Introduction to Molecular Mechanisms in Notch Signal Transduction and Disease Pathogenesis

Benedetto Daniele Giaimo and Tilman Borggrefe

Abstract

The Notch signaling pathway plays a pivotal role in development, physiology and diseases such as cancer. In this chapter, we first give an overview of the different molecular mechanisms that regulate Notch signaling. Each subject is covered in more depth in the subsequent chapters of this book. Next, we will use the inflammatory system as an example to discuss the physiological function of Notch signaling. This is followed by a discussion of recent advances in the different pathophysiological roles of Notch signaling in leukemia as well as a wide range of solid cancers. Finally, we discuss how information about pathogenic mutations in Notch pathway components, combined with structural biological data, are beginning to provide important biological and mechanistic insights about the pathway.

Keywords

Notch · Transcription · Cancer · Inflammation

B. D. Giaimo (✉) · T. Borggrefe (✉)
Institute of Biochemistry, University of Giessen, Giessen, Germany
e-mail: Benedetto.Giaimo@biochemie.med.uni-giessen.de; Tilman.Borggrefe@biochemie.med.uni-giessen.de

Abbreviations

A	Alanine
ACC	Adenoid cystic carcinoma
ADAM	A Disintegrin And Metalloprotease
ANKs	Ankyrin
AML	Acute Myeloid Leukemia
AOS	Adams-Oliver Syndrome
ASCL1	Achaete-scute homolog 1
AVD	Aortic valve disease
AVS	Aortic valve stenosis
BAV	Bicuspid aortic valve
BCC	Basal cell carcinoma
BMDM	Bone marrow derived macrophages
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BRD4	BromoDomain-containing 4
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CDK9	Cyclin-dependent kinase 9
CLL	Chronic lymphocytic leukemia
COA	Coarctation of the aorta
CR	Cysteine-rich
CSCs	Cancer stem cells
cSCC	Cutaneous squamous cell carcinoma
DKO	Double knockout
DLL	DELTA-LIKE
DN-MAML	Dominant-Negative Mastermind
DSL	DELTA, SERRATE, LAG-2
E	Glutamic acid

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EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-Mesenchymal Transition
EP300	E1A Binding Protein P300
ER	Endoplasmic reticulum or estrogen receptor
ERK	Extracellular signal-Regulated Kinase
ETS1	E26 avian leukemia oncogene 1
F	Phenylalanine
FBXW7	F-box and WD repeat domain-containing 7
FOXA2	Forkhead box A2
G	Glycine
GABPA	GA Binding Protein Transcription Factor Alpha Subunit
GBM	Glioblastoma
GOF	Gain-Of-Function
GSI	γ-Secretase Inhibitor
HCC	Hepatocellular carcinoma
HCS	Hadju-Cheney syndrome
HD	Heterodimerization Domain
HDACs	Histone deacetylases
HER2	Human epidermal growth factor receptor 2
Hes1	Hairy and Enhancer of Split 1
HEY1	Hairy/enhancer-of-split related with YRPW motif 1
HLH	Helix-loop-helix
HLHS	Hypoplastic left heart syndrome
H&NHLHS	head and neck
I	Isoleucine
IκB	Inhibitor of kappa B
IKKα	Inhibitor of Kappa-B Kinase subunit alpha
IL1R	Interleukin 1 receptor
IL4	Interleukin 4
IL6	Interleukin 6
IL10	Interleukin 10
IL12	Interleukin 12
IL13	Interleukin 13
IFNγ	Interferon γ
IRAK2	Interleukin 1 receptor-associated kinase-like 2
IRF8	Interferon-regulatory factor 8
JAG	JAGGED
K	Lysine
KO	Knockout
KMT2D	Lysine methyltransferase 2D
L	Leucine
LNR	Lin-12/Notch Repeat
LOF	Loss-Of-function
LPS	Lipopolysaccharide
lSCC	Lung squamous cell carcinoma
MAML	MASTERMIND-LIKE
MAPK	Mitogen-activated protein kinase
MCL	Mantle cell lymphoma
MINT	Msx2-interating protein
miR	microRNA
MST	Mammalian sterile 20-like kinase
mTOR	mammalian target of rapamycin
Myc	myelocytomatosis proto-oncogene
nCC	Noncutaneous carcinoma
NCoR	Nuclear receptor corepressor
NECD	Notch extracellular domain
NEXT	Notch EXtracellular Truncation
NF-κB	Nuclear Factor-κB
NICD	Notch intracellular domain
NRR	Negative regulatory region
NSCLC	Non-small cell lung cancer
OLIG2	Oligodendrocyte transcription factor
P	Proline
PanIN	Pancreatic intraepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PDZ	PSD-95/Dlg/ZO-1
PEST	proline (P), glutamic acid (E), serine (S) and threonine (T)
PI3K	PhosphatidylInositol 4,5-bisphosphate 3-Kinase
PIM	Proto-Oncogene, Serine/Threonine Kinase
PIN1	Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1
POFUT1	Protein O-fucosyltransferase 1
PR	Progesterone receptor
Ptcra	invariant preTα chain of the pre-T cell receptor
PTEN	Phosphatase and tensin homolog

PTM	Post-translational modification
R	Arginine
RAM	RBPJ-associated module
RAS	Rat sarcoma virus oncogene
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
RCC	Renal cell cancer
RUNX1	Runt related transcription factor 1
RUNX3	Runt related transcription factor 3
S	Serine
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SHARP	SMRT/HDAC1 Associated Repressor Protein
SLE	Systemic lupus erythematosus
SMRT	Silencing mediator for retinoid or thyroid-hormone receptors
SMZL	Splenic marginal zone lymphoma
SPEN	Split ENds family transcriptional repressor
SPOC	Spen paralog and ortholog C-terminal domain
T	Threonine
T-ALL	T-cells acute lymphoblastc leukemia
TAD	Trans-activation domain
TAV	Tricuspid aortic valve
TCR	T-cell receptor
TGFβ	Transforming growth factor beta
TKO	Triple knockout
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TM	Transmembrane
TMZ	Temozolomide
TNBC	Triple-negative breast cancer
TNFα	Tumor necrosis factor alpha
TORC1/2	mTOR signaling complex 1/2
V	Valine
Vegfr1	Vascular endothelial growth factor receptor 1
YAP/TAZ	Yes-associated protein and WW domain containing transcription regulator 1
ZNF143	Zinc finger protein 143

1 Overview on Notch signaling

The Notch mutant phenotype was first described over a hundred years ago by John Dexter, who noticed the appearance of notches at the wing margins of fruit flies *Drosophila melanogaster*. Thomas Hunt Morgan identified the alleles of the corresponding genes (Morgan 1917). Several decades later, the *Notch* gene, encoding a transmembrane receptor controlling *Drosophila* neurogenesis, was identified (Artavanis-Tsakonas et al. 1983; Wharton et al. 1985; Kidd et al. 1986; del Amo et al. 1993). Soon after that, it became apparent that the *Notch* gene is evolutionary conserved and controls a plethora of developmental decisions, regulating homeostasis as well as development and differentiation of several different tissues and cell types during both embryonic and postnatal life. Thus, it is one of a few signaling pathways, like Wnt, transforming growth factor beta (TGFβ) and Hedgehog that is repeatedly used in multicellular organisms throughout embryonal and adult development. In Integration of *Drosophila* and Human Genetics to Understand Notch Signaling Related Diseases, Yamamoto and colleagues introduce how biological and genetic experiments in *Drosophila* contributed to the identification of key players in Notch signaling, and further discuss how mechanistic information obtained in flies can be translated to understand Notch signaling related genetic disorders in human. Notch signaling has also been implicated in carcinogenesis, of which we will highlight in this chapter and further dedicate several chapters in this book (The Notch3 Receptor and Its Intracellular Signaling-Dependent Oncogenic Mechanisms and Notch in Leukemia).

