Rumi (Harvey et al. 2016;

Kakuda and Haltiwanger 2017). Pofut1 adds O-fucose and Poglut1 adds O-glucose on specific residues of Notch EGFs. Fringe proteins can extend O-fucose sites by addition of GlcNAc. Glycosylation have different outcomes on receptor–ligand interactions, depending on which kind of sugar is added and which residue is modified (Harvey et al. 2016; Kakuda and Haltiwanger 2017). A comprehensive map of glycosylated site in Notch receptors and structural studies on Notch–ligands interactions identified glycosylated residues of Notch and confirmed their key role in establishing the interaction with ligands (Harvey et al. 2016; Kakuda and Haltiwanger 2017; Luca et al. 2015, 2017). Hartiwanger's group recently developed O-fucose analogs that are incorporated by Pofut1 in Notch1 and inhibit its interaction with DLL1 and DLL4, but not JAG1. This is because Pofut1 adds the analogs, instead of physiological O-fucose, to residues that are important for Notch–ligand interaction (Schneider et al. 2018). The analogs inhibited Notch1 signaling in mammalian cells, zebrafish, and blocked Notch-dependent differentiation of T-cells (Schneider et al. 2018). The potent Notch inhibitory activity and especially their selectivity toward specific ligands make O-fucose analogs appealing for therapeutic intervention. Also, these specific analogs were designed in such a way that they do not affect the physiological biosynthesis of fucose, which is instead affected by other analogs (Schneider et al. 2018). Glycosylation also plays a role in the correct maturation and transport of Notch to the cell membrane. Depletion of *Pofut1* was found to suppress the constitutive activation of certain Notch1 mutants in T-ALL cell lines by reducing the transport of Notch1 to the cell membrane (McMillan et al. 2017). Similar results were obtained by depletion of *Pofut1* in Kras-dependent myeloid leukemia cells and mouse models (Kong et al. 2019). Other agents, like inhibitors of sarco/endoplasmic reticulum calcium ATPase (SERCA) or heat shock protein 90 (Hsp90), which block the correct maturation of Notch in the ER, showed similar results in T-ALL cell lines and mouse models (Roti et al. 2013; Wang et al. 2017). Interestingly, these agents inhibited Notch without severe toxicity in mouse models. It is possible that because of their structural defects certain Notch mutants are more sensitive to impaired maturation compared to wild type. Therefore, inhibition of Pofut1, SERCA, or Hsp90 might allow a more specific targeting of selected Notch mutants.

Recent work on the structural resolution of Notch1 receptor and its ligands DLL4 and JAG1 showed that different regions of Notch extracellular domain are required for the interaction with different ligands. DLL4 mainly interacts with Notch1 EGF 11–12, whereas EGF 8–10 also significantly contributes to the interaction with JAG1 (Luca et al. 2015, 2017). Further, measurement of forces in Notch–ligand interaction showed that DLL4 and JAG1 require different tension forces in their binding to Notch1 (Luca et al. 2017). Recent studies also showed that the N-terminal region of Notch ligands can interact with lipids present on the cell membrane of the Notch-expressing cells (Kershaw et al. 2015; Suckling et al. 2017). Interestingly, Notch ligands lipid-binding preference varies and might represent another regulatory mechanism for specific Notch–ligand interaction (Suckling et al. 2017; Shilo and Sprinzak 2017). Given that the lipid composition of the cell membrane is heterogeneous, the position of Notch in different subdomains of the cell membrane might also affect its interaction with ligands. These findings have profound implications in the design of Notch-targeting therapies. Antibodies against Notch and ligands or engineered Notch receptors and ligands are currently under development and will have to carefully take the requirements for Notch–ligand interaction into account to make these functional and specific.

Notch in the Endocytic Maze

An increasing number of evidences showed that ubiquitination of Notch mediated by different ubiquitin ligases orchestrates the degradation and the ligand-independent activation of Notch. This process involves the endocytosis of Notch receptor and its sorting in different endocytic

compartments (Shimizu et al. 2014; Steinbuck et al. 2018; Wilkin et al. 2008; Alfred and Vaccari 2018; Yamada et al. 2011; Hori et al. 2011; Schneider et al. 2013). This mechanism has mainly been described in *Drosophila*, however the evidence of a similar regulation in mammals and its relevance in cancers is increasing. The amount of full-length Notch receptors at the cell membrane could be regulated through lysosomal-dependent degradation. This process seems to be mediated by the *Drosophila* HECT E3 ubiquitin ligase, suppressor of Deltex (Su(dx)), and its mammalian ortholog Itch/AIP4 since both were found to poly-ubiquitinate the intracellular domain of Notch and lead the receptor to endosomal internalization and lysosomal-dependent degradation (Shimizu et al. 2014; Wilkin et al. 2004; Chastagner et al. 2008; Yao et al. 2018). Other ubiquitin ligases, including c-Cbl and Nedd4, were also showed to have analogous functions (Wilkin et al. 2004; Jehn et al. 2002; Platonova et al. 2015). It is not completely clear whether this negative regulatory machine directly induces the endocytosis of Notch from the cell membrane or diverts Notch from a constitutive recycling route or other endocytic pathways (Shimizu et al. 2014; Wilkin et al. 2004). Depletion of components of the *Drosophila* recycling retromer machinery was found to cause accumulation of Notch in endosomes and ectopic ligand-independent activation (Gomez-Lamarca et al. 2015), suggesting recycling and endocytic degradation of Notch might be linked and both contribute to Notch turnover. Numb, a conserved adaptor protein, also plays a role in regulating Notch endocytic degradation, likely by facilitating the interaction between Itch/AIP4 and Notch (McGill et al. 2009). Interestingly, Numb was found downregulated in breast cancer cell lines and primary breast tumor cells leading to increased Notch activation (Pece et al. 2004; Stylianou et al. 2006), thus confirming the importance of this degradative regulatory mechanism. Recently, it was showed that Numb overexpression reduces metastasis and tumor growth in breast cancer mouse models (Zhang et al. 2016). Another recent finding showed that Vasorin, a protein frequently overexpressed in glioma stem-like cells in hypoxic conditions, blocks Numb-dependent degradation and stabilizes Notch at the cell membrane for activation (Man et al. 2018). Importantly, silencing of Vasorin reduced Notch and tumor growth in glioblastoma mouse models (Man et al. 2018). Similarly, inhibition of PI3K-AKT was found to cause the lysosomal degradation of Notch upon ubiquitination by c-Cbl in T-ALL cells (Platonova et al. 2015). These recent findings suggest that the lysosomal-dependent degradation of Notch is conserved and is an important regulatory mechanism for the homeostasis of Notch pathway in different cellular contexts. Targeting Notch degradation might represent a strategy to inhibit or reactivate Notch.

Different studies in *Drosophila* have reported that endocytosis and ubiquitination can not only lead to Notch degradation but also to activation in the endosomes. In *Drosophila*, Deltex, a ring-finger ubiquitin ligase, was found to mono-ubiquitinate Notch and sort it for lysosomal-dependent proteolytic activation (Shimizu et al. 2014; Wilkin et al. 2008; Yamada et al. 2011; Hori et al. 2011). In this way, Dx competes with Su(dx) for the endocytic sorting of Notch (Shimizu et al. 2014; Wilkin et al. 2008). This form of endosomal activation is ligand-independent and requires γ-secretase cleavage, but not S2 cleavage (Shimizu et al. 2014; Wilkin et al. 2008; Gupta-Rossi et al. 2004), which might be bypassed thanks to the acidic ionic environment or lysozymes present in the lumen of endosomes which could unmask and cleave S2 site (Steinbuck et al. 2018; Vaccari et al. 2010; Kobia et al. 2014). This was also supported by the observation that genetic and pharmacological inhibition of the vacuolar H+ ATPase, which is responsible of the acidification of endosomes, reduces Notch endocytic activation in *Drosophila* tissues and mammalian breast cancer cell lines (Vaccari et al. 2010, 2008; Kobia et al. 2014; Faronato et al. 2015). Dx has five mammalian orthologs of which three can bind to Notch, such as DTX1, DTX2, and DTX4 (Matsuno et al. 1998; Chastagner et al. 2017). Old literature showed that mammalian Dx proteins act either as positive

