TITLE: NOTCH PATHWAY INHIBITION SIGNIFICANTLY REDUCES RHABDOMYOSARCOMA INVASIVENESS AND MOBILITY IN VITRO.

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Running title: Notch pathway in rhabdomyosarcoma.

KEY WORDS: Rhabdomyosarcoma, Notch, Hes1, Hey1, cancer, GSI.

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STATEMENT OF TRANSLATIONAL RELEVANCE:

RMS patients with metastatic disease continue to have very poor prognosis, despite the indication of aggressive therapies. Moreover, the major cause of death in these patients is the formation of distant metastases. This work states that the Notch pathway is broadly expressed in RMS tumors and its inhibition in vitro using γ-secretase inhibitors clearly impairs the mobility and invasiveness of RMS cells. This work proposes the Notch pathway as a candidate for the development of targeted therapies focused on reducing metastases in this neoplasia.
ABSTRACT

**Purpose:** Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children and can be divided into two main subtypes: embryonal and alveolar RMS. Patients with metastatic disease continue to have very poor prognosis although aggressive therapies and recurrences are common in advanced localized disease. The oncogenic potential of the Notch pathway has been established in some cancers of the adult and in some pediatric malignancies. **Experimental Design:** A real-time PCR assay was used to ascertain the expression of several Notch pathway components in a wide panel of RMS and cell lines. Four γ-secretase inhibitors (GSIs) were tested for pathway inhibition and the degree of inhibition was assessed by analysis of Hes1 and Hey1 expression. The putative effects of Notch pathway inhibition were evaluated by wound-healing, matrigel/transwell invasion, cell-cycle and apoptosis assays. **Results:** The Notch pathway was widely expressed and activated in RMS and underwent substantial inhibition when treated with GSIs or transfected with a dominant negative form of MAML1. RMS cells showed a significant decrease in its mobility and invasiveness when the Notch pathway was properly inhibited; conversely, its inhibition had no noticeable effect on cell cycle or apoptosis. **Conclusion:** Pharmacological or genetic blockage of the pathway significantly reduced invasiveness of RMS cell lines, thereby suggesting a possible role of the Notch pathway in the regulation of the metastatic process in RMS.
INTRODUCTION

Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children. Regarding histopathologic criteria, RMS can be divided into two main subtypes: embryonal and alveolar RMS (eRMS and aRMS, respectively). The majority of aRMS (80–85%) contain one of the reciprocal chromosomal translocations: either t(2;13)(q35;q14) or t(1;13)(p36;q14). These translocations generate the novel fusion genes PAX3-FOXO1 and PAX7-FOXO1, respectively (1, 2). However, no characteristic translocations have been described in eRMS. The eRMS is typically characterized by loss of heterozygosity on the short arm of chromosome 11 (11p15.5) (3) and gains in chromosomes 2, 7, 8, 11, 12, 13 and 17 are also common in this subtype (4).

The Notch signaling pathway is an evolutionary conserved pathway that plays a critical role in tissue development in organisms ranging from nematodes to mammals. The highly conserved Notch gene family encodes 4 cell surface receptors (Notch1, Notch2, Notch3 and Notch4). When the Notch receptor is activated by its ligands (Delta and Jagged in vertebrates), the Notch intracellular domain (NICD) is released by the γ-secretase complex (5) and translocates to the nucleus where it binds to CSL transcription repressors, converting them into transcriptional activators. The targets of these transcription factors in vertebrates are typified by Hes and Hey genes (6, 7).

The oncogenic potential of the Notch pathway was first described in acute T-cell lymphoblastic leukemia (T-ALL) in the late 1980’s (8). An abnormal up-regulation of the Notch pathway has also been reported in ovarian (9), breast (10) and other cancers (11). With respect to pediatric malignancies, Notch signaling appears to contribute essentially to osteosarcoma metastasis (12) and proliferation (13); Notch signaling also
promotes medulloblastoma cancer stem cell survival (14) and contributes to angiogenesis in neuroblastoma (15).

