

Chapter 11

Notch Signaling in Pediatric Soft Tissue Sarcoma



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Abstract Etiology, biology, response to treatment, and outcome greatly differ between adult and childhood cancers. Soft tissue sarcoma encompasses a heterogeneous group of pediatric sarcomas characterized by a high capacity to invade neighboring tissues. Although in the last years the overall survival in childhood cancers has improved to over 70% for the nonmetastatic forms, subgroups of young patients with metastatic and aggressive disease still show a poor outcome. Moreover, survivors often suffer from long-term morbidity due to the effects of therapy. It is widely accepted that soft tissue sarcomas of childhood develop from mesenchymal progenitor cells affected by chromosomal aberrations and mutations in genetic and epigenetic pathways during development. Therefore, pathways driving tissue differentiation are particularly relevant. Among these, the Notch signaling pathway plays one of the major roles. Notch signaling is evolutionarily conserved among species, working as a cell-to-cell communication system strictly defining cell fate, stem cell renewal, and tissue homeostasis during embryo development and in postnatal life. In the present chapter, we describe recent insights on Notch deregulation in the most prominent pediatric soft tissue sarcomas: rhabdomyosarcomas, Ewing sarcomas, and synovial sarcomas. We also summarize the challenges and opportunities in inhibiting Notch signaling for the treatment of this group of tumors.

Keywords Notch signaling · Notch receptors · Gamma-secretase · Soft tissue sarcoma · Rhabdomyosarcoma · Ewing sarcoma · Synovial sarcoma

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Abbreviations

| | |
|------------|------------------------------------|
| DLL1, 3, 4 | Delta-like 1, 3, 4 |
| ES | Ewing sarcoma |
| GEMM | Genetically engineered mice models |
| GSI | Gamma secretase inhibitors |
| MAML1 | Mastermind-like 1 |
| NEC | Notch extracellular domain |
| NEXT | Notch extracellular truncation |
| NICD | Notch intracellular domain |
| NTM | Notch transmembrane domain |
| RMS | Rhabdomyosarcoma |
| SS | Synovial sarcoma |

11.1 Introduction

11.1.1 *Childhood Versus Adult Cancers*

Conversely to adult tumors, whose pathogenesis is related to environment-/age-dependent genetic and epigenetic alterations, pediatric cancers originate from progenitor cells in which developmental pathways governing embryonic life are deregulated. In line with this, tumors of childhood often contain a clonal population of presumably tumor-initiating cells expressing fusion products of genes that guide tissue development.

Increasing knowledge of the landscape of molecular networks involving genetic and epigenetic mechanisms acting in childhood cancers have opened the way to the discovery of novel potential approaches to treat the disease.

Crucial developmental pathways involved in pediatric tumor biology are Sonic Hedgehog (SHH), Wntless (WNT), and Notch signaling. These pathways are fundamental for proper cell differentiation and tissue lineage commitment of progenitor cells and, more importantly, cooperate and cross talk each other (reviewed in [1–6]). Considering the crucial role of Notch signaling in developmental processes, it is not surprising that it has been found affected in several diseases ([7–15] and reviewed in [16]).

An oncogenic role of Notch signaling has been highlighted for the first time in pediatric acute T-cell leukemia (T-ALL). Indeed, two groups demonstrated that (i) mutations of the Notch1 receptor resulted in the constitutive production of an activated form of Notch1, i.e., the Notch1 intracellular domain, in patients with T-ALL [17], and that (ii) this Notch1 constitutive activation is sufficient for tumorigenesis [18]: an observation confirmed later also in adult cancers [19]. In the last few years, the deregulation of Notch signaling has been shown to be involved in several types of pediatric solid tumors. Recently, we and others have shown Notch signaling abnormalities are pathogenetic

events in pediatric soft tissue sarcomas, a heterogeneous group of solid tumors affecting mainly soft tissue and bone of young patients.

As for adult cancers, where several clinical trials with Notch signaling inhibitors are being evaluated, the modulation of the Notch signaling is under preclinical study as an anticancer strategy in this type of pediatric tumors.

11.1.2 Pediatric Soft Tissue Sarcomas

Pediatric soft tissue sarcomas include a group of tumors derived from the mesenchymal compartment that are highly heterogeneous in terms of clinical behavior and genomic alterations [20].

Collectively, they represent about 8–10% of all childhood tumors and about 15% of tumors outside the central nervous system [21]. Multimodal approach with chemotherapy and surgery is the usual treatment of pediatric soft tissue sarcoma, while radiation is rarely used in young children due to its side effects on a growing organism [22]. Advances in treatments have improved the overall survival in all childhood cancers to over 70% today. However, although the prognosis of soft tissue sarcoma has improved considerably, a group of patients still shows a dismal prognosis. Indeed, metastatic forms and subsets of tumors harboring specific oncogenic mutations/chromosomal translocations are often incurable. Additionally, young survivors often suffer from long-term side effects linked to therapy. An additional clinical challenge to eradicate soft tissue sarcomas is due to the high ability of tumor cells to invade the neighboring tissues [22].

Therefore, the scientific community is focusing on finding a therapy that is more specific and less toxic for these young patients. This can be achieved only through the knowledge of the molecular pathogenetic mechanisms responsible for the development and maintenance of these tumors.

The three major groups of pediatric soft tissue sarcomas include rhabdomyosarcoma (RMS), Ewing sarcoma (ES), and synovial sarcoma (SS). Although they have different and peculiar characteristics, experimental evidences clearly indicate that all can develop from mesenchymal progenitor cells affected by chromosomal aberrations and/or gene mutations. It is widely accepted that the dysregulation of the major embryonic developmental molecular pathways plays a fundamental role in the pathogenesis of pediatric soft tissue sarcomas. In agreement, small populations of cells that remain undifferentiated and maintain self-renewal capacity seem to represent the tumor ancestor cells unresponsive to therapy [23, 24].

Therefore, the modulation of developmental pathways regulating stem cell properties, such as the Notch pathway, might be a potential strategy to improve the clinical response of this type of tumors affecting young patients.

In the last several years, we and others have reported preclinical experimental proofs of principle indicating Notch signaling modulation as a potential approach to reduce the tumorigenesis of pediatric soft tissue sarcomas.

11.1.3 Structure of Notch Receptors and Ligands

The Notch pathway is one of the fundamental signaling pathways strictly defining developmental processes regulating cell fate and tissue differentiation and homeostasis in embryo and in the postnatal life. The pathway signals through cell-to-cell interaction between a signal-sending cell (expressing Notch ligands) and a signal-receiving cell (expressing Notch receptors) (Fig. 11.1) [25, 26]. This type of cell communication relies on the particular structure of ligands and receptors.

