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ANTI-TUMOR EFFECTS OF THE NOTCH PATHWAY IN GASTROINTESTINAL STROMAL TUMORS

Amaury G. Dumont

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ANTI-TUMOR EFFECTS OF THE NOTCH PATHWAY IN GASTROINTESTINAL
STROMAL TUMORS

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ANTI-TUMOR EFFECTS OF THE NOTCH PATHWAY IN GASTROINTESTINAL
STROMAL TUMORS

A
DISSERTATION

Presented to the Faculty of
The University of Texas Health Science Center at Houston
and

The University of Texas M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences

in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

Amaury.G.Dumont, Pharm.D.

Houston, Texas

December, 2012

DEDICATION

*This work is dedicated to my wife, Sarah, who encouraged me in this journey, never stop to believe in me, and most of all, gave me our two extraordinary children, Ethan and Eden.
You are an amazing physician-scientist;
I love you.*

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*Nature seems unaware of our intellectual need for convenience and unity, and very often
takes delight in complication and diversity.*

Santiago Ramón y Cajal, 1906 - Nobel lecture

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Publication No _____

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Supervisory professor: Dennis P. Hughes, M.D., Ph.D.

Gastrointestinal Stromal Tumors (GIST) are sarcomas driven by gain-of-function mutations of *KIT* or *PDGFRA*. Although, the introduction of tyrosine kinase inhibitors has dramatically changed the history of this disease, evidences emerge that inhibition of KIT or PDGFRA are not sufficient to cure patients. The developmental pathway Notch has a critical role in the cell fate, regulating cell proliferation and differentiation. Dysregulation of Notch pathway has been implicated in a wide variety of cancers functioning as a tumor promoter or a tumor suppressor in a cell context dependent manner.

Given that Notch activation deregulates the morphogenesis of mesenchymal cells in the GI track, that Notch acts as a tumor suppressor in neuroendocrine tumors, and finally that the cell of origin of GIST are the Interstitial Cell of Cajal that arise from a mesenchymal origin with some neuroendocrine features, we hypothesized that Notch pathway signaling may play a role in growth, survival and differentiation of GIST cells. To test this hypothesis, we

genetically and pharmacologically manipulated the Notch pathway in human GIST cells. In this study, we demonstrated that constitutively active intracellular domain of Notch1 (ICN-1) expression potently induced growth arrest and downregulated KIT expression. We have performed a retrospective analysis of 15 primary GIST patients and found that high mRNA level of *Hes1*, a major target gene of Notch pathway, correlated with a significantly longer relapse-free survival.

Therefore, we have established that treatment with the FDA approved histone deacetylase inhibitor SAHA (Vorinostat) caused dose-dependent upregulation of *Notch1* expression and a parallel decrease in viability in these cells. Retroviral silencing of downstream targets of Notch with dominant negative *Hes-1* as well as pharmacological inhibition of Notch pathway with a γ -secretase inhibitor partially rescued GIST cells from SAHA treatment.

Taken together these results identify anti-tumor effect of *Notch1* and a negative cross-talk between Notch1 and KIT pathways in GIST. Consequently, we propose that activation of this pathway with HDAC inhibitors may be a potential therapeutic strategy for GIST patients.

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LIST OF ABBREVIATIONS

ANK	ankyrin repeats
ATP	adenosine triphosphate
BCR-ABL	breakpoint cluster region-abelson
CML	chronic myelogenous leukemia
CR	complete response
CT	computed tomography
DFS	disease-free survival
DT	desmoid tumor
EORTC	european organisation for research and treatment of cancer
ETV1	ETS translocation variant 1
ESFT	ewing's sarcomas family tumors
FDA	food and drugs administration
GFP	green fluorescent protein
GIST	gastrointestinal stromal tumor
HES	hairy and enhancer of split 1
HSP90	heat shock protein 90
IC50	half-maximal inhibitory concentration
ICC	interstitial cells of Cajal
ICC-MY	myenteric interstitial cells of Cajal
ICC-IM	intra-muscular interstitial cells of Cajal
ICN	intracellular domain of notch

IM	imatinib mesylate (Gleevec)
KIT	c-KIT; cellular homolog of v-KIT oncogene (HZ4 feline sarcoma virus)
LINE-1	long interspersed nuclear element one
LMS	leiomyosarcomas
LNR	lin12/Notch repeats
MAM	mastermind like
MAPK	mitogen-activated protein kinase
MMTV	mouse mammary tumor virus
MP	myenteric plexus
NLS	nuclear localization sequence
OS	overall survival
PBS	phosphate buffered saline
PDGFRA	platelet-derived growth factor receptor-alpha
PI	propidium iodide
PI3K	phosphatidylinositol 3 kinase
PE	phycoerythrin
PR	partial response
RMS	rhabdomyosarcoma
SAHA	suberoylanilide hydroxamic acid (vorinostat)
SCF	stem cell factor
SD	stable disease
SDH	succinate dehydrogenase
shRNA	short hairpin RNA

SLMV	synaptic-like microvesicles
STAT	signal transducer and activator of transcription
STR	sort tandem repeated
TAD	transcription transactivation domain
TKI	tyrosine kinase inhibitor
UTMACC	university of texas - md anderson cancer center

Chapter 1. Introduction and Background

1. Gastrointestinal stromal tumors as a paradigm in translational research

Gastrointestinal stromal tumors (GIST) are soft tissue sarcomas primarily occurring in the stomach (60%) or small intestine (35%), but may also affect the colon, rectum, esophagus or retroperitoneum [1, 2]. Their incidence has been estimated to be between ten and 15 cases per million people, giving approximately 4,000 to 5,000 new cases annually in the United States [3-5]. The median age at diagnostic is 60 years [1] and GISTs rarely affect children with less than 3% of cases before the age of 21 years [6]. The term ‘GIST’ was introduced by Mazur and Clark in 1983 to distinguish specific stromal tumors from other mesenchymal neoplasms of the Gastrointestinal (GI) tract such as leiomyosarcomas (LMS), leiomyomas, leiomyoblastoma, and schwannomas [7]. At that time, it was clear that GIST had a specific natural history and was generally resistant to conventional chemotherapy and radiation, with less than 5% of response in advanced patients [8, 9]. Although some old studies reported higher response rates, it is possible to speculate that old studies that have reported higher response rates to conventional chemotherapy in GIST included, in fact, some LMS patients. Prior the area of target therapies, the majority of GIST patients developed recurrences within five years despite complete surgical resection, preferentially into the peritoneal cavity or the liver [1]. In this population, the median survival was only 15 months. Long term survivals were rare, for example, a study from MD Anderson Cancer Center (MDACC) has found that no more than 10% of patients were disease free after forty months of follow-up [10]. The origin of GIST remained controversial until 1998, when Kindblom and colleagues from Göteborg University, observed striking morphological and immunophenotypic resemblance between GIST cells and the Interstitial Cells of Cajal (ICC), which serve as the pacemaker

cell of the gastrointestinal tract [11]. Ultrastructural similarities, including filopodia-like cytoplasmic projections, large mitochondria, large Golgi, associated with strong immunoreactivity for CD117 (KIT) and CD34 antibodies provided evidences that GIST are likely to arise from ICC or ICC precursors. The same year, Hirota and colleagues, made the seminal discovery that the large majority of GIST contain activating mutations in the proto-oncogene *KIT* [12]. In this study, on 6 patients with GIST, 5 harbored mutation in the juxta-membrane domain of KIT. These mutations constitutively activated KIT receptor without the requirement of the stem cell factor (SCF) ligand. These ‘gain-of-function’ mutations proved to be oncogenic as they induced malignant transformation in a lymphoid cell model, suggesting a major role of KIT mutations in GIST development. The functional key role of *KIT* mutations in GIST has been since supported by multiple lines of evidence. First, the activated phosphorylated KIT is found by immunohistochemistry in virtually all GIST samples [13]. Then, rare patients with germ-line mutations of *KIT* develop multiple familial GIST [14-17]. Engineered mouse models expressing *KIT* mutations develop GIST-like tumors [18, 19]. And lastly, the inhibition of KIT with imatinib resulted in a decrease of proliferation of GIST [20]. These findings allowed a major paradigm shift in GIST treatment with the possibility to target a specific molecular abnormality resulting in an unprecedented clinical success of a targeted therapy in solid tumor.

2. Oncogenic mutations in GIST

The proto-oncogene *KIT* is located on chromosome 4q12 and encodes for a type III transmembrane receptor tyrosine kinase. The gene *KIT* was first cloned in 1987 as the homolog of the Hardy-Zuckerman feline sarcoma virus oncogene v-KIT [22]. The protein of 145 kilodaltons contains five extracellular immunoglobulin domains involved in ligand binding and subsequent homodimerization, a single transmembrane domain, a juxtamembrane domain with inhibitory functions, a kinase domain (ATP binding pocket and activation loop) separated by a kinase insert and a carboxy-terminal tail region (Figure 1) [22].

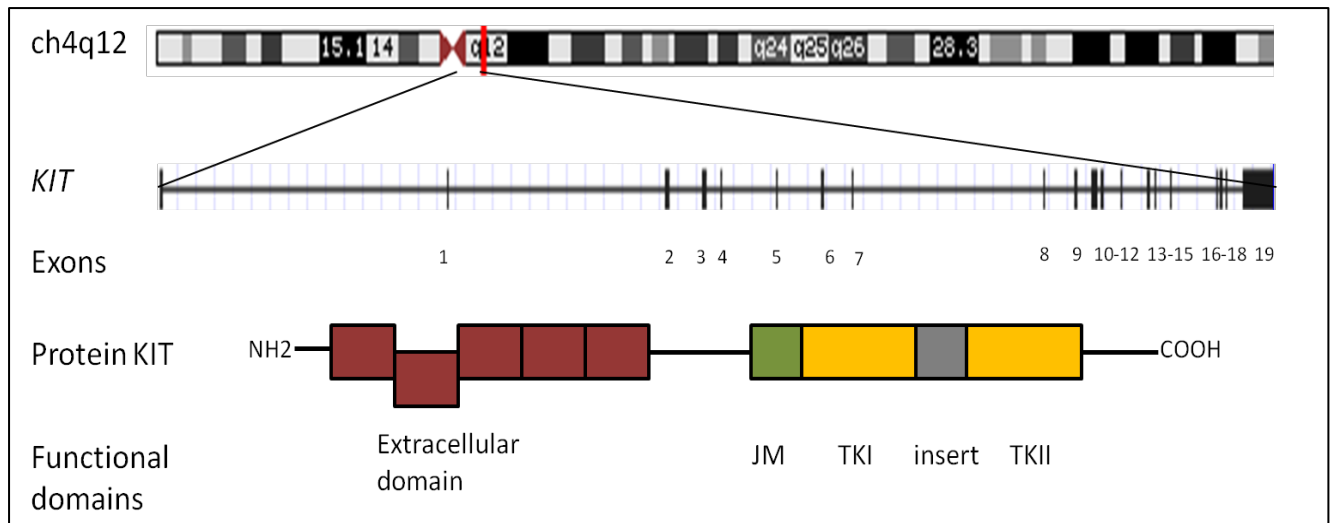


Figure 1. Schematic illustration of the gene and protein structure of KIT

KIT is located on chromosome 4q12 and contains 19 exons that encodes for an extracellular ligand-binding domain, a juxtamembrane region, an ATP binding site (tyrosine kinase I), a kinase insert and an activation loop (tyrosine kinase II).

In normal conditions, SCF binds KIT receptor and activates the kinase domain through transphosphorylation of tyrosine residues [23, 24]. Downstream signaling effectors includes the three following pathways: the phosphatidylinositol 3'-Kinase (PI3K), the mitogen-activated protein Kinase (MAPK) and Janus kinase/ signal transducers and activators of

transcription (JAK/STAT) pathways [13, 25, 26]. AKT activation through PI3K, regulates multiple target proteins such as Bad, MDM2, p27, FOXO, and mTOR, leading protein translation, survival and proliferation. MAPK pathway activates transcription factor including MYC, ELK, CREB, JUN and FOS (Figure 2).