The mechanisms of how Notch signaling pathway regulates a wide range of functions can be grouped in three main categories: lateral inhibition, lateral induction and lineage decisions. During lateral inhibition, equipotent cells establish a hierarchy mediated by NOTCH receptors and ligands. During these signaling events, one cell “A” signals more to the adjacent ones preventing them to adopt the same “A” cell

fate. In the lateral induction model, non-equipotent cells are involved. In particular, one group of cells signals to another group determining the acquisition of different cell fates. Finally, in the lineage decision model, asymmetrical cell division allows daughter cells to adopt different cell fates by the differential expression and/or segregation of NOTCH receptors or modulators of the Notch pathway. These models are described in depth in "Modeling the Notch Response", "Integration of *Drosophila* and Human Genetics to Understand Notch Signaling Related Diseases", "Notch and Stem Cells" and "Notch and Senescence" of this book. T-cell differentiation is a well-characterized example of the lineage decision model that was investigated in depth. In particular, loss-of-function (LOF) of Notch leads to a complete block in T-cell development (Radtke et al. 1999), whereas gain-of-function (GOF) of Notch, by introducing a constitutive-active form of Notch into hematopoietic progenitor cells, leads to T-cells acute lymphoblastic leukemia (T-ALL) in mice (Pear et al. 1996). In fact, the human *NOTCH1* gene was identified in T-ALL patients as a hot spot of chromosomal translocations (Ellisen et al. 1991). The role of Notch in the early stages of T-cell development is discussed by Osborne and colleagues in "Notch and T Cell Function – A Complex Tale" of this book. Regarding pathogenesis, Chiang and colleagues discuss the aspects of Notch signaling in leukemogenesis (Notch in Leukemia) and Screpanti and colleagues focus on NOTCH3 related functions (The Notch3 Receptor and Its Intracellular Signaling-Dependent Oncogenic Mechanisms).

2 Molecular Mechanisms Controlling the Notch Signal Transduction Pathway

At the molecular level, the Notch signaling pathway is a seemingly simple pathway that does not involve any second messengers. Ligand-triggered activation of the NOTCH receptor leads to the release of the cleaved NOTCH intracellular domain (NICD) that drives the signaling response (Fig. 1). NOTCH receptors are single-pass transmembrane proteins that are synthesized in the endoplasmic reticulum (ER) and processed in the Golgi apparatus. During their maturation, NOTCH receptors are proteolytically processed by cleavage at the S1 site (Blaumueller et al. 1997; Logeat et al. 1998; Lake et al. 2009) and further post-translationally modified (discussed in detail in "Regulation of Notch Function by *O*-Glycosylation" of this book), producing the mature heterodimeric NOTCH receptor that is exposed on the plasma membrane. In mammals, four NOTCH receptors (NOTCH1-4) are expressed in a tissue- and cell-type specific manner. Mature NOTCH receptors consist of a NOTCH extracellular domain (NECD) and an intracellular portion (NICD) which are connected by a transmembrane (TM) domain. The NECD is characterized by epidermal growth factor (EGF)-like repeats that vary in number among the different isoforms, followed by three Lin-12/Notch repeats (LNR) and finally by a hydrophobic region required for the heterodimerization of the receptor. The LNR and heterodimerization (HD) domains form a negative regulatory region (NRR) that prevents ligand-independent cleavage of the receptor at the S2 cleavage site (Sanchez-Irizarry et al. 2004). The NOTCH-TM domain contains the S3 cleavage site which is the target of the γ-secretase complex that releases the NICD (Fortini 2002). The NICD is characterized by an N-terminal RBPJ (recombination signal binding protein for immunoglobulin kappa J region)-associated module (RAM) followed by ankyrin repeats (ANKs) that together form the RBPJ-interacting region (Tamura et al. 1995). These domains are followed by a transactivation domain (TAD) required for transcriptional activation and by a proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST)-rich domain involved in regulating the turnover of the NICD protein. It must be noted that the TAD is not conserved in all NOTCH proteins but it is specifically found within NOTCH1 and NOTCH2, suggesting different mechanisms of transcriptional activation used by the different NOTCH proteins.

Similar to NOTCH receptors, the NOTCH ligands are single-pass transmembrane proteins. They are members of two different families: the DELTA/DELTA-LIKE and the

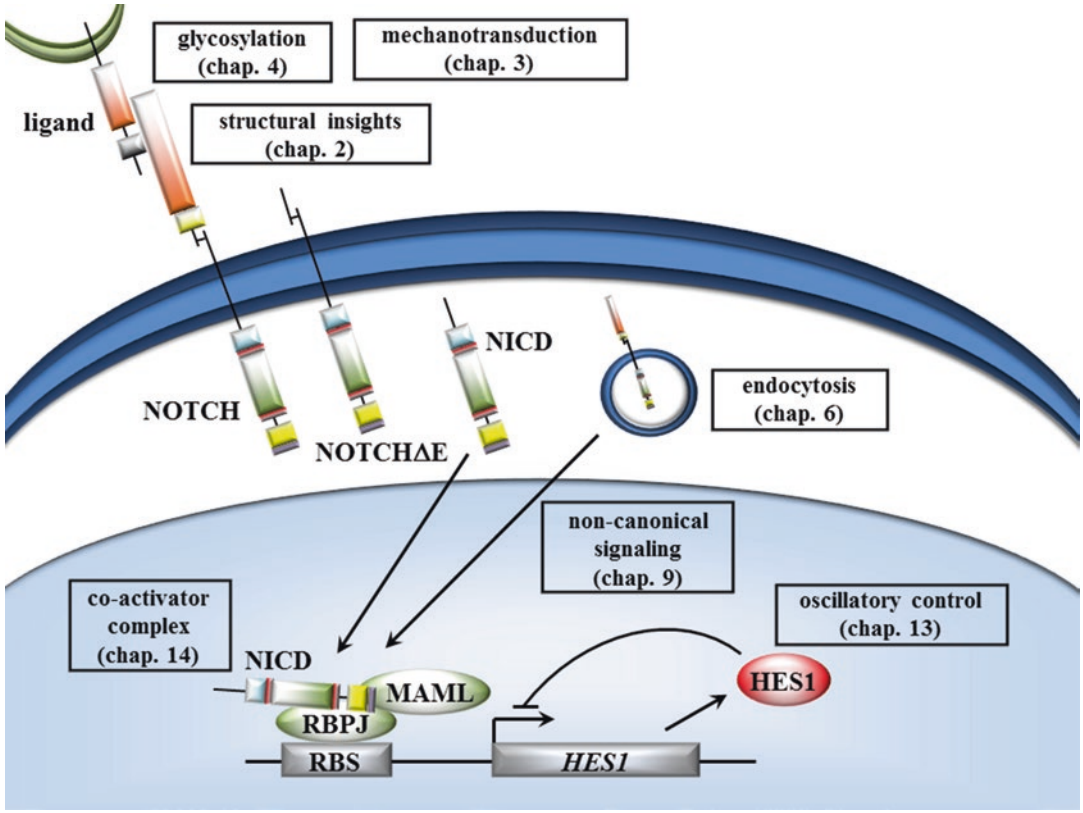

Fig. 1 Overview of the Notch signaling cascade. Ligand binding to NOTCH receptor leads to proteolysis-dependent release of the NOTCH intracellular domain (NICD). Structural aspects of the ligand/receptor interaction are discussed in "Structural Insights into Notch Receptor-Ligand Interactions" whereas mechanotransduction of the signal and glycosylation of the NOTCH receptor are discussed in "The Molecular Mechanism of Notch Activation" and "Regulation of Notch Function by *O*-Glycosylation", respectively. The first cleavage, mediated by ADAM metalloproteases, generates an intermediate proteolytic product called NEXT (Notch EXtracellular Truncation) which is substrate for a γ-secretase complex that releases the NICD. The NICD subqsequently translocates into the nucleus where it interacts with the transcription factor RBPJ and cofactor MAML leading to activation of Notch target genes (see "CSL-Associated Corepressor and Coactivator Complexes" in this book). Several Notch target genes are involved in feedback regulation of the Notch pathway, as in the case of *HES1* which also regulates its own expression leading to an oscillatory control (see Oscillatory Control of Notch Signaling in Development" in this book). The Notch pathway is also regulated by endocytosis and vesicle trafficking of the NOTCH receptor (see "Endocytic Trafficking of the Notch Receptor" in this book) which can lead to degradation or ligand independent activation of the pathway (see "Mechanisms of Non-canonical Signaling in Health and Disease: Diversity to Take Therapy up a Notch?" in this book)

SERRATE/JAGGED families. The *Drosophila* genome encodes one member of each family (*Delta* and *Serrate*) while mammalian ligands are more complex as three members of the DELTA family [DELTA-LIKE (DLL) 1, 3 and 4] and two members of the JAGGED family (JAG1 and 2) are encoded. All NOTCH ligands present with a DSL (DELTA, SERRATE, LAG-2) domain that contains the NOTCH receptor-interacting region followed by EGF repeats which vary in number among the different members of the families (Parks et al. 2006; D'Souza et al. 2008). Compared to the DELTA family, only the JAGGED family presents a cysteine-rich (CR) region proximal to the TM domain. Additionally, the intracellular domain of some Notch ligands is characterized by a PDZ (PSD-95/Dlg/ZO-1) domain that supports interactions with proteins of the adherens junctions (Mizuhara et al. 2005).