Notch receptors While in the fruit fly *Drosophila melanogaster* only a single Notch gene exists (reviewed in [27]), in mammals four Notch receptors have been identified, i.e., Notch 1–4 [28]. They are encoded by four different gene loci on chro-

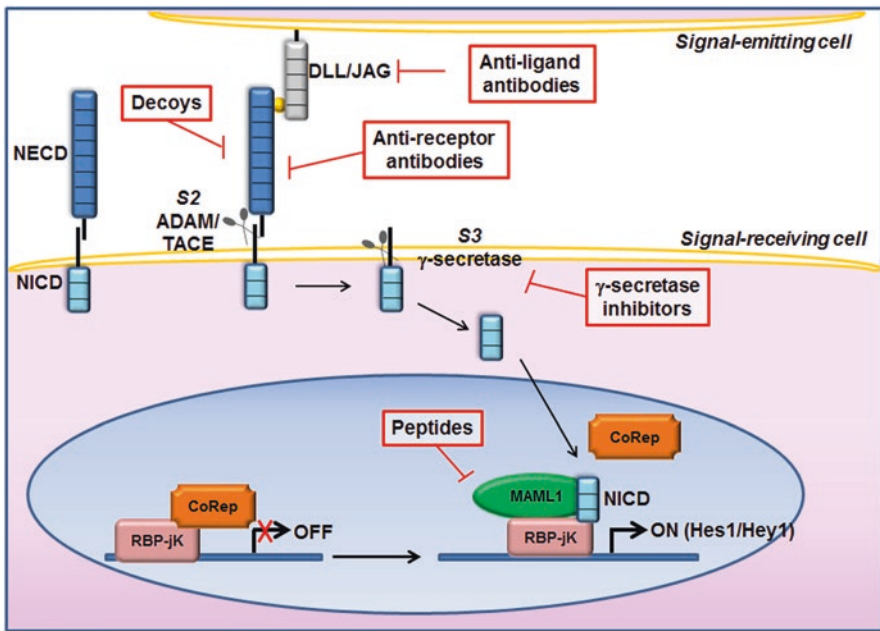


Fig. 11.1 After proteolytic processing maturation, Notch receptors are expressed on the cell membrane as an extracellular domain (NECD) non-covalently associated with a transmembrane portion and an intracellular domain (NICD). Notch signaling is initiated by a Notch receptor-Delta/Jagged-type (DLL/JAG) ligand interaction between two neighboring cells *in trans*, which induces two successive proteolytic cleavages. The first one is operated on the S2 site by “a disintegrin and metalloprotease” 10 (ADAM10) or ADAM17, which is followed by an S3 cleavage by a presenilin complex (γ -secretase). The S3 cleavage gives rise to the NICD fragment that translocates into the nucleus, where it binds to a protein complex containing recombination signal-binding protein Jk (RBP-Jk) relieving the repressor complex (CoRep). This event modulates chromatin activity recruiting activators such as MAML1 and converts RBP-Jk from a transcriptional repressor to an activator, leading to the transcription of hairy/enhancer of split (Hes) and Hey family genes, which work as transcriptional repressors. Several stages of the Notch signaling pathway are prone to pharmacological intervention. Decoys, anti-ligand antibodies, anti-receptor-antibodies, γ -secretase inhibitors, and peptide inhibitors are labeled in the red boxes

mosome (Chr) 9, Chr 1, Chr 19, and Chr 6, respectively, and are about 60% homologous to each other. Each Notch paralog is translated as a single-pass transmembrane protein that is subjected to posttranslational modifications before being expressed on the surface of the cells: a single-chain precursor is cleaved by furin-like proteases in the Golgi compartment (S1 cleavage), resulting in an N-terminal extracellular domain (NECD) and a C-terminal portion encompassing both a Notch transmembrane (NTM) and intracellular domain (NICD). The two fragments are non-covalently reassembled on the Golgi membranes and, then, expressed on the surface of the plasma membrane ([29] and reviewed in [30]).

The NECD is formed by a number of epidermal growth factor (EGF)-like repeats responsible for the binding of ligands [31]. Important under a functional point of view, a specific number of EGF repeats characterize each Notch receptor, Notch1 containing 36 EGF repeats [32], whereas Notch2 presenting 35 EGF repeats [33], Notch3 34 EGF repeats [34], and Notch4, the shorter Notch receptor, only 29 EGF repeats [35]. A negative regulatory region (NRR), composed of three cysteine-rich Lin12/Notch repeats (LNR) [36, 37], followed by a juxtamembrane hydrophobic region, is responsible for the heterodimerization of the NECD and the NTM-NICD portions of the receptor. The LNR regulates the auto-inhibition of the Notch receptor preventing the receptor from being cleaved without binding to the ligand [37, 38].

The intracellular region NICD contains a module, named RAM, which recognizes the recombination signal-binding protein Jk (RBP-Jk) supporting the transcriptional role for the NICD that can interact with the transcriptional coactivator RBP-jK in the CSL complex (RBP-jK/CBF-1/KBF2 in mammals) [39]. The RAM region is followed by seven ankyrin (ANK) repeats important for the interaction with CSL and other transcriptional regulators [40, 41], two nuclear localization signals (NLS) [42], a transactivation domain (TAD) [43], and a C-terminal PEST sequence (rich in proline, glutamic acid, serine, and threonine) [44]. The PEST sequence is highly important since it can be phosphorylated, thus regulating the ubiquitination of the NICD and, consequently, its stability and signaling ability [44]. Notably, the strength of the TAD sequence in transactivating gene transcription is different among the paralogs being strong for Notch1, weak for Notch2, and strong but highly specific for Notch3, while Notch4 does not have a TAD [43, 45]. These differences in the structure and activity explain the diverse and somewhat divergent functions of the Notch receptor family.

Notch ligands Only two canonical ligands of the Delta-Serrate family are expressed in *Drosophila*, while mammalian cells express three ligands of the Delta family, Delta-like 1 (DLL1), DLL3, and DLL4 [46–48], and two of the Serrate family, JAG1 and JAG2 [49, 50]. All the five mammalian ligands are type I transmembrane proteins containing an N-terminal region and a cysteine-rich domain (DSL for Delta, Serrate, and LAG-2), followed by a number of EGF-like repeats. In particular, the N-terminal region with DSL and the first two EGF-repeats are responsible for the interaction with the EGF-like repeats of Notch receptors ([51, 52] and reviewed in [25]). The structure of the intracellular region of the canonical ligands is not conserved among species and regulates ligand interactions with the cellular cytoskeleton.

Additional noncanonical ligands can interact with and activate Notch receptors, either transmembrane or soluble proteins, such as DLK1, DLK2, DNER, the EGF-like protein7 (EGFL7), or the F3/contactin ([53, 54] and reviewed in [25, 55, 56]). They do not contain a DSL domain but are all characterized by the presence of EGF-like repeats.

Another level of complexity is added by the posttranslational modifications of Notch receptors, operated in the cytoplasmic compartment, which strictly regulate their half-life, selectivity, and activity [25, 57]. Among those are the glycosylation, ubiquitylation, phosphorylation, and acetylation.

Fringe glycosyltransferases, firstly identified in *Drosophila*, glycosylate specific EGF-like repeats of the Notch heterodimer in the Golgi compartment [58–60]: a modification that affects the affinity of the receptor for the ligands, specifically preventing Jagged-dependent activation [61, 62]. Three mammalian fringe enzymes are known, i.e., lunatic fringe (LFNG), manic fringe (MFNG), and radical fringe (RFNG) [63]. It is arguable that dysregulation of these enzymes can lead to imbalance in the expression/activity of Notch components since it can induce the Notch receptors to be cleaved with higher rate than in normal tissue (reviewed in [64]), as demonstrated for breast cancer cells [65].