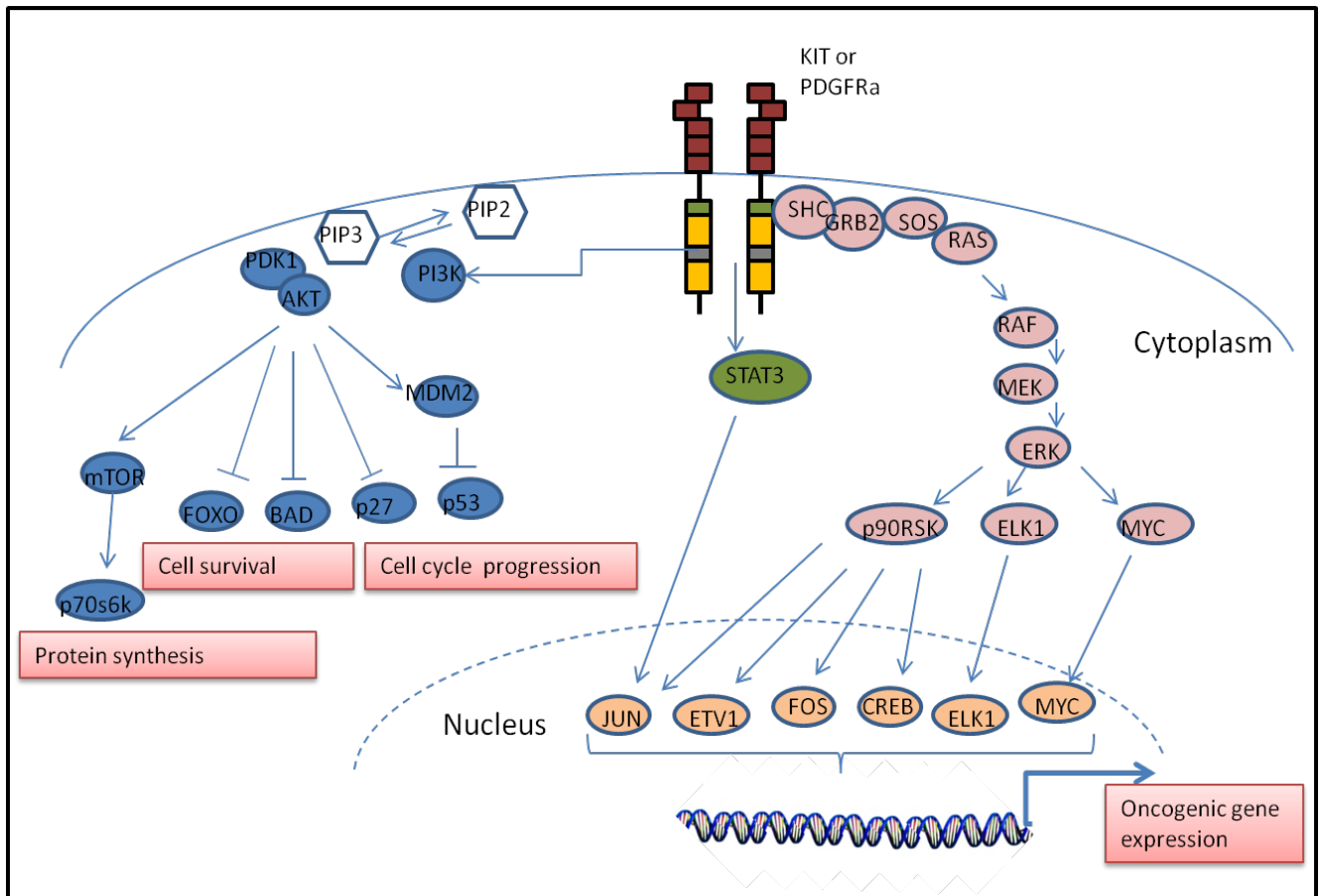


Figure 2. KIT and PDGFRa signaling in GIST

KIT activation by ligand binding or gain-of-function mutation, results in activation of RAS/RAF, PI3K and STAT pathways. The signal amplification leads changes in gene expression through MYC, ELK, CREB, JUN, ETV1 and FOS. In parallel, AKT activation modifies protein translation, cell cycle entry and apoptosis.

In GIST, gain-of-function mutations of *KIT* result in kinase activation in absence of ligand.

The gene contains several hotspots, the majority of mutations occurring in exon 11. Those mutations disrupt the juxtamembrane domain, forcing the kinase domain to switch into the

active conformation [27]. Mutations consist of substitutions, deletion, and insertions and are found in two thirds of GIST patients [28, 29]. The second most frequent mutations are in exon 9 that encodes the extracellular domain of KIT and affect between 8 to 12 % of GIST patients [30]. In presence of these mutations, the conformation of KIT changes to mimic the homodimerization that occurs after ligand binding [24]. It is important to realize that mutations in exon 9 imitate wild-type KIT activation resulting in a more resistant phenotype. Clinically, exon 9 mutations are found preferentially in the small and large intestine whereas exon 11 mutations are commonly seen in the stomach [31]. Uncommon primary mutations (<2%) have been observed in the kinase domain (the ATP binding domain or the activation loop) [32]. Of the GIST that not harbor *KIT* mutation, approximately 30% have a mutually-exclusive activating mutations of the gene that encode for the receptor for platelet-derived growth factor-alpha (*PDGFRA*) [33-36]. *PDGFRA* is a homologue of *KIT* and is located in adjacent loci on chromosome 4q. This protein is also a tyrosine kinase receptor with comparable functions. Mutations in *PDGFRA* occur in the juxtamembrane domain (exon 12), the ATP binding domain (exon 14) or the activation loop (exon 18). It has been reported that GIST lacking *KIT* or *PDGFRA* mutations may have mutations in *BRAF* [37]. Additionally, defects in succinate dehydrogenase (SDH) complex caused by germ-line mutations in *SDH* genes have been recently described in *KIT* or *PDGFRA* wild-type familial GIST patients [38, 39].

3. Chromosomal alterations in GIST

Although *KIT* or *PDGFRA* mutations play a key function during the initiation and development of GIST, other molecular alterations have essential roles during tumor

progression. This is illustrated by the fact that patients with germline *KIT* mutations develop familial GIST only after the age of 40 years indicating that though the mutation was present since birth, this time was required to acquire additional genomic aberrations [17, 40]. An additional argument is the description of ‘micro-GIST’ of less than 10 mm in size that are frequently found in the general population after surgery or during autopsy (between 2.9-35% of all patients) [41-44]. These micro-GIST appear inactive but harbor oncogenic *KIT* mutations indicating that these mutations alone may be insufficient for complete tumor progression.

Investigators have found that almost all adult GIST have cytogenetic aberrations [45, 46]. In GIST, losses of chromosomes are more frequent than gains [47]. In particular, loss of chromosome 14q, 1p and 22q appear to be frequent in GIST, observed in 40-67% [48-52]. This suggests that these three chromosomal regions may contain tumor suppressor genes important in malignant transformation of GIST. Clinically, the loss of 1p is most likely to be found in intestinal GIST and associated with aggressive clinical outcomes where the loss of 14p is more characteristic of gastric tumor and favorable prognosis. Additional losses on chromosomes, 9p, 11p, 13q, 15q and 17p have been also reported and are associated with malignancy [47]. Gains touch preferentially chromosomes 8q, 3q and 17q [53, 54]. Not surprisingly, accumulation of chromosomal alterations correlated with a worse prognosis [54]. Based on this observation, a recent study has proposed a gene signature on ‘genome complexity’ to classified low and high risk GIST patients [55].

4. Epigenetic alterations in GIST

The contribution of DNA methylation in the GIST malignancy has been suggested. In one study, the hypomethylation of long interspersed nuclear element one (LINE-1), a surrogate for global methylation, correlated with aggressiveness of GIST as well as chromosomal unbalance [56]. In general in tumors, the global hypomethylation observed in repetitive sequences coexists with a regional hypermethylation of CpG islands within specific gene promoter regions [57, 58]. In GIST, a genome-wide DNA methylation analysis of GpC islands has identified the hypermethylation of *REC8*, *PAX3* and *p16* promoter regions and a correlation with worse prognosis [59].

The role of small non-coding RNAs in the development of GIST remains unclear but initial data indicate that miR-221, miR-222 and miR-494 are down regulated in GIST and may negatively regulate KIT expression [60, 61]. On the other hand, upregulation of miR-196a was shown to drive malignant progression in GIST [62]. Additionally, our team has recently presented data indicating that Imatinib exposure is associated with differential miRNA expression profiles in GIST patient samples. In particular, a miRNA-449b increase was noted and appeared to target the pRb-E2F1 pathway [63].

5. Quest for the origin: The Interstitial Cells of Cajal

The recent success in the management of GIST has been the accomplishment of more than a century of research. At the end of the XIX century, the Spanish pathologist Santiago Ramón y Cajal using staining with silver chromate and methylene blue identified interstitial cells within the muscular layers of the GI wall [64, 65]. In his crucial work, he hypothesized that these cells formed a complex system at the interface between the autonomic nervous system and the smooth muscle of the GI tract [66]. This initial description was followed early by the

intuition that these cells referred as ‘the interstitial cells of Cajal’ represented pacemaker cells of the GI track similar to the pacemaker cells of the heart [67, 68]. Nevertheless, the rigorous scientific demonstration of a pacemaker function came decades later with the development of murine models where ablation of ICC resulted in suppression of the electrical slow wave activity [68-71]. A breakthrough came when investigators found that ICC expressed the receptor tyrosine kinase KIT (CD117) [72, 73]. This discovery gave a tremendous opportunity to gain insight of into the biology of ICC [73, 74]. Developmental studies that used to rely only on ultrastructure analysis became much powerful [75, 76]. Using KIT antibodies, it was now possible to follow the development of ICC within the GI track and determined their embryological origin [77]. With an elegant quail and chick chimeric model, a study suggested that ICC derived from mesenchymal precursors instead of neural crest cells as previously thought [78]. Moreover, the lack of neural crest markers gives additional arguments in favor of a mesodermal origin. Immunohistochemical studies and confocal images using KIT antibodies revealed different types of ICC [79, 80]. The two main ICC are the intramuscular ICC (ICC-IM) within the circular and longitudinal muscle layers mediating neurotransmission and the myenteric ICC (ICC-MY) surround the neuronal myenteric plexus and generating electrical slow waves (Figure 3). These two populations of ICC regulating electrical and secretory signals in the muscular layers confirm remarkably the Cajal’s initial vision that the interstitial cells are a complex network at the interface of the nervous system and the smooth muscle tissue.

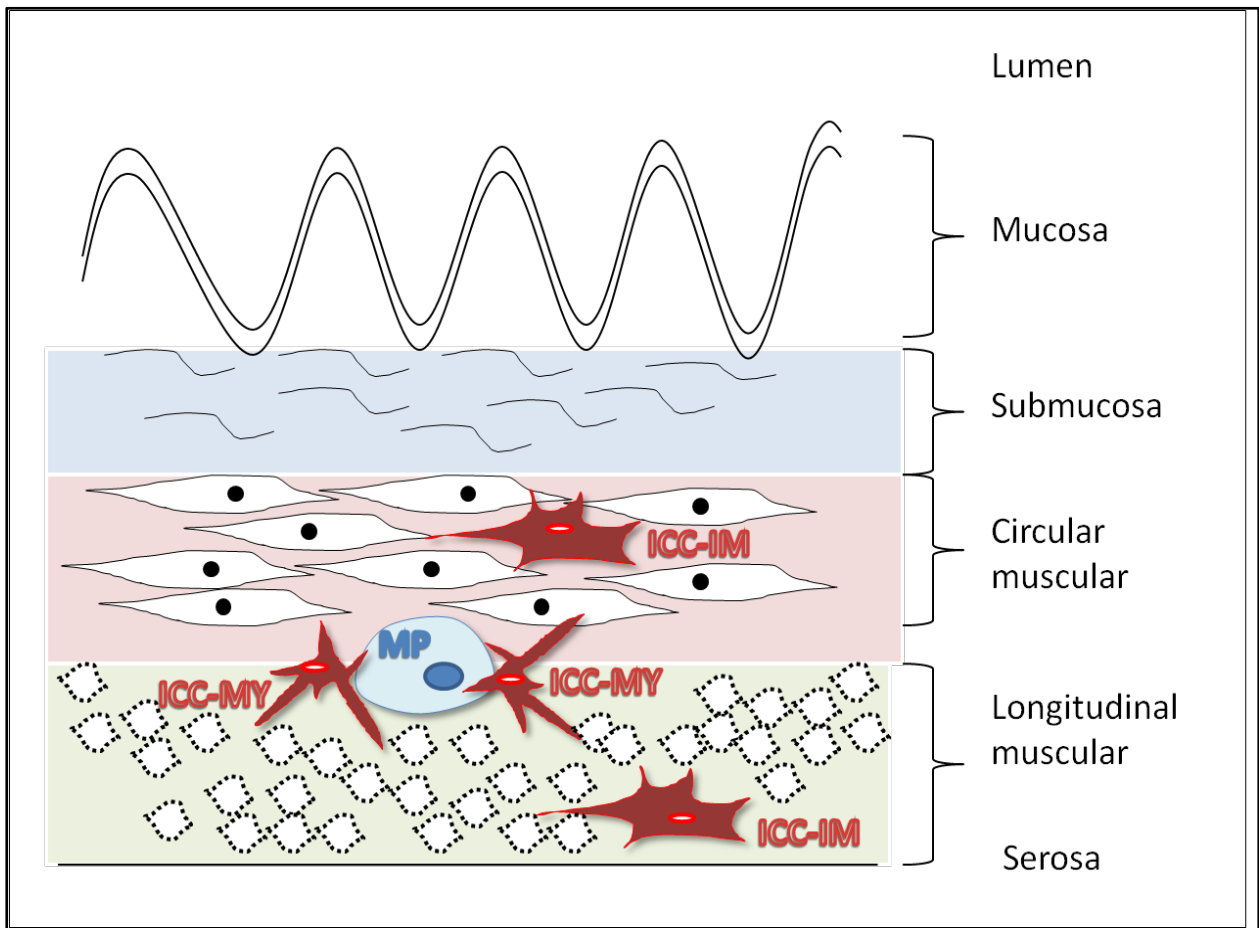


Figure 3. Schematic depicting the localization of ICC through the gastrointestinal wall layers

Cartoon showing localization of Intra-muscular ICC (ICC-IM) and Myenteric ICC (ICC-MY) throughout the GI tract; MP myenteric plexus.