In the last decade, the use of pharmacologic inhibitors of the γ-secretase complex, which inhibit activation of the four Notch receptors, has been reported. Gamma-secretase inhibitors (GSIs) block the generation of the active form of Notch: the Notch intra-cytoplasmatic domain (NICD) (16). In recent decades, several GSIs have been actively studied as potential inhibitors of the generation of the β-amyloid peptide associated with Alzheimer’s disease (17). More recently, some GSIs have begun to be studied in phase I trials for patients with advanced breast cancer and acute T-cell leukemias (18, 19).

The possible role of the Notch pathway in RMS remains unknown. Since the regulation of Notch signaling is involved in satellite cell activation and in cell fate determination during postnatal myogenesis (20) and since RMS are embryonal tumors whose cells exhibit gene expression profiles resembling those of fetal muscle cells (21), we hypothesized that the Notch pathway could play a major role in maintaining the immature muscle status characteristic of RMS. In the present work, Notch pathway expression and activation profiles were characterized for the first time in a wide panel of RMS tumors and cell lines. The reduction by several γ-secretase inhibitors in the oncogenic potential of RMS was evaluated by wound-healing, matrigel/transwell invasion, cell cycle and apoptosis assays.
METHODS

Tumor samples

Samples were collected from a non-selected cohort of 37 children with rhabdomyosarcoma. Twenty-seven samples were from patients referred for diagnosis and/or treatment at the Vall d’Hebron Hospital Oncology Unit and 10 were a kind donation from Dr Albert Chetcuti (Tumour Bank, Oncology Research Unit, Children’s Hospital at Westmead, Australia). Informed consent has been previously obtained from all parents or legal guardians.

Cell cultures and drug treatments

Cells were cultured in MEM media with Earle’s Salts (PAA Laboratories, Haidmannweg, Austria), supplemented with 10% FCS (PAA), 2mM L-glutamine (PAA), 1mM sodium pyruvate (PAA), 1X NEAA (PAA), 100U/ml penicillin (PAA) and 0.1mg/ml streptomycin (PAA), and maintained at 37°C in a 5% CO2 water-jacketed incubator. All GSI inhibitors were diluted in DMSO and added to the culture media at the desired concentration. Doses were as follows: 25μM for DAPT, 200nM for GSI-X, 8nM for GSI-XX and 2nM for GSI-XXI. The untreated plates were supplemented with an equivalent volume of DMSO. The inhibitors GSI-X, GSI-XX and GSI-XXI were purchased from Calbiochem (San Diego, CA, USA) and DAPT from Sigma-Aldrich (St. Louis, MO, USA). Cell lines RH-30, CW-9019 and HTB-82 were obtained from American Type Culture Collection (ATCC).

RNA isolation, retrotranscription and Real-time PCR

Total RNA was isolated using a Quick-prep micro RNA isolation kit (Qiagen, Valencia, CA, USA); RNA integrity was quantified and verified by an ABI PRISM
7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Samples of 2μg of total RNA were reverse-transcribed using random primers (Invitrogen, Carlsbad, CA, USA). The reaction mixture was incubated for 60 min at 37ºC with 200U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was based on the TaqMan assay (Applied Biosystems). A 40-cycle PCR was performed to detect the four Notch receptors (assays Hs01062014_m1, Hs01050708_m1, Hs00166432_m1 and Hs00270200_m1 for Notch1, Notch2, Notch3 and Notch4, respectively) and the effectors Hes1 and Hey1 (assays Hs00172878_m1 and Hs00232618_m1, respectively). The housekeeping gene TBP (assay Hs00172424_m1) was used as internal control. Quantification of relative levels of each mRNA analyzed was performed by the method of Livak and Schmittgen (22). All samples were tested in triplicate.