or negative regulators of Notch in different contexts (Matsuno et al. 1998; Sestan et al. 1999; Yamamoto et al. 2001; Izon et al. 2002). An interesting recent work showed that DTX4 enhances ligand-dependent activation of Notch1 by favoring its endocytosis and S2 cleavage (Chastagner et al. 2017). Dx role needs further investigation; however, it is possible that different mammalian Dx proteins have distinct regulatory functions and their role might depend on the cellular context or their interactions with other regulators. In *Drosophila* it was found that interaction of Dx with Kurtz (Krz), the ortholog of the human non-visual B-arrestin 2, is critical for the sorting of Notch to endosomal degradation or activation (Hori et al. 2011; Mukherjee et al. 2005). Dx–Krz–Notch complex seems to act as a platform for the recruitment of other regulators (Hori et al. 2011; Schneider et al. 2013; Mukherjee et al. 2005). It is very likely that Su(dx) may also join this complex and Krz acts as a switch between Dx and Su(dx) and in turn, between endosomal degradation and activation. Notch endocytic trafficking is also regulated by Endosomal Sorting Complex Required for Transport (ESCRT). It was showed that *Drosophila ESCRT* mutants, which block different steps of the endocytic trafficking, lead to aberrant ligand-independent activation of Notch (Vaccari et al. 2008). Similarly, knockout of the ESCRT1 component Tumor-susceptibility-gene-101 leads to the endosomal activation of Notch in human cell lines (Leitch et al. 2014). Also, Shrub, a subunit of the ESCRT III complex, was found to contribute to the Dx–Krz–Notch complex (Hori et al. 2011; Schneider et al. 2013). These observations suggest that ESCRTs contribute to the endocytic sorting of Notch, and that defect in the endocytic machinery could lead to uncontrolled ligand-independent Notch signaling, something that could be happening in cancer cells. New regulators of the endocytic trafficking of Notch have been identified in recent years. Cis-inhibition is a known mechanism by which cis-interaction between Notch receptors and ligands expressed in the same cell inhibits Notch signaling (Sprinzak et al. 2010; del Álamo et al. 2011). An elegant work from Wu-Min Deng's group showed that ligand-independent activation of Notch can be inhibited by cis-inhibition in different *Drosophila* tissues in mutant and physiological conditions (Palmer et al. 2014). Similarly, Crumbs (Crb), a conserved large transmembrane protein involved in cell polarization, was found to inhibit Notch ligand-independent activation by blocking Notch at the cell surface in *Drosophila* epithelial wing tissue and Crb depletion leading to upregulation of Notch (Nemetschke and Knust 2016; Das and Knust 2018). Recent work reported for the first time that cis-activation occurs in vitro in mammalians cells in the absence of Notch ligands in trans (Nandagopal 2019). However possible, it is not clear if this process is linked to endocytic regulation of Notch.

The physiological function of the endocytic regulation of Notch is not fully understood, but intriguing hypotheses are rising. It is possible that this mechanism acts as a regulatory network that tunes the ligand-dependent activation against different environmental changes and stress conditions, since this was found to regulate the amount of ligand-dependent signaling in *Drosophila* in response to temperature variation (Shimizu et al. 2014). For instance, hypoxia and nutrients availability might represent stress conditions in which the endocytic regulation ensures Notch signaling homeostasis in mammals. This mechanism could also control Notch signaling in contexts in which ligand binding is challenging. This could be the case of circulating cells as hemocytes and lymphocytes. Indeed, recent works suggest that the activation of Notch in CD4+ and CD8+ T-cells is ligand-independent and likely linked to endosomal regulation (Sorrentino et al. 2019; Steinbuck and Winandy 2018). It is also very interesting that depletion of different endocytic components leads to uncontrolled activation of Notch, which might be relevant to aberrant Notch signaling in diseases. Tuberous sclerosis, a dominant genetic disease which causes the growth of benign tumors, is caused by mutations in *Tuberous Sclerosis* 1 and 2 (TSC1 and 2) and characterized by upregulation of Notch. TSC1 and 2 are lysosomal-associated regulators that

were first believed to regulate mTOR; however, recent studies suggest that TSC1 and 2 might be direct regulators of Notch (Ma et al. 2010; Karbowniczek et al. 2010; Cho et al. 2017), and it is possible this involves endosomal regulation. These mechanisms have been mainly described in *Drosophila*; however, it would be interesting and significant to further explore these mechanisms in the mammalian system and in diseases. Notch is a highly conserved pathway and is likely that similar mechanisms are found in mammals and might help to elucidate Notch regulation in physiological and, importantly, in disease conditions.

Exosomes and other types of extracellular vesicles have received great attention in recent years because of their capacity to transfer signaling molecules between cells. Notch1 together with γ-secretase was found in exosomes secreted by *Tsc1*-null cells (Patel et al. 2016). Notch1 containing exosomes were delivered to healthy cells where the transported Notch was activated leading to the acquisition of a *Tsc1*-like phenotype in recipient cells (Patel et al. 2016). Contrarily, ligands were also found to be transported via exosomes and to cis-inhibit Notch in the recipient cells (Sheldon et al. 2010). Since exosomes originate from late endosomes, a link between the endocytic trafficking of Notch and its transport into exosomes is possible. Similarly, Notch2 was found in ARMM vesicles (arrestin domain-containing protein 1–mediated microvesicles), which buds from the cell membrane, and to be transported and activated in recipient cells (Wang and Lu 2017). Interestingly, Itch and ADAM10 were involved in the loading of Notch to ARMMs and were also incorporated in the vesicles (Wang and Lu 2017). Therefore, Notch might also deliver its signaling in nonadjacent cells via extracellular vesicles. Notch signaling in recipient cells is likely to be ligand independent.

Endocytic trafficking of Notch seems to tightly regulate Notch homeostasis and deletion of endocytic regulators, leading to uncontrolled Notch signaling. A better understanding of Notch endocytic regulation might reveal mechanisms by which Notch is deregulated in cancer and provide new means for Notch-targeting therapies. The endocytic regulation of Notch can lead either to activation or degradation of Notch; therefore targeting this regulation might provide ways to inhibit or enhance Notch signaling.

Notch as a Metabolic Reprogrammer

Metabolism reprogramming is now considered a major hallmark of cancer, through which cancer cell can adapt and survive to different environmental changes, develop resistance to treatments, and modulate antitumor immunity. These mechanisms are tightly entangled with Notch. Metabolic reprogramming mediated by Notch has been reported in different hematological (Kong et al. 2019; Jitschin et al. 2015; Kishton et al. 2016). and solid tumors (Bayin et al. 2017; Bhola et al. 2016)

In physiological conditions, Notch regulates cell size, glucose uptake, and glycolysis through activation of PI3K/Akt or directly by transcriptional regulation of metabolic genes, including c-Myc (Ciofani and Zúñiga-Pflücker 2005; Wang et al. 2011). Interestingly, more recent evidence showed that Notch can reprogram metabolism by direct transcriptional regulation of mitochondrial DNA. It was observed that NICD is recruited to mitochondrial DNA and upregulates respiratory chain components to favor pro-inflammatory activation of macrophages (Xu et al. 2015). Also, mitochondrial metabolism seems important to sustain cancer cells and Notch might be linked to it (Kong et al. 2019; Herranz et al. 2015). Up to date, Notch-dependent metabolic regulation has been reported to sustain survival of T-cell progenitors, CD4+ memory T-cells, and activation of macrophages (Ciofani and Zúñiga-Pflücker 2005; Xu et al. 2015; Maekawa et al. 2015), and might be involved in the metabolic regulation of other immune cells, given Notch's important role in immunity. Several studies showed that tumors counteract T-cells antitumor responses by hampering T-cells glycolytic metabolism (Molon et al. 2016). It is not yet known how this is achieved, but recent reports showed this might be

via Notch downregulation in T-cells (Zhao et al. 2016b). Further studies are needed to confirm this link, but targeting T-cell metabolism or Notch might represent a way to counteract tumor-mediated immunosuppression.

Metabolism reprogramming seems responsible for the development of cancer cell resistance to therapies. Therapeutic inhibition of Notch in T-ALL leads to reduction of glutamine usage, which should hamper T-ALL survival (Herranz et al. 2015). However, in response to Notch inhibition, T-ALL cells activate autophagy for the recovery of nutrients to sustain their metabolism, possibly leading to resistance. This resistant mechanism can be counteracted by inhibition of glutaminolysis and autophagy, since this was showed to increase efficacy of Notch inhibition in T-ALL (Herranz et al. 2015). Resistance to therapeutic inhibition of PI3k/mTOR, often observed in triple-negative breast cancers, was found to be caused by activation of mitochondrial metabolism via Notch1 (Bhola et al. 2016). Pharmacological inhibition of Notch reduced tumor formation and resistance in triple-negative breast cancer xenografts (Bhola et al. 2016).

Differences in the metabolic profile of cancer cells versus healthy cells might be critical to design targeting strategies that affect cancer cell metabolism and spare normal cells. Both normal T-cells and T-ALL were thought to rely on aerobic glycolysis promoted by PI3K and c-Myc (Ciofani and Zúñiga-Pflücker 2005; Palomero et al. 2007). However, analysis of primary T-ALL and normal T-cells showed that their metabolism is different and this is because of Notch. In T-ALL, Notch promotes glycolysis, but also induces activation of AMPK, which favors mitochondrial metabolism over glycolysis, which seems to promote T-ALL survival (Kishton et al. 2016).

Tumor microenvironment influences metabolism reprogramming and heterogeneity. Stroma cells were found to promote glycolysis and survival in B-cell chronic lymphocytic leukemia via activation of Notch and its transcription target c-Myc (Jitschin et al. 2015). Differential activation of Notch was found to regulate the metabolic status in glioblastoma stem cell subpopulations (Bayin et al. 2017). In this study, cells with high activation of Notch relied on aerobic glycolysis and resided in vascular microenvironment, whereas cells with low Notch depended on anaerobic metabolism and resided in hypoxic microenvironment. Importantly, reactivation of Notch in the second group of cells reversed their metabolism from anaerobic to aerobic and abolished their resistance to hypoxia (Bayin et al. 2017).