As mentioned earlier, the initial observations done in 1998 that GIST cells and ICC shared phenotypical similarities leading the hypothesis that ICC are the cell-of-origin of GIST has been confirmed by the development of genetically engineered mice model expressing KIT mutations. In these mice ICC hyperplasia and GIST-like tumors were observed [18, 19]. Additionally, several studies have shown that ICC and GIST have similar gene expressions, in particular with high expression of nestin, DOG1, PKC θ , Ano1, and ETV1 [80-86].

Importantly, many studies have revealed that GIST expressed a neuroendocrine phenotype suggesting a neuroendocrine differentiation [87, 88]. In particular, specific hormone production such as synaptic vesicle protein 2 (SV2), synapsin 1, synaptobrevin, amphiphysin and ghrelin have been found in GIST. Furthermore, ultrastructural studies by electron microscopy showed numerous clear vesicles similar to synaptic-like microvesicles (SLMV).

In addition of ICC, KIT is highly expressed in mast cells, melanocytes, germ cells and hematopoietic progenitors [21]. As a result, KIT has critical functions for the developmental processes of melanogenesis, gametogenesis and haematopoiesis.

6. Association with other primary malignancies

GIST can be associated with other primary tumors; in particular, in the well characterized hereditary conditions such as Carney's triad (gastric GIST, paraganglioma, and pulmonary chondroma), Carney-Stratakis's dyad (paraganglioma and GIST) and Neurofibromatosis type 1 [89-91]. Nevertheless, outside these syndromes, our group reported that GIST may often be associated with other neoplasms, in particular, gastric, genitourinary and other sarcomas [92]. By studying 783 patients with GIST from MDACC during a 12 year follow-up, we found that approximately 20% of patients developed additional cancers, a higher percentage than that expected in the general population. In a subsequent study, using a large international cohort including 10 institutions, we identified and described a new syndrome that associated GIST with Desmoid Tumor (DT) [93]. With a statistical analysis, we showed

that the associations between GIST and DT were not random. More details of this study are provided in the appendix of this dissertation.

7. Targeted therapy in GIST

The identification of *KIT* activation through mutations as the primary oncogenic mechanism driving GIST offered a tremendous therapeutic opportunity. Indeed, imatinib mesylate (IM) (Gleevec), a tyrosine kinase inhibitor (TKI) had been developed to inhibit the fusion kinase BCR-ABL caused by the Philadelphia chromosome translocation in patient with chronic myelogenous leukemia (CML) but have also showed activity against KIT and PDGFR- α [94, 95]. The IC₅₀ of this molecule is 0.1 μ M for KIT. IM is orally available, well absorbed with a bioavailability of 98%, and metabolized by cytochrome P450 [96]. The first report of clinical response with imatinib in GIST was published in April, 2001. The article described the case of a remarkable response in a 50-year-old patient with metastatic GIST [97]. Following the report, clinical trials were launched and established the efficacy and safety of imatinib in a record time. Clinical efficacy results for patients with advanced GIST from clinical trials are detailed in Table 1. In February 2002, the US Food and Drugs Administration (FDA) approved imatinib for treatment of patients with metastatic and unresectable GIST [103].

Study	Year	N	IM dose (mg)	Response Rate (%)	2-year PFS (%)	Ref
EORTC Phase I	2001	36	400-1000	56	not reported	van Oosterom et al.[98]
US B2222 Phase II	2002	73	400	50	48	Demetri et al.[99]
		74	600	58	52	
EORTC Phase II	2003	27	800	70	73 (1-year PFS)	Verweij et al.[100]
EORTC Phase III	2004	473	400	50	44	Verweij et al.[101]
		473	800	51	52	
Intergroup S0033 Phase III	2008	345	400	45	41	Blanke et al.[102]
		349	800	45	46	

Table 1. Efficacy results from clinical trials in patients with advance GIST.

IM : imatinib, PFS: Progression Free survival.

The initial response to imatinib is observed in up to 80% of patients but varies depending upon the mutation. Patient harboring a KIT exon 11 mutation experience a significantly higher response rate and a longer relapse free survival and overall survival than patient with wild-type tumor or exon 9 mutation [107, 108]. Despite the dramatic clinical success, the long-term efficacy of imatinib is limited by the development of resistance and tumor progression. Secondary resistance to imatinib after initial response is commonly caused by secondary mutations of KIT, decreasing imatinib-binding and restoring KIT pathway activity [109-112]. These mutations are concentrated in two ‘hotspots’: the ATP binding domain pocket, encoded by the exon 13 and 14, and the activation loop encoded by the exon 17 and 18. Molecular and functional analyses of the different types of secondary mutations

have provided a better understanding of structural events that are taking place [113-115]. These mutations are different but they all affect the 3 dimensional structure of KIT leading to alteration of binding with imatinib.

The use of imatinib in adjuvant setting following complete resection of primary GIST has been subsequently approved by the FDA for delayed tumor recurrence in patients at high risk [104]. The recent result from a controlled trial presented by the Scandinavian Sarcoma Group demonstrated that the RFS was better for a 3 years adjuvant treatment with IM than 1 year [105, 106]. As a result, IM 400mg/day for 3 years duration after surgery become the new standard for high-risk patients. The optimal duration of adjuvant treatment remains unknown but a large increase in relapse was observed after IM discontinuation supporting the idea that an even longer duration may have a clinical benefit.

In the case of imatinib-refractory GIST, the second line TKI, Sunitinib malate (Sutent), is the only FDA-approved treatment, but with limited clinical benefit prior to disease progression [116]. Other TKIs, such as nilotinib (Tasigna), sorafenib (Nexavar), dasatinib (Sprycel), regorafenib (Stivarga), crenolanib, masitinib are currently tested but due to TKI cross-resistances, are unlikely to have a major clinical impact on resistant GIST [117]. For those patients, it is crucial to find alternative strategies beyond KIT inhibition. The present work proposes to evaluate, *in vitro*, the role of alternative pathways in the biology of GIST cells with the ultimate objective to develop new therapeutic options to treat this tumor.

8. Notch pathway in normal development

Notch signaling is an extremely evolutionally conserved pathway that contributes in the normal development of multicellular organisms by influencing cell-fate decisions during

embryogenesis and postnatal development [118, 119]. In 1917, Thomas Hunt Morgan was the first to describe fruit flies with 'notched' wings, following two years later by the description of a mutation causing this phenotype in a gene termed *Notch* [120, 121]. It is only sixty years later that the fly *Notch* gene was cloned simultaneously by the teams of Artavanis-Tsakonas and Young [122, 123]. The gene encodes a large transmembrane polypeptide translated as a full-length protein but subsequently cleaved in the trans-Golgi by a furin-like convertase to create a non-covalent heterodimeric receptor [124]. The extracellular domain contains several Epidermal Growth Factor (EGF)-like domains and cysteine-rich Lin12/Notch repeats (LNR). The cytoplasmic part contains a RAM domain, six ankyrin repeats (ANK), two nuclear localization sequences (NLS), a transcription transactivation domain (TAD) and a C-terminal PEST sequence (Figure 4) [125, 126]. Two ligands for the Notch receptor have been described in *Drosophila melanogaster*, delta and serrate. In mammals, the Notch family contains four receptors (Notch1, Notch2, Notch3, Notch4) and five ligands (delta-like-1-3-4 and Jagged-1-2) [127-131]. The activation of Notch pathway is initiated by the ligand-receptor binding that changes the receptor conformation. This three dimensional modification allows two consecutive proteolytic cleavages, first by metalloproteases (ADAM10 or ADAM17) and then a γ -secretase complex [132]. The γ -secretase complex is composed by the five following subunits: nicastrin, presenilin1 and 2, Pen-2 and Aph1 [133, 134]. These cleavages allow the release of the intracellular domain of Notch (ICN) into the cytoplasm. The ICN translocates to the nucleus and attaches to the transcription factor CSL. In parallel, ICN recruits the co-activators mastermind-like (MAM) and histone acetyltransferase p300 to remove the inhibitory effect of CSL and activate the transcriptional activity of Notch target genes (Figure

4) [135]. The best characterized Notch targets are from the Hes family (Hairy and Enhancer of Split) transcription factors which are basic helix-loop-helix repressors [136, 137]. Importantly, in addition of this canonical pathway, several lines of evidences indicate the existence of CSL independent Notch signaling [138, 139]. In physiological conditions, the Notch pathway participates to balance homeostasis via the control of cell-fate decisions, maintenance of undifferentiated state or promotion of differentiation depending on the cell context [140]. For example, a forced Notch signaling, *in vivo*, has shown to prevent neurogenesis differentiation and to maintain neural stem-cells. Conversely, in skin, Notch activation enhances terminal differentiation and cell-cycle arrest [141-143]. In the digestive tract, a recent study has shown that constitutive Notch activity induced mesenchymal cell loss and deregulated morphogenesis indicating a probable role of Notch in the development of digestive tract mesenchyme [144].

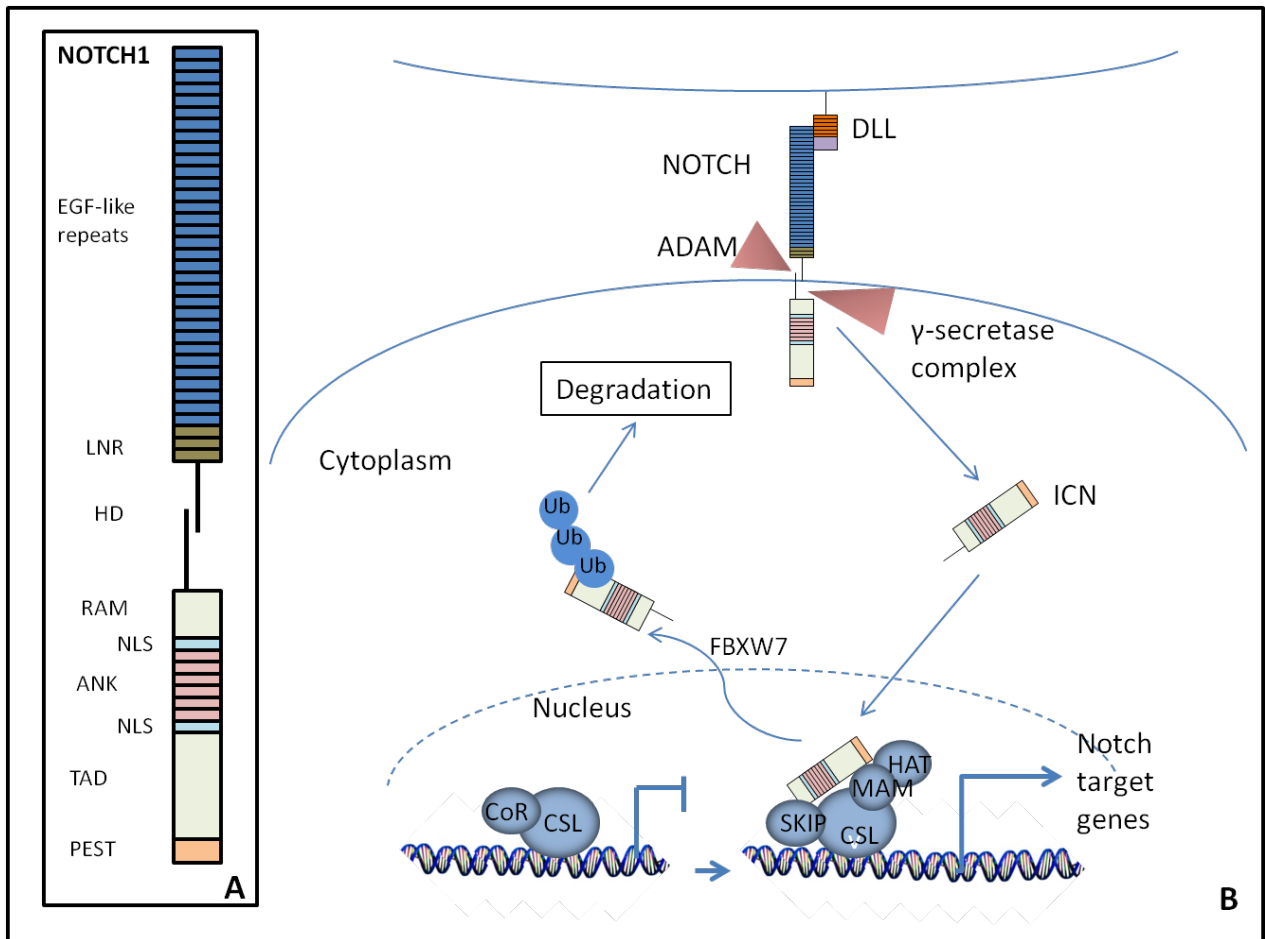


Figure 4. Simplified view of Notch signaling

A. structure of Notch1 proteins, includes epithelial-growth factor (EGF) like repeats, Lin12/Notch repeats (LNR), heterodimerization domain (HD), RAM domain, two nuclear localization signals (NLS), six ankyrin repeats (ANK), trans-activation domain (TAD) and a PEST sequence. B. canonical Notch signaling is activated following ligand-receptor interaction. Two cleavages mediated by metalloproteases and the γ -secretase complex liberate the intracellular domain of Notch (ICN) in the cytoplasm. Before Notch activation CSL is bound to DNA with co-repressor (CoR). Then ICN translocates into the nucleus, binds CSL and recruits co-activators (HAT, histone acetyltransferase; MAM, mastermind-like; SKIP, ski-interaction protein) which lead transcriptional activation of Notch target genes. The signal ends by ubiquitylation (Ub) of ICN and proteasomal degradation.