**Western blot analysis and immunohistochemistry**

Western blot: Cells were homogenized in lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM PMSF, 1mM EDTA, 5mg/ml aprotinin, 5mg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) and incubated for 4 min at 90°C and the total protein content was measured (DC assay kit, Bio-Rad Laboratories). Following the addition of 5% β-mercaptoethanol, 0.001% bromophenol blue and 20% glycerol, 12.5μg of protein per lane were loaded in 12% SDS-PAGE and then blotted onto PVDF membranes (Bio-Rad Laboratories). Membranes were then incubated with rabbit polyclonal antibody anti-Hes1 AB5702 (Millipore, Billerica, MA, USA) diluted 1:1000 and anti-α-tubulin (Cell Signaling, Danvers, MA, USA) diluted 1:2000 was used as a loading control.
Immunohistochemistry (IHC): Patient samples were obtained from the Vall d’Hebron Hospital Pathology Department (kindly provided by Dr. Nuria Toran). Paraffin-embedded tissues were sliced, deparaffinized and rehydrated, followed by antigen retrieval in 10 mM citrate buffer (DAKO). Endogenous peroxidase activity was quenched using 1% hydrogen peroxide. Samples were blocked and incubated overnight with anti-Hes1 antibody (AB5702, Millipore) diluted 1:200. After incubation with peroxidase conjugated secondary antibody and peroxidase substrate, samples were counterstained with hematoxylin for 10 s, dehydrated and mounted.

**Wound-healing and transwell assays**

For wound-healing assay, cells were grown until confluence in the presence of the appropriate GSI (48 hours). Cell monolayers were then scratched with a pipette tip and placed in complete growth medium with the appropriate GSI. Over 37.5 hours, serial images of selected fields were acquired every 15 minutes by a live cell imaging cell-R workstation (Olympus, Hamburg, Germany) coupled to a bright-field microscope. The scratched area was measured using the ImageJ software (NIH, freely available at http://rsb.info.nih.gov/ij/) and the healing velocity was calculated as a quotient between the scratched area and time required for the wound to heal.

For the matrigel/transwell assay, cells were pre-treated with GSI for 48 h and harvested using trypsin. Once pre-treated, $10^5$ cells were resuspended in serum-free MEM media with the appropriate GSI and plated in the upper chamber previously coated with BD Matrigel™ (BD-Biosciences, Erembodegem, Belgium) in an 8 µm pore size transwell (Corning, Schiphol, The Netherlands). Following incubation at 37°C, remaining cells were removed from the upper chamber with a cotton swab, and cells migrated to the lower surface of the membrane were stained in 5ng/ml of Hoechst33342. Cells were then stained with 0.2% crystal violet, lysed in 10% acetic
acid and the absorbance at 590nm was determined as a value proportional to the number of cells on each membrane lower surface. All analyses were made in triplicate.

**Cell cycle and apoptosis**

One million cells were grown for 3 days in MEM media supplemented with the appropriate GSI or DMSO (control) and harvested with trypsin. For cell cycle, cells were fixed in 70% ethanol, treated with 100μg/ml RNase A and stained for 1 hour at room temperature with 1μg/ml propidium iodide. Apoptosis was detected using the annexin-V apoptosis kit (BD Biosciences) following manufacturer’s instructions. Cells were analyzed in a FacsCalibur cytometer (BD Biosciences).

**Transfection with dnMAML1**

Cells were transfected using FuGENE 6 transfection reagent (ROCHE, Basel, Switzerland) with dominant negative dnMAML1-GFP or GFP alone both cloned in the plasmidic vector pEGFP (Clontech Laboratories, CA, USA). Stably-transfected cells were selected for vector incorporation over 3 weeks in MEM culture media containing 2.0mg/ml G418 (Sigma-Aldrich).
RESULTS

Notch pathway is consistently expressed and activated in RMS tumors

Expression of the four Notch receptors and two downstream effectors of the Notch pathway were studied by quantitative PCR in 37 RMS primary tumor samples. The results showed a wide distribution of the Notch receptors and downstream effectors in the rhabdomyosarcoma samples studied (Figure 1). Levels of the RNAs studied in tumoral tissue (aRMS and eRMS) were compared with those of adult and fetal muscle (AM and FM) (2 pools of three samples were analyzed for both AM and FM). The most striking finding in tumors was the increase in the expression of Notch2, a receptor that showed very obvious upregulation in both aRMS and eRMS. Notch3 showed slight upregulation in both aRMS and eRMS not as clear as Notch2. Conversely, Notch1 and Notch4 showed no significant expression increase in RMS (Figure 1A). Hes1 expression was slightly upregulated in eRMS compared with both adult and fetal muscle, while Hey1 showed an increase in aRMS and eRMS compared with adult muscle levels (Figure 1A). IHC showed cytoplasmic and nuclear staining of Hes1 in a majority of RMS samples (Figure 1B) compared with prostate carcinoma as positive control (23).