A major breakthrough in the Notch field was the recent elucidation of the molecular structure of the NOTCH/ligand complex (see "Structural Insights into Notch Receptor-Ligand Interactions" of this book). Genetic and biochemical studies already revealed that Notch receptor glycosylation is pivotal for its function. Reassuringly, the structures showed that sugars are in the midst of the receptor/ligand structure. This aspect and the complex regulation by NOTCH glycosylation are discussed in "Regulation of Notch Function by *O*-Glycosylation". In addition to glycosylation, the exact molecular mechanisms of receptor/ligand interactions and the signal triggering mechanisms are discussed in "Structural Insights into Notch Receptor-Ligand Interactions" by Handford and colleagues considering the lipid environment and in "The Molecular Mechanism of Notch Activation" by Gordon and colleagues considering mechano-transduction and pulling-forces between two adjacent cells that express NOTCH ligand and NOTCH receptor.

The signaling cascade activated upon ligand binding is remarkably simple; in fact, two consecutive proteolytic cleavages of the NOTCH receptor release the NICD from the membrane. First, ADAM (a disintegrin and metalloprotease) metalloproteases (Brou et al. 2000; Mumm et al. 2000) cleave off the majority of the NECD; this is known as the S2 cleavage. Subsequently, the intracellular domain of the remaining Notch receptor (NICD) is liberated by an intramembrane cleavage mediated by the γ-secretase complex, a process known as S3 cleavage. The intricate regulation of receptor cleavage and endocytic trafficking as part of this process is discussed in detail by Klein and colleagues in "Endocytic Trafficking of the Notch Receptor" of this book. Upon activation, the NICD translocates into the nucleus, associates with transcription factor RBPJ and activates the expression of Notch target genes (Fig. 1). Pivotal cofactors within the RBPJ/NICD complex are MASTERMIND-LIKE (MAML) proteins which are required for the complex to be fully functional (Wu et al. 2000; Lin et al. 2002; Wu et al. 2002; Nam et al. 2003; Nam et al. 2006; Wilson and Kovall 2006); this trimeric complex recruits several additional coactivators such as acetyltransferase EP300 [E1A Binding Protein P300, (Oswald et al. 2001; Hansson et al. 2009; Jung et al. 2013)]. This is known as the canonical pathway of Notch activation and these nuclear events are discussed in "CSL-Associated Corepressor and Coactivator Complexes". Regarding non-canonical Notch signaling, which is represented for example by RBPJ-independent events, Vaccari and colleagues elucidate these aspects of Notch signaling in "Mechanisms of Non-canonical Signaling in Health and Disease: Diversity to Take Therapy up a Notch?". Interestingly, the protein half-life of the NICD is pivotal for amplitude and duration of the Notch response. Several post-translational modifications (PTMs) of the NICD, such as phosphorylation, acetylation and methylation are key in this process, and they culminate in the ubiquitin-dependent proteasomal degradation of the NICD, thereby terminating the Notch response (Fryer et al. 2002; Fryer et al. 2004; Palermo et al. 2012; Hein et al. 2015; Borggrefe et al. 2016). This is particularly relevant in pathophysiological conditions such as leukemogenesis. Here, stabilizing NOTCH mutations are found in several leukemias, such as T-ALL and chronic lymphocytic leukemia (CLL). Additionally, the NOTCH ubiquitin-ligase FBXW7 (F-box and WD repeat domain-containing 7) is frequently mutated in leukemia patients. The interested reader is referred to our recent review (Borggrefe et al. 2016) as well as "The Notch3 Receptor and Its Intracellular Signaling-Dependent Oncogenic Mechanisms" and "Notch in Leukemia" of this book.

In the absence of a Notch signal, the central transcription factor RBPJ remains in the nucleus bound to its target nucleotide sequence and recruits corepressors to prevent the expression of Notch target genes. In the last few years several groups including our have set out to characterize the composition of the RBPJ corepressor and coactivator complexes (Oswald et al. 2001; Hansson et al. 2009; Jung et al. 2013; Kao et al. 1998; Oswald et al. 2002; Oswald et al. 2005; Salat et al. 2008; Borggrefe and Oswald 2009; Moshkin et al. 2009; Liefke et al. 2010; Mulligan

et al. 2011; Yatim et al. 2012; Oswald et al. 2016; Xu et al. 2017 and "The Notch Interactome: Complexity in Signaling Circuitry" and "Oscillatory Control of Notch Signaling in Development" of this book) and to unveil their structure (Nam et al. 2003; Nam et al. 2006; Wilson and Kovall 2006; Kovall and Hendrickson 2004; Kovall 2007; VanderWielen et al. 2011; Collins et al. 2014; Contreras et al. 2015; Yuan et al. 2016 and "Structural Insights into Notch Receptor-Ligand Interactions" of this book). These studies resulted in two important findings: First, the Notch signaling pathway is not based on a simple ON/OFF-state concerning Notch target gene expression; second, the individual RBPJ/NICD complex does not operate alone but functions as homodimer and may collaborate with other DNA binding proteins. The first observation is supported by the characterization of the protein interaction network of SHARP [SMRT (silencing mediator for retinoid or thyroid-hormone receptors)/HDAC1 (histone deacetylase)-associated repressor protein; also known as mouse MINT (Msx2-interating protein) or SPEN (Split ENds family transcriptional repressor)] which, focusing on its SPOC (Spen paralog and ortholog C-terminal) domain, unveiled an interesting and surprising scenario (Oswald et al. 2016). In fact, while previously SHARP was exclusively considered as a transcriptional repressor (Oswald et al. 2002; Oswald et al. 2005; Salat et al. 2008), proteomics studies revealed that SHARP does not exclusively interact with the corepressor NCoR (nuclear receptor corepressor) complex but also with the coactivator KMT2D (lysine methyltransferase 2D) complex (Oswald et al. 2016). These observations identified SHARP as a key regulator of the Notch pathway where NCoR and KMT2D compete for the same binding site of SHARP (Oswald et al. 2016). Thus, it is likely that SHARP is a central chromatin regulator tuning the output of the Notch response by balancing histone methylation and deacetylation.

The second observation is based on the identification of NICD homodimers that are required to specifically induce a subset of Notch target genes such as *Hes1* (hairy and enhancer of split 1), *Myc* (myelocytomatosis proto-oncogene) and *Ptcra* [invariant preTα chain of the pre-TCR (T-cell receptor)] that are characterized by paired RBPJ binding sites oriented and spaced in a specific manner (Nam et al. 2007; Liu et al. 2010; Hass et al. 2015). Additionally, genome-wide studies unveiled that NOTCH1 and RBPJ binding occurs at sites that are also bound by additional transcription factors such as AML1 [acute myeloid leukemia 1, also known as RUNX1 (Runt related transcription factor 1)], ETS1 (E26 avian leukemia oncogene 1), GABPA (GA binding protein transcription factor alpha subunit) and ZNF143 [Zinc finger protein 143, (Wang et al. 2011a; Ngondo-Mbongo et al. 2013; Wang et al. 2014)], suggesting that several transcriptional factors synergize to fine-tune the expression of Notch target genes. Alternatively, competitive binding may have different transcriptional outputs in regard to the expression of Notch target genes.

Apart from chromatin regulation prior to the Notch response and combinatorial activities of several transcription factors, differential gene regulation is achieved by different promoter structures and feedback loops, which can result in oscillatory mechanisms that play key roles in development (Fig. 1). One particularly well-studied example is the basic helix-loop-helix (HLH) transcription factor HES1, encoded by a prototypic Notch target gene. Kageyama and colleagues discuss in depth the function of HES1 in "Oscillatory Control of Notch Signaling in Development" of this book.

3 Notch in Inflammation

Notch signaling has been shown to play important roles in both innate and adaptive immunity. In innate immunity, Notch signaling promotes the differentiation of specific cell types as well as supports the activation of specific cells. Macrophages are key mediators of innate immunity but are also involved in supporting specific aspects of the adaptive immunity. Based on the activating stimulus, macrophages polarize into so called M1 or M2 states: while M1 macrophages are involved in supporting inflammatory responses by producing inflammatory molecules such as interleukin 12 (IL12), IL6 or tumor

necrosis factor alpha (TNFα), M2 macrophages regulate the resolution of inflammation by producing anti-inflammatory molecules such as IL10 or TGFβ (Porta et al. 2015; Kapellos and Iqbal 2016; Patel et al. 2017). Polarized macrophages can be further distinguished in M2a, M2b or M2c based on the different gene expression profile and the activating stimulus, for example IL4 and IL13 induce the M2a phenotype, the M2b is induced by exposure to immune complexes with Toll-like receptors (TLRs) or interleukin 1 receptor (IL1R) while M2c is induced by IL10 (Mantovani et al. 2004).