9. The oncogenic role of Notch signaling

The oncogenic function of Notch was first described in T-cell acute lymphoblastic leukemias (T-ALL). A rare translocation fuses the chromosome 7 and 9 that result in a juxtaposition of the *T-cell receptor β* (*TCR β*) gene with a truncated *Notch1* gene [145, 146]. This translocation occurs in <1% of T-ALL cases, and results in expression of ICN1 that leads to activation of the Notch pathway. The oncogenic role of Notch was further confirmed by the fact that mice transplanted with hematopoietic progenitor cells expressing ICN1 developed T-ALL [147]. In addition, it has been observed that proviral integration, in particular the Moloney murine leukemia virus, into Notch genes may also result in T-cell leukemia [148, 149]. Nevertheless, the definitive clinical relevance of Notch deregulation in T-cell malignancy was confirmed 13 years later by the description of gain-of-function mutations in *Notch1* occurring in more than 50% of T-ALL [150]. These mutations affect the heterodimerization domain and the PEST domain increasing a ligand-independent cleavage and ICN1 stability, respectively, resulting in activation of Notch pathway in both cases. Furthermore, a recent whole-genome sequencing analysis of chronic lymphoblastic leukemia (CLL) has found recurrent activating *Notch1* mutations in the PEST domain [151]. Although these mutations were less frequent than in T-ALL, they were associated with poor prognosis of CLL.

In solid tumors, the oncogenic role of Notch was originally described in mouse mammary tumor virus (MMTV) breast cancer model where retroviral insertions adjacent of *Notch4* (*int3*) transform mammary cells [152]. In addition, *Notch1* has also been found to function as an oncogene in mice breast tumor models [153]. In human breast carcinomas, studies

reported a high expression of Notch1, Notch ligands and Hes-1 associated with poor overall survival [154, 155].

In non-small cell lung cancer (NSCLC), gain-of-function mutations of *Notch1* and a loss of Numb, an inhibitor of the Notch pathway, are frequently observed and correlated with poor clinical outcomes [156]. *Notch3* may also have a role, as a study reported that 40% of NSCLC over-expressed Notch3 [157]. Additionally, inhibition of the Notch3 using a dominant-negative or by γ -secretase inhibitor, reduced growth in soft agar and increased the apoptosis. Similarly, in pancreatic cancer, activation of Notch pathway is frequently found in patient samples and seems involved in tumor initiation and maintenance [158, 159]. Specific silencing of *Notch1* or γ -secretase inhibition reduced pancreatic tumor cell growth [160]. Additional studies have implicated Notch pathway in the carcinogenesis, tumor maintenance and drug resistance in other solid tumors including colorectal, cervical, ovarian cancers and melanomas [161-170].

Several studies have indicated that the molecular basis for the oncogenic role of Notch may be caused by cross-talk with other pathways including Her2, phosphatidylinositol 3-kinase / AKT, Ras, p53 and β catenin / Wnt signaling [171-175]. These interactions promote various oncogenic roles such as cell cycle entry, inhibition of apoptosis, regulation of angiogenesis, and epithelial to mesenchymal transformation [176].

10. The tumor suppressive role of Notch signaling

From studies on the skin, researchers started to understand that the role of Notch pathway is not exclusively oncogenic but may suppress tumors in some tissues. Indeed, expression of Notch receptors and ligands induced cell-cycle arrest and terminal differentiation in the skin

[141, 143, 177]. In consequence, mice with tissue-specific ablation of *Notch1* resulted in skin hyperplasia and spontaneous basal-cell carcinoma tumors [178]. Consistent with this observation, the expression of *Notch1*, *Notch2* and *Jag1* are low in human basal cell carcinomas. In addition of basal cell carcinomas, two high-throughput next generation sequencing studies fortuitously revealed that 10 to 15% of patients with squamous cell carcinomas of the head and neck (HNSCC) harbor inactivating mutations of *Notch*. In addition, loss of heterogeneity (LOH) at the *Notch1* locus was detected in some tumors analyzed for gene copy number resulting in inactivation of both allele, providing evidence for an important tumor suppressor effect of *Notch1* in HNSCC [179, 180].

A good example of the versatility of Notch signaling in carcinogenesis is in lung cancer. In this organ the impact of Notch pathway is predictable by a dichotomy between neuroendocrine and non-neuroendocrine lung tumors. In contrast with what is observed in NSCLC, in small-cell lung cancer (SCLC) cell growth is inhibited by an over-expression of activated *Notch1* and *Notch2* [181, 182]. Transduction of intracellular *Notch1* leads to an increase in *Hes1*, down-regulation of *Mash1* and a potent G1 arrest with p21 induction. In other neuroendocrine tumors (NET) such as carcinoid and medullary thyroid cancer, Notch signaling is minimally present and several observations strongly support a tumor suppressor role for the Notch pathway [183-185]. Finally, the group of Dr. Zweidler-McKay has reported that in B cell malignancies as well as in neuroblastoma, Notch can act as a tumor suppressor [186-188]. In those cell lines, expression of constitutively active, truncated forms of the 4 Notch receptors (ICN1-4) as well as *HES* genes inhibited growth and induced apoptosis.

11. Notch pathway in sarcomas

Given that the Notch pathway plays a pivotal role in the development of the mesoderm, different groups have recently studied the deregulation of this pathway in the sarcomagenesis. As it is the case for other malignancies, the role of Notch in sarcomas is highly contingent on the cellular context (Table 2). We provide in this paragraph a comprehensive review of Notch pathways in sarcomas.

Sarcomas	Effect	Mechanisms	Ref
Rhabdomyosarcoma	Oncogene	Notch1 inhibition reduces proliferation, and promotes differentiation in vitro and in vivo Notch3 inhibition reduces cell growth in vitro and in vivo Notch inhibition via dnMAM reduces invasiveness and mobility in vitro Hes 1 reverses cellular quiescence and suppress differentiation in vitro	Belyea et al, [189] Raimondi et al, [190] Roma et al, [191] Sang et al, [192]
Ewing's sarcoma	Tumor suppressor	Activation of Notch1 inhibits cell proliferation in vitro Activation of Notch1, 2, 3, Jag1 inhibits cell proliferation in vitro Expression of Notch3, Jag1 or Hey1 Induces p53 and p21 in vitro	Baliko et al, [193] Bennani-Baiti et al, [194] Ban et al, [195]
Osteosarcoma	Oncogene	HES1 increases invasion and metastasis Inhibition via CBF1 si RNA suppresses growth and induces p21 in vitro and in vivo Inhibition via dnMAM decreases tumor growth <i>in vivo</i>	Zhang et al, Tanaka et al, [196] Engin et al, [197]
Angiosarcoma	Tumor suppressor	Notch1 knockout mice develop angiosarcoma in the liver	Dill et al, [198]
Undifferentiated pleomorphic sarcoma	Oncogene	Inhibition of Notch decrease self-renewal in vivo	Wang CY et al, [199]

Table 2. Implication of Notch pathway in sarcomas.

A computational search on PubMed was performed using the keywords: Sarcoma*& Notch (September 1st, 2012). On the 81 abstracts evaluated 11 studies were found to be relevant.

11.1. Notch pathway in Rhabdomyosarcomas

Rhabdomyosarcoma (RMS) is the most frequent type of soft tissue sarcoma in children. These tumors are divided in two main histologic subtypes: embryonal (60%) and alveolar (20%) [200, 201]. Although the exact cell of origin remains uncertain, it is accepted that RMS arise from skeletal muscle precursors that failed to differentiate. In normal myogenesis, Notch pathway inhibits differentiation and promotes stem cell maintenance [202-204]. The first report implicating Notch in RMS tumorigenesis came from a study showing that *Hes1* controlled the reversibility of cellular quiescence. In this study, *Hes1* mRNA was increased in 21 primary RMS tumors in comparison with skeletal controls. Inactivation of *Hes1* using a dominant negative *Hes1* or a gamma-secretase inhibitor (GSI) led to differentiation and inhibited proliferation of RMS cells [192]. Subsequent studies confirmed the role of Notch pathway in RMS tumorigenesis *in vivo*, by showing that inhibition of Notch via Notch1 shRNA or by GSI decreased tumor growth [189]. The pathway has also been implicated in RMS mobility and invasiveness. A recent study has found increased expression of Notch2, Notch3, *Hes1* and *Hey1* in a wide panel of RMS primary tumors [191]. *In vitro*, the genetic and pharmacological inhibition through GSI and dominant negative MAM decreased mobility and invasiveness of RMS cells. Additional molecular details have been provided by Raimondi et al. They found that inhibition of Notch3 induced cell differentiation, hyper-phosphorylation of p38 and increase of p21. Notch3 downregulation was sufficient to reduce tumor growth *in vitro* and *in vivo* [190]. All together, these reports provide evidence for an oncogenic role of Notch in RMS and support new therapeutic approaches targeting this pathway.

11.2. Notch pathway in Ewing's sarcomas

Ewing's sarcomas family of tumors (ESFT) arises mainly in bone and less commonly in soft tissues [205]. The origin of ESFT is a subject of intense debate. The initial thought was in favor of a neuroectodermal origin, but many now consider that ESFT arise from a mesenchymal stem cell (MSC) with expression of neuroectodermal markers [206]. They are characterized by chromosomal translocations that combine *EWS* and *ETS* family transcription factors. Although that ESFT express Notch receptors, it has been reported that Notch was inactive [194, 195]. Additionally, forced expression of activated Notch1, 2 and 3 induced cell cycle arrest and p21 upregulation. A different study has found a similar result: that expression of active Notch-1 reduced the proliferation of ESFT cells [193]. These findings suggest tumor suppressor effects of Notch signaling in ESFT.

11.3. Notch pathway in other sarcomas

In osteosarcoma (OS), studies found overexpression of Notch2, Jagged1, Hey1, and Hey2 [196, 197] in comparison with normal tissue. Inhibition with GSI, CBF1 siRNA, or dnMAM prevented the growth of OS cells and upregulated p21. These findings were replicated in a xenograft models and suggested a contribution of Notch in the pathogenesis of OS. Additionally, a study identified a critical role of Notch in osteosarcoma invasion and metastasis.

Recent reports have implicated Notch pathway in two additional sarcomas. First, *Notch1* knockout mice developed spontaneous angiosarcoma in the liver endothelium suggesting a tumor suppressor effect on *Notch1* [198]. Lastly, in undifferentiated pleomorphic sarcomas (malignant fibrous histiocytoma), preliminary data suggest that the Notch pathway regulates self-renewal of tumor initiating cells [199]. It is important to mention that before the publication of the current work, the role of Notch in GIST was unknown.