Notch pathway activation correlates with invasive phenotype in RMS cells

Although the RMS cell lines analyzed significantly expressed Notch1, Notch2 and Notch3, no significant association was observed between their expression and the oncogenicity of the three cell lines analyzed (data not shown). Conversely, when expression of the Notch downstream effectors Hes1 and Hey1 was studied in the three RMS cell lines, they showed low values in HTB-82 (derived from eRMS), moderate values in CW-9019 (aRMS with PAX7-FOXO1 translocation) and high values in RH-
30 (aRMS bearing the PAX3-FOXO1 translocation). Thus, expressing results as a fold increase relative to the values found in HTB-82 (Figure 2A), Hes1 showed 24.2-fold higher expression in CW-9019, and 37.5-fold higher in RH-30. The same increasing pattern was observed for Hey1 (12.3-fold higher in CW-9019 and 34.8-fold higher in RH-30). When the invasive capabilities of the three cell lines were tested by matrigel/transwell invasion assay (Figure 2B), the results verified the correlation between the expression of the aforementioned Notch targets and the invasiveness of each cell line. Thus, invasiveness of the HTB-82 line was very low, with no significant invasion being found even at 48 hours. In contrast, the cell line CW-9019 showed noticeably invasive potential. At 8 hours, there was no significant invasion, but the number of cells migrated to the lower chamber rose considerably at 24 and 48 hours. Finally, the putatively most invasive cell line showed very high invasiveness detected as soon as at 8 hours. At longer times (24 and 48 hours), the invasion was so great that the lower chamber was saturated with cells and no significant differences were observed between 24 and 48 hours. When the wound-healing capability of the three cell lines was tested, the results revealed that while the RH-30 cell line completed the healing in approximately 18 hours, the CW-9019 cell line needed approximately 3 days to close the wound completely and the time required was even longer than a week for HTB-82. A significant image of each cell line was taken at 48 hours post-wounding (Figure 2C). At that time, cell line HTB-82 was very far from closing the wound, CW-9019 near to complete closure and RH-30 was completely closed.

**Notch pathway can be pharmacologically inhibited using γ-secretase inhibitors (GSIs) in RMS cells**
The effects of the inhibitors on the Notch pathway were evaluated by quantification of Hes1 and Hey1 mRNA expression. Hes1 mRNA expression showed a significant decrease in the 3 cell lines treated with DAPT (25μM) GSI-XX (8nM) and GSI XXI (2nM). The GSI-X (200nM) produced minor or null inhibition in the three cell lines analyzed (Figure 3A). Hey1 expression showed a similar inhibition pattern, with a significant decrease in the three cell lines treated with GSI-XX and GSI-XXI, while DAPT rendered only a significant reduction in Hey1 expression in the cell lines RH-30 and CW-9019. The GSI-X rendered a minor or null reduction, only significant in the cell line RH-30 (Figure 3B).

Although total inhibition was not achieved, Hes1 protein also decreased significantly when cells were treated with GSIs, especially with GSI-XXI (Figure 3C). No significant effect was observed when cells were treated with GSI-X or DAPT (not shown).

**Notch pathway inhibition severely impairs cell mobility and invasiveness in RMS cells**

When the 3 rhabdomyosarcoma cell lines were treated with GSIs, a significant reduction was observed in their mobility that could be measured in a wound-healing assay (Figure 4). The delay observed is illustrated in figure 4A for the cell line RH-30. Two images per treatment taken immediately post-wounding and 18 hours later are shown. It can be clearly seen that while cells treated with DMSO (control) closed the wound in approximately 18h, cells treated with all GSIs, particularly with DAPT, GSI-XX and GSI-XXI, did not. GSI-X, though presenting delay versus the control, showed a lesser effect on wound-healing time than the other three inhibitors tested. This lessening of wound-healing capability of cells treated with GSIs is demonstrated as a
reduction in healing velocity to approximately half that of the control in the 3 cell lines treated with GSI-XX and GSI-XXI. DAPT produced significant inhibition; however, while the reduction in the RH-30 cell line was approximately half that of the control, the reduction in the other two cell lines was only moderate. Finally, with GSI-X treatment, the reduction was very slight or null for all three cell lines (Figure 4B).