In bone marrow derived macrophages (BMDMs) from *Rbpj* conditional knockout (KO) mice (*Rbpj*$^{flox/flox}$*;Mx1-Cre*) the expression of lipopolysaccharide (LPS)-induced genes is inhibited (Xu et al. 2012). RBPJ positively regulates LPS-mediated transcription via the canonical Notch signaling pathway as treatment with inhibitors of the γ-secretase complex (GSIs), that block the activation of the Notch pathway, *Adam10* or *Notch1* deficiencies impair gene expression of LPS targets (Xu et al. 2012). Mechanistically RBPJ controls the expression of IRAK2 (interleukin-1 receptor-associated kinase-like 2) protein that supports a cascade that culminates with the synthesis of IRF8 (interferon-regulatory factor 8) (Xu et al. 2012), a key transcription factor of the inflammatory gene expression program (Mancino et al. 2015). The control of this program in macrophages is more complex as LPS treatment also leads to upregulation of Notch target genes, such as *HES1* and *HEY1* (*Hairy/enhancer-of-split related with YRPW motif 1*), which are involved in a negative feedback loop that controls the expression of pro-inflammatory cytokines (Hu et al. 2008). Importantly, treatment with interferon γ (IFNγ) leads to downregulation of *HES1* and *HEY1* gene expression. This suggests a mechanism how IFNγ may augment the production of pro-inflammatory cytokines (Hu et al. 2008). As these studies pointed out to the RBPJ-dependent induction of *Il12* gene upon LPS stimulation (Xu et al. 2012; Hu et al. 2008), another study could demonstrate that this effect does not involve the transcriptional activity of the NICD/RBPJ complex as overexpression of a dominant negative form of MAML (DN-MAML) does not influence the expression of *Il12* in BMDMs (Boonyatecha et al. 2012). The reasons for this contrasting results are still not clear but it must be noted that another study could show that the pro-inflammatory cytokine IL6 is positively and directly regulated by the Notch signaling pathway upon treatment of BMDMs with LPS and IFNγ. In fact, *Il6* expression is downregulated by GSIs and upregulated by overexpression of NICD1 upon LPS and IFNγ treatment and finally the *Il6* locus is bound by NOTCH1 (Wongchana and Palaga 2012). Fung and colleagues observed that *NOTCH3* expression increases during differentiation of human monocytes into macrophages in culture, while *DLL4* expression increases upon pro-inflammatory stimulation of human macrophages (Fung et al. 2007). Of note, the LPS-mediated *DLL4* induction is dependent on TLR4 (Toll-like receptor 4) and NF-κB (nuclear factor-κB) pathways and triggers the Notch signaling cascade that finally increases the pro-inflammatory properties of human macrophages (Fung et al. 2007). Similarly, also *JAG1* is induced upon LPS stimulation of human macrophages in an NF-κB-dependent manner (Foldi et al. 2010) as well as *Notch1* induction is observed upon macrophages activation and GSIs pretreatment leads to reduced expression of pro-inflammatory genes upon stimulation with LPS and IFNγ (Palaga et al. 2008), suggesting Notch signaling as an important determinant of macrophages-mediated inflammatory responses. Myeloid-specific LOF of *Notch1*, obtained from *LysMCre;Notch1*$^{flox/flox}$ mice, leads to decreased macrophages recruitment at wounds as well as GSIs treatment results in failure of *Vegfr1* (vascular endothelial growth factor receptor 1) induction upon macrophages stimulation with LPS and INFγ (Outtz et al. 2010).

In peritoneal macrophages, Notch signaling determines a switch from pro-inflammatory cytokines (TNFα and IL6) to anti-inflammatory cytokines (IL10) production upon stimulation with LPS in a way that is dependent on the PEST domain of NICD proteins (Zhang et al. 2012). This pro-inflammatory inhibitory effect of Notch signaling is based on the inhibition of the MAPK

(mitogen-activated protein kinase) pathway leading to reduced transcriptional activity of NF-κB (Zhang et al. 2012). In contrast, another study observed that Notch signaling increases pro-inflammatory properties of macrophage derived Raw 264.7 cells upon LPS stimulation by promoting nuclear translocation of NF-κB (Monsalve et al. 2009). The reasons for the differences observed in these studies are not clear and more work is required to better dissect the role of Notch signaling upon LPS stimulation in these cells.

RBPJ controls also the M2 polarization of macrophages as RBPJ KO macrophages from *Rbpj*$^{flox/flox}$*;Lyz2-Cre* mice treated with chitin, a major structural component of fungi and helminthes that induce the M2 polarization, present impaired expression of genes associated with the M2 phenotype (Foldi et al. 2016). It must also be noted that *Rbpj* KO results in M2 polarization of BMDM upon LPS stimulation (Wang et al. 2010a), suggesting that RBPJ may play different roles in the M1 vs M2 polarization based on the activating stimulus. Additionally, stimulation of macrophages with IL4, an interleukin that drives the M2 polarization, leads to upregulation of *Jag1* (Outtz et al. 2010).

Interestingly, in a mouse model of systemic lupus erythematosus (SLE), Notch signaling is required to induce macrophage polarization versus the M2b phenotype through PI3K (phosphatidylInositol 4,5-bisphosphate 3-kinase)/AKT-ERK (Extracellular signal-regulated kinase)-1/2 and p38 MAPK signaling pathways (Zhang et al. 2010).

In summary, an important role for Notch in inflammation is evident, but further studies are required to differentiate between direct and indirect effects and to clarify how the Notch pathway orchestrates different polarization of macrophages.

4 Dysregulation of Notch Signaling in Diseases

Accurate regulation of the Notch signaling pathway is required for development, differentiation and homeostasis of a wide variety of tissues during both adult and embryonic life (see "The Notch3 Receptor and Its Intracellular Signaling-Dependent Oncogenic Mechanisms", "Notch and Neurogenesis", "Notch and Stem Cells", "Notch and Senescence", "Control of Blood Vessel Formation by Notch Signaling" and "Notch and T Cell Function – A Complex Tale" in this book) and dysregulation of Notch signaling is associated with many diseases (see "Integration of *Drosophila* and Human Genetics to Understand Notch Signaling Related Diseases", "Mechanisms of Non-canonical Signaling in Health and Disease: Diversity to Take Therapy up a Notch?", "The Notch3 Receptor and Its Intracellular Signaling-Dependent Oncogenic Mechanisms", "Notch and Senescence", "Control of Blood Vessel Formation by Notch Signaling" and "Notch in Leukemia" in this book).

Notch signaling has been associated with several congenital disorders, for example Notch LOF has been linked to Alagille and Adams-Oliver syndromes (AOS) whereas Notch GOF results in Hadju-Cheney syndrome [HCS, see "Integration of *Drosophila* and Human Genetics to Understand Notch Signaling Related Diseases" in this book and Masek and Andersson 2017]. In addition, missense mutations that affect the structure of NOTCH receptors have been found in genetic diseases. For example, *NOTCH3* mutations that affect specific domains of the NECD have been linked to CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy). There are several instances where somatic mutations of *NOTCH* or Notch pathway components or modulators lead to cancer. We will briefly discuss the current knowledge about Notch signaling in cancer and the interested reader is referred to "The Notch3 Receptor and Its Intracellular Signaling-Dependent Oncogenic Mechanisms" and "Notch in Leukemia" of this book and other recent reviews (Aster et al. 2017).

4.1 Notch in Leukemia

In 1991, recurring mutations in the *NOTCH1* gene were first described in patients with T-ALL,

thus implicating Notch signaling directly in leukemogenesis (Weng et al. 2004). Those mutations lead to a C-terminal truncation of the intracellular NOTCH1 protein, thereby removing the destabilizing PEST domain and leading to increased NICD1 half-life (Weng et al. 2004). Other *NOTCH1* activating mutations in T-ALL have also been identified in the NECD leading to constitutive cleavage of the NOTCH receptor (Weng et al. 2004). Similar activating *NOTCH1* mutations were also identified in CLL (Puente et al. 2011), in line with previous data showing activation of the Notch pathway in CLL (Rosati et al. 2009). These data suggest Notch signaling as a potential therapeutic target in the treatment of T-ALL and CLL and lead to some clinical trials in the last years.