The lysosomal degradation or, conversely, the recycling to the plasma membrane of the cleaved Notch is regulated by polyubiquitylation, a process governed by several E3 ubiquitin ligases such as Deltex, β -arrestin/Kurtz, Itch, NEDD4 (neural precursor cell expressed developmentally downregulated 4), Cbl (casitas B-lineage lymphoma), and Fbw7/Sel-10 ([66–69] and reviewed in [70]). The inclusion of Notch in the early endosomes can be regulated by Numb, a cytoplasmic negative regulator of the pathway [71], and it is followed by proteasome-mediated degradation [72]. The phosphorylation of NICD to the ANK and/or PEST domain along with acetylation modulates the stability and the activity of the cleaved receptor [73–77]. Further, NICD can interact in the cytoplasm with several molecules among which Nemo-like kinase NLK, which suppresses Notch signaling [78], or Pin1, which conversely amplifies Notch activation [79–82].

11.1.4 Notch Signaling Pathway

The Notch signaling is critical in embryos during the differentiation of stem cells when a ligand-expressing cell interacts with a Notch-expressing cell and, then, the former undergoes differentiation while the latter remains in an undifferentiated state [30]. However, the results of this cell-to-cell communication highly depend on the molecular, cellular, and environmental contexts, making a simple mechanism extremely versatile [83–85].

When a canonical ligand on a cell binds to the specific EGF-like repeats of a Notch receptor on a neighboring cell (in *trans*), the resulting mechanical stretch favors the cleavage (at site S2) of the heterodimeric portion just outside the plasma membrane by the a disintegrin and metalloprotease 10 (ADAM10) or 17 (ADAM17) [86].

A requirement for this process is the ubiquitination and subsequent endocytosis of the ligand (reviewed by [87]). Then, the remaining membrane-tethered intermediate, named NEXT (Notch extracellular truncation), is subsequently cleaved in an intracellular region (at sites S3 and S4) by a γ -secretase complex formed by four subunits [88–93]. This last cleavage results in an intracellular activated form, NICD, which translocates to the nucleus, binds the CSL complex (RBP-Jk/CBF-1/KBF2 in mammals) and activates the transcription of canonical Notch target genes [94]. To do so, the CSL/Notch complex recruits several transcriptional coactivators such as Mastermind-like 1 (MAML1) and the acetyltransferases CBP/p300 or PCAF/GCN5 ([41, 95–97] and reviewed in [98]). The canonical target genes belong to the basic helix-loop-helix (bHLH) families of hairy/enhancer of split (Hes) and Hey (subfamily of Hes, related with YRPW motif) repressors [25]. The result is the transcriptional repression of multiple differentiation genes. Interestingly, conversely to the classical view based on the recruitment of NICD by RBP-jK already bound to the DNA in a repressor state [83, 99], more recently the group of Tajbakhsh demonstrates that in mammalian myoblasts (i) NICD recruits free RBP-jK to the chromatin on specific enhancers, while (ii) the amount of RBP-jK constitutively bound to the DNA is unaffected by Notch activation [100]. This finding further highlights the importance of the cellular and molecular context for the regulation and effects of Notch signaling pathway. In addition to the Hes and Hey genes, Notch signaling can activate in a context-/tissue-dependent manner the transcription of, among others, Deltex or members of NF- κ B family, the cyclin-dependent kinase inhibitor p21^{Cip1}, cyclin D1 or MYC [101–106]. Notch signaling can be also activated in a noncanonical way that can be (i) independent from CSL, (ii) independent from the S3 cleavage, or (iii) in the absence of Notch cleavage and NICD formation (reviewed in [55, 107, 108]). Finally, ligand-receptor interactions on the same cell can be also in *cis* and results in inhibition of the signaling [109–112]. Importantly, the structural molecular features of Notch components that allow several types of modifications concurring to the diverse mechanisms of signalization represent a platform for therapeutical interventions with modulators of the pathway (Fig. 11.1). Notably, being Notch signaling tissue- and context-specific and paralogs similar but not identical, the signal triggered by different Notch receptors in different tissues is somewhat specific and can be even opposite (reviewed in [25]).

11.2 Notch Signaling Deregulation in Pediatric Soft Tissue Sarcomas

11.2.1 Notch Signaling in Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft tissue tumor of childhood of myogenic origins accounting for about 8% of all pediatric tumors [113]. Despite the expression of the master regulators of skeletal muscle differentiation such as MYOD and myogenin, also used for diagnostic purposes to exclude other small round blue

cell tumors, RMS cells do not differentiate in multifiber structures and proliferate indefinitely ([114, 115] reviewed in [116]). To date, we and others have shown that the modulation of differentiation represents a potential approach to restore the cell cycle checkpoints inhibiting tumor cell proliferation [117, 118]. However, as shown in genetically modified mice models (GEMM) of spontaneous RMS, this sarcoma could originate from a heterogeneous group of mesenchymal-derived cells, even if mesenchymal precursors with different degrees of skeletal muscle commitment have been implicated as the major tumor-prone subset [119–124]. Two major histological subtypes are included in pediatric RMS: the embryonal (ERMS) and the alveolar (ARMS) variants. ERMS represents about 70–75% of all cases of pediatric RMS and primarily affects young children arising in the head and neck and retroperitoneum and showing, when nonmetastatic, a good prognosis with an overall survival of about 80% [125, 126]. ERMS is characterized by somatic gene mutations in the *RAS* gene family, *TP53*, *FGFR4*, *PIK3CA*, *CTNNB1*, *FBXW7*, and *BCOR*, associated with genomic instability including loss of imprinting and loss of heterozygosity of specific chromosomal regions, among which the Chr. 11p.15 region, and gain of regions of chromosomes 2, 7, 8, 11, 12, 13, and 20 [127–129]. Moreover, ERMS pathogenesis has been related to mutation/dysfunction of components of one of the major developmental pathways, i.e., Hedgehog [130–133]. Interestingly, the *MYOD* gene has been shown to be mutated in a group of older adolescent with an aggressive form of ERMS [134]. The p.Leu122Arg substitution leads to a MYOD protein capable to activate gene transcription in a “MYC-like” manner, once more highlighting the strong involvement of malfunction of myogenic factors in RMS. Collectively, these findings emphasize the heterogeneous molecular features of the ERMS variant. An about 20% of ARMS behave clinically and show molecular alterations similar to the ERMS subtype [127, 135], whereas the majority of ARMS is characterized by clonal cell populations with specific chromosomal translocations, defining a subset of RMS clinically and molecularly different from fusion-negative RMS [127, 135].

The most frequent chromosomal translocations in ARMS are t(2;13) (q35;q14) or t(1;13) (q36;q14), which result in the expression of the two oncogenic proteins PAX3-FOXO1 and PAX7-FOXO1, respectively [136, 137]. Both are transcription factors formed by the DNA-binding domain of PAX3/7 and the transactivation domain of FOXO1. The result is a constitutive activation of a PAX3/7 transcriptional gene profile. In addition, PAX3-FOXO1 acquires transcriptional ability that is absent in PAX3 alone (reviewed in [138]). Fusion-positive ARMS affects mainly older children and adolescents arising in legs and trunk. The expression of the fusion proteins is a negative prognostic factor per se independent from histology, identifying a subset of patients at high risk frequently with metastatic disease at diagnosis. Fusion-positive ARMS but also metastatic fusion-negative RMS represent a challenge for clinicians since they are often unresponsive to treatments with a high chance to recur. The demonstration of the expression of the fusion products is entering the clinical practice to help in the risk stratification of patients, and, more recently, the Shipley group demonstrated that those patients characterized by a PAX3-FOXO1 protein expression are at ultrahigh risk showing a 5-year overall

survival (OS) less than 15% [139]. Taken together, these clinical data indicate that to halt the disease, it is imperative to hamper PAX3-FOXO1 activity. Despite improvements in the therapeutic strategies, the outcome of high-risk patients remains poor. Therefore, the need of a deeper knowledge of the mechanisms underlying the development and progression/recurrence of RMS is urgent. However, transcription factors such as PAX3-FOXO1 are difficult to target. Therefore, targeting PAX3-FOXO1 downstream molecules could be an acceptable approach to block its signaling. The developmental networks appear to be good targets due to their involvement in the differentiation of mesenchymal cells in addition to the PAX3 program. In particular, Notch signaling plays one of the major roles among the crucial regulators of skeletal muscle differentiation, maintenance, and homeostasis, both in embryo and in the postnatal life [140].