Furthermore, the three cell lines suffered severe impairment in the matrigel/transwell invasion assay when treated with some GSIs (Figure 5), suggesting that the Notch pathway could also play a role in their invasiveness. In that assay, the observed effects of the four Notch inhibitors tested were also unequal. Thus, cells treated with GSI-X showed only a minor reduction in their invasiveness (slightly lower than 80% compared to the DMSO control). On the other hand, cells treated with GSI-XX and GSI-XXI showed a remarkable reduction in invasiveness in the three cell lines (all values under 30% compared to the control, respectively). Finally, DAPT produced cell line-dependent effects, with moderate effect on RH-30 invasiveness, but a remarkably effect on CW-9019 and HTB-82 (Figure 5B).

**Down-regulation of Notch pathway has no noticeable effect on cell cycle and apoptosis**

Notch pathway inhibition had no significant effect on cell cycling and apoptosis, and all the drugs applied (in a 3 days treatment) produced the same results in treated and non-treated RMS cells (data not shown), confirming that the inhibition observed in cell motility and invasion can not be attributable to changes in cell proliferation or in apoptosis.

**Specificity (dnMAML1)**
Seeking to rule out the possibility of the effects observed in cells treated with GSIs being attributable to the involvement of other pathways and therefore demonstrate that these effects resulted from specific Notch inhibition, we genetically manipulated Notch signaling activity by transfecting a construct containing dominant negative MAML1 (dnMAML1) or empty vector (pEGFP). MAML1 is a co-activator that cooperates with NICD and CSL transcription factors (CBF1, Su(H) and Lag-1) to activate transcription of Notch target genes (24,25). It has been demonstrated that truncated versions of MAML-1 can maintain an association with the complex that behaves in a dominant negative fashion and depresses transcription of Notch target genes (26). In the RH-30 cell line stably transfected with dnMAML1, Hes1 mRNA expression fell to under 30% of the levels found in controls (Figure 6A). Likewise, the levels of Hes1 protein underwent a decrease in cells transfected with dnMAML1 (Figure 6B). This attenuation of Hes1 expression produced effects on cell mobility and invasiveness. Thus, RH-30 cells (not transfected) or transfected with GFP alone (control) closed the wound significantly faster than cells transfected with dnMAML1. This lessening of wound-healing capability in cells transfected with dnMAML1 is demonstrated as a reduction in healing velocity (Figure 6C). Finally, and paralleling the results previously shown with GSI treatments, the RH-30 cell line expressing the dnMAML1 underwent substantial inhibition of its invasiveness on a matrigel/transwell assay (Figure 6D) which was seen to fall to a level under 20% of that of control cells.
DISCUSSION

Notch signaling is known to play a fundamental role in the normal development of multicellular organisms, particularly in processes that imply cell-fate determination. More recently, some advances have been made in elucidating the role of the Notch pathway in adult cancer. However, little is known on the role of the Notch pathway in the oncogenicity of pediatric tumors. Only very recent works on medulloblastoma (14), neuroblastoma (15) and osteosarcoma (12, 13) have clarified some aspects of the Notch pathway in these neoplasias; however, the overall role of this pathway in pediatric malignancies remains unclear. In addition, the possible role of the Notch pathway on the oncogenicity of RMS remains unknown. RMS patients with metastatic disease continue to have a very poor prognosis, although aggressive therapies are indicated. Moreover, the major cause of death in these patients is the formation of distant metastasis. The cellular components that control metastasis in RMS should be identified before the development of targeted therapies focused on reducing metastasis in this neoplasia.