A role for metabolism in cancer has been known since early studies; however, this has gained attention and been explored only in recent times. Further investigation will be needed to crack down metabolism reprogramming in cancer and its link with Notch. Nevertheless, current evidence provides a rationale for Notch/metabolism-targeting to increase antitumor immunity, counteract therapy resistance, and adaptation of cancer cells.

Notch for Immunotherapy

Accumulating evidence has shown that Notch is heavily involved in shaping the immune system in physiological conditions and the pro-tumoral immune microenvironment in cancer (Grazioli et al. 2017; Hossain et al. 2018). Together with the rising enthusiasm for the use of immunotherapy for cancer, this provided a strong rationale for the evaluation of Notch-targeting strategies as immunomodulators and opened up a new research direction in the Notch-in-cancer field, which previously mainly focused on targeting stem-like and bulk tumor cells.

Notch is crucial in the development and maintenance of different immune cells both in the adaptive, specific, and long-lasting as well as innate, fast, and unspecific immunity. Notch determines the specification and lineage of adaptive T-cells CD4+, CD8+, and Natural killer cells in the thymus and the survival, function, and differentiation to memory lineage of peripheral T-cells. At the same time, Notch also regulates the differentiation of innate immune myeloid cells (granulocytes, macrophages, dendritic cells [DCs]), and crosstalks between myeloid cells

and T-cells during immune responses. Some of these processes, including differentiation of T-cells and crosstalks between immune cells, are mediated by Notch ligands. For example, it was showed that the expression of different ligands in DCs stimulates the differentiation of CD4+ T-cells into different lineages during immune responses (Kassner et al. 2010; Biktasova et al. 2015; Meng et al. 2016). However, other processes might rely on ligand-independent Notch signaling. It was recently showed that Notch activation in CD4+ T-cells is ligand-independent and involved Notch endocytosis (Steinbuck et al. 2018). This form of activation is triggered by stimulation of T-cell receptor (TCR)/CD28 receptor and PI3K pathway followed by downstream events that facilitate the proteolytic cleavage of Notch (Steinbuck et al. 2018).

In the tumor microenvironment, protumor and antitumor immune cells coexist and antagonize each other. Notch is important for both protumor and antitumor immunity and for their crosstalk. CD4+ T-helper 1 and CD8+ cytotoxic T-cells are the main weapons of our immune system against cancer because they can recognize and induce cell death in malignant cells. Unfortunately, tumors are very skilled in evading our body immune response by different means: immunosuppressive molecules, inhibitory ligands, and suppressive cell types. Different studies showed that Notch is decreased in tumor-infiltrating T-cells, and reactivation of Notch enhances antitumor immunity in mouse models (Sierra et al. 2014; Huang et al. 2011; Sugimoto et al. 2010). In particular, a pivotal work by Paulo Rodriguez's research group demonstrated that Notch1 and Notch2 are downregulated in tumor-infiltrating CD8+ T-cells and, strikingly, ectopic expression of Notch1 NICD in CD8+ T-cells enhanced their cytotoxic response and antitumor activity in vivo in mouse models (Sierra et al. 2014). These findings suggest that Notch is targeted by tumor-mediated immunosuppression and let to the idea that reactivation of Notch in T-cells might protect them from the tumor-mediated immunosuppression and boost their antitumoral activity. Therefore, Notch-targeting therapies are worth exploring for immunotherapy.

Bortezomib, a FDA-approved proteasome inhibitor for multiple myeloma, mantle cell lymphoma, and NSCL cancer, was found to favor antitumor immunity by rescuing Notch1 and Notch2 in CD8+ cells from the tumor-mediated immune suppression and enhance the production of effectors and stimulatory cytokines (Thounaojam et al. 2015; Pellom et al. 2017). These findings led Shanken and colleagues to apply bortezomib for adoptive T-cell therapy. They successfully showed that treatment with bortezomib-sustained T-cell function after transfer of the treated T-cells in the host mice and reduced tumor burden in human renal carcinomas xenografts (Shanker et al. 2015). Despite the success of this study there have been no further advances in this direction. Only low doses of bortezomib seems to elicit a positive effect on immune cells, while high doses were reported to suppress immune cells (Berges et al. 2008), suggesting that the effect of proteosomal degradation inhibition on Notch pathway in T-cells might be complex and needs further investigation. Indeed, it is not yet clear how bortezomib have an impact on T-cells. Some studies reported that this regulation might rely on the crosstalk between NICD and Nuclear Factor kB (NFkB), which together can enhance CD8+ effector function (Thounaojam et al. 2015), or to positively regulate miR155, the suppression of which seems to downregulate Notch in T-cells (American Association of Immunologists 2018, 2019). Given that Notch turnover, which is mediated by proteosomal and lysosomal degradation, is key to ensure the fine regulation of Notch, it is also possible that bortezomib might rescue Notch receptor or one of its regulators from proteosomal degradation, thus increasing Notch activation in T-cells. Further mechanistic description of bortezomib-dependent Notch modulation will be needed for the safe use of this agent for immunomodulation.

Another way in which tumors suppress the immune response is through the production of adenosine in the tumor microenvironment. This molecule stimulates the adrenergic receptors A2AR, A2BR, A1R, and A3R and have different regulatory effects depending on the receptor and

the cell in which it is expressed (Vijayan et al. 2017; Leone and Emens 2018). Adenosine was found to have a direct suppressive effect on CD8+ through the stimulation of the adenosine receptor A2AR (Ohta et al. 2006). Conversely, several studies have shown that genetic or pharmacological inhibition of A2AR, using A2AR antagonists, restores antitumor immunity and counteracts adenosine-mediated immunosuppression (Waickman et al. 2012; Beavis et al. 2013a, b). These compounds also showed to enhance the effect of checkpoint inhibitors (PD1, PDL1, and CTL4) in preclinical mouse models and a number of A2AR antagonists are now in clinical development (Willingham et al. 2018; Iannone et al. 2014; Mittal et al. 2014; Beavis et al. 2015). Also, A2AR inhibition was found to potentiate the efficacy of adoptive CAR-T cell therapy in HER2+ mouse models, likely because of boosting of T-cell effector function and resistance (Beavis et al. 2017). Morello and Miele's research groups recently showed that stimulation of A2AR inhibits the activation of Notch1 and in turn the production of INF-γ and Granzyme B in CD8+ cells (Sorrentino et al. 2019). Importantly, treatment with an A2AR antagonist restored Notch1 and the effector production, suggesting inhibition of A2AR might enhance CD8+ effector function through Notch (Sorrentino et al. 2019). This is very interesting because it shows that adenosine affect CD8+ effector function via Notch, thus placing Notch at the core of the adenosine-mediated immunoregulation and A2AR antagonists mechanism of action. Also, this study proposed that A2AR-mediated regulation of Notch might involve its endocytic regulation, similarly to what was found in CD4+ T-cells (Steinbuck et al. 2018). Because of their effect on releasing the "brakes" of antitumor immune response, as PD1/PDL1/CTL4, A2AR antagonists have been referred as the "next generation of checkpoint inhibitors." (Leone et al. 2015). In light of their recent link with Notch, A2AR antagonists might turn out to be one of the first examples of Notch-modulating immunotherapy. Further studies on how adenosine receptors regulate Notch will be required to maximize the therapeutic application of adenosine receptor antagonists and avoid unwanted off-target effects. As we discussed, both Notch and A2AR, or more generally adenosine receptors, are expressed in different sets of cells within the tumor microenvironment, and the function of their crosstalk might vary.

Notch is not only involved in the intrinsic properties of T-cells, but also in the crosstalk between T-cells and other regulatory immune cells. In physiological conditions, myeloid cells differentiate in several regulatory immune cell types (macrophages, dendritic cells, granulocytes), which are recruited by inflammation and control the immune response. The tumor microenvironment releases signals that perturb the differentiation of myeloid cells, leading to the generation of myeloid-derived suppressive cells (MDSCs), dendritic cells (DCs), and tumor-associated macrophages (TAMs), which suppress the antitumoral immune response (Hossain et al. 2018). Recent papers showed that Notch ligands play a major role in both the specification of these pro-tumoral immune cells and their crosstalk with T-cells. It was observed that pro-tumoral MDSCs overexpress JAG1 and JAG2 and have a decreased expression of DLL1 and DLL4 (Sierra et al. 2014, 2017). On the contrary, it was showed that expression of DLL1 or DLL4, but not JAG1 or JAG2, in DCs stimulates T-cell effector and memory functions (Kassner et al. 2010; Biktasova et al. 2015; Meng et al. 2016). Also, expression of JAG1 or JAG2 in DCs correlates with PD-1 expression in tumor-infiltrating CD8+ effector and memory T-cells, whereas expression of DLL1 or DLL4 correlates with the expression of Notch receptors (Tchekneva et al. 2019). These observations suggest that JAG1/2 and DLL1/4 generally favor pro-tumoral and anti-tumoral immunity, respectively. Indeed, targeting of Notch ligands had a positive outcome in preclinical mouse models. Systemic administration of JAG1/2 blocking antibodies improved antitumor immune response, inhibited MDSCs, and enhanced adoptive T-cell therapy in lung, colon, melanoma, and thymoma mouse models (Sierra et al. 2017). On the same lines, engineered DLL1 multivalent clustered construct or JAG1 monovalent construct, which stimulates DLL1 signaling and inhibits JAG1 signaling,

respectively, improved antitumor immune response and reduced PD1 expression in pancreatic and lung cancer mouse models (Huang et al. 2011; Tchekneva et al. 2019). The significance of these studies is that targeting Notch ligands might represent a way to modulate the immune response in the tumor microenvironment and the development of antagonistic antibodies or engineered Notch ligands might be an attractive therapeutic strategy.