To date, several recent experimental findings by our group and other laboratories demonstrate that Notch signaling pathway is deregulated in RMS (Table 11.1). The first evidence of an implication of a Notch component in RMS stems from the work of Sang et al. [151] showing that the Notch target gene *HES1*, encoding for a transcriptional repressor, was able to halt the muscle-like differentiation when expressed in fibroblasts engineered with a plasmid encoding MYOD. This effect was reversed by treatment with a γ -secretase inhibitor (GSI), which blocks the cleavage of Notch receptors, or by silencing a corepressor working with HES1, i.e., TLE1/groucho. *HES1* transcripts were then shown to be overexpressed in RMS tumors and cell lines compared to normal skeletal muscle tissue. Then, the authors elegantly demonstrated that inhibition of the HES1 function using either a mutant HES1, defective

Table 11.1 Notch signaling in STS

| Tumor | Notch deregulated component | Functions | Ref. |
|------------------|-----------------------------|------------------------------|------------|
| Synovial sarcoma | Notch1, JAG1, and TLEs | Oncogenic | [141] |
| | TLE1 | Oncogenic | [142, 143] |
| | TLE1 | Oncogenic | [144] |
| Ewing sarcoma | MFNG | | [145] |
| | MNFG and Notch1 | Regulator of differentiation | [146] |
| | JAG1 and HEY1 | Onco-suppressor | [147] |
| | Notch1 and Notch3 | Onco-suppressor | [148] |
| | HEY1 and Notch1 | Onco-suppressor | [149] |
| Rhabdomyosarcoma | DLL1, Notch1, and Notch3 | Oncogenic | [150] |
| | HES1 | Oncogenic | [151] |
| | Notch2 and HEY1 | Oncogenic | [152] |
| | Notch1 and HEY1 | Oncogenic | [153] |
| | Notch3 and HES1 | Oncogenic | [154] |
| | RBP-jK | Oncogenic | [155] |
| | DLL1, JAG1, Notch3 | Oncogenic | [156] |
| | JAG1 | Oncogenic | [157] |
| DIII1 | Oncogenic | [123] | |

in the DNA binding, or a dominant-negative HES1 form, lacking the domain that mediates the interaction between HES1 and its corepressors, halted cell proliferation and facilitated muscle-like differentiation of fusion-positive ARMS cell lines [151]. Similar results, associated to a diminution of the levels of HES1, were obtained inhibiting Notch signaling with a GSI, establishing that the effects seen were, at least in part, dependent from the Notch signaling activation.

Subsequently, the group of Gallego published a report showing a general deregulation in transcripts of the Notch pathway in 37 primary RMS samples, irrespective of the fusion status [152]. The authors showed significant upregulation of *Notch2* and *HEY1* compared to normal muscles. No overt difference in the levels of *Notch4* and *Notch1* transcripts in RMS compared to control tissues was seen, while *HES1* transcripts resulted modestly overexpressed in ERMS. However, the expression of the HES1 protein by immunohistochemistry was more elevated in RMS either fusion-negative or fusion-positive compared to muscle tissues. Interestingly, HES1 expression levels well correlated with the invasive capabilities of RMS cells with the lowest expression in low-invasive ERMS cell lines, and highest expression in PAX3-FOXO1 cells, which are the most invasive subtype [152]. The importance of Notch signaling in the invasive features of RMS cells was then confirmed either (i) inhibiting the γ -secretase-dependent cleavage of Notch receptors with several GSIs or (ii) transfecting RMS cells with a dominant-negative form of MAML1 (dnMAML1), which forms inactive RBP-jK/NICD/MAML1 complexes on DNA [158]. In both cases, HES1 transcript and protein levels were negatively affected by each of the two approaches, supporting the view of a Notch-dependent direct or indirect mechanism for HES1 overexpression. In a more recent work, Belyea et al. [153], interrogating previously published gene expression datasets [135], showed a marked upregulation of *HEY1* transcripts in ERMS compared not only to muscle tissues but also to ARMS samples. The results were confirmed in ERMS cell lines with respect to fusion-positive ARMS cells. The authors investigated the protein levels of HEY1 along with those of nuclear Notch1 in primary samples by immunohistochemistry and found that both were remarkably higher in ERMS compared to ARMS or to normal muscle tissue. HEY1 or Notch1 genetic depletion through shRNAs led to impaired ERMS cell proliferation in vitro and enhanced expression of the differentiation gene myogenin, particularly when cells were cultured in differentiation medium (low serum). However, despite the upregulation of myogenin and the phenotypic changes from round- to spindle-shaped cells, only a few myofiber-like structures were formed in these experimental conditions. Since Notch1 downregulation induced HEY1 decrease, suggesting that HEY1 was directly or indirectly targeted by Notch1 signaling in ERMS cells, the Notch1-HEY1 axis seems to be a regulator of cell cycle rather than of terminal differentiation in the ERMS context [153]. These effects were phenocopied by two GSIs and, more importantly, rescued in GSI-treated cells by vector-induced NICD forced expression, supporting the hypothesis of a Notch1-specific effect. Moreover, these approaches worked also in in vivo models of ERMS xenografts, which showed reduced tumor growth for those formed by cells depleted of Notch1 or in animals treated with a GSI [153]. This last treatment resulted in the reduction of Notch1 levels in tumor samples, confirming

the involvement of the Notch paralog signaling in the development of tumor masses [153]. Recently, the RBP-jK transcription factor has been shown to indicate a trend for a bad prognosis in RMS patients [135], and its modulation in ERMS cells clarified that Notch signaling aberrant functions in ERMS relies partly on a canonical signaling [155]. In fact, RBP-jK knockdown in ERMS cells downregulated HES1 expression and reduced colony formation in soft agar, while its overexpression behaved in the opposite manner [155]. ERMS cells depleted of RBP-jK formed smaller tumors *in vivo* and showed downregulation of pro-proliferative markers associated with upregulation of the cyclin-dependent inhibitor p21^{Cip1} [155].