12. Histone deacetylase inhibitors and Notch pathway

As mentioned earlier, growing evidence showed that genetic deregulation in cancer are not the only events that are essential for cancer formation and progression. In fact, epigenetic modifications seem to play a central role in cancer [207, 208]. It has been widely recognized that the remodeling of chromatin, and in particular histone modifications including acetylation, methylation, phosphorylation, ubiquitination and sumoylation, regulate gene expression [209]. In particular, histone acetylation and deacetylation have been shown to play a major role in tumor formation. The two enzymes responsible for acetyl modifications are the histone deacetylases (HDAC) that remove the acetyl-group and histone acetyltransferases (HAT) that acetylate histone tails leading closed state chromatin in the first case and open chromatin state in the second (Figure 5).

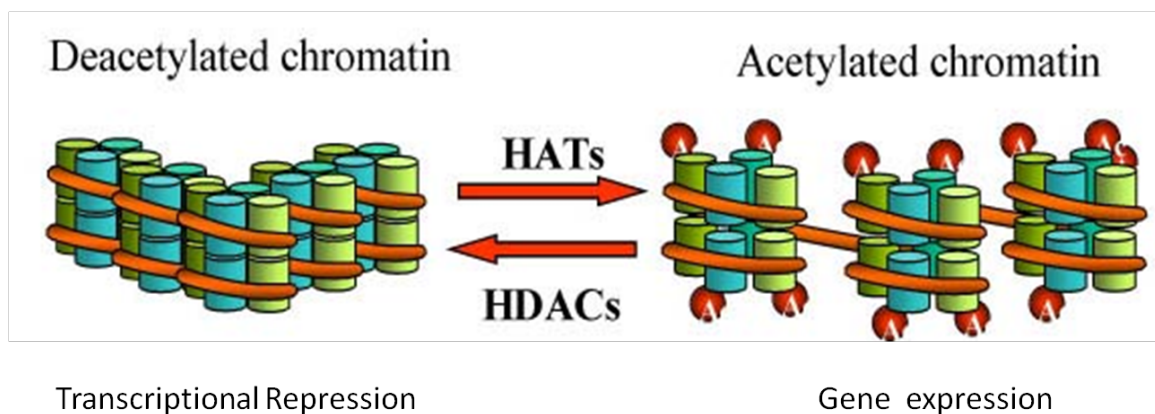


Figure 5. Role of histone acetyl modifications in gene expression

Reciprocal reactions, acetylation and deacetylation are catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC). Acetylation of chromatin by HAT results in open chromatin conformation and transcriptional activation of a subset of genes while histone deacetylation by HDAC results in packed chromatin and transcriptional repression. Figure modified from Gillet et al. *Retrovirology* 2007 4:18, Open Access article distributed under the terms of the Creative Commons Attribution License.

To date, eighteen HDAC have been identified in human and grouped into four classes. Class I HDAC includes HDAC-1-2-3 and-8. They are highly conserved and ubiquitously expressed. They are involved in many cellular processes such as proliferation, cell cycle, apoptosis and DNA damage response. The Class II HDAC contains HDAC -4-5-6-7-9 and -10. They are generally expressed in a tissue-specific manner and are implicated in the developmental process. The class III HDAC contains Sirtuins. They are broadly expressed and have multiple biological functions, such as regulation of oxidative stress, metabolism and aging. The class IV HDAC contains HDAC-11 [210]. Aberrant HDAC activities have been reported in multiple human cancers. As a result HDAC are currently considered as a target to treat cancers with epigenetic aberrations. Two HDAC inhibitors, vorinostat (SAHA) and romidepsin are currently approved by the FDA for the treatment of cutaneous T cell lymphoma. Although, the biologic mechanisms of action of HDAC inhibitors are not fully understood, a large number of studies have demonstrated that HDAC inhibitors induced cell death in a variety of tumors [211, 212]. The death-receptor extrinsic pathway as well as the mitochondrial intrinsic death pathway have been shown to be involved in this process [213-216]. Importantly, effects on non-histone proteins, including the chaperone HSP90 have been characterized [217].

Suberoylanilide hydroxamic acid (SAHA) is a pan-HDAC inhibitor. A recent study supported that SAHA displayed antiproliferative effects in GIST cells [218]. Furthermore, several *in vitro* studies have shown that diverse histone deacetylase (HDAC) inhibitors up-regulated *Notch1* in various neuroendocrine tumors (carcinoid tumors, pheochromocytomas, and medullary thyroid cancers) and in parallel, decreased tumor cell growth [219-222].

These observations support that HDAC inhibition could be an effective strategy to activate the Notch-1 pathway and inhibit growth and hormonal secretion in NET.

13. Rational, significance of the study and specific aims

Although imatinib has dramatically changed the history of GIST treatment, it is clear that inhibition of KIT signaling is not enough to cure patients. Primary and secondary resistance to TKI in GIST underscore the need for new therapeutic targets beyond KIT. The developmental pathway Notch has a critical role in cell fate, regulating cell proliferation and differentiation. Dysregulation of the Notch pathway has been implicated in a wide variety of cancers. Given that (i) Notch activation deregulates the morphogenesis of mesenchymal cells in the GI track, (ii) Notch acts as a tumor suppressor in neuroendocrine tumors, (iii) the cell of origin of GIST are the Interstitial Cell of Cajal that likely arise from a mesenchymal origin although sharing common features with neuroendocrine tumors, we hypothesized that Notch pathway may have an inhibitory effect in GIST cells. In addition, given that HDAC inhibitors have been shown to up-regulate *Notch1* in neuroendocrine tumors, and had antiproliferative effects in these cells, we hypothesized that HDAC inhibitors may activate Notch pathway and could offer a therapeutic opportunity to treat GIST patients, particularly after failure of tyrosine kinase inhibitors.

The present study described in this dissertation aspires to explore the role of Notch pathway in the pathogenesis of GIST. To address this general objective, we carried out the following research aims:

Aim 1: To determine the impact of Notch pathway in GIST cells and to examine a potential cross-talk between KIT signaling and Notch pathway.

Aim 2: To determine the relative prognostic potential of Notch pathway members for predicting clinical outcome of GIST patients.

Aim 3: To Investigate the anti-tumor effects of the HDAC inhibitor SAHA and its impact on the Notch pathway *in vitro*.

Chapter 2. Material and Methods

1. Cell culture

Three human cell lines have been used in the current study. The GIST-T1, which has an imatinib-sensitive *KIT* mutation in exon 11 (V560-Y579del), was established by Dr. Takahiro Taguchi from a patient with metastatic imatinib-naïve GIST [223]. The imatinib-sensitive cell line GIST882, which has homozygous missense mutations in *KIT* exon 13 (K642E), was established by Dr. George Demetri from a patient with primary, imatinib-naïve GIST [20]. Imatinib-resistant cell line GIST48IM, which harbors homozygous *KIT* exon 11 mutations (V560D) and a heterozygous secondary exon 17 mutation (D820A), was established by Dr. Jonathan Fletcher from a GIST patient that has progressed on IM, after initial clinical response [224]. GIST-T1, GIST882, and GIST48IM cells were kindly provided by Drs. Andrew Godwin (Fox Chase Cancer Center), Jonathan Fletcher (Brigham And Women's Hospital), and Anette Duensing (University of Pittsburgh Cancer Institute), respectively. The p53 status of these cell lines have been recently published, and suggest that both GIST-T1 and GIST882 harbor defective p53 with homozygous deletions whereas GIST48 cells are wildtype for p53 [225]. *KIT* mutations were confirmed by direct sequencing. Potential contaminations by other cell lines were ruled out using short tandem repeated (STR) DNA fingerprinting with an AmpF ℓ STR Identifiler kit according to the manufacturer's instructions (Applied Biosystems). STR profiles were compared to known fingerprints. Cells were maintained at 37°C in a humidified incubator containing 95% atmospheric air and 5% CO₂ as described previously [226]. GIST-T1 and GIST882 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Cellgro), supplemented with 10% fetal bovine serum (FBS, PAA), and 1% penicillin/streptomycin (Cellgro).

GIST48IM cells were maintained in Ham's media (F-10, Thermo scientific), supplemented with 20% FBS, 2 mM L-glutamine (VWR International), 1% penicillin/streptomycin, 0.1% amphotericin (Amresco), 10 µg/ml gentamycin (Amresco), 0.5% MITO+ serum extender (Becton, Dickinson and Co) and 1% bovine pituitary extract (Becton, Dickinson and Co). The cell lines were regularly tested for mycoplasma contamination using MycoAlert detection Kit (Lonza).

2. Drug treatment

Suberoylanilide Hydroxamic Acid (SAHA) and imatinib were purchased from the University of Texas-MD Anderson Cancer Center Pharmacy. γ -secretase inhibitor, GSI XXI was purchased from Calbiochem. The drugs were dissolved in DMSO (Fisher) at 10 mMol/L and filtered through 0.22-micron filters, and aliquots were stored in -20°C, protected from light.

3. RNA purification, cDNA synthesis and real-time reverse transcription – PCR

Total RNA was isolated with RNeasy minikit (QIAGEN) as described by the manufacturer. Samples were desalted and concentrated by centrifugation columns. Omniscript Reverse Transcription kit (QIAGEN) was used to synthesize cDNA, using 2µl RT buffer, 2µl dNTP mix, 1µl oligo-dT primer, 1µl Rnas Inhibitor, 1µl Omniscript Reverse Transcriptase, incubated for 60 minutes at 40°C. The newly synthesized cDNA was diluted in a final volume of 200 µl.

Real-time quantitative RT-polymerase chain reaction (RT-PCR) was performed by mixing 13µl SYBR Green buffer (Bio-Rad), 1 µl of forward and reverse primers (Invitrogen) and

RNase-free water to a total volume of 23 μ l. In total, 2 μ l of the cDNA template was added and the reaction mix heated to 95°C for 10 minutes. Amplification was carried out on an ABI5700 (Applied Biosystems) for 40 cycles with a denaturation temperature of 95°C for 15 seconds and an annealing and extension temperature of 60°C for 1 minute. The genes analyzed were: *Notch1*, *Hes1* and *KIT*. The designed primer sequences are listed in Table 3. The reference gene *GAPDH* was used for normalization. All experiments were performed in triplicate at least three times and the results were analyzed using the C_t method and recorded as relative expression level [227].

Genes	Forward (F)	Reverse (R)
<i>Notch1</i>	5'-CGGGTCCACCAGTTTGAATG-3'	5'-GTTGTATTGGTTCGGCACCAT-3'
<i>Hes1</i>	5'-ACGACACCGGATAAACCAAA-3'	5'-CGGAGGTGCTTCACTGTCAT-3'
<i>KIT</i>	5'-AGCAAATCCATCCCCACACC-3'	5'-GGCTTGAGCATCTTTACAGCGAC-3'

Table 3. List of primer sequences used for RT-Q-PCR.

4. Plasmids and retroviral constructs

A murine stem cell virus-based retroviral vector MigR1 coexpressing green fluorescent protein (GFP) as an expression marker was used to modulate Notch signaling in GIST cells (Figure.5) [228]. The intracellular active domain of human *Notch1* (ICN1) or full-length human *Hes1* or *Hes5* was inserted into the MigR1 vector. The dominant-negative *Hes1* (dnHes1) and mastermind (dnMAM) were similarly generated as described previously [187, 229, 230]. All the sequences have been confirmed by sequencing. Plasmids were transfected using lipofectamine 2000 (Invitrogen). The viruses were produced with Amphotropic Phoenix packaging cells. This cell line is based on the 293T cell line transduced to stably produce gag- pol, and envelope proteins.

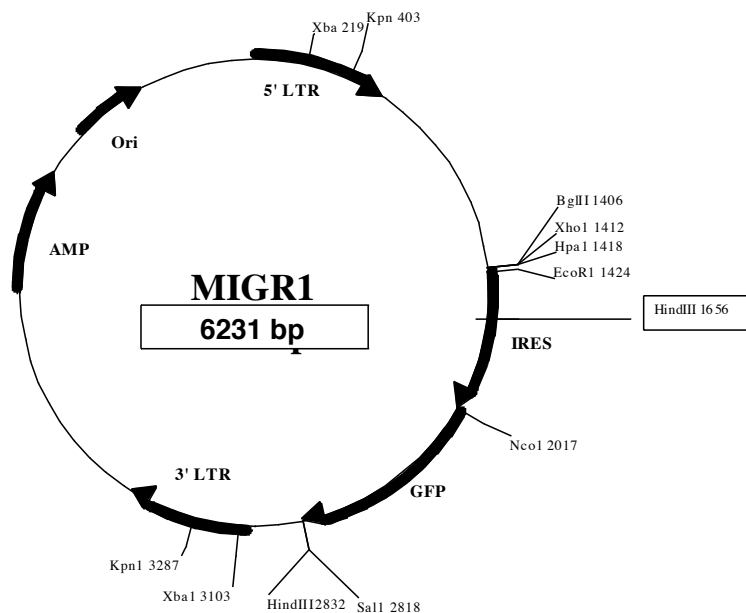


Figure 6. MigR1 sequence
MSCV-IRES-EGFP retroviral
construct

5. Flow cytometric analysis

For GFP analysis, adherent GIST cells were trypsinized and suspended in PBS starting 2 days from transduction. Samples were analyzed with FL-1 channel. For measurement of KIT and Notch1 surface expression, adherent cells were resuspended in FACS buffer (PBS, 2% FBS, 0.1% sodium azide) and incubated with phycoerythrin-conjugated (PE) KIT or a Notch1 monoclonal antibody (eBioscience) for 30 minutes. To control for nonspecificity, GIST cells were similarly stained with a PE-conjugated isotype anti-IgG1 monoclonal antibody (FastImmune). Transduced cells were identified as GFP+.