Notch is also important for the differentiation of MDSCs, DCs, and TAMs in the tumor microenvironment. Anti-JAG1/2 seems to inhibit MDSCs or to induce their switch to a non-immunosuppressive phenotype (Sierra et al. 2017). It is not clear how this is achieved, but it is possible that inhibition of Jagged in MDSCs or adjacent cells ultimately modulate Notch in MDSCs (Sierra et al. 2017). In DCs Notch stimulation positively modulates their response to pro-inflammatory signals (Gentle et al. 2012). Majority of TAMs downregulate Notch and acquire a M2-anti-inflammatory phenotype; however, reactivation of Notch in TAMs favors their M1-pro-inflammatory phenotype and ameliorate antitumor immunity (Xu et al. 2015; Wang et al. 2010).

Targeting Notch demonstrated a remarkable effect on antitumor immunity and has a promising future. Since Notch plays a different role in different cells in the tumor microenvironment, the main challenge of systemic immunomodulation will be to design strategies that selectively target Notch in the desired immune cells. In line with this idea, targeting of ligands seems an attractive strategy to modulate the crosstalk between immune cells in the tumor microenvironment. On the other hand, since Notch seems regulated in a ligand-independent way in T-cells, it might be interesting to explore strategies to selectively target this unique mode of activation. The important role of Notch in immunomodulation also highlights that Notch-targeting therapies directed to cancer and stroma cells will have to be selective enough to not affect Notch in immune cells. For example, pan-Notch GSIs have a demonstrated immunosuppressive activity and this might play in favor of the tumor.

Notch in ACTion

In the era of immunotherapy, adoptive T-cell therapy (ACT) is one of the most exciting T-cell-based technologies and Notch is at the frontline of its development. ACT is based on the in vitro generation of T-cells, which are able to recognize tumor-specific antigens and are then transferred in the patients where they will trigger a potent antitumor immune response (Garber 2018). T-cells for ACT are either generated and instructed in vitro from tumor infiltrating T-cells taken from the patient or are engineered T-cells, which present a transgenic T-cell receptor (TCRs) or a chimeric-antigen receptor (CARs) (Garber 2018). ACT has shown remarkable results in clinical trials in B-ALL and melanoma (Dudley et al. 2008; Besser et al. 2010; Brentjens et al. 2013; Grupp et al. 2013). However, this technology has some limitations, which need to be addressed to expand its use to other tumors and increase its effectiveness and safety. The limits of ACT are the low number of T-cells recovered from the patient, tumor-specific antigen recognition, and immune suppression in the tumor microenvironment. In particular, increasing the number of cells is critical for ACT because only a limited number of T-cells can be isolated from the patient. CARs recognize specific antigens on the surface of cells, while TCRs have a broader recognition potential, because they recognize peptides from antigen-presenting cells. However, both can lose antigen recognition because of change of antigens expressed in tumor cells and suppression of antigen-presenting cells in the tumor microenvironment. Also, this might give rise to unspecific immune responses if the antigen recognition is not cancer-cell specific. Finally, all kinds of ATC, as endogenous T-cells, have to counteract immunosuppression in the tumor microenvironment. Given the direct involvement of Notch signaling in T-cell intrinsic functions, tolerance, and differentiation, Notch modulation is an attractive strategy to address ACT limitations. In the previous chapter we saw that Notch ligands are critical for T-cells maintenance in the tumor microenvironment. To generate a higher number of T-cells for ACT, different groups exploited Notch-induced

differentiation by culturing induced pluripotent stem cells (iPSC) on DLL1-expressing stroma cells (Lei et al. 2011) or, more recently, hemato-vascular mesodermal progenitors on DLL4-expressing stroma cells (Kumar et al. 2019) obtaining a high number of T-cells and increasing their in vitro expansion capacity. Recent studies also tried to generate T-stem memory cells for ACT by coculturing activated T-cells from mouse or humans with DLL1-expressing stroma cells (Kondo et al. 2017). These cells, named iTscm, had features of memory cells, like self-renewal and rapid response to antigens, lower expression of inhibitory ligands (PD1, CTL4), and showed stronger antitumor effect in humanized Epstein–Barr virus transformed-tumor model mice (Kondo et al. 2017, 2018). Importantly, iTscm can be generated from tumor-infiltrating T-cells from the patient, thus overcoming the need of engineered antigen recognition.

Till date, a number of agents has shown the ability to increase T-cell tolerance via Notch signaling against immune suppression and these could be employed to improve ACT resistance. As we mentioned, bortezomib potentiated ACT in human renal carcinomas xenografts (Shanker et al. 2015). Also, it was showed that treatment with an A2AR antagonist increases the activation and effector function of CARs and their efficacy in HER2+ cancer mouse models (Beavis et al. 2017). These compounds represent a potential asset that can be applied to boost ACT resistance against tumoral immune suppression; however, there is no yet evidence that these treatments will enhance ACT in human tumors.

SynNoches: Sin or Miracle?

The mechanism of activation of Notch receptor is fascinating, having the extracellular domain responding to external clues and triggering the release of NICD to deliver intracellular responses. This inspired researchers to build synthetic Notch receptors, called synNotches, which have customizable extracellular and intracellular domains linked by the transmembrane domain of Notch, thus allowing customizable extra- to intracellular signaling. Recently, synNotches have been extensively applied to ACT to improve the antigen recognition of engineered T-cells and for many other applications, such as delivery of drugs or pro-immunity signals in the tumor microenvironment. Wendell Lim and his group were the firsts to design a synNotch receptor, which, upon recognition of a specific antigen, triggers the expression of a CAR in the same T-cell, which recognizes a second antigen (Roybal et al. 2016; Morsut et al. 2016). They showed that these engineered T-cells were able to recognize and kill cancer cells that express both antigens and not only one of them, in vitro and in vivo in mouse models (Roybal et al. 2016). This strategy could improve the efficacy of engineered T-cells especially in solid tumors that do not express a specific antigen, where the recognition of multiple antigens instead of one will greatly increase the chances of targeting. Further, this could avoid the unspecific targeting of healthy cells that express one of the antigens present in cancer cells, which could cause severe side effects. Using synNotch technology, T-cells were also engineered to drive a plethora of other functions, such as delivery of therapeutic molecules (antibodies, cytotoxic proteins, apoptosis inducers) to increase the antitumor effect, pro and suppressive immune signals (cytokines, ligands, master regulators, adjuvants) to regulate the immune response in the tumor microenvironment (Morsut et al. 2016), thus showing the versatility of this technology. SynNotch-CARs have opened up a new platform for molecule targeting and delivery which seems to have almost unlimited possibilities. Several investigators are now using synNotches to establish new therapeutic strategies. ROI is a potential target for CAR therapy since it is expressed in different solid tumors; however this antigen is also expressed on stroma cells, thus arising the possibility of severe toxicity upon ROI targeting. Recently, T-cells were engineered with a synNotch recognizing the tumor antigen EpCam or B7H3, which triggers the expression of a CAR specific for ROI, thus allowing the specific targeting of tumor cells only and sparing of ROI+ stroma cells (Srivastava et al. 2019). Another group designed

synNotch-CAR T-cells which express an antibody fragment against Ax1, an antigen expressed in different tumors, which led to increased cytokine production and targeting of Ax1-expressing tumor cells in mouse models (Cho et al. 2018).

It was also proposed that synNotches could be broadly used to modify the cellular microenvironment in different contexts (Morsut et al. 2016). Since Notch machinery is ubiquitously expressed, this technology could be applied to different cell types. For example, the development and organization of tissues is controlled by cell–cell communication which produce specific morphological signals and Notch is well known to play a role in tissue patterning and morphogenesis. A recent study used synNotches to engineer morphological signals between cells to lead the self-organization of multicellular structures for tissue engineering (Toda et al. 2018), therefore synNotches could be used to customize morphological or reprogramming signals. More generally, synNotches were used to study cell–cell interactions in different *Drosophila* tissues, suggesting this technology might be extensively used to study developmental processes in which cell–cell interactions are critical, including cell competition, differentiation (He et al. 2017), tissue morphogenesis, and tumorigenesis. Other applications of synNotches include platforms to identify and study transmembrane receptors which are activated by proteolysis similarly to Notch (Hayward et al. 2019).

SynNotches applied to T-cells engineering have shown remarkable therapeutic applications with promising clinical perspectives. However, this technology is young and will need further establishment and evaluation before reaching clinical development. Application of synNotches to tissue engineering are also very intriguing, but due to the important involvement of Notch in tissue morphogenesis, safety will have to be carefully addressed. This is valid for all applications that will aim to use synNotches as synthetic modulators in biological processes. On the other hand, SynNotches could be a very powerful tool to study these processes. SynNotches have surely shown to be incredibly versatile and their employment in different technologies can be easily foreseen.