The metastatic behavior is recognized as extremely important for the response to therapy and outcome of RMS patients, and metastasis formation has been related to Notch activation in cancer [159–161]. Therefore, starting from the findings of a correlation of HES1 or HEY1 levels with cell invasion *in vitro* in RMS cell lines, the Rome group further clarified the role of Notch1 and HES1 in the invasiveness of RMS cells [162]. Pharmacological treatment with a GSI of one fusion-negative- and one PAX7-FOXO1- and one PAX3-FOXO1-positive cell line led to a marked decrease of cell adhesion on two different substrates and negatively modulated N-cadherin and α 9-integrin transcriptional expression, together with those of the Notch target gene HES1, resulting in the lowering of protein levels [162]. These findings were in agreement with the observation that Notch1 and Notch3 upregulate N-cadherin in melanoma cells [163, 164]. In patients with RMS, a positive correlation between N-cadherin and α 9-integrin with HES1 was seen. In line with the hypothesis of an involvement of Notch signaling in this phenomenon, RMS cells transfected with a plasmid expressing a dominant-negative form of MAML1 [152] showed a response similar to that of cells treated with a GSI. Conversely, RMS cells in which an exogenous DLL1 was forcedly overexpressed, thus leading to Notch signaling over-activation, enhanced the expression of all the three genes. These effects appeared quite specific since the level of the usual partner of α 9-integrin, *i.e.*, β 1-integrin, was unaffected. Interestingly from a translational point of view, the authors showed that cell adhesion on fibronectin and the invasive capabilities of the cells *in vitro* were markedly reduced using an anti-N-cadherin-blocking antibody, whereas anti- α 9-integrin-blocking antibody was able to impair only the tumor cell adhesive properties. Chromatin-immunoprecipitation assays demonstrated a possible direct regulation of Notch1 on the two gene promoters. However, HES1 seemed also to bind those promoters, but its role in regulating these genes should be clarified in future studies. This pro-invasive role of Notch signaling in RMS seems to be counteracted by the restoration of the expression of miR-203, a microRNA often downregulated epigenetically by promoter hypermethylation in RMS primary samples and cell lines and re-expressed after treatment with the DNA methyltransferase 1 inhibitor 5-AZA [157]. When miR-203 was re-expressed *in vitro* in one ERMS and one PAX3-FOXO1 ARMS cell line, it inhibited cell proliferation inducing the myogenic conversion of the tumor cells, decreasing the levels of the transcription factor p63, an inducer of JAG1 and of HES1. Similar results were obtained silencing p63. These findings suggest that the promyogenic role of miR-203 relies, at least in part, on its

ability to down-modulate p63. Moreover, miR-203 forced expression blocked both cell migration and invasion. Tumor growth *in vivo* was also hampered in RMS cells overexpressing miR-203 or in ERMS-xenografted mice treated with 5-AZA. It could be interesting to evaluate whether the re-expression of miR-203 could have similar effects *in vivo* also in PAX3-FOXO1 ARMS cells, which are less prone to differentiate.

Previously, our findings unveiled a role for Notch3 in RMS [154]. Genetic down-regulation of Notch3 by silencing in fusion-negative and fusion-positive RMS cell lines overexpressing nuclear Notch1–3-activated forms compared to myoblasts resulted in a blockade of cell cycle in the G1 phase and formation of myofiber-like structures even when the cells were cultured in medium containing serum. In agreement with this phenotype, p21 was upregulated together with members of the differentiation machinery such as myogenin, MHC, and troponin. Moreover, p38MAPK, AKT, and mTOR were activated as during myogenesis. In parallel, HES1 levels were decreased suggesting that Notch3 can have a direct or indirect effect on its expression. Concordantly, HES1 depletion mimicked, as already reported by Sang et al. [151], as well as reinforced the effects of Notch3 silencing, while, conversely, its forced overexpression partially overcame them. Moreover, silencing Notch3 even in a fraction of cells inhibited tumor growth *in vivo*. Interestingly, (i) the depletion of Notch1, which was also hyperactivated in RMS cell lines, reduced the proliferation of the cells and, only in fusion-negative cells, favored the formation of some myotube-like structures, but was ineffective in fusion-positive cells; and (ii) the knockdown of Notch2, whose levels were higher in myoblasts, reduced the expression of myogenin and led to HES1 levels upregulation.

Consistent with a role of Notch3 in RMS, tumor cells forcedly expressing an exogenous N3ICD form proliferated faster *in vitro* and formed more colonies in soft agar irrespective of their fusion status [156]. Notably, the antiproliferative effects of a GSI were counteracted by N3ICD overexpression. We also confirmed that N3ICD influences tumor growth *in vivo* showing that PAX3-FOXO1/N3ICD xenografted cells produced bigger masses with a higher expression of Ki67 and HES1 [156]. Of note, we also showed that HES1 and Notch3 protein levels correlated with those of Ki67 in samples from RMS patients [156].

Since a very low number of mutations of Notch paralogs have been found in RMS primary samples [128, 129, 165], it is arguable that the hyperactivation of Notch receptors in tumor cells could be due to other reasons such as to the binding to the Notch ligands. As a matter of fact, downregulating DLL1 and JAG1, whose transcripts were found expressed in RMS cell lines [153] and primary specimens [135, 155], led to the inhibition of cell proliferation of ERMS and PAX3-FOXO1 ARMS cells associated with the lowering of N3ICD and HES1 levels [154, 156]. Summarizing all these results, it appears clear that a general dysregulation of the Notch signaling characterizes the RMS setting opening the way to potential targeted therapy for this sarcoma.

One of the characteristics of Notch signaling is the capacity to cross talk with several key pathways that regulate stem cell fate and are involved in cancer pathogen-

esis and maintenance. The Hedgehog pathway is one of the major regulators of the myogenesis in vertebrates, by maintaining the expression of the myogenic regulatory factors (MRFs) such as MYF5 and modulating survival and proliferation of developing myoblasts [166]. In particular, it supports the proliferation of myogenic precursors repressing terminal differentiation and apoptosis [167–169]. A dysregulation of Hedgehog seems to be one of the drivers of ERMS development, as highlighted by studies in humans and GEMM [170–173]. Recently, alterations of Hedgehog signaling have been recently shown to be interconnected to that of Notch in the pathogenesis of ERMS [132]. In this work the authors demonstrated that in mice heterozygous for the negative regulator of Hedgehog signaling *ptch1*, which spontaneously develop ERMS, the cells of origin of the tumor are derived from those expressing the Notch ligand *Dll1* and concomitantly negative for *Myf5*, *myogenin*, and *Pax3* expression [132]. This type of cells is prone to undergo myogenic differentiation but is not yet stably committed. These results, on one hand, imply that Hedgehog and Notch cross talk to define the fate of some cells during myogenesis, and on another hand highlight the importance of the molecular degree of differentiation and commitment for subsets of cells to behave as tumor-initiating RMS cells, as already demonstrated by the group of Keller [122, 174]. Importantly under a translational point of view, Hedgehog signaling activation is able to induce HES1 expression in both mesodermal and neural cells independently from Notch, suggesting combinatorial inhibition of the two pathways [175].

Several points on the impact of Notch signaling deregulation in RMS remain to be investigated among which the expression of protein levels of Notch ligands in RMS patients, its role in the invasiveness and metastasis in *in vivo* models, and its effects in GEMM of RMS. However, it appears evident that this signaling pathway could be activated in both ARMS and ERMS thus representing a potential target for therapy in both RMS variants.