In this report, we provide evidence of Notch pathway expression and activation in RMS. One criterion to be met before a possible role of a pathway in a tissue can be considered is that this pathway must be expressed and activated in that tissue. It is noteworthy that all RMS tumors (n=37) analyzed presented expression of several Notch receptors and downstream Notch targets. Upregulation of Notch2 was particularly striking while upregulation of Notch3, Hes1 and Hey1 was also significant. The possible correlation between Notch pathway activation and the invasiveness and mobility of each RMS cell line analyzed is of particular interest. The HTB-82 cell line is derived from an eRMS, bears no translocation and is associated with the most benign phenotype of the disease. Its invasiveness is minimal and Notch activation is practically
absent. By contrast, the RH-30 cell line is derived from an aRMS bearing the PAX3-FOXO1 translocation (representing the most aggressive phenotype). This cell line presents a highly-invasive phenotype in culture and is the cell line with the highest Notch pathway activation. Finally, the CW-9019 cell line is derived from an aRMS bearing the PAX7-FOXO1 translocation (moderately aggressive phenotype) and presents considerable invasiveness, although less than the RH-30 cell line. Its levels of Notch activation are also considerable and situated between the other two cell lines. These results raise the possibility that Notch pathway activation could be responsible for the triggering, at least in part, of metastatic mechanisms in the invasive cells, particularly in rhabdomyosarcoma cell lines. Using the three aforementioned RMS cell lines as models for the study of Notch activation and its possible role in the promotion of oncogenicity, some GSIs were used to test the effects of Notch inhibition on the oncogenicity of these cell lines. Gamma-secretase inhibitors were first assayed for possible use as therapeutic agents in Alzheimer’s disease (27). However, the use of GSIs reveals a concomitant Notch proteolytic inhibition and, over recent decades, these inhibitors have also been tested for anti-oncogenic effects (28). In the present work, we demonstrate the usefulness of several GSIs for Notch pathway inhibition in three RMS cell lines. One of the 4 drugs tested (GSI-X) produced only moderate inhibition but the other three drugs (DAPT, GSI-XX and GSI-XXI) yielded better results. This GSI-mediated Notch inhibition in the three cell lines analyzed – although total inhibition was not achieved – led to severe lessening of cell mobility and invasiveness in vitro, suggesting that the Notch pathway is responsible, at least partially, for the control of the mechanisms that allow cells to migrate and invade, thereby pointing to a possible future use of the drugs tested – particularly GSI-XX and GSI-XXI – as a therapeutic agents in the fighting against metastasis in RMS. Treatment with GSIs produces considerable
gastrointestinal toxicity (29) that could be reduced, at least in part, by concurrent treatment with corticosteroids (30). Moreover, GSI-XX and -XXI may have a more favorable therapeutic profile than the most commonly used DAPT, since they are effective at lower concentrations in vitro.

Around 55 membrane proteins are proteolyzed by γ-secretase (28), a fact to be borne in mind before the effects observed in γ-secretase inhibition are attributed to a single pathway. Therefore, in our opinion, all the results obtained should be validated using a γ-secretase-independent inhibition methodology. For this reason, we provide a model in which Notch proteolysis remains untouched, while NICD cannot activate transcription of Notch target genes owing to the expression of a dominant negative MAML1 transcription factor. Using this methodology, the effects seen on cells were practically identical to those observed with GSI treatment, which confirms more specifically that Notch inhibition suffices to produce the effects described with GSI treatment, particularly on invasiveness.

Although our study demonstrates a critical role of the Notch pathway in the activation of the process that leads to cell mobility and invasiveness in cell culture, further study will be needed in RMS to elucidate its implications in the more intricate tumor microenvironment. In our opinion, one of the challenges for the future is to create RMS animal models to characterize the pathway in vivo and attempt the possible use of GSIs – while avoiding toxicity – as a possible complementary targeted therapy against RMS, particularly in cases with a high probability of relapse.
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FIGURE LEGENDS

Figure 1: Notch pathway is widely expressed in RMS. (A) Box-plot depicting the expression of the four Notch receptors and expression of Hes1 and Hey1 assessed by real-time PCR in RMS tumors. Results are normalized using AM values as a baseline (value log2 = 0). AM: adult muscle, FM: fetal muscle, ERMS: embryonal rhabdomyosarcoma, ARMS: alveolar rhabdomyosarcoma. Asterisks: mean values significantly higher than levels obtained in AM (*) or FM (**). (B) Hes1 IHC in 3 samples of RMS (RMS 1, 2 and 3). Prostate carcinoma (PC) was used as positive control. Horizontal bar: 100 μm.