For cell-cycle analysis, approximately 3×10^5 cells were cultured with SAHA for 72 hours. Floating and adherent cells were collected and incubated overnight protected from light at 4°C with 0.005% Propidium iodide (PI) and 0.1% Triton X-100 diluted in PBS. In this assay PI binds the cellular DNA to distinguish apoptotic cell populations, and cell-cycle phases. Apoptotic cells are identified as hypodiploid cells (sub-G1 phase), where cells with

diploid DNA are in G0- or G1-phase, cells with supra-diploid in S-phase, and cells with tetraploid DNA are in mitosis or G2. Events were acquired on a FACSCalibur flowcytometer or FACSCanto II (Becton, Dickinson and Co.) and results were analyzed with FlowJo v.7.6 software (Tree Star). Flow cytometric analyses were repeated at least three times with consistent results.

6. Analysis of cell proliferation

Cell proliferation assays were performed using a Promega CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. This assay detects reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into formazan, which occurs in viable cells after the addition of phenazine methosulfate (PMS). The absorbance ABS at 490nm reflects the proportion of living cells in culture. Briefly, 4000 cells/well were seeded onto 96-well plates, and incubated at 37°C for 24 hr. Cells were treated with DMSO or SAHA (1, 100, 500, 1000, 2000, 10000 nM in a 100 µL volume/well for 72 hr incubation, MTS and PMS were combined and added to each well. ABS at 490nm was quantified with Bio-Tek microplate reader A3100 (Bio-Tek Instruments) to calculate the percent viability. Direct cell counting were done by counting Nuclei (5-25 µm) using an automated Vi-Cell Analyzer (Beckman Coulter) as described previously or Mozi Z cell counter (Orflo) [231]. These experiments were repeated three times.

7. Western blotting analysis

GIST cells were harvested and washed with PBS; pellets were lysed on ice for 5 minutes in a lysis buffer with complete Protease Inhibitor Cocktail (Roche Diagnostic). The protein concentration was measured using a Bio-Rad Protein Assay (Bio-Rad). Cell lysates were diluted 1:2 with 10 mM DTT sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Forty micrograms of protein was resolved using SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes by using iBlot Blotting System (Invitrogen). Membranes were blocked with 5%, non-fat milk dissolved in 0.05% Tween-20 in PBS (PBS-T) for one hour, and washed thrice with 0.05% PBS-T for 10 minutes. The membranes were incubated for one hour with primary antibodies in 5% milk-PBS-T. Membranes were washed with 0.05% PBS-T thrice for 10 minutes before incubation with horseradish peroxidase-conjugated secondary antibodies for an hour at room temperature as previously described [226, 230]. The primary antibodies used incubated as follow: rabbit anti-pKIT (Tyr719) (1:1000, Cell Signaling), rabbit anti-AcH3K9 (1:1000, Cell signaling), and rabbit anti-H3 (1:1000, Cell signaling). Actin served as control for equal protein loading. Anti-rabbit IgG ECL antibody (1:2000, GE healthcare) was used as a secondary antibody. Proteins bands were detected by enhanced chemiluminescence (Thermo Scientific). Western blot analysis were repeated at least three times with consistent results.

8. Patients, tumor specimens and study approval

All GIST specimens were obtained from patients with pre-treated GIST enrolled in a previously described IRB approved prospective phase II study of neoadjuvant and adjuvant imatinib at MDACC [232]. The diagnoses of GIST were confirmed by experienced soft-

tissue pathologists on the basis of clinicopathologic evaluation and marker studies. Comprehensive patient and tumor variables were recorded and updated into a database until December 2011. Tumor size was considered the greatest primary tumor diameter in any dimension by computed tomography (CT). Experiments involving recombinant DNA were approved by Institutional Biosafety Committees at MDACC and the University of Miami. *In vivo* work was approved by MDACC Animal Care and Use Committee.

9. Gene expression data

For gene expression analysis, RNA from 37 GIST samples were measured using whole human genome oligo arrays with 44-K 60-mer probes (Agilent Technology). Normalization values (quantile intensity values) with the median of all samples have been done. Heatmap has been generated with Matlab software (MathWorks). Some of these data have been previously published [54].

10. Xenograft GIST mouse model

A retroviral vector encoding Firefly Luciferase and GFP was used for the transduction of GIST-T1 cells. Stably transduced cells were sorted and tested, *in vitro* for light emission in presence of D-Luciferin (Xenogen). The orthotopic xenograft model was generated by transplantation of GIST-T1-Luc⁺ cells into the stomach wall of NOD/SCID IL-2-R-gamma^{-/-} mice purchased from the Jackson Laboratory. Methoxyflurane-anesthetized mice were imaged weekly using a bioluminescence imaging IVIS 200 system (Xenogen).

11. Statistical analysis

For *in vitro* analysis, the significance of the results was assessed using the Student-*t* test. All experiments were conducted at least three times. Statistical analyses were performed using the Prism 5 software program (GraphPad). For patient sample analysis, the relapse-free survival (RFS) was defined as the time from surgical resection to disease recurrence; and the overall survival (OS) as the time from surgical resection to death. To control for the known prognostic factors, the χ^2 test was used for categorical variables and the Student-*t* test for continuous variables. To evaluate the association between *Hes1* mRNA expression and patient outcome, Kaplan-Meier curves were created, and log-rank tests were used to compare RFS and OS curves among patients. To evaluate associations between the expression of two genes, we used linear regression analyses and Pearson correlation. A value of $P < 0.05$ was considered to be statistically significant.

Chapter 3. Results: Impact of Notch pathway in GIST

As previously mentioned, the cell of origin of GIST, are ICC or cells in this lineage that represent the interface between the neural system of the gut and the endocrine system. GIST and ICC have similar characteristics and particularly some neuroendocrine differentiation phenotypes. We, therefore reasoned that Notch signaling may function as a tumor suppressor for GIST, as it does for neuroendocrine tumors. We therefore examined the impact of Notch signaling in GIST cells and tumor samples since any tumor suppressor function might have potential benefits to treat GIST.

1. The active form of *Notch1* transduction decreases GIST cell proliferation

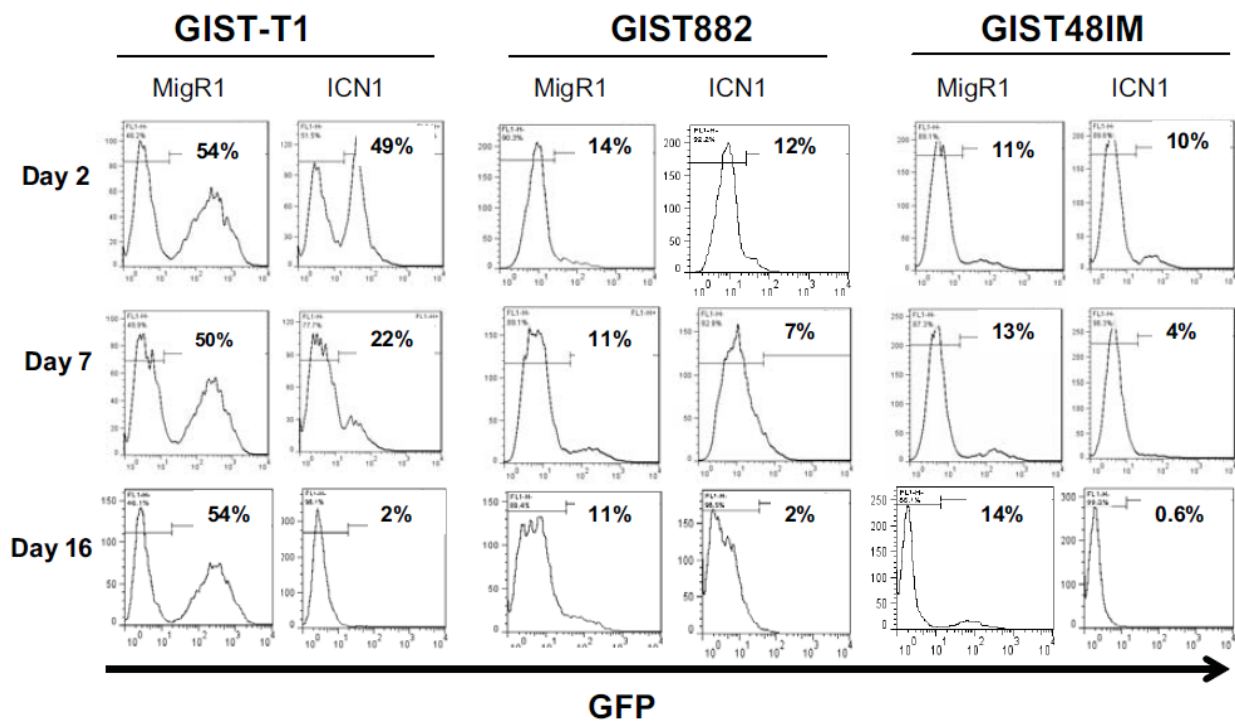
To determine the effect of Notch signaling on GIST cells, we transduced imatinib-sensitive GIST-T1 and GIST882 cells, and imatinib-resistant GIST48IM cells with either an empty vector expressing GFP alone (MigR1), or co-expressing a constitutively active truncated intracellular portion of Notch receptor 1 (ICN1). We used the percentage of GFP+ cells at different times after transduction as an indirect measure of cell growth (Figure.7A). ICN1 expression potently induced growth arrest in the three GIST cell lines irrespective of their sensitivity or resistance to imatinib (Figure.7B). The proportion of GFP+ ICN-expressing cells decreased compared to GFP- cells (relative decrease of 93%, 84%, and 95% in GIST-T1, GIST882, GIST48IM, respectively) 16 days after transduction ($P<0.01$). In contrast, proliferation of cells transduced with control empty vector was not affected.

Since Hes1 and Hes5 are downstream target molecules of Notch signaling under the control of the ICN/CSL/MAML complex and are essential in regulation of neuronal differentiation,

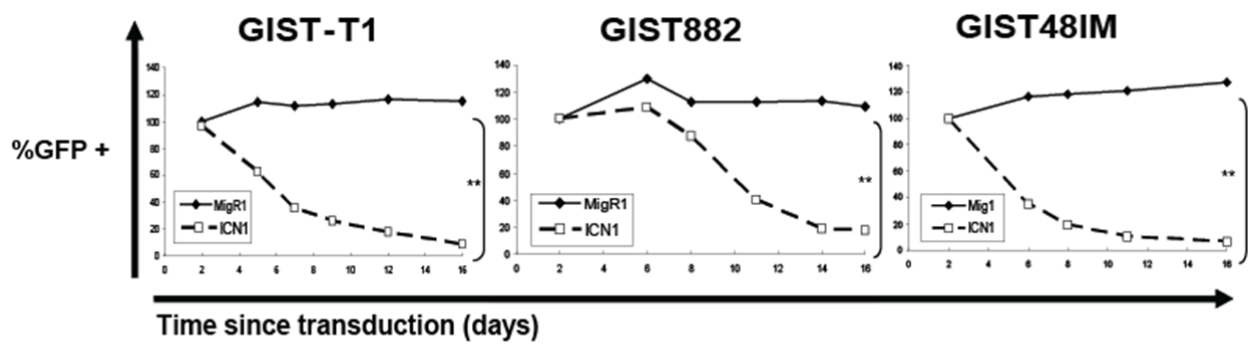
we asked whether expression of these is sufficient to recapitulate the inhibitory effect of ICN1 in GIST-T1 cells. To answer this question, *Hes1* or *Hes5* were cloned into MigR1 and retrovirally expressed these vectors in GIST-T1 cells. Although the transduction of *Hes1* and *Hes5* into GIST-T1 cells had a growth-inhibitory effect, this effect did not fully recapitulate the growth inhibition observed with transduction of ICN-1 (Figure.7C).

To evaluate the reduced viability in GIST cells, we then examined the morphology of GIST-T1 and GIST48IM after viral transduction under the microscope. Especially, we confirm that the vector alone (MigR1) did not affect the phenotype of the cells. In contrast, the forced expression of ICN1, resulted in cellular condensation consistent with typical apoptotic cell death (Figure 8). The transduction with *Hes5* did not appear to cause cell death in the two cells line morphologically observed.