GSIs can be used to prevent the activation of the Notch pathway by blocking the release of the NICD from the membrane. However, this approach is unfortunately limited due to two reasons: 1) GSIs cause severe gastrointestinal side effects due to the important role of Notch signaling in differentiation of the highly proliferating gut epithelium; 2) Drug resistance to GSIs also fairly frequently arises and it is associated with mutational loss of *PTEN* [phosphatase and tensin homolog, (Palomero et al. 2007)] or *FBXW7* (O'Neil et al. 2007) and dependent on BRD4 [bromodomain-containing protein 4, (Knoechel et al. 2014)] as well as on miR (microRNA)-223 (Kumar et al. 2014). The problems encountered with the clinical use of GSIs pointed out the need for a better dissection of the molecular mechanisms that define the Notch signaling response with the final goal to identify additional potential therapeutic targets to block Notch signaling or its oncogenic target genes. This will be of benefit not exclusively for T-ALL and CLL as aberrant Notch signaling is also observed in acute myeloid leukemia [AML, (Thiel et al. 2017)], mantle cell lymphoma [MCL, (Kridel et al. 2012)] and splenic marginal zone lymphoma [SMZL, (Rossi et al. 2012)].

4.2 Notch in Solid Tumors

NOTCH1 was originally identified as an oncogene in leukemia but surprisingly *NOTCH* genes have also been found to have tumor suppressive roles in other contexts (Table 1). In this section we will discuss the different functions of Notch signaling in different types of solid tumors.

4.2.1 Notch in Glioblastoma

Glioblastoma (GBM) represents one of the most aggressive forms of brain tumor and the Notch signaling pathway has been implicated in the molecular pathogenesis of gliomas. NOTCH1 receptor as well as JAG1 and DLL1 ligands are upregulated in GBM cell lines and in primary human gliomas and their knockdown results in decreased luciferase activity, using a Notch-dependent reporter assay (Purow et al. 2005). When human cell lines, transfected with *NOTCH1* siRNAs, were intracranially injected into recipient mice, an increased survival was observed compared to controls (Purow et al. 2005). In line with these observations, GSIs treatment of GBM neurospheres reduces their proliferation while overexpression of an active form of NOTCH2 has the opposite effect (Fan et al. 2010). This phenotype is linked to cancer stem cells (CSCs), as GSIs treatment downregulates the expression of CSCs markers such as CD133, NESTIN, BMI1 (B lymphoma Mo-MLV insertion region 1 homolog) and OLIG2 (oligodendrocyte transcription factor 2). The most striking observation is that GSIs treatment reduces the mortality in mouse models (Fan et al. 2010), suggesting Notch signaling as a good candidate for therapeutic intervention. Even if GSIs lead to increased apoptosis of GBM neurosphere cells, as revealed by increased cleaved CASPASE-3 (Fan et al. 2010), the molecular mechanisms behind are poorly defined. Similarly, expression of DN-MAML reduces the proliferation of GBM cells but the same study pointed out to a cell type-specific dependence on different NOTCH receptors (Chen et al. 2010). Given the poor outcomes of GSIs in clinical applications, it will be important to identify additional targets that may be used to modulate the Notch pathway. One of this targets is potentially RBPJ which is upregulated in brain CSCs (Xie et al. 2016). Knockdown of RBPJ in CSCs has a stronger effect compared to GSIs in term of proliferation and it significantly increases

Table 1 Mutations in FBXW7, NOTCH1 or RBPJ associated with tumors and/or genetic diseases. The list includes insertions, deletions, missense and nonsense mutations. ACC adenoid cystic carcinoma; AOS: Adams-Oliver syndrome; AVD: aortic valve disease; AVS: aortic valve stenosis; BAV: bicuspid aortic valve; CLL: B-cells chronic lymphocytic leukemia; COA: coarctation of the aorta; cSCC: cutaneous squamous cell carcinoma; HLHS: hypoplastic left heart syndrome; H&N: head and neck; lSCC: lung squamous cell carcinoma; MCL: mantle cell lymphoma; nCC: noncutaneous carcinoma; RCC: renal cell cancer; SCLC: small cell lung cancer; SMZL: splenic marginal zone lymphoma; T-ALL: T-cells acute lymhoblastic leukemia; TAV: tricuspid aortic valve; TNBC: triple-negative breast cancer

Gene	Domain	Disease	Reference
FBXW7	F-box	Melanoma, SCLC	George et al. (2015) and Aydin et al. (2014)
	WD40 repeats	Melanoma, SCLC, T-ALL	George et al. (2015), Aydin et al. (2014) and Larson-Gedman et al. (2009)
NOTCH1	EGF repeats	ACC, AOS, AVD, AVS, BAV, bladder cancer, breast cancer (TNBC), COA, cSCC, HLHS, lSCC, MCL, nCC, SCLC, TAV	George et al. (2015), Iascone et al. (2012), Wang et al. (2011b), Foffa et al. (2013), Mohamed et al. (2006), McBride et al. (2008), Kridel et al. (2012), Kent et al. (2013), Ducharme et al. (2013), Garg et al. (2005), Wang et al. (2015), McKellar et al. (2007), Stittrich et al. (2014), Stoeck et al. (2014), Southgate et al. (2015) and Rampias et al. (2014)
	LNR repeats	AOS, AVD, bladder cancer, breast cancer (luminal B), breast cancer (TNBC), cSCC, endometrial cancer, HLHS, RCC	Iascone et al. (2012), Wang et al. (2011b), Garg et al. (2005),Wang et al. (2015), Stittrich et al. (2014), Stoeck et al. (2014), Southgate et al. (2015) and Rampias et al. (2014)
	HD domain	ACC, AOS, AVS, BAV, bladder cancer, breast cancer (TNBC), cervical adenocarcinoma, colon adenocarcinoma, cSCC, glioblastoma, H&N, MCL, melanoma, neuroendocrine carcinoma, pancreatic cancer, SCLC, T-ALL	George et al. (2015), Larson-Gedman et al. (2009), Wang et al. (2011b), Foffa et al. (2013), McBride et al. (2008), Kridel et al. (2012), Wang et al. (2015), Stoeck et al. (2014), Southgate et al. (2015), Rampias et al. (2014), Weng et al. (2004), Breit et al. (2006), Malecki et al. (2006), Zhu et al. (2006), Mansour et al. (2006), Mansour et al. (2007) and De Keersmaecker et al. (2008)
	RAM domain	BAV, cSCC	Wang et al. (2011b) and Mohamed et al. (2006)
	ANK repeats	AOS, bladder cancer, cSCC, SCLC, T-ALL	George et al. (2015), Wang et al. (2011b), Stittrich et al. (2014) , Southgate et al. (2015), Rampias et al. (2014) and Zhu et al. (2006)
	TAD/ PEST domain	BAV, breast cancer (ER^+, PR^+, $HER2^+$), breast cancer (TNBC), CLL, COA, MCL, SCLC, SMZL, T-ALL	George et al. (2015), Larson-Gedman et al. (2009), McBride et al. (2008), Kridel et al. (2012), Wang et al. (2015), Stoeck et al. (2014), Weng et al. (2004), Breit et al. (2006), Zhu et al. (2006), Mansour et al. (2006), De Keersmaecker et al. (2008), Rossi et al. (2012), Puente et al. (2011), Bea et al. (2013), Bittolo et al. (2017), D'Agaro et al. (2017), Fabbri et al. (2011), Pozzo et al. (2017) and Pozzo et al. (2016)
RBPJ	NTD	AOS	Hassed et al. (2012)

the life-span of tumor-bearing hosts (Xie et al. 2016). The differences between GSIs treatment and RBPJ knockdown depend on the fact that RBPJ regulates also a Notch-independent transcriptional program and the effect of RBPJ is based on its interaction with CDK9 (cyclin-dependent kinase 9) to support transcriptional elongation (Xie et al. 2016). It must be also noted that a difference in regard to Notch activity in different GBMs cannot be excluded and that this difference is likely dependent on the *P53* status; in fact, cells with a mutated *P53* background seem to be more sensitive to Notch inhibition compared to cells with a wild type *P53* background (Chen et al. 2010). In line with that, *P53* wild type GBM cells present with low Notch activity as revealed by GSI treatment and DN-MAML overexpression (Xu et al. 2017).