11.2.2 Notch Signaling in Ewing Sarcoma

Ewing sarcoma (ES) is the second most common bone and soft tissue sarcoma of childhood. It arises most commonly in adolescents showing a median age of 15 years, even if cases of ES in neonates and infants have been reported [176, 177]. The most frequently affected sites are the lower extremities and pelvis for bone and the trunk and extremities for soft tissue disease. It is an aggressive malignancy, metastatic at diagnosis in about 25% of young patients [176]. Improvements in therapy have enhanced the survival rates for localized forms, but the outcome and disease-free survival of patients with metastatic disease remain poor [178–180]. ES often shows gains of chromosomes 8, 12, 20, and 1q, losses of 1p36 and 16q, and homozygous deletion of *CDKN2A*, but the mutation rate is low and mostly involves *STAG2* or *TP53* (5–20% of cases), making finding actionable therapeutic targets difficult ([181–183] and reviewed in [180]). In about 90% of cases, ES is characterized by typical chromosomal translocations t(11;22)(q24;q12) resulting in the fusion

of the amino-terminal-encoding portion of EWSR1 to the carboxyl-terminal DNA-binding domain of the FLI1 gene of the ETS family genes, generating the EWS-FLI1 fusion product with transcriptional regulatory functions ([184, 185] and reviewed in [186]). The translocation can involve several different portions of the genes, due to different breakpoints in each of the sequences, but without effects on the prognosis [187, 188]. Variants of fusion products involving or not EWS have also been observed in a number of cases (reviewed in [186]). When expressed in a “permissive” cell of origin context, i.e., mesenchymal- and neural crest-derived progenitors, EWS-FLI1 shows transforming capacity [185, 189–192]. EWS-FLI1 is a transcription factor with higher potency compared to FLI1 that binds to ETS consensus sequences across the genome [185, 193] and whose mechanism of action has been recently unraveled. It binds several types of chromatin regions, from promoters to intra- and intergenic regions, repressing but also inducing a high number of genes [194–197] with a function that can be context-dependent [198]. When exogenously expressed in murine fibroblasts, EWS-FLI1 induced the transcription of the Notch signaling enzymatic component Mfng [145], a result in agreement with transcript *MNFG* upregulation found in ES patients (Table 11.1) [146], even if in human ES cells, the transcriptional effect on *MNFG* is weaker [199]. Recently, the group of Kovar unveiled the mechanism through which EWS-FLI1 was able to overcome cell cycle arrest in a context of wild-type *TP53* [147]. The authors demonstrated that, by repressing the expression of the Notch ligand *JAG1*, EWS-FLI1 reduced the activation of Notch3 necessary for the induction of the Notch target gene *HEY1* that, in turn, stabilized and activated p53 [147]. Indeed, in *TP53* wild-type ES cell lines, (i) *EWS-FLI1* silencing promoted p53 and p21^{Cip1} expression followed by cell cycle arrest; (ii) this effect was associated with the induction of *JAG1* and *HEY1*, often barely expressed in ES primary samples; (iii) Notch2 and Notch3 were expressed in both ES cell lines and primary samples, and Notch3 resulted activated only in *TP53* wild-type cells by *JAG1*; and (iv) in EWS-FLI1-depleted cells, *JAG1* or *HEY1* silencing, treatment with a GSI, or expression of the negative regulator of Notch, NUMB, prevented p53 and p21^{Cip1} induction, while forced expression of either exogenous *JAG1*, *HEY1*, or N3ICD reversed the effects. Therefore, in ES cells with wild-type *TP53*, Notch signaling seems to act as an onco-suppressor stabilizing p53 with an unknown mechanism involving *HEY1*. Interestingly, when Notch signaling was inhibited in the presence of EWS-FLI1, no *HEY1* expression was observed, suggesting that the pathway could be inactive under these cell conditions [147]. This was consistent with the observation of a lack of nuclear expression of NICD and *HES1* in ES tumors, despite the mRNA upregulation of the latter [148]. Moreover, the transcriptional overexpression of *HES1* was independent from Notch activation and also from EWS-FLI1 expression.

ES pathogenesis implies an aberrant chromatin remodeling due to the influence of the fusion proteins on epigenetic machinery (reviewed in [186]). Accordingly, pharmacological inhibition of the lysine demethylase LSD1 (or KDM1A), upregulated in a large cohort of sarcomas including ES, led to p53 expression in ES cell lines

through the methylation of Lys 4 on histone H3 (H3K4) followed by cell cycle arrest [200]. In other cell systems, LSD1 is able, as a component of a corepressor complex with the deacetylase SIRT1, to inhibit Notch signaling by recruiting the RBP-jK complexes and repressing the expression of Notch target genes, including *HEY1* [201–203]. Starting from this observation, the same group sheds light on the mechanism of p53 induction after EWS-FLI1 depletion showing that ectopically expressed HEY1 prevented the expression of the deacetylase SIRT1, which in turn was responsible for the posttranslational modification that leads to p53 destabilization and deactivation [149]. This effect was obtained also by ectopic expression of NICD, demonstrating that it is Notch-dependent, and also demonstrated in other cell contexts in which Notch signaling can act similarly, such as B-cell tumors and primary human keratinocytes lacking *HEY1* expression. Consistently, genetic and pharmacologic inhibition of SIRT1 was sufficient to increase p53 acetylation and target genes activation, in ES cells in the presence of EWS-FLI1, resulting in tumor cell death, while its overexpression reverted the phenotype [149]. An antitumorigenic effect was also seen in vivo after pharmacological treatment of xenografted zebrafish models. Finally, the screening of about 400 ES human tumor samples by immunohistochemistry showed that SIRT1 expression could be correlated to disseminated disease due to the highest levels of staining in metastatic patients. Thus, on one hand, this work unveils a novel epigenetic Notch-dependent mechanism to regulate cell cycle and on the other hand points to SIRT1 as a pharmacologically targetable factor in ES. Although EWS-FLI1 is necessary for tumorigenesis, it requires a “permissive” cellular background for transformation. Among the involved adjuvant molecules is CD99 [204], a cell surface protein involved in cell migration, proliferation, and differentiation [205, 206]. As a matter of fact, EWS-FLI1 is able to upregulate CD99 that, in turn, facilitates the oncogenic function of the fusion protein [204, 207, 208]. However, although CD99 contributes to the oncogenic phenotype defined by the fusion gene, EWS-FLI1 is able to induce a neuroblastic phenotype while CD99 counteracts this effect [204]. Since ES cells are unable to completely differentiate, a recent work demonstrates that a network CD99-miR-34a-Notch-NF-kB underpins the mechanism underlying the anti-differentiative phenotype and suggests novel avenues for intervention [150].

The work showed that CD99, by inducing the expression of the Notch ligand DLL1, resulted in Notch1 and Notch3 activation paralleled by a concomitant activation of NF-kB, all effects prevented by CD99 depletion or GSI treatment. In turn, the CD99-dependent activity of NF-kB, or NF-kBp65 forced overexpression in a CD99 knock-down context, affected the neural phenotype due to the presence of EWS-FLI1, whereas, conversely, its silencing enhanced the proneural differentiation [150]. Elegantly, the authors then demonstrate that all the molecular and phenotypic effects of CD99 depletion, including Notch components regulation, can be phenocopied by a microRNA previously involved in ES and able to regulate Notch signaling, i.e., miR-34a [209–212], which was induced by CD99 knockdown. Thus, the presence of CD99 prevented miR-34a expression thus allowing Notch and NF-kB activation [150]. Interestingly, Notch

and NF- κ B pathways cross talk in several systems mainly in a noncanonical way (reviewed in [213]), which is in agreement with the inactivation of canonical Notch signaling found in ES, despite the expression of Notch receptors [147]. Strikingly, the effects of CD99 expression spread to neighboring cells through exosomes bearing CD99 from ES cells, and, consequently, when CD99 was depleted, exosomes lacking CD99 and containing high levels of the induced miR-34a carried a proneural signal to the target cells. These important results are in agreement with a previous report showing that Notch signaling inhibition induced neuroectodermal differentiation of tumor xenografts in ES with low impact on tumor cell proliferation [146]. Taken together, the reported findings further complicate the scenario of an role of Notch signaling in ES, showing an antiproliferative but also anti-differentiative role for this pathway. The predominance of a canonical versus noncanonical Notch signaling activation depends on the molecular context of the cells and deserves further investigations.