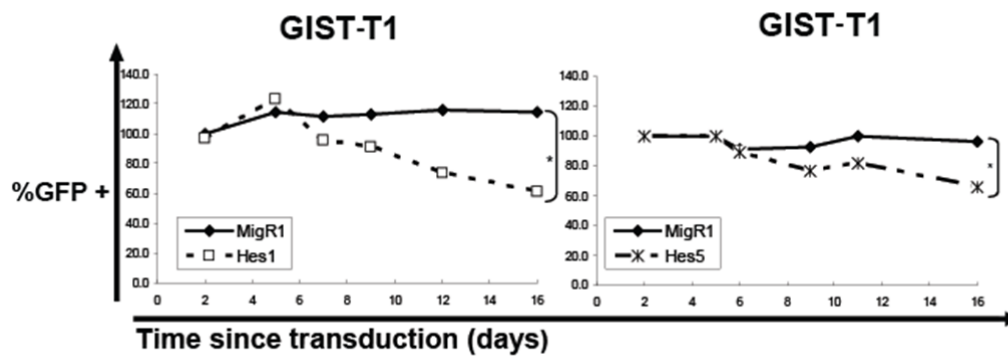
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Figure 7. Intracellular domain of Notch1 (ICN1) inhibits GIST cells proliferation

A. Representative histograms of GFP (marker for transduced cells) are shown in the three GIST cell lines at days 2, 7 and 16 after transduction with MigR1 or ICN1. Three independent experiments were performed, and one representative result is shown. GFP, green fluorescent protein; MigR1, empty vector; ICN1, intracellular domain of Notch1.

B. Graph of the percentage of GFP+ GIST cells over time after stable retroviral transduction of vector alone (MigR1) or containing ICN1 (normalized to day 2 after transduction). GIST-T1, GIST882, and GIST48IM cells were transduced with the control vector or ICN1, statistical differences: $**P<0.01$.

C. Graphs of the percentage of GFP+ GIST-T1 cells over time (normalized to day 2) after retroviral transduction with MigR1 alone, Hes1 or Hes5, statistical differences: $*P<0.05$. GFP, green fluorescent protein; MigR1, empty vector; ICN1, intracellular domain of Notch1.

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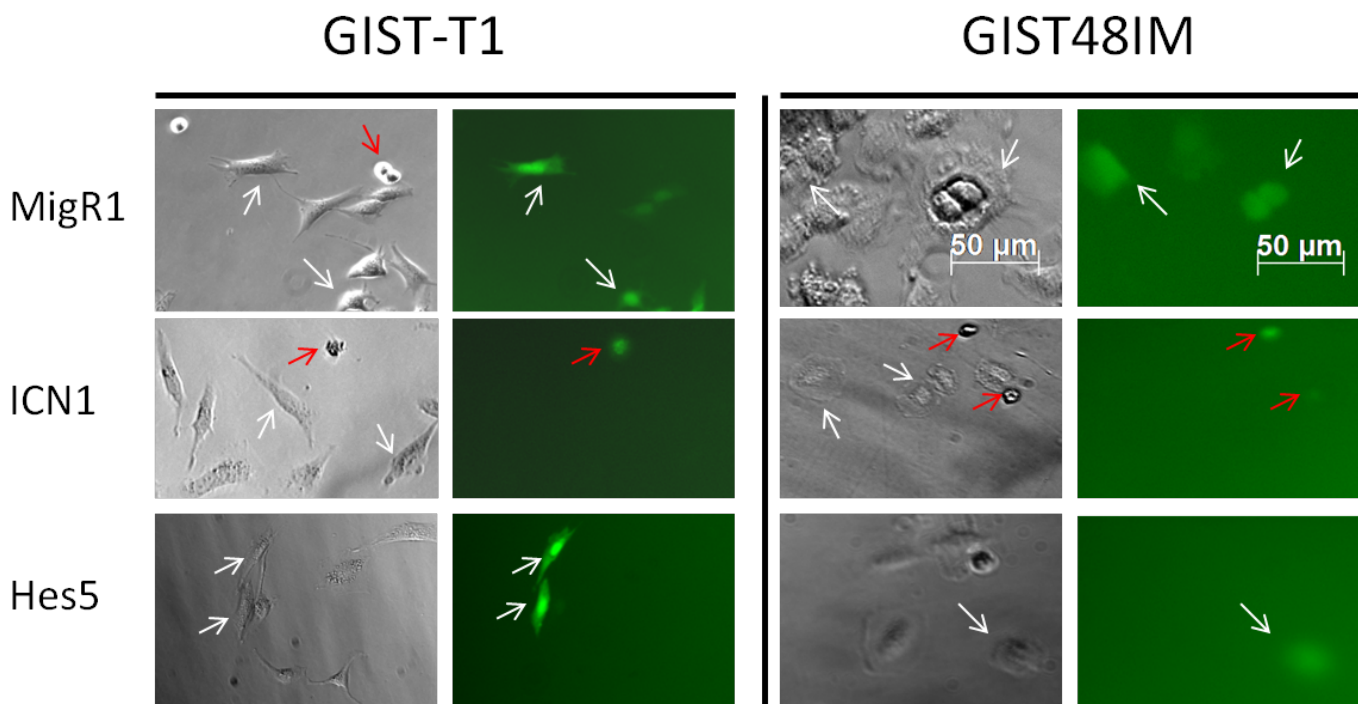


Figure 8. Morphologic features after viral transduction

GIST-T1 and GIST48IM were transduced with GFP tag vectors and the morphologic features were evaluated by microscopy. Representative micrographs of GIST-T1 (left) and GIST48IM (right) 6 days after transduction without sorting. Cells were transduced with the vector alone (MigR1), ICN1 and Hes5 showing cellular condensation in presence of ICN1. Original magnification x200. White arrow: live cells; Red Arrow: dead cells.

2. Active form of Notch1 decreases KIT protein expression in GIST cells

To further elucidate the mechanism of growth arrest in GIST cells, and given that GIST cell present a classical phenomenon of oncogene addiction for KIT, we investigated the effects of ICN1 forced expression on KIT expression. We transduced the three GIST cell lines with either empty MigR1 or MigR1 containing ICN1. Six days after transduction, we analyzed the cell-surface KIT expression by flow cytometry using a monoclonal antibody against KIT conjugated with phycoerythrin (PE). We observed that activation of Notch1 decreased the KIT protein expression in the three GIST cell lines (Figure.9A and Figure.9B).

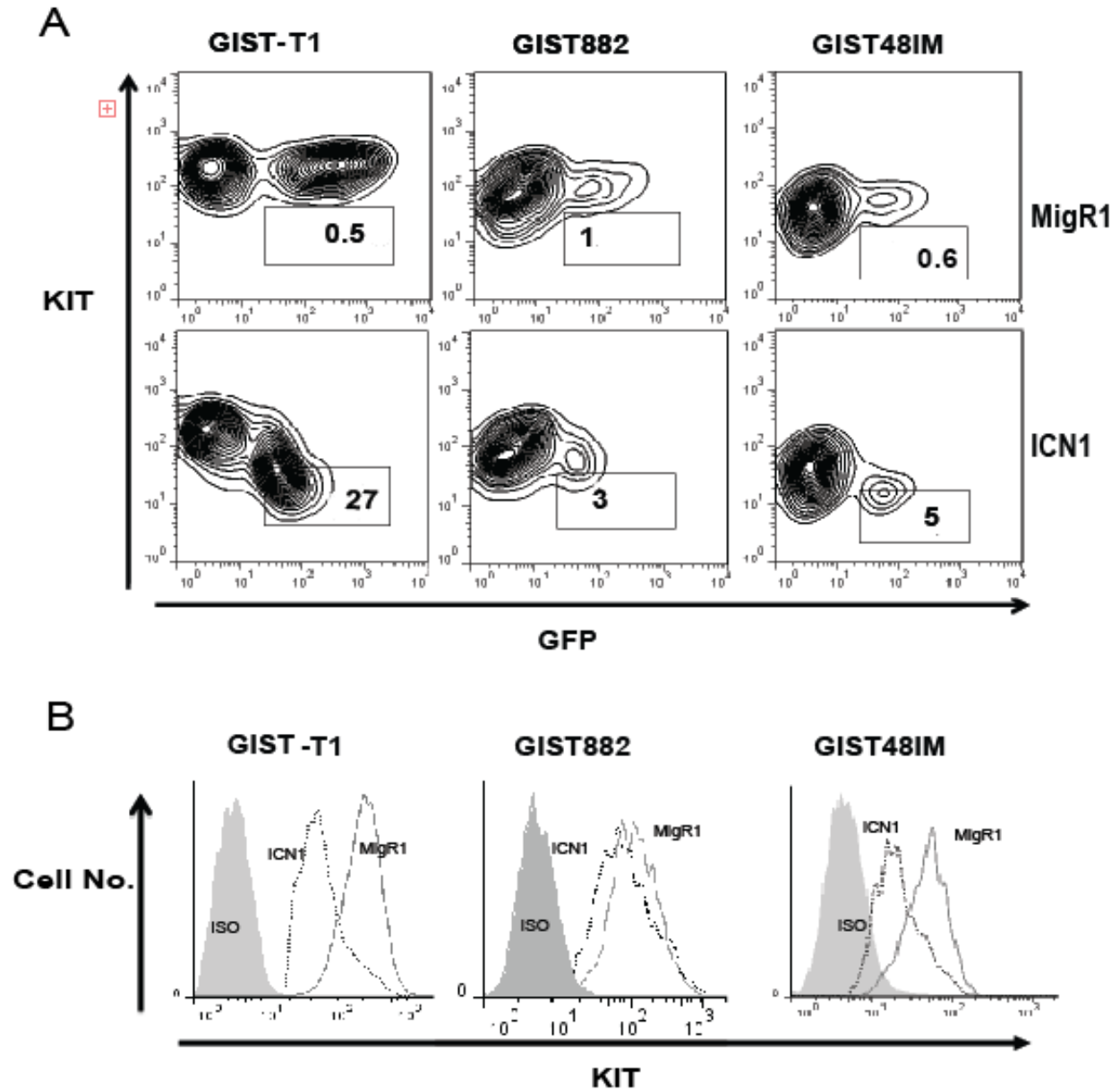


Figure 9. ICN1 decreases KIT protein expression

A. the GIST cell lines were transduced with MigR1 or ICN1 as described above. After transduction, KIT expression on the surface of the cells was evaluated using flow cytometry. The KIT expression in the GFP+ (transduced cells) cells is indicated in each cell specific gate. KIT expression was lower in GFP+ cells (transduced cells) than GFP- cells (parental cell lines) for the constructs containing ICN1 but not for the empty vector. Three independent experiments were performed, with similar results: one representative result is shown.

B. comparison of the KIT expression in the GFP+ cells transduced with MigR1 alone and ICN1 recorded in Mean Fluorescence Intensity (MFI). A PE-conjugated IgG antibody was used as an isotype control (ISO). KIT expression was lower in the ICN1-transduced cells

than in the vector control cells. From Dumont et al, copyright 2012, with permission from Oxford University Press.

3. Dominant negative Hes1 transduction increases KIT protein and mRNA expression in GIST-T1 cells

Since we found that ICN1 forced expression decreased KIT expression, we next determined whether a specific *Hes1* silencing would impact KIT expression. We blocked Hes1 by transducing GIST-T1 cells with a construct containing dominant negative Hes1 (dnHes1) or empty vector (MigR1). The inhibition of Hes1 via dnHes1 resulted in a two fold increase in KIT protein level and 3 fold increase in mRNA level (Figure. 10A, 10B, and 10C).

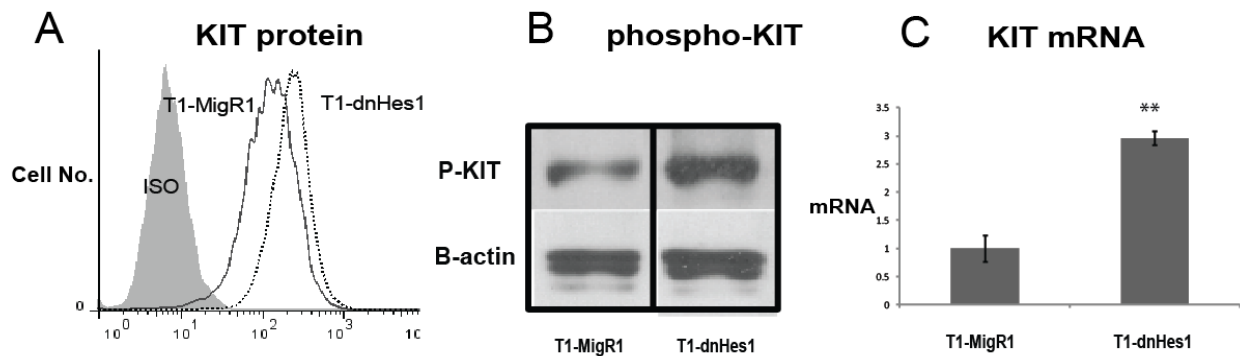


Figure 10. Effects of Hes1 silencing on KIT expression

A. comparison of KIT expression in the vector control (MigR1) versus dnHes1 GIST-T1 cells by flow cytometric analysis.

B. Western blot analysis of pKIT showing higher protein expression in dnHes1 transduced GIST-T1 cells. Actin served as control for equal protein loading.