Gliomas are usually treated by surgical intervention aimed to remove the tumor mass followed by radiotherapy and chemotherapy but, while an initial response to radiotherapy is visible, gliomas are refractory (Grossman and Batara 2004; Furnari et al. 2007), probably associated to radiation resistance of CSCs (Bao et al. 2006). GSIs treatment increases the sensitivity of glioma stem cells to clinical doses of radiation while GOF of active forms of NOTCH1 or NOTCH2 protects them from apoptosis upon radiation (Wang et al. 2010b). Importantly, when CSCs were subjected to NOTCH1 or NOTCH2 knockdown before radiation, they showed a reduced tumorigenic activity in mouse models (Wang et al. 2010b), suggesting that a combined therapy, based on radiotherapy and GSIs may be used as a therapeutic approach. In line with these data, Gilbert and colleagues could show that GSIs treatment significantly reduces the recovery of neurospheres treated with Temozolomide (TMZ), a chemotherapeutic agent used to treat gliomas (Gilbert et al. 2010). Given that the neurospheres number was reduced only when TMZ was added before GSIs, one can imagine that Notch signaling, in gliomas, is a mechanism that is activated as part of a resistance upon chemotherapy. Additionally, Gilbert and colleagues could show that combined TMZ and GSIs treatment reduces tumorigenicity in mouse models (Gilbert et al. 2010).

In summary, although the oncogenic role of Notch is clear, Notch inhibition alone remains ineffective in therapeutic terms. Thus, a combination therapy seems to be highly desirable and targeting the CSCs or preventing the tumor plasticity may lead the way.

4.2.2 Notch in Breast Cancer

Interestingly, Notch signaling has been linked to the triple-negative breast cancer (TNBC), negative for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Foulkes et al. 2010). Similar to the subset of mutations identified in leukemia, Notch activating mutations are found in TNBC at the C-terminal PEST domain of NOTCH1, NOTCH2 and NOTCH3 (Wang et al. 2015). The prolyl-isomerase PIN1 (Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1) is a positive regulator of the Notch signaling pathway (Rustighi et al. 2009) and it supports Notch signaling in TNBC cells by antagonizing the FBXW7-dependent degradation of NICD1 and NICD4 (Rustighi et al. 2014). Overexpression of NUMB, a negative regulator of the Notch signaling pathway, in TNBC cells reduces the epithelial-mesenchymal transition (EMT), a process associated with cancer progression and metastasis and suppresses tumor growth in xenografts mouse models (Zhang et al. 2016a). In line with these observations, NUMB expression is lost in several breast cancer cell lines including lines established from TNBC (Stylianou et al. 2006), as well as in primary samples, leading to increased Notch signaling (Pece et al. 2004).

Mechanistically, Notch signaling regulates cell proliferation in TNBC by directly modulating the expression of CYCLIN D1 (encoded by the *CCND1* gene). In fact, NOTCH1 binds to the *CCND1* locus and LOF of the Notch ligand JAG1 leads to downregulation of *CCND1* associated with cell cycle defects (Cohen et al. 2010).

TNBC frequently presents with alterations in the PI3K/AKT/mTOR (mammalian target of rapamycin) pathway (Lehmann et al. 2011; Banerji et al. 2012; Cancer Genome Atlas 2012) but pharmacological inhibition of this pathway proved to be ineffective. Recently, Bhola and col-

leagues showed that inhibition of PI3K/mTOR or TORC1/2 (mTOR signaling complex 1/2) in TNBC cells enriches for CSCs and leads to increased expression of NICD1 and JAG1 as well as increased Notch activity (Bhola et al. 2016). Importantly, inhibition of Notch signaling decreases the induction of CSCs upon PI3K/mTOR or TORC1/2 inhibition (Bhola et al. 2016), suggesting a possible combined therapy. In line with this, monoclonal antibodies that prevent Notch signaling activation can reduce tumor growth of TNBC xenografts and increase the efficacy of the chemotherapeutic agent docetaxel in mice (Qiu et al. 2013).

In MCF7 cells (ER^+ PR^+ $HER2^-$), Notch controls a metabolic switch involved in tumorigenesis (Landor et al. 2011). Mechanistically, this process is controlled by PIM (Proto-Oncogene, Serine/Threonine Kinase) kinases that phosphorylate NOTCH1 increasing both its nuclear localization and activity (Santio et al. 2016). Notch signaling is also upregulated upon anti-estrogen treatment of ER^+ patient derived samples and xenografts (Simoes et al. 2015). Additionally, MCF7 cells that undergo EMT upon irradiation, present increased expression of Notch pathway components, namely NOTCH2, DLL4 and JAG1 (Kim et al. 2016). Interestingly, pharmacological inhibition of Notch signaling with GSIs or knockdown of NOTCH2, DLL4 or JAG1 leads to reduced EMT upon radiation of MCF7 cells (Kim et al. 2016), supporting the idea that Notch signaling may contribute to radiation resistance.

In summary, Notch might be a valuable lead target for future therapeutic approaches in TNBC, possibly making use of combined therapies.

4.2.3 Notch in Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading cause of cancer death and it is believed that it develops from pancreatic intraepithelial neoplasia (PanIN). Notch signaling plays a dual role in pancreatic cancer: on one hand it is oncogenic in PDAC whereas it acts as a tumor suppressor in PanIN.

In PDAC, several Notch pathway components are upregulated including *NOTCH2*, *NOTCH3* and *JAGGED1* (Miyamoto et al. 2003) whereas, using a conditional pancreatic mouse model based on the expression of the RAS (Rat sarcoma virus oncogene) mutant K-RAS^{G12D} (*Pdx1-Cre;LSL-*$Kras^{G12D}$), Hanlon and colleagues observed an increase of PanIN upon inactivation of *Notch1* (*Pdx1-Cre;LSL-*$Kras^{G12D}$*;*$Notch1^{flox/flox}$, (Hanlon et al. 2010)), supporting the tumor suppressive role of Notch signaling in PanIN. This conclusion is further supported by the observation that conditional inactivation of *Notch1*, in the $Ptf1a^{+/Cre}$*;LSL-*$Kras^{+/G12D}$ ($Ptf1a^{+/Cre}$*;LSL-*$Kras^{+/G12D}$*;*$Notch1^{flox/flox}$) background, slightly reduces survival (Mazur et al. 2010a). In contrast, the same study pointed out that *Notch2* might play an entirely different role. In fact, its conditional inactivation ($Ptf1a^{+/Cre}$*;LSL-*$Kras^{+/G12D}$*;*$Notch2^{flox/flox}$) leads to decreased PanIN and PDAC development associated with increased survival (Mazur et al. 2010a), suggesting a different and opposing role for the different NOTCH receptors in pancreatic cancer. However, De La O and colleagues observed the opposite in regard to the role of *Notch1*. Its conditional GOF (*Pdx1-CreERT;Rosa26-NICD1*) in the $Kras^{G12D}$ background leads to increased PanIN (De La O et al. 2008). These discrepancies are potentially explained by the different genetic approaches used (loss- versus gain-of-function). Thus, it is possible that different NOTCH receptors are involved in different steps of pancreatic tumorigenesis. In line with this hypothesis, conditional LOF of *Lunatic Fringe* in the *Pdx1-Cre;LSL-*$Kras^{G12D}$ background ($Lfng^{flox/flox}$*;Pdx1-Cre;LSL-*$Kras^{G12D/+}$), that encodes for an O-fucosylpeptide 3-β-N-acetylglucosaminyltransferase known to modify the epidermal growth factor repeats of NOTCH proteins, caused increased NOTCH3 activation during PDAC initiation and progression but activation of NOTCH1 only at a later time point, suggesting that *Lunatic Fringe* is a tumor suppressor (Zhang et al. 2016b). It must also be noted that conditional expression of DN-MAML in $Kras^{G12D}$ background (*p48-Cre;LSL-*$Kras^{G12D}$*;*$Rosa26^{dn\text{-}MAML/+}$), that blocks the canonical activity of all NOTCH receptors, delays PanIN development (Thomas et al. 2014). In agreement with the above, GSIs treatment efficiently blocks Notch signaling and reduces pro-

liferation of both PanIN and PDAC cell lines. GSIs also attenuate PDAC development in mouse models (Plentz et al. 2009). Surprisingly, GSIs treatment of the PDAC mouse model *LSL-Kras$^{G12D/+}$;p53$^{R172H/+}$;Pdx-Cre$^{tg/+}$* only modestly increases survival but, when used in combination with the chemotherapeutic agent gemcitabine, a significant increase in survival is observed (Cook et al. 2012). Similarly, GSIs treatment enhances radiosensitivity in xenografts (Bi et al. 2016). A significant reduction in tumor volume was also observed when anti-DLL4 antibodies, in combination with gemcitabine, were used in pancreatic xenografts models (Yen et al. 2012). Furthermore, genetic inactivation of FBXW7, the E3-ubiquitin ligase that supports the degradation of the NICD, in the *p48-Cre;LSL-Kras^{G12D}* mouse model increases pancreatic tumorigenesis (Zhang et al. 2016c).