Discussion and Conclusion

Notch is a fascinating signaling pathway. From its discovery in *Drosophila* to Notch entry in cancer immunotherapy, Notch field saw a continuous revolution. However, it seems that we still have not unravel all the secrets and potentials of this pathway. In this chapter, we discussed current and new Notch-targeting therapies with their exciting promises and challenges. Notch pleiotropic nature seems to be both the advantage and the challenge of Notch-targeted therapies. Targeting Notch allows to virtually modulate any aspect of cancer; however, this means that Notch-targeting must be highly specific toward the desired target. This chapter highlighted different factors that are critical to ensure the specificity of Notch-targeting. Due to the complexity of its regulation, Notch can be modulated in many different ways. Understanding the mechanism by which Notch is modulated in different sets of cells within the tumor microenvironment will be crucial to predict whether Notch-targeting therapies will be effective and to identify new druggable targets. Understanding whether Notch function is pro- or anti-tumoral is essential, especially because Notch is differently expressed in subsets of cells within the tumor and the microenvironment. The first Notch-targeted therapies were designed to inhibit Notch; however, it is now becoming clear that in certain situations Notch should be favored instead of inhibited. Recent investigation on Notch regulation has revealed alternative ways in which Notch can be activated or inhibited. For example, the endocytic regulation of Notch lead to either degradation or activation and this might be an attractive mechanism to inhibit or reactivate Notch. Finally, preclinical and clinical trials demonstrated that certain patients/tumors are more responsive to Notch-targeting therapies. Therefore, selection of patient responders and identification of signatures should be implemented for the rational use of Notch-targeting therapies. The new means of Notch-targeting and their applications to new fields hold promising perspectives and it will be exciting to see which advances they will bring to cancer therapy.

References

Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ et al (2011) Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science 333:1154–1157

Alfred V, Vaccari T (2018) Mechanisms of non-canonical signaling in health and disease: diversity to take therapy up a Notch? Springer, Cham, pp 187–204

American Association of Immunologists. AN, Thounaojam MC, Thomas PL, Shanker A. The journal of immunology: official journal of the American Association of Immunologists. Williams & Wilkins, 2018. https://www.jimmunol.org/content/200/1_Supplement/57.2.abstract. Accessed 9 Oct 2019.

American Association of Immunologists. AN, Thounaojam M, Chaudhuri E, Dash C, Shanker A. The journal of immunology: official journal of the American Association of Immunologists. Williams & Wilkins, 2019. https://www.jimmunol.org/content/202/1_Supplement/136.18.abstract. Accessed 9 Oct 2019.

Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284:770–776

Aster JC, Pear WS, Blacklow SC (2017) The varied roles of Notch in cancer. Annu Rev Pathol Mech Dis 12:245–275

Bayin NS, Frenster JD, Sen R, Si S, Modrek AS, Galifianakis N et al (2017) Notch signaling regulates metabolic heterogeneity in glioblastoma stem cells. Oncotarget 8:64932–64953

Beavis PA, Milenkovski N, Stagg J, Smyth MJ, Darcy PK (2013a) A $_{2A}$ blockade enhances anti-metastatic immune responses. Onco Targets Ther 2:e26705

Beavis PA, Divisekera U, Paget C, Chow MT, John LB, Devaud C et al (2013b) Blockade of A2A receptors potently suppresses the metastasis of CD73+ tumors. Proc Natl Acad Sci U S A 110:14711–14716

Beavis PA, Milenkovski N, Henderson MA, John LB, Allard B, Loi S et al (2015) Adenosine receptor 2A blockade increases the efficacy of anti–PD-1 through enhanced antitumor T-cell responses. Cancer Immunol Res 3:506–517

Beavis PA, Henderson MA, Giuffrida L, Mills JK, Sek K, Cross RS et al (2017) Targeting the adenosine 2A receptor enhances chimeric antigen receptor T cell efficacy. J Clin Invest 127:929–941

Bender MH, Gao H, Capen AR, Clay JM, Hipskind PA, Reel JK et al (2013) Novel inhibitor of Notch signaling for the treatment of cancer. In: Experimental and molecular therapeutics. American Association for Cancer Research, pp 1131–1131

Berges C, Haberstock H, Fuchs D, Miltz M, Sadeghi M, Opelz G et al (2008) Proteasome inhibition suppresses essential immune functions of human CD4+ T cells. Immunology 124:234–246

Besser MJ, Shapira-Frommer R, Treves AJ, Zippel D, Itzhaki O, Hershkovitz L et al (2010) Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. Clin Cancer Res 16:2646–2655

Bhola NE, Jansen VM, Koch JP, Li H, Formisano L, Williams JA et al (2016) Treatment of triple-negative breast cancer with TORC1/2 inhibitors sustains a drug-resistant and Notch-dependent cancer stem cell population. Cancer Res 76:440–452

Biktasova AK, Dudimah DF, Uzhachenko RV, Park K, Akhter A, Arasada RR et al (2015) Multivalent forms of the Notch ligand DLL-1 enhance antitumor T-cell immunity in lung cancer and improve efficacy of EGFR-targeted therapy. Cancer Res 75:4728–4741

Borgegård T, Gustavsson S, Nilsson C, Parpal S, Klintenberg R, Berg A-L et al (2012) Alzheimer's disease: presenilin 2-sparing γ-secretase inhibition is a tolerable Aβ peptide-lowering strategy. J Neurosci 32:17297–17305

Bray SJ (2016) Notch signalling in context. Nat Rev Mol Cell Biol 17:722

Bray SJ, Gomez-Lamarca M (2018) Notch after cleavage. Curr Opin Cell Biol 51:103–109

Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG et al (2013) CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med 5:177ra38

Carrieri FA, Murray PJ, Ditsova D, Ferris MA, Davies P, Dale JK (2019) CDK 1 and CDK 2 regulate NICD 1 turnover and the periodicity of the segmentation clock. *EMBO Rep* 20. https://doi.org/10.15252/embr.201846436

Chastagner P, Israël A, Brou C (2008) AIP4/itch regulates Notch receptor degradation in the absence of ligand. PLoS One 3:e2735

Chastagner P, Rubinstein E, Brou C (2017) Ligand-activated Notch undergoes DTX4-mediated ubiquitylation and bilateral endocytosis before ADAM10 processing. Sci Signal 10:eaag2989

Chiorean EG, LoRusso P, Strother RM, Diamond JR, Younger A, Messersmith WA et al (2015) A phase I first-in-human study of enoticumab (REGN421), a fully human delta-like ligand 4 (Dll4) monoclonal antibody in patients with advanced solid tumors. In: Clinical cancer research. American Association for Cancer Research Inc, pp 2695–2703

Cho J-H, Patel B, Bonala S, Manne S, Zhou Y, Vadrevu SK et al (2017) Notch transactivates Rheb to maintain the multipotency of TSC-null cells. Nat Commun 8:1848

Cho JH, Okuma A, Al-Rubaye D, Intisar E, Junghans RP, Wong WW (2018) Engineering Axl specific CAR and SynNotch receptor for cancer therapy. Sci Rep 8:3846

Ciofani M, Zúñiga-Pflücker JC (2005) Notch promotes survival of pre–T cells at the β-selection checkpoint by regulating cellular metabolism. Nat Immunol 6:881–888

Collu GM, Hidalgo-Sastre A, Brennan K (2014) Wnt–Notch signalling crosstalk in development and disease. Cell Mol Life Sci 71:3553–3567

Couch JA, Zhang G, Beyer JC, de Zafra CLZ, Gupta P, Kamath AV et al (2016) Balancing efficacy and safety of an anti-DLL4 antibody through pharmacokinetic modulation. Clin Cancer Res 22:1469–1479

Das S, Knust E (2018) A dual role of the extracellular domain of *Drosophila* Crumbs for morphogenesis of the embryonic neuroectoderm. Biol Open 7:bio031435

del Álamo D, Rouault H, Schweisguth F (2011) Mechanism and significance of cis-inhibition in Notch signalling. Curr Biol 21:R40–R47

Dexter JS (1914) The analysis of a case of continuous variation in Drosophila by a study of its linkage relations. Am Nat 48:712–758

Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U et al (2008) Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J Clin Oncol 26:5233–5239

Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD et al (1991) TAN-1, the human homolog of the Drosophila Notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 66:649–661

Falo-Sanjuan J, Lammers NC, Garcia HG, Bray SJ (2019) Enhancer priming enables fast and sustained transcriptional responses to Notch signaling. Dev Cell 50:411–425.e8

Faronato M, Nguyen VTM, Patten DK, Lombardo Y, Steel JH, Patel N et al (2015) DMXL2 drives epithelial to mesenchymal transition in hormonal therapy resistant breast cancer through Notch hyper-activation. Oncotarget 6:22467–22479

Fleming RJ (1998) Structural conservation of Notch receptors and ligands. Semin Cell Dev Biol 9:599–607

Fryer CJ, White JB, Jones KA (2004) Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol Cell 16:509–520