11.2.3 *Notch Signaling in Synovial Sarcoma*

Synovial sarcoma (SS) is a soft tissue sarcoma developing most commonly in the lower limbs of adolescents and young adults and showing a high metastatic potential ([214]; and reviewed in [215, 216]). It accounts for about 10–20% of all soft tissue sarcomas in young patients [217]. SS includes three histological subtypes: monophasic (only spindle cells), biphasic (both spindle and epithelial cells), and poorly differentiated. In addition to the soft tissue adjacent to the joints (i.e., synovial), it can develop in extra-synovial tissues. Localized disease can be treated by surgical intervention followed by adjuvant radiotherapy, but it often shows early and even late recurrences with 50% 10-year disease-free survival [218]. Molecularly, SS is characterized by the chromosomal translocation t(X,18; p11,q11) involving *SS18* (previously *SYT*) on chromosome 18q11 and either *SSX1*, *SSX2*, or very rarely *SSX4* on chromosome Xp11. The results are fusion proteins formed by almost all the SS18 sequence with the C-terminal portion of the SSX paralogs. That SS18-SSX proteins are the oncogenic drivers of the malignancy was demonstrated by the observations that their expression in vitro is sufficient to transform the cells, while their silencing reverts the malignant phenotype [219, 220]. SS is considered to be derived from mesenchymal stem cells in which the fusion proteins behave as oncogenes [221, 222]. In agreement with the importance of a specific cell of origin for tumorigenesis, the SS1-SSX oncoprotein induces spontaneous SS in transgenic mice in vivo with 100% penetrance when expressed in mesenchymal-derived progenitors expressing *Myf5* [223]. However, conversely to myogenic sarcomas, no expression of myogenic markers has been unveiled in SS murine models or in SS patients. The evidence of the presence of the fusion both in primary and metastatic lesions and the apoptotic effects linked to its depletion concur to suggest a master role for SS18-SSX in the development of SS [220, 224]. Although SS18 is a transcriptional activator and SSX functions as a repressor and both bind several partners, SS18-SSX does not contain a DNA-binding domain making difficult the identification of direct target genes [219]. However, it acts as a

transcriptional regulator controlling gene expression by chromatin remodeling (reviewed in [225]). Indeed, both SS18 and SS18-SSX associate with the SWI/SNF chromatin remodeling complex, which in normal cells/tissues facilitates gene transcriptional programs creating nucleosome-depleted regions at core promoters and regulatory regions [226–229]. The inclusion of SS18-SSX fusion products in the SWI/SNF complex dysregulates the function of the complex [229]. This is due to the repressor intrinsic properties of the SSX portion that can interact with gene repressor complexes, thus behaving in an opposite manner compared to SS18 itself [230]. SS shows no additional chromosomal imbalance in young patients; however it is characterized by a high expression of components of molecular pathways strictly involved in early embryogenesis. Among these are WNT, Hedgehog, BMP, and Notch pathways. Studies aimed at unveiling binding partners for the SS18-SSX factor demonstrated an interaction of the SSX portion with the corepressor TLE1 (Table 11.1) [144]. *TLE* genes encode for TLE1–4 proteins that are corepressors and, in particular TLE1, components of the Notch signaling the regulate stemness of embryonic progenitors during development. As a matter of fact, TLE1 is recruited by the Notch target HES1 on promoters to prevent gene expression [231]. SS18-SSX/TLE1 complex was found linked to ATF2, a transcriptional activator and DNA-binding protein, and was able to turn the ATF2 activator program in a repressor program [144]. The ultimate result is the repression of apoptotic/cell cycle blocker genes *EGR1*, *p21Cip1*, and *ATF3* and the promotion of tumor cell survival, which was impaired by SS18-SSX silencing. The intrinsic mechanism of this effect on ATF2 was related to the interaction of SS18-SSX with the polycomb repressor complexes PRC2 and PRC1 [232], whose repressor activity was further enhanced by the presence of TLE1 in the complex. A deregulated transcript expression of TLE1 has been found by expression profiling experiments in primary SS [141] and the nuclear expression of the protein confirmed by immunohistochemistry [142, 233]. To date, the evidence of an overexpression of TLE1 has currently entered the clinical use to discriminate among other soft tissue sarcomas [143]. In addition to TLE1, also other Notch-related factors have been shown to be upregulated in SS, such as Notch1 and JAG1 [141], although no evidence for functional roles for these proteins in SS pathogenesis has been described so far. However, results from a randomized Phase I/II clinical trial using the GSI RO4229097 in association with the Hedgehog inhibitor vismodegib for adult and adolescent patients with advanced and metastatic sarcomas, among which SS (Table 11.2), will give some information about the potentiality of Notch signaling inhibition in SS.

11.3 Approaches to Inhibit Notch Signaling

Considering the structure, regulation, and function of Notch components, several steps of the signaling pathway can be targeted for inhibition.

11.3.1 γ -Secretase Inhibitors (GSI)

The most widely used approach to hamper Notch signaling is based on the inhibition of γ -secretase activity resulting in Notch cleavage blockade. GSI showed anti-tumorigenic activities in various cancer cells in preclinical models, and some of them are currently in clinical trials for oncologic diseases, mostly for adult patients. However, over the last years, several Phase I/II studies have been started involving also pediatric and adolescent oncologic patients (Table 11.2).

MK-0752 is a clinical GSI that was evaluated in several Phase I clinical trials for treatment in pediatric and adult malignancies (Table 11.2) [234–236]. Another GSI, RO4929097 [237], was evaluated in several NCI-sponsored Phase I/II clinical trials for treatment of solid tumors and T-ALL (Table 11.2). RO4929097 has been used in combinatorial adjuvant regimens with other anticancer drugs, and it is recently in Phase I/II associated with vismodegib, an inhibitor of Hedgehog signaling, for treatment of advanced and metastatic sarcomas for adults and pediatric patients (Table 11.2).

The Phase II clinical trial with the GSI PF-03084014 for pediatric patients is ongoing for desmoid tumors and aggressive fibromatosis and is progressing to a Phase II for T-ALL and solid tumors (Table 11.2) [238]. In preclinical models, GSIs have shown also anti-angiogenic effects that could contribute to their efficacy in vivo. However, (i) GSI are unable to discriminate among Notch receptors and (ii) γ -secretase have a plethora of targets, and, thus, these chemicals can have off-target effects in vivo [239]. Among these, the most evident is the goblet cell metaplasia of the small intestine due to Notch2 inhibition in the intestinal epithelial stem cells compartment. Even if this effect can be partly prevented by coadministration of glucocorticoids, often the treatment with GSI requires a lowering in the doses and intermittent administration. Moreover, the evidence of Notch target inhibition in tumor tissue, to decide the dose escalation, is often difficult since the modulated clinical targets not always are the Notch targets found in preclinical studies but can depend on the tissue-context of the patient.