Finally, both JAG2 and NOTCH1 have been linked to cell migration of pancreatic cancer cells but this mechanism does not seem to require Notch downstream signaling as GSIs treatment has no effect on PDAC cell migration (Hu et al. 2015).

In summary, Notch signaling plays a key role in pancreatic cancer and a better dissection of the molecular mechanisms involved in this context may lead to develop more effective therapies.

4.2.4 Notch in Hepatocellular Carcinoma

Notch plays an oncogenic role in hepatocellular carcinoma (HCC). *NOTCH1* (Cantarini et al. 2006; Zhu et al. 2017) and *NOTCH3* (Hu et al. 2013) are upregulated and inhibition of Notch signaling with antibodies directed against NOTCH2 or JAG1 in a mouse model of liver cancer has a tumor suppressive effect (Huntzicker et al. 2015) while liver specific GOF of NICD2 leads to HCC (Dill et al. 2013). Similar results were observed in mice upon liver specific overexpression of NICD1 and Notch pathway activation is observed in human HCC (Villanueva et al. 2012). Knockdown of *NOTCH1* reduces the migration and invasion of HCC cells (Hu et al. 2014) without influencing cell viability (Zhou et al. 2013) and, in line with these data, GSIs treatment reduces invasion of HCC cells but surprisingly also their viability (Zhou et al. 2012), suggesting that cell viability may be regulated by a different member of the NOTCH family.

POFUT1 (protein O-fucosyltransferase 1), a glycosyltransferase that modifies the EGF repeats of NOTCH receptors promoting ligand interaction, is upregulated in HCC and its expression correlates with poor prognosis (Ma et al. 2016). *POFUT1* knockdown reduces cell growth, proliferation and migration of HCC cells, associated with reduced activation of the Notch pathway (Ma et al. 2016), suggesting POFUT1 as a possible therapeutic target in HCC. Hyperactivation of the Notch pathway in HCC is also mediated by the upregulation of JAG1, caused by the loss of the transcriptional repressor *RUNX3* (Nishina et al. 2011). In addition, RUNX3 also physically interacts with the NICD1/RBPJ complex and decreases its transactivating capacity in HCC cells (Gao et al. 2010). Another study pointed out to a link between IKKα [IκB (inhibitor of kappa B) kinase subunit alpha] and Notch signaling in HCC (Liu et al. 2012). IKKα is upregulated in HCC tumor samples and inactivates the transcription factor FOXA2 (forkhead box A2) by phosphorylation leading to downregulation of NUMB (Liu et al. 2012). Recently, a crosstalk between the Notch and Hippo pathways was described as a mechanism involved in HCC pathogenesis (Kim et al. 2017). Double KO (DKO) of mammalian sterile 20-like kinase 1 and 2 (MST1/2), involved in inhibition of the Hippo pathway by phosphorylation of the transcription factors YAP/TAZ (Yes-associated protein and WW domain containing transcription regulator 1), results in HCC (Song et al. 2010) associated with activation of Notch signaling which forms a positive feedback loop with YAP/TAZ (Kim et al. 2017). GSI treatment leads to reduced HCC in the MST1/2 DKO mouse model and while these data suggest an oncogenic role for Notch signaling in HCC, Wnt pathway plays the opposing role having a tumor suppressive function in HCC (Kim et al. 2017), suggesting the involvement of several different signaling pathways in HCC pathogenesis.

Notch signaling plays a positive role in HCC CSCs as its inhibition reduces their invasion and migration (Luo et al. 2016) and it may also be important in radio-resistance of HCC CSCs. In fact, $CD133^+$ HCC CSCs exhibit upregulation of ADAM17, associated with increased Notch signaling, upon irradiation (Hong et al. 2016).

Of note, some reports also provide evidence for a tumor suppressive role of Notch signaling in HCC. Liver specific deletion of all the three members of the *Retinoblastoma protein* family [*Rb*, *p107* and *p130*; triple knockout (TKO) mice] leads to HCC associated with increased expression of Notch pathway components due to upregulation of E2F transcription factors with transactivation capacity (Viatour et al. 2011). Although this suggests that Notch signaling may be an oncogenic driver, GSIs treatment of TKO mice enhances HCC development, revealing a tumor suppressive role of Notch signaling (Viatour et al. 2011). The Sage laboratory could also show that TKO liver progenitors do not show increased expression of *Notch1*, *Hes1*, *Hey1* or *Nrarp* Notch target genes, suggesting that deregulation of Notch signaling by *Rb* family members is cell type-specific and occurs during tumor progression (Viatour et al. 2011).

As consequence, Notch signaling may be an important player in HCC and a better comprehension of its function in this disease may lead to significant improvement of the current therapies.

4.2.5 Notch in Lung Cancer

Lung cancer is the leading cause of cancer-associated mortality worldwide. Based on histopathology and molecular characteristics two main subtypes can be distinguished: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).

SCLC is distinguished from NSCLC by its characteristic small-cell phenotype that reflects its origin from the neuroendocrine lineage. SCLC is highly refractory to chemotherapy. Recent whole-genome sequencing studies of SCLC have identified recurrent mutations in the *NOTCH1-4* genes (George et al. 2015), suggesting that Notch needs to be inactivated for SCLC development. As the Notch signaling pathway is a physiological regulator of neuronal and neuroendocrine differentiation, mutations in *NOTCH* genes are likely responsible for the characteristic neuroendocrine phenotype of SCLC.

In cancer, lineage specification genes often provide survival advantages of which cancer cells become dependent on - similar as they become addicted to - activated oncogenes (Garraway and Sellers 2006). In line with an addiction of SCLC to the neuroendocrine lineage, the Notch target gene *ASCL1* (*achaete-scute homolog 1*), encoding for a transcription factor which is physiologically required to establish the lineage of neuroendocrine cells in the lung (Borges et al. 1997), was previously shown to be required for the continued survival of SCLC cells (Osada et al. 2005; Jiang et al. 2009). Thus, in this setting, Notch signaling most likely plays a tumor suppressive role and it would be attractive to reactivate Notch target genes to induce the cancer initiating cells to differentiate into a different lineage to block its malignancy. Only then, conventional chemotherapeutic agents could eliminate this devastating cancer cells.

4.2.6 Notch in Skin Cancer and Melanoma

Counterintuitively, Notch may also play the role of a tumor suppressor in other contexts. Notch signaling has a tumor suppressive function in the skin as conditional inactivation of *Notch1* leads to epidermal and corneal hyperplasia followed by the development of skin tumors (Nicolas et al. 2003). Similar results were obtained by skin specific deletion of *Notch1*, mediated by *Pdx1-Cre*, using the RAS mutant $Kras^{+/LSL\text{-}G12D}$ mouse model (Mazur et al. 2010b). The same study also pointed out a specific tumor suppressive role for *Notch1* as genetic depletion of *Notch2* does not support carcinogenesis (Mazur et al. 2010b). Demehri and colleagues showed that *Notch1* depletion in epidermal keratinocytes induces tumorigenesis in a non-cell autonomous manner (Demehri et al. 2009). Similarly to *Notch1* LOF, conditional expression of *dn-Maml* driven by *SM22-Cre* in the skin leads to development of cutaneous squamous cell carcinoma [SCC, (Proweller et al. 2006)]. In line with these data, mesenchymal deletion of the

Notch signaling effector *Rbpj* results in skin tumor (Hu et al. 2012). Notch signaling may also play a tumor suppressive role in human skin cancer as several Notch pathway components are downregulated in human basal cell carcinoma [BCC, (Thelu et al. 2002)]. This hypothesis is further supported by the identification of mutations in human *NOTCH1* in cutaneous SCC that impair the Notch function (Wang et al. 2011b).

At molecular level, data from keratinocytes and SCC cell lines suggest that *NOTCH1* is under the positive control of P53 (Lefort et al. 2007), which is frequently mutated in skin SCC (Backvall et al. 2004). This positive function of P53 is counteracted by EGFR (epidermal growth factor receptor) signaling as its inhibition promotes *P53* expression and, consequently, *NOTCH1* expression with increased Notch signaling (Kolev et al. 2008). Of note, EGFR inhibition in SCC cells induces differentiation and, when EGFR inhibition is combined with inhibition of the Notch signaling pathway, increased apoptosis is observed (Kolev et al. 2008).