Fu W, Lei C, Yu Y, Liu S, Li T, Lin F et al (2019) EGFR/Notch antagonists enhance the response to inhibitors of the PI3K-Akt pathway by decreasing tumor-initiating cell frequency. Clin Cancer Res 25:2835–2847

Gallahan D, Callahan R (1997) The mouse mammary tumor associated gene INT3 is a unique member of the NOTCH gene family (NOTCH4). Oncogene 14:1883–1890

Garber K (2018) Driving T-cell immunotherapy to solid tumors. Nat Biotechnol 36:215–219

Gavai AV, Quesnelle C, Norris D, Han W-C, Gill P, Shan W et al (2015) Discovery of clinical candidate BMS-906024: a potent pan-notch inhibitor for the treatment of leukemia and solid tumors. ACS Med Chem Lett 6:523–527

Gentle ME, Rose A, Bugeon L, Dallman MJ (2012) Noncanonical Notch signaling modulates cytokine responses of dendritic cells to inflammatory stimuli. J Immunol 189:1274–1284

George J, Lim JS, Jang SJ, Cun Y, Ozretić L, Kong G et al (2015) Comprehensive genomic profiles of small cell lung cancer. Nature 524:47–53

Gomez-Lamarca MJ, Snowdon LA, Seib E, Klein T, Bray SJ (2015) Rme-8 depletion perturbs Notch recycling and predisposes to pathogenic signaling. J Cell Biol 210:303–318

Gomez-Lamarca MJ, Falo-Sanjuan J, Stojnic R, Abdul Rehman S, Muresan L, Jones ML et al (2018) Activation of the Notch signaling pathway in vivo elicits changes in CSL nuclear dynamics. Dev Cell 44:611–623.e7

Gordon WR, Arnett KL, Blacklow SC (2008) The molecular logic of Notch signaling--a structural and biochemical perspective. J Cell Sci 121:3109–3119

Grazioli P, Felli MP, Screpanti I, Campese AF (2017) The mazy case of Notch and immunoregulatory cells. J Leukoc Biol 102:361–368

Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR et al (2013) Chimeric antigen receptor–modified T cells for acute lymphoid leukemia. N Engl J Med 368:1509–1518

Gupta-Rossi N, Six E, LeBail O, Logeat F, Chastagner P, Olry A et al (2004) Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. J Cell Biol 166:73–83

Gutierrez A, Look AT (2007) NOTCH and PI3K-AKT pathways intertwined. Cancer Cell 12:411–413

Haapasalo A, Kovacs DM (2011) The many substrates of presenilin/γ-secretase. J Alzheimers Dis 25:3–28

Habets RA, de Bock CE, Serneels L, Lodewijckx I, Verbeke D, Nittner D et al (2019) Safe targeting of T cell acute lymphoblastic leukemia by pathology-specific NOTCH inhibition. Sci Transl Med 11:eaau6246

Harvey BM, Rana NA, Moss H, Leonardi J, Jafar-Nejad H, Haltiwanger RS (2016) Mapping sites of O-glycosylation and fringe elongation on Drosophila Notch. J Biol Chem 291:16348–16360

Hayward AN, Aird EJ, Gordon WR (2019) A toolkit for studying cell surface shedding of diverse transmembrane receptors. elife 8. https://doi.org/10.7554/eLife.46983

He L, Huang J, Perrimon N (2017) Development of an optimized synthetic Notch receptor as an in vivo cell-cell contact sensor. Proc Natl Acad Sci U S A 114:5467–5472

Herranz D, Ambesi-Impiombato A, Sudderth J, Sánchez-Martín M, Belver L, Tosello V et al (2015) Metabolic reprogramming induces resistance to anti-NOTCH1 therapies in T cell acute lymphoblastic leukemia. Nat Med 21:1182–1189

Hori K, Sen A, Kirchhausen T, Artavanis-Tsakonas S (2011) Synergy between the ESCRT-III complex and Deltex defines a ligand-independent Notch signal. J Cell Biol 195:1005–1015

Hossain F, Majumder S, Ucar DA, Rodriguez PC, Golde TE, Minter LM et al (2018) Notch signaling in myeloid cells as a regulator of tumor immune responses. Front Immunol 9. https://doi.org/10.3389/FIMMU.2018.01288

Huang Y, Lin L, Shanker A, Malhotra A, Yang L, Dikov MM et al (2011) Resuscitating cancer immunosurveillance: selective stimulation of DLL1-Notch signaling in T cells rescues T-cell function and inhibits tumor growth. Cancer Res 71:6122–6131

Iannone R, Miele L, Maiolino P, Pinto A, Morello S (2014) Adenosine limits the therapeutic effectiveness of anti-CTLA4 mAb in a mouse melanoma model. Am J Cancer Res 4:172–181

Izon DJ, Aster JC, He Y, Weng A, Karnell FG, Patriub V et al (2002) Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1. Immunity 16:231–243

Izumchenko E, Sun K, Jones S, Brait M, Agrawal N, Koch WM et al (2014) Notch1 mutations are drivers of oral tumorigenesis. Cancer Prev Res 8:277–286

Jehn BM, Dittert I, Beyer S, Von Der Mark K, Bielke W. c-Cbl binding and ubiquitin dependent lysosomal degradation of membrane associated Notch1 downloaded from. JBC Papers in Press, 2002. http://www.jbc.org/. Accessed 13 Sept 2018.

Jimeno A, Moore KN, Gordon M, Chugh R, Diamond JR, Aljumaily R et al (2019) A first-in-human phase 1a study of the bispecific anti-DLL4/anti-VEGF antibody navicixizumab (OMP-305B83) in patients with previously treated solid tumors. Investig New Drugs 37:461–472

Jitschin R, Braun M, Qorraj M, Saul D, Le Blanc K, Zenz T et al (2015) Stromal cell–mediated glycolytic switch in CLL cells involves Notch-c-Myc signaling. Blood 125:3432–3436

Kakuda S, Haltiwanger RS (2017) Deciphering the fringe-mediated Notch code: identification of activating and inhibiting sites allowing discrimination between ligands. Dev Cell 40:193–201

Karbowniczek M, Zitserman D, Khabibullin D, Hartman T, Yu J, Morrison T et al (2010) The evolutionarily conserved TSC/Rheb pathway activates Notch in tuberous sclerosis complex and Drosophila external sensory organ development. J Clin Invest 120:93–102

Kassner N, Krueger M, Yagita H, Dzionek A, Hutloff A, Kroczek R et al (2010) Cutting edge: plasmacytoid dendritic cells induce IL-10 production in T cells via the Delta-like-4/Notch axis. J Immunol 184:550–554

Kershaw NJ, Church NL, Griffin MDW, Luo CS, Adams TE, Burgess AW (2015) Notch ligand delta-like1: X-ray crystal structure and binding affinity. Biochem J 468:159–166

Kidd S, Kelley MR, Young MW (1986) Sequence of the notch locus of Drosophila melanogaster: relationship of the encoded protein to mammalian clotting and growth factors. Mol Cell Biol 6:3094–3108

Kishton RJ, Barnes CE, Nichols AG, Cohen S, Gerriets VA, Siska PJ et al (2016) AMPK is essential to balance glycolysis and mitochondrial metabolism to control T-ALL cell stress and survival. Cell Metab 23:649–662

Kobia F, Duchi S, Deflorian G, Vaccari T (2014) Pharmacologic inhibition of vacuolar H+ ATPase reduces physiologic and oncogenic Notch signaling. Mol Oncol 8:207–220

Kondo T, Morita R, Okuzono Y, Nakatsukasa H, Sekiya T, Chikuma S et al (2017) Notch-mediated conversion of activated T cells into stem cell memory-like T cells for adoptive immunotherapy. Nat Commun 8:15338

Kondo T, Imura Y, Chikuma S, Hibino S, Omata-Mise S, Ando M et al (2018) Generation and application of human induced-stem cell memory T cells for adoptive immunotherapy. Cancer Sci 109:2130–2140

Kong G, You X, Wen Z, Chang Y-I, Qian S, Ranheim EA et al (2019) Downregulating Notch counteracts KrasG12D-induced ERK activation and oxidative phosphorylation in myeloproliferative neoplasm. Leukemia 33:671–685

Kopan R, Ilagan MXG (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137:216–233

Kumar A, Lee JH, Suknuntha K, D'Souza SS, Thakur AS, Slukvin II (2019) NOTCH activation at the hemato-vascular mesoderm stage facilitates efficient generation of T cells with high proliferation potential from human pluripotent stem cells. J Immunol 202:770–776

Kummar S, O'Sullivan Coyne G, Do KT, Turkbey B, Meltzer PS, Polley E et al (2017) Clinical activity of the γ-secretase inhibitor PF-03084014 in adults with Desmoid tumors (aggressive fibromatosis). J Clin Oncol 35:1561–1569

Lee D, Kim D, Bin CY, Kang K, Sung E-S, Ahn J-H et al (2016) Simultaneous blockade of VEGF and Dll4 by HD105, a bispecific antibody, inhibits tumor progression and angiogenesis. MAbs 8:892–904

Lei F, Zhao B, Haque R, Xiong X, Budgeon L, Christensen ND et al (2011) In vivo programming of tumor antigen-specific T lymphocytes from pluripotent stem cells to promote cancer immunosurveillance. Cancer Res 71:4742–4747