Figure 2: Notch activation correlates with RMS cell-line invasiveness. A: Relative quantification (RQ) of Hes1 and Hey1 in the three RMS cell lines HTB-82, CW-9019 and RH-30. B: Evaluation of HTB-82, CW-9019 and RH-30 invasiveness in a matrigel/transwell assay at 8, 24 and 48 hours. *: In A and B, the value is significantly higher than the level obtained in the HTB-82 cell line with p < 0.001. C: Representative pictures of the wound-healing assay taken 48 hours after wounding of the three RMS cell lines indicated. The 2 parallel lines on the images delimit the wounded zone. Horizontal bar: 100 μm.

Figure 3: Notch activation could be significantly inhibited using GSIs in RMS cell lines. Effects of GSIs on mRNA expression of Hes1 (A) and Hey1 (B) in RMS cell lines expressed as levels relative to untreated RH-30 (DMSO). Significance of differences compared to control: * p<0.05. All RNA measurements were taken in triplicate. C: Effects of GSIs on Hes1 protein amount in the RMS cell lines RH-30.
(20μg of protein/well), CW9019 (40μg of protein/well) and HTB-82 (80μg of protein/well). Alpha-tubulin was used as a protein loading control. GSI: γ-secretase inhibitor XXI.

Figure 4: Inhibition of Notch activation severely impairs wound-healing. A: Representative images of the wound-healing assay in presence of the indicated GSIs just after wounding (0h) and 18 hours later (18h) in the RH-30 cell line. Horizontal bar: 100 μm. B: Effect of the GSIs on healing velocity in three RMS cell lines RH-30, CW-9019 and HTB-82. Each bar represents the mean of healing velocity in eight independent samples in μm$^2$ per hour. Significance of differences with control: * p < 0.05; ** p < 0.005.

Figure 5: Inhibition of Notch activation severely impairs invasiveness in RMS cell lines. A: Photocomposition showing one representative field per treatment on the lower surface of the transwell membrane in RH-30 cells. Cells were stained with Hoechst33342. The inhibitor is indicated in the upper right corner of each picture. Merged image: Hoechst33342 and visible light. B: Bars represent the percentage of invasion for the 3 cell lines analyzed compared to the control (DMSO) when the GSI indicated below each bar were added to the media. Each bar represents the mean of three independent wells. Horizontal bar = 100 μm. Significance of differences with control: *: p < 0.05; **: p < 0.005.

Figure 6: Effects of Notch downstream target silencing using dominant negative MAML1 (dnMAML1) in RH-30 cells. A: Effects of dnMAML1 on Hes1 expression assessed by real-time PCR expressed as a percentage and compared to the control (RH-
30). **B**: Western blot showing the effect of dnMAML1 expression on Hes1 protein amount. Alpha-tubulin was used as a loading control. **C**: Wound-healing assay showing the reduction in healing velocity in cells expressing dnMAML1. **D**: Effects of dnMAML1 on matrigel/transwell invasion assay. In A, C and D, *: Significance of differences with control (p < 0.005).
Figure 2

(A) Hes1 □ Hey1

(B) 8h □ 24h □ 48h

(C)

Downloaded from clincancerres.aacrjournals.org on April 1, 2017.
Figure 3

(A) and (B) show the relative expression levels of genes under different conditions. The graphs compare the expression of RH-30, CW-9019, and HTB-82 in DMSO, DAPT, X, XX, and XXI treatments.

(C) Represents Western blot analysis for Hes-1 and α-tubulin in RH-30, CW-9019, and HTB-82 cell lines treated with DMSO and GSI.
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A

B

Healing velocity (um/hour)

DMSO  DAPT  GSI-X  GSI-XX  GSI-XXI

0h

18h

RH-30  CW-8002  HTS-82

*  **  ***
Figure 6

A

Hes1 mRNA relative expression (%)

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B

GFP    dnMA ML1

Hes1

α-tubulin

C

Healing velocity (μm²/hour)

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Invasiveness (%)

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* indicates significant difference.
Clinical Cancer Research

NOTCH PATHWAY INHIBITION SIGNIFICANTLY REDUCES RHABDOMYOSARCOMA INVASIVENESS AND MOBILITY IN VITRO

Josep Roma, Anna Masia, Jaume Reventos, et al.

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