11.3.2 Antibodies Against Notch Signaling Components

Although all Notch paralogs have similar mechanisms of signalization, paralog-specific and even opposite downstream effects have been reported [154, 240–246]. Therefore, specific monoclonal antibodies against individual receptors or ligands have been developed so far. Although no Notch monoclonal antibody has been evaluated in pediatric tumors, some of them are being evaluated in clinical trials for adult tumors. The binding of the Notch component by the antibody results in the blockade of interaction between the receptor and the ligand and hampers the activation of the signaling. Among the antibodies against DLL4, the ligands responsible for the sprouting of endothelial cells and formation of new vessels that have been evaluated in

Table 11.2 Completed and ongoing clinical trials with γ -secretase inhibitors in pediatric/young adult oncologic patients

| Compound | Combined | Clinical trials. Gov Identifier | Clinical studies | Cancer type | Patients age |
|-------------|---|---------------------------------|------------------|--|----------------------------------|
| MK0752 | | NCT00106145 | Phase I | Breast and advanced solid tumors | 18 years and older |
| MK0752 | | NCT00100152 | Phase I | T-ALL | 12 months ^a and older |
| PF-03084014 | | NCT01981551 | Phase II | Desmoid tumors/aggressive fibromatosis | 18 years ^a and older |
| PF-03084014 | | NCT00878189 | Phase I | Advanced solid tumors T-ALL | 16 years and older |
| RO4929097 | | NCT01269411 | Phase I | Gliomas | 18 years and older |
| RO4929097 | WBRT SRS | NCT01217411 | Phase I/II | Breast cancer, lung cancer, melanoma | 18 years and older |
| RO4929097 | Dexamethasone | NCT01088763 | Phase I | Leukemia, solid tumors, lymphoma | 1 year to ^a 21 years |
| RO4929097 | Vismodegib | NCT01154452 | Phase I/II | Advanced or metastatic sarcoma | 18 years and older |
| RO4929097 | Carboplatin/paclitaxel | NCT01238133 | Phase I | Breast cancer | 18 years and older |
| RO4929097 | Cisplatin, vinblastine, and temozolomide | NCT01196416 | Phase I/II | Recurrent or metastatic melanoma | 18 years and older |
| RO4929097 | Cediranib maleate | NCT01131234 | Phase I | Advanced solid tumors | 18 years and older |
| RO4929097 | | NCT01232829 | Phase II | Metastatic pancreas cancer | 18 years and older |
| RO4929097 | Gemcitabine hydrochloride | NCT01145456 | Phase I | Advanced solid tumors | 18 years and older |
| RO4929097 | Temozolomide and radiation therapy | NCT01119599 | Phase I | Malignant glioma | 19 years and older |
| RO4929097 | Ketoconazole, rifampin, midazolam, hydrochloride, omeprazole, tolbutamide, dextromethorphan, hydrobromide | NCT01218620 | Phase I | Adult solid neoplasm | 18 years and older |
| RO4929097 | Bicalutamide | NCT01200810 | Phase II | Prostate cancer | 18 years and older |

(continued)

Table 11.2 (continued)

| Compound | Combined | Clinical trials. Gov Identifier | Clinical studies | Cancer type | Patients age |
|-----------|-------------------------------|---------------------------------|------------------|--|--------------------|
| RO4929097 | | NCT01141569 | Phase II | Renal cell carcinoma | 18 years and older |
| RO4929097 | | NCT01192763 | Phase I | Pancreatic cancer | 18 years and older |
| RO4929097 | Letrozole | NCT01208441 | Phase I | Breast cancer | 18 years and older |
| RO4929097 | Exemestane, goserelin acetate | NCT01149356 | Phase I | Metastatic breast cancer | 18 years and older |
| RO4929097 | | NCT01175343 | Phase II | Metastatic epithelial ovarian cancer, fallopian tube cancer, and primary peritoneal cancer | 18 years and older |
| RO4929097 | Capecitabine | NCT01158274 | Phase I | Solid tumors | 18 years and older |
| RO4929097 | | NCT01116687 | Phase II | Colon cancer, rectal cancer | 18 years and older |
| RO4929097 | Cetuximab | NCT01198535 | Phase I | Metastatic colorectal cancer | 18 years and older |
| RO4929097 | | NCT01070927 | Phase II | Non-squamous non-small cell lung cancer | 18 years and older |
| RO4929097 | Bevacizumab | NCT01189240 | Phase I/II | Glioma | 18 years and older |
| RO4929097 | Erlotinib hydrochloride | NCT01193881 | Phase I | Lung cancer | 18 years and older |
| RO4929097 | Vismodegib | NCT01071564 | Phase I | Breast cancer | 18 years and older |
| RO4929097 | | NCT01193868 | Phase II | Advanced non-small cell lung cancer | 18 years and older |
| RO4929097 | Temsirolimus | NCT01198184 | Phase I | Advanced solid tumors | 18 years and older |
| RO4929097 | | NCT01096355 | Phase I | Solid malignancies | 18 years and older |

<http://clinicaltrials.gov>

T-ALL T-cell acute lymphoblastic leukemia/lymphoma

^aEnrollment of children

clinical trials for adult malignancies are MEDI0639 (NCT01577745, recruiting Phase I), OMP-21M18 (NCT01189929; NCT01952249; NCT01189942; NCT01189968 Phase I and Ib), and REGN421 (Phase I completed, showing good tolerability and two partial responses [247]). The specific antibody OMP-52M51 against Notch1 is in clinical trial Phase I, NCT01778439; NCT01703572) and the antibody OMP-59R5 against Notch2/3 in Phase I/II trials (NCT01647828; NCT01859741).

11.3.3 Blocking Peptides

Preclinical studies demonstrated that it is possible to interfere with the transcriptional machinery of Notch signaling with inhibitory peptides. This is the case of a dnMAML1 used to block the RBP-jK-dependent transcription due to Notch activation. Stapled peptides competing with MAML are able to prevent gene transcription in murine models of T-ALL [248, 249]. One characteristic of this stapled peptide is the ability to bind also to preassembled Notch1–CSL complexes to inhibit the binding of the endogenous MAML1 [249]. These peptides have relatively small size and are highly cell-permeable. However, if they target only the transcriptional activity of Notch signaling, they can be ineffective in cancers in which the Notch pathway works in a noncanonical way. Nonetheless, dnMAML peptides could act also sequestering NICD in the cytoplasm, thus hampering also noncanonical roles of the cleaved protein.

11.3.4 Decoys

Soluble forms of the extracellular domains of Notch receptors and their ligands have been studied as decoys to inhibit the signaling. Decoys function by binding to endogenous ligands or receptors preventing the endogenous counterpart to be bound, and, since it lacks intracellular domains, the signaling of the pathway is completely abrogated [250–252]. Interestingly, endogenous soluble Notch ligands can be produced by metalloproteases, but their physiologic role still needs to be clarified [253, 254].

11.4 Conclusions

In conclusion, we summarized the role of Notch signaling in pediatric soft tissue sarcomas, giving an overview of the potentiality in targeting the pathway. Notch signaling plays a major role in the determination and homeostasis of tissues of mesenchymal origin in the embryo and postnatal life. Here we highlighted a role of Notch signaling deregulation in pediatric soft tissue sarcomas in the preclinical setting, reporting evidence that Notch modulation regulates cell proliferation, differentiation, and motility/invasion of tumor cells. To date, the majority of approaches against Notch signaling activation rely on the use of GSI even if promising monoclonal antibodies and cell-permeable small molecules are being developed for adult cancers. It is arguable that the pharmacokinetics properties and the biodistribution of decoys and antibodies are the limiting factors for their therapeutic application. Interestingly, for those patients with tumors in which Notch pathway works as a tumor suppressor, such as in EWS, agents stimulating its activity or downstream effects should be considered. In summary, potentially a Notch-based therapy might

represent one of the future personalized strategies for young patients with soft tissue sarcomas.

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