Leitch CC, Lodh S, Prieto-Echagüe V, Badano JL, Zaghloul NA (2014) Basal body proteins regulate Notch signaling through endosomal trafficking. J Cell Sci 127:2407–2419

Leone RD, Emens LA (2018) Targeting adenosine for cancer immunotherapy. J Immunother Cancer 6:57

Leone RD, Lo Y-C, Powell JD (2015) A2aR antagonists: next generation checkpoint blockade for cancer immunotherapy. Comput Struct Biotechnol J 13:265–272

Lim JS, Ibaseta A, Fischer MM, Cancilla B, O'Young G, Cristea S et al (2017) Intratumoural heterogeneity generated by Notch signalling promotes small-cell lung cancer. Nature 545:360–364

Luca VC, Jude KM, Pierce NW, Nachury MV, Fischer S, Garcia KC (2015) Structural biology. Structural basis for Notch1 engagement of Delta-like 4. Science 347:847–853

Luca VC, Kim BC, Ge C, Kakuda S, Wu D, Roein-Peikar M et al (2017) Notch-jagged complex structure implicates a catch bond in tuning ligand sensitivity. Science 355:1320–1324

Ma J, Meng Y, Kwiatkowski DJ, Chen X, Peng H, Sun Q et al (2010) Mammalian target of rapamycin regulates murine and human cell differentiation through STAT3/p63/Jagged/Notch cascade. J Clin Invest 120:103–114

Maekawa Y, Ishifune C, Tsukumo S, Hozumi K, Yagita H, Yasutomo K (2015) Notch controls the survival of memory CD4+ T cells by regulating glucose uptake. Nat Med 21:55–61

Man J, Yu X, Huang H, Zhou W, Xiang C, Huang H et al (2018) Hypoxic induction of vasorin regulates Notch1 turnover to maintain glioma stem-like cells. Cell Stem Cell 22:104–118.e6

Marambaud P, Shioi J, Serban G, Georgakopoulos A, Sarner S, Nagy V et al (2002) A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. EMBO J 21:1948–1956

Massard C, Michiels S, Ferté C, Le Deley M-C, Lacroix L, Hollebecque A et al (2017) High-throughput genomics and clinical outcome in hard-to-treat advanced cancers: results of the MOSCATO 01 trial. Cancer Discov 7:586. https://doi.org/10.1158/2159-8290.CD-16-1396

Massard C, Azaro A, Soria J-C, Lassen U, Le Tourneau C, Sarker D et al (2018) First-in-human study of LY3039478, an oral Notch signaling inhibitor in advanced or metastatic cancer. Ann Oncol 29:1911–1917

Matsuno K, Eastman D, Mitsiades T, Quinn AM, Carcanciu ML, Ordentlich P et al (1998) Human deltex is a conserved regulator of Notch signalling. Nat Genet 19:74–78

McAuliffe SM, Morgan SL, Wyant GA, Tran LT, Muto KW, Chen YS et al (2012) Targeting Notch, a key pathway for ovarian cancer stem cells, sensitizes tumors to platinum therapy. Proc Natl Acad Sci 109:E2939–E2948

McGill MA, Dho SE, Weinmaster G, McGlade CJ (2009) Numb regulates post-endocytic trafficking and degradation of Notch1. J Biol Chem 284:26427–26438

McMillan BJ, Zimmerman B, Egan ED, Lofgren M, Xu X, Hesser A et al (2017) Structure of human POFUT1, its requirement in ligand-independent oncogenic Notch signaling, and functional effects of Dowling-Degos mutations. Glycobiology 27:777–786

Meng L, Bai Z, He S, Mochizuki K, Liu Y, Purushe J et al (2016) The Notch ligand DLL4 defines a capability of human dendritic cells in regulating Th1 and Th17 differentiation. J Immunol 196:1070–1080

Mittal D, Young A, Stannard K, Yong M, Teng MWL, Allard B et al (2014) Antimetastatic effects of blocking PD-1 and the adenosine A2A receptor. Cancer Res 74:3652–3658

Molon B, Calì B, Viola A (2016) T cells and cancer: how metabolism shapes immunity. Front Immunol 7:20

Morgan TH (1917) The theory of the gene. Am Nat 51:513–544

Morgan KM, Fischer BS, Lee FY, Shah JJ, Bertino JR, Rosenfeld J et al (2017) Gamma secretase inhibition by BMS-906024 enhances efficacy of paclitaxel in lung adenocarcinoma. Mol Cancer Ther 16:2759–2769

Morsut L, Roybal KT, Xiong X, Gordley RM, Coyle SM, Thomson M et al (2016) Engineering customized cell sensing and response behaviors using synthetic Notch receptors. Cell 164:780–791

Mukherjee A, Veraksa A, Bauer A, Rosse C, Camonis J, Artavanis-Tsakonas S (2005) Regulation of Notch signalling by non-visual β-arrestin. Nat Cell Biol 7:1191–1201

Nandagopal N., Santat L. A. & Elowitz M. B. (2019) Cis-activation in the Notch signaling pathway. Elife 8:e37880

Nemetschke L, Knust E (2016) *Drosophila* Crumbs prevents ectopic Notch activation in developing wings by inhibiting ligand-independent endocytosis. Development 143:4543–4553

Ntziachristos P, Lim JS, Sage J, Aifantis I (2014) From fly wings to targeted cancer therapies: a centennial for notch signaling. Cancer Cell 25:318–334

O'Rourke CJ, Matter MS, Nepal C, Caetano-Oliveira R, Ton PT, Factor VM et al (2020) Identification of a pan-gamma-secretase inhibitor response signature for Notch-driven cholangiocarcinoma. Hepatology 71(1):196–213

Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashev D, Wong MKK et al (2006) A2A adenosine receptor protects tumors from antitumor T cells. Proc Natl Acad Sci U S A 103:13132–13137

Palmer WH, Jia D, Deng W-M (2014) Cis-interactions between Notch and its ligands block ligand-independent Notch activity. elife 3. https://doi.org/10.7554/eLife.04415

Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M et al (2007) Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med 13:1203–1210

Patel B, Patel J, Cho J-H, Manne S, Bonala S, Henske E et al (2016) Exosomes mediate the acquisition of the disease phenotypes by cells with normal genome in tuberous sclerosis complex. Oncogene 35:3027–3036

Pece S, Serresi M, Santolini E, Capra M, Hulleman E, Galimberti V et al (2004) Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. J Cell Biol 167:215–221

Pellom ST, Dudimah DF, Thounaojam MC, Uzhachenko RV, Singhal A, Richmond A et al (2017) Bortezomib augments lymphocyte stimulatory cytokine signaling in the tumor microenvironment to sustain CD8+T cell antitumor function. Oncotarget 8:8604–8621

Pikman Y, Alexe G, Roti G, Conway AS, Furman A, Lee ES et al (2017) Synergistic drug combinations with a CDK4/6 inhibitor in T-cell acute lymphoblastic leukemia. Clin Cancer Res 23:1012–1024

Platonova N, Manzo T, Mirandola L, Colombo M, Calzavara E, Vigolo E et al (2015) PI3K/AKT signaling inhibits NOTCH1 lysosome-mediated degradation. Genes Chromosom Cancer 54:516–526

Qiu M, Peng Q, Jiang I, Carroll C, Han G, Rymer I et al (2013) Specific inhibition of Notch1 signaling enhances the antitumor efficacy of chemotherapy in triple negative breast cancer through reduction of cancer stem cells. Cancer Lett 328:261–270

Ran Y, Hossain F, Pannuti A, Lessard CB, Ladd GZ, Jung JI et al (2017) γ-Secretase inhibitors in cancer clinical trials are pharmacologically and functionally distinct. EMBO Mol Med 9:950–966

Ridgway J, Zhang G, Wu Y, Stawicki S, Liang W-C, Chanthery Y et al (2006) Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature 444:1083–1087

Roti G, Carlton A, Ross KN, Markstein M, Pajcini K, Su AH et al (2013) Complementary genomic screens identify SERCA as a therapeutic target in NOTCH1 mutated cancer. Cancer Cell 23:390–405

Roybal KT, Rupp LJ, Morsut L, Walker WJ, McNally KA, Park JS et al (2016) Precision tumor recognition by T cells with combinatorial antigen-sensing circuits. Cell 164:770–779

Schneider M, Troost T, Grawe F, Martinez-Arias A, Klein T, Akbar MA et al (2013) Activation of Notch in lgd mutant cells requires the fusion of late endosomes with the lysosome. J Cell Sci 126:645–656

Schneider M, Kumar V, Nordstrøm LU, Feng L, Takeuchi H, Hao H et al (2018) Inhibition of Delta-induced Notch signaling using fucose analogs. Nat Chem Biol 14:65–71

Schott AF, Landis MD, Dontu G, Griffith KA, Layman RM, Krop I et al (2013) Preclinical and clinical studies of gamma secretase inhibitors with docetaxel on human breast tumors. Clin Cancer Res 19:1512–1524

Sestan N, Artavanis-Tsakonas S, Rakic P (1999) Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. Science 286:741–746