Recently, the involvement of Notch signaling in melanoma has gained attention. NOTCH receptors and ligands as well as Notch effectors are upregulated in melanomas (Balint et al. 2005; Massi et al. 2006) and Notch signaling inhibition, via GSIs or expression of DN-MAML, suppresses melanoma cell growth (Balint et al. 2005). In line with this, GOF of the active form of *NOTCH1* increases melanoma cell growth as well as enhances primary melanoma and lung metastasis in adult mice (Balint et al. 2005; Liu et al. 2006). In addition, *FBXW7* was found to be mutated in melanoma patients and these mutations compromise the function of FBXW7 protein leading to accumulation of the active form of NOTCH1 (Aydin et al. 2014). At mechanistic level, active NOTCH1 stabilizes the Wnt signaling effector protein β-CATENIN rather than acting through RBPJ. Indeed, LOF of *β-CATENIN* in melanoma cells mirrors the proliferative defects observed upon LOF of Notch signaling (Balint et al. 2005). Such non-canonical functions of the intracellular active form of Notch affecting other conserved signaling pathways have been recently reviewed (Borggrefe et al. 2016) and are also discussed by Vaccari and colleagues in "Mechanisms of Non-canonical Signaling in Health and Disease: Diversity to Take Therapy up a Notch?" of this book. There is another study by Liu and colleagues showing that NOTCH1 increases melanocyte growth by activating the MAPK and PI3K/AKT signaling pathways (Liu et al. 2006), suggesting that Notch signaling is involved in melanoma by regulating crosstalk with even more signaling pathways.

In conclusion, Notch signaling may be a valuable target also for the treatment of melanoma and skin cancer. However, in the case of skin cancer, this will be particularly challenging because of the tumor-suppressive function of Notch.

5 Mutational Spectrum of Notch Pathway Components

Several mutations involving Notch pathway components have been identified in cancer and genetic disorders as discussed in the previous sections and selectively summarized in Table 1. One striking observation is that the same protein domains are mutated in different diseases (see also "Integration of *Drosophila* and Human Genetics to Understand Notch Signaling Related Diseases" of this book), suggesting that common molecular mechanisms are probably used to confer pathogenicity.

A number of structure biological studies have fully or partially solved the molecular structure of key Notch signaling components, allowing us to understand the effect of disease-linked mutations in the context of 3D protein structure. For example, mutations occurring in the *FBXW7* gene (Fig. 2a and Table 1), encoding for the E3 ubiquitin ligase involved in the degradation of the NICD, are frequently found in melanoma, SCLC and T-ALL. These mutations can compromise the activity of FBXW7, leading to increased protein stability of the NICD and of the other FBXW7 substrates (Aydin et al. 2014). *RBPJ* has been reported to be mutated in AOS, a genetic disease characterized in most of the patients by terminal limb malformations (Hassed

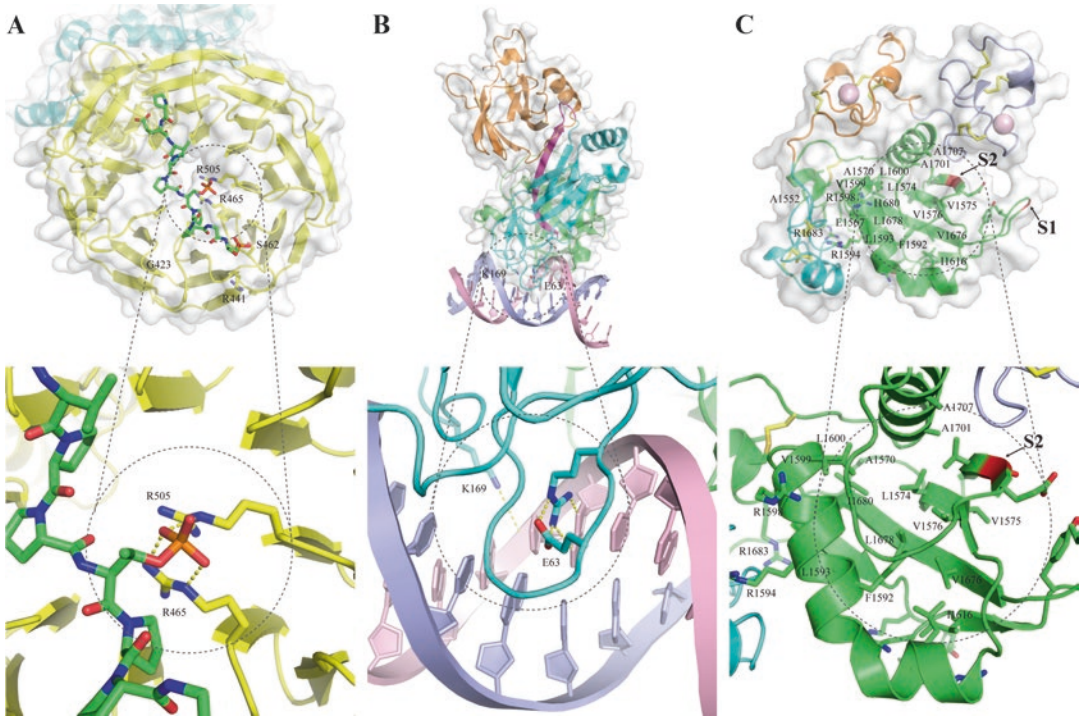

Fig. 2 Examples of pathogenic mutations in key Notch pathway components in the context of protein structure. (**a**) Structure of the WD40 repeats of FBXW7 (PDB ID, 5V4B). Residues for which mutations have been identified in diseases are indicated. (**b**) Structure of the transcription factor RBPJ in complex with the DNA (PDB ID, 3IAG). Indicated are residues mutated in AOS. (**c**) Structure of the NRR of NOTCH1 (PDB ID, 3ETO). Indicated are residues for which mutations have been identified in diseases and that have been functionally analyzed. A: alanine; E: glutamic acid; F: phenylalanine; G: glycine; I: isoleucine; K: lysine; L: leucine; R: arginine; S: serine; V: valine

et al. 2012). The AOS-associated missense mutations identified in the *RBPJ* gene (Fig. 2b) compromise its DNA binding ability (Hassed et al. 2012) and mutations in *NOTCH1* and *DLL4* have been also identified in AOS patients (Meester et al. 2015; Stittrich et al. 2014). The reader is also referred to "Integration of *Drosophila* and Human Genetics to Understand Notch Signaling Related Diseases" for an in-depth review of genetic mutations of Notch pathway components. Chromosomal translocations and aberrations involving *FBXW7* and *RBPJ* are also linked to diseases. *FBXW7* is translocated in renal cell cancer [RCC; (Kuiper et al. 2009)] while *RBPJ* in the proximal 4p deletion syndrome (Nakayama et al. 2014).

Mutations occurring in the *NOTCH1* gene are clustered in different regions (Table 1). Among them, mutations that occur in the LNR repeats, HD and PEST domains are seen in many types of diseases as well as genetic disorders. Typically, mutations involving the LNR repeats and HD domain lead to disruption of the negative regulatory region (Fig. 2c) and promote ligand-independent cleavage of the receptor, leading to increased Notch signaling. Similarly, mutations that influence the structure of the PEST domain lead to increased half-life of the NICD resulting in aberrant transcriptional activity. Similarly to *FBXW7* and *RBPJ*, also the *NOTCH1* gene is subjected to chromosomal translocations that impair its activity (Ellisen et al. 1991).

Thus, there are indeed viable genetic mutations of the Notch receptor or Notch signaling components, that could in future provide even more insights in Notch-related pathologies, not only in the context of development but also in the cancer context.

6 Perspectives

Given the important function of the Notch signaling pathway in cancer as well as in genetic diseases, it will be important to deeper understand its regulation focusing on the molecular basis that characterize this signaling cascade. This approach will allow in the future the development of new and more efficient therapies that can overcome the limitations of the current approaches, primarily the side effects and resistance observed by using GSIs. New cancer therapies might be based on small molecule inhibitors of Notch modulators or Notch pathway components to reactivate the tumor suppressive function or to block the oncogenic activities of the pathway depending on the different pathological contexts. Another fascinating alternative would be the use of antibodies aimed to stimulate or block the activation of NOTCH receptors, an approach that seems to be promising. This can be achieved by using antibodies directed against NOTCH receptors (Aste-Amezaga et al. 2010; Wu et al. 2010; Canalis et al. 2017), ligands (Billiard et al. 2011; Lafkas et al. 2015; Xu et al. 2016; Wang et al. 2017) or the γ-secretase complex (Hayashi et al. 2012). Similar approaches can be employed to modulate the Notch function in macrophages as inflam-mation is one the key processes that drive tumorigenesis. In conclusion, more work is needed to deeply understand the regulation of the Notch signaling pathway and modulate its activity for clinical use.

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