Shanker A, Pellom ST, Dudimah DF, Thounaojam MC, de Kluyver RL, Brooks AD et al (2015) Bortezomib improves adoptive T-cell therapy by sensitizing cancer cells to FasL cytotoxicity. Cancer Res 75:5260–5272

Sheldon H, Heikamp E, Turley H, Dragovic R, Thomas P, Oon CE et al (2010) New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. Blood 116:2385–2394

Shilo B, Sprinzak D (2017) The lipid-binding side of Notch ligands. EMBO J 36:2182–2183

Shimizu H, Woodcock SA, Wilkin MB, Trubenová B, NAM M, Baron M (2014) Compensatory flux changes within an endocytic trafficking network maintain thermal robustness of Notch signaling. Cell 157:1160–1174

Sierra RA, Thevenot P, Raber PL, Cui Y, Parsons C, Ochoa AC et al (2014) Rescue of Notch-1 signaling in antigen-specific CD8+ T cells overcomes tumor-induced T-cell suppression and enhances immunotherapy in cancer. Cancer Immunol Res 2:800–811

Sierra RA, Trillo-Tinoco J, Mohamed E, Yu L, Achyut BR, Arbab A et al (2017) Anti-jagged immunotherapy inhibits MDSCs and overcomes tumor-induced tolerance. Cancer Res 77:5628–5638

Song Y, Li L, Ou Y, Gao Z, Li E, Li X et al (2014) Identification of genomic alterations in oesophageal squamous cell cancer. Nature 509:91–95

Sorrentino C, Hossain F, Rodriguez PC, Sierra RA, Pannuti A, Osborne BA et al (2019) Adenosine A2A receptor stimulation inhibits TCR-induced Notch1 activation in CD8+T-cells. Front Immunol 10:1–12

Sprinzak D, Lakhanpal A, Lebon L, Santat LA, Fontes ME, Anderson GA et al (2010) Cis-interactions between Notch and Delta generate mutually exclusive signalling states. Nature 465:86–90

Srivastava S, Salter AI, Liggitt D, Yechan-Gunja S, Sarvothama M, Cooper K et al (2019) Logic-gated ROR1 chimeric antigen receptor expression rescues T cell-mediated toxicity to normal tissues and enables selective tumor targeting. Cancer Cell 35:489–503.e8

Steinbuck MP, Winandy S (2018) A review of Notch processing with new insights into ligand-independent Notch signaling in T-cells. Front Immunol 9:1230

Steinbuck MP, Arakcheeva K, Winandy S (2018) Novel TCR-mediated mechanisms of Notch activation and signaling. J Immunol 200:997–1007

Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A et al (2011) The mutational landscape of head and neck squamous cell carcinoma. Science 333:1157–1160

Stylianou S, Clarke RB, Brennan K (2006) Aberrant activation of Notch signaling in human breast cancer. Cancer Res 66:1517–1525

Suckling RJ, Korona B, Whiteman P, Chillakuri C, Holt L, Handford PA et al (2017) Structural and functional dissection of the interplay between lipid and Notch binding by human Notch ligands. EMBO J 36:2204–2215

Sugimoto K, Maekawa Y, Kitamura A, Nishida J, Koyanagi A, Yagita H et al (2010) Notch2 signaling is required for potent antitumor immunity in vivo. J Immunol 184:4673–4678

Tanis KQ, Podtelezhnikov AA, Blackman SC, Hing J, Railkar RA, Lunceford J et al (2016) An accessible pharmacodynamic transcriptional biomarker for Notch target engagement. Clin Pharmacol Ther 99:370–380

Tchekneva EE, Goruganthu MUL, Uzhachenko RV, Thomas PL, Antonucci A, Chekneva I et al (2019) Determinant roles of dendritic cell-expressed Notch Delta-like and Jagged ligands on anti-tumor T cell immunity. J Immunother Cancer 7:95

Thounaojam MC, Dudimah DF, Pellom ST, Uzhachenko RV, Carbone DP, Dikov MM et al (2015) Bortezomib enhances expression of effector molecules in anti-tumor CD8+ T lymphocytes by promoting Notch-nuclear factor-κB crosstalk. Oncotarget 6:32439–32455

Toda S, Blauch LR, Tang SKY, Morsut L, Lim WA (2018) Programming self-organizing multicellular

structures with synthetic cell-cell signaling. Science 361:156–162
Vaccari T, Lu H, Kanwar R, Fortini ME, Bilder D (2008) Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. J Cell Biol 180:755–762
Vaccari T, Duchi S, Cortese K, Tacchetti C, Bilder D (2010) The vacuolar ATPase is required for physiological as well as pathological activation of the Notch receptor. Development 137:1825–1832
Vijayan D, Young A, Teng MWL, Smyth MJ (2017) Targeting immunosuppressive adenosine in cancer. Nat Rev Cancer 17:709–724
Villalobos VM, Hall F, Jimeno A, Gore L, Kern K, Cesari R et al (2018) Long-term follow-up of Desmoid fibromatosis treated with PF-03084014, an oral gamma secretase inhibitor. Ann Surg Oncol 25:768–775
Waickman AT, Alme A, Senaldi L, Zarek PE, Horton M, Powell JD (2012) Enhancement of tumor immunotherapy by deletion of the A2A adenosine receptor. Cancer Immunol Immunother 61:917–926
Wang Q, Lu Q (2017) Plasma membrane-derived extracellular microvesicles mediate non-canonical intercellular NOTCH signaling. Nat Commun 8:709
Wang Y-C, He F, Feng F, Liu X-W, Dong G-Y, Qin H-Y et al (2010) Notch signaling determines the M1 versus M2 polarization of macrophages in antitumor immune responses. Cancer Res 70:4840–4849
Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D et al (2011) The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. Immunity 35:871–882
Wang Z, Hu Y, Xiao D, Wang J, Liu C, Xu Y et al (2017) Stabilization of Notch1 by the Hsp90 chaperone is crucial for T-cell leukemogenesis. Clin Cancer Res 23:3834–3846
Wharton KA, Johansen KM, Xu T, Artavanis-Tsakonas S (1985) Nucleotide sequence from the neurogenic locus Notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell 43:567–581
Wilkin MB, Carbery A-M, Fostier M, Aslam H, Mazaleyrat SL, Higgs J et al (2004) Regulation of notch endosomal sorting and signaling by Drosophila Nedd4 family proteins. Curr Biol 14:2237–2244
Wilkin M, Tongngok P, Gensch N, Clemence S, Motoki M, Yamada K et al (2008) Drosophila HOPS and AP-3 complex genes are required for a Deltex-regulated activation of notch in the endosomal trafficking pathway. Dev Cell 15:762–772
Willingham SB, Ho PY, Hotson A, Hill C, Piccione EC, Hsieh J et al (2018) A2AR antagonism with CPI-444 induces antitumor responses and augments efficacy to anti-PD-(L)1 and anti-CTLA-4 in preclinical models. Cancer Immunol Res 6:1136. https://doi.org/10.1158/2326-6066.CIR-18-0056
Xu J, Chi F, Guo T, Punj V, Lee WNP, French SW et al (2015) NOTCH reprograms mitochondrial metabolism for proinflammatory macrophage activation. J Clin Invest 125:1579–1590
Yamada K, Fuwa TJ, Ayukawa T, Tanaka T, Nakamura A, Wilkin MB et al (2011) Roles of Drosophila Deltex in Notch receptor endocytic trafficking and activation. Genes Cells 16:261–272
Yamamoto N, Yamamoto S, Inagaki F, Kawaichi M, Fukamizu A, Kishi N et al (2001) Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. J Biol Chem 276:45031–45040
Yan M (2011) Therapeutic promise and challenges of targeting DLL4/NOTCH1. Vasc Cell 3:17
Yao W, Shan Z, Gu A, Fu M, Shi Z, Wen W (2018) WW domain-mediated regulation and activation of E3 ubiquitin ligase Suppressor of Deltex. J Biol Chem 293:16697–16708
Yen WC, Fischer MM, Axelrod F, Bond C, Cain J, Cancilla B et al (2015) Targeting notch signaling with a Notch2/Notch3 antagonist (Tarextumab) inhibits tumor growth and decreases tumor-initiating cell frequency. Clin Cancer Res 21:2084–2095
Zhang J, Shao X, Sun H, Liu K, Ding Z, Chen J et al (2016) NUMB negatively regulates the epithelial-mesenchymal transition of triple-negative breast cancer by antagonizing Notch signaling. Oncotarget 7:61036–61053
Zhao Z-L, Zhang L, Huang C-F, Ma S-R, Bu L-L, Liu J-F et al (2016a) NOTCH1 inhibition enhances the efficacy of conventional chemotherapeutic agents by targeting head neck cancer stem cell. Sci Rep 6:24704
Zhao E, Maj T, Kryczek I, Li W, Wu K, Zhao L et al (2016b) Cancer mediates effector T cell dysfunction by targeting microRNAs and EZH2 via glycolysis restriction. Nat Immunol 17:95–103
Zhou R, Wang S, Wen H, Wang M, Wu M (2019) The bispecific antibody HB-32, blockade of both VEGF and DLL4 shows potent anti-angiogenic activity in vitro and anti-tumor activity in breast cancer xenograft models. Exp Cell Res 380:141–148