C. RT-PCR analysis confirmed that mRNA KIT level was up-regulated in dnHes1 cells. Relative KIT mRNA expression levels in relation to GAPDH mRNA levels. Statistical difference: ** $P < 0.01$.

1. GIST patients with high levels of *Hes1* mRNA expression have better clinical outcomes than patients with low levels of expression

The previous finding that Notch1 signaling has an anti-tumor effect in GIST cells prompted us to determine whether baseline expression of members from this pathway is related to relapse-free (RFS) or overall survival (OS) in GIST patients. In particular, as the previous data showed an inhibitory effect of *Notch1* in GIST cells, we hypothesized that expression of Notch family members may be associated with improved clinical outcome in patients. We evaluated the clinical predictive value of *Notch1* and *Hes1* mRNA expression by performing quantitative RT-PCR in samples from 15 pre-imatinib GIST patients who underwent tumor resection followed by adjuvant treatment with imatinib. Patient characteristics are listed in Table 4.

Characteristic		Overall cohort (15)	High <i>Hes1</i> (7)	Low <i>Hes1</i> (8)	<i>P</i>
Age (y)		54 (29-84)	53	48	0.29
Tumor size (cm)		8.5 (3.2-18.8)	6.9	12.5	0.43
		n(%)	n	n	
Gender	Male	8(53)	4	4	0.99
	Female	7(47)	3	4	
Site of disease	Stomach	9(60)	6	3	0.30
	Small bowel	6(40)	1	5	
Metastatic		4(27)	1	3	0.38
GIST genotype	KIT exon 11	12(80)	5	7	0.87
	KIT exon 9	1(7)	0	1	
	PDGFRA	1(7)	1	0	
	Wilde type	1(7)	1	0	

Table 4. GIST patient and tumor characteristics (n=15).

Statistical tests are based on *P* value calculation. χ^2 test was used for binary/categorical variables; t-test was used for continuous variables. From Dumont et al, copyright 2012, with permission from Oxford University Press.

The primary tumor sites were the stomach and small bowel. The *Notch1* mRNA transcript levels were almost undetectable in all the 15 samples (Figure.11A). In contrast, *Hes1* mRNA expression was variable in the cohort (Figure.11B). We then performed a Kaplan-Meier analysis in the patient cohort after grouping them into two categories, based on *Hes1* expression. Patients with high *Hes1* expression had longer RFS than did those with low expression (median of 37 months versus median not reached at >80 months; *P*=0.005 (log-rank test)) (Figure. 11C). However, because of the small size of the cohort and small number of patient deaths, the difference in overall survival between the two group was not significantly different (median not reached at >80 months in either groups) (Figure.11D).

In the current cohort some tumors express very low levels of *Notch1* mRNA while elevated *Hes1* mRNA expression. This observation may indicate that *Hes1* is uncoupled from *Notch1*. To clarify this point, we used additional gene expression data from 37 GIST samples (Figure 12A). We then performed linear regression analyses of mRNA level of *Hes1* versus several *Notch* receptors (*Notch1*, *Notch2*, *Notch4*) (Figure 12B). The result shows that *Hes1* expression do not correlate with Notch receptor expression (Pearson correlations, $p>0.05$). An evaluation of the Notch receptors activation at the protein level would be essential to determine in GIST tumors if *Hes1* mRNA level is strictly under the control of the Notch pathway or alternatively may be regulated by other signaling pathways, epigenetic regulations or non-coding RNAs.

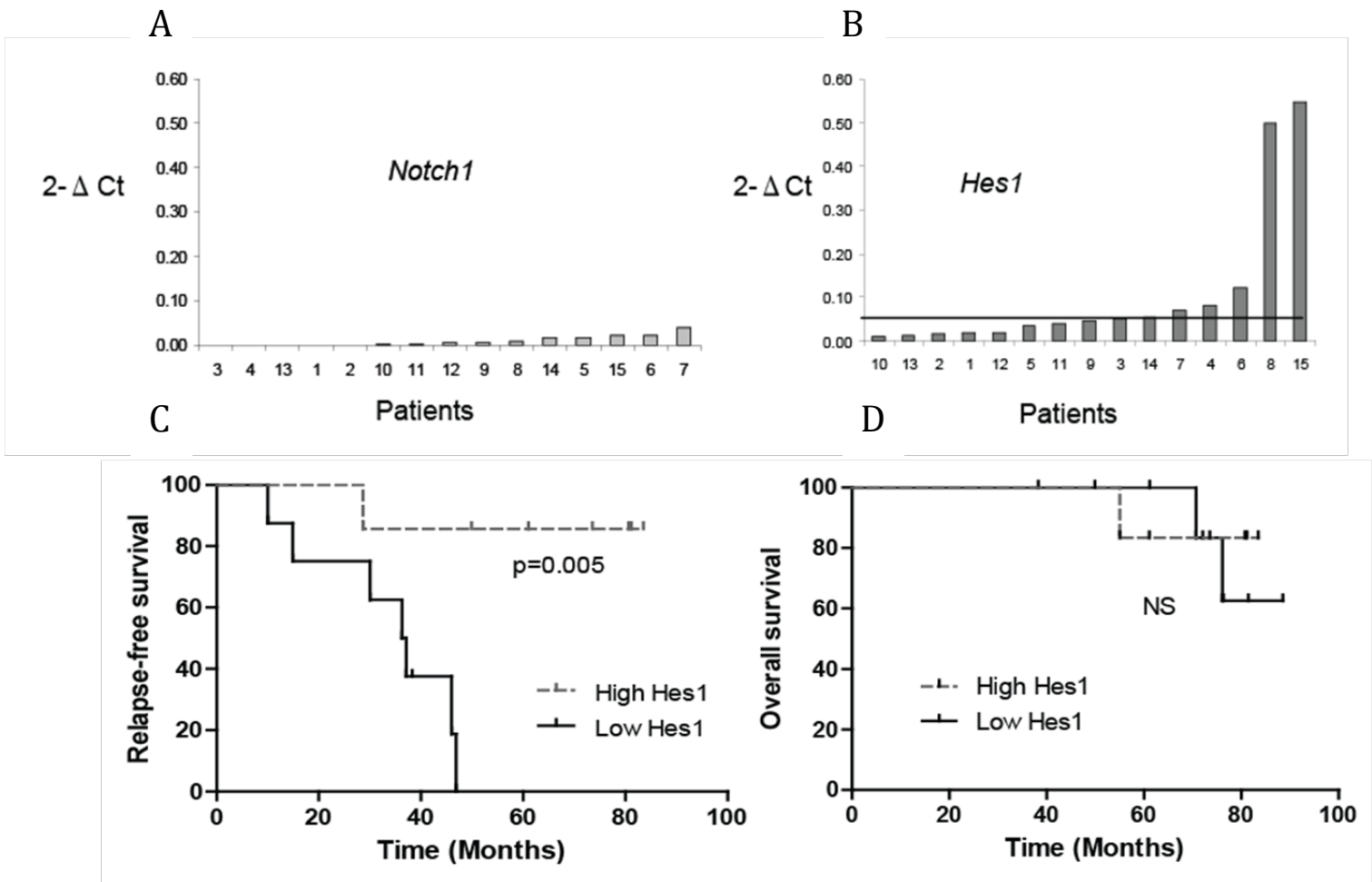
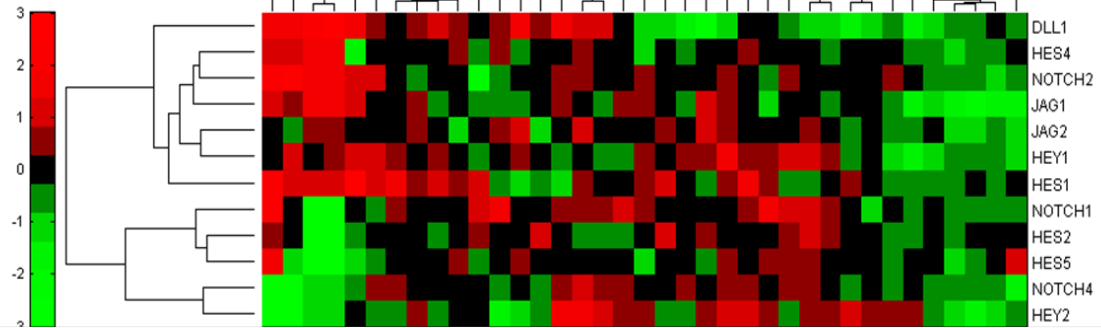


Figure 11. Hes1 mRNA level predicts the relapse of patients with GIST

A. Real-time RT-PCR for *Notch1* and *Hes1* in the 15 GIST samples. Experiments were performed in triplicate. The data are presented as the relative expression levels using GAPDH ($2^{-\Delta C_T}$).

B. Kaplan-Meier analysis for relapse-free survival (RFS) in GIST patients with high versus low *Hes1* mRNA expression (n = 15; $P = 0.005$ (log rank test)). C, Kaplan-Meier analysis for overall survival (OS) in GIST patients with high versus low *Hes1* mRNA expression (n = 15; $P =$ not significant (log rank test)). From Dumont et al, copyright 2012, with permission from Oxford University Press.

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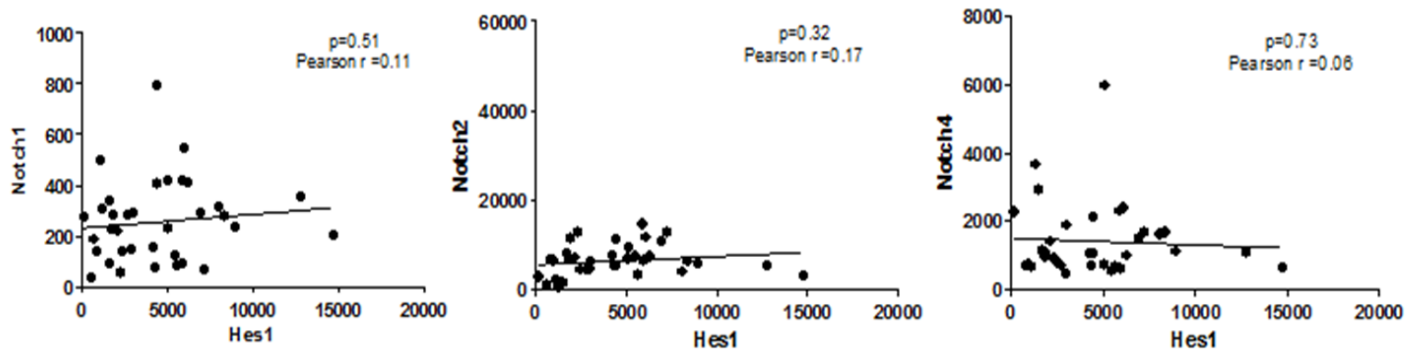


Figure 12. Correlation of Hes1 and Notch receptor mRNA expression in GIST tumor samples

A. Heatmap of gene expression levels from 37 GIST human samples. Relative gene expression are show in red (high expression) and green (low expression).

B. No statistical correlation of *Hes1* mRNA level and *Notch 1, 2 and 4* mRNA levels was found, statistics are by Pearson's correlation test.

Chapter 4. Results: Effects of the histone deacetylase inhibitor

SAHA in GIST cells

Histone acetylation and deacetylation are processes used by the cell to modulate transcriptional activity, cell proliferation, differentiation and apoptosis. Aberrant expression of HDAC has been reported in a number of cancers. As a result, histone deacetylase (HDAC) inhibition appeared to be an appealing strategy in cancer. Although a clinical activity has been proven in particular in T-cell lymphoma, the mechanisms of action of HDAC inhibitors are not completely elucidated. In neuroendocrine tumors, including carcinoid tumors, pheochromocytomas, and medullary thyroid cancers, studies have shown that HDAC inhibitors can up-regulate Notch1. In addition, a recent study provided evidence that HDAC inhibitors, including suberoylanilide hydroxamic acid (SAHA), display antiproliferative effects in GIST cells. SAHA inhibits class I and II histone deacetylases [233]. We hypothesized that part of the activity of SAHA in GIST may be through modulation of Notch pathway. Here we investigate the anti-tumor effects of the HDAC inhibitor SAHA and the impact on the Notch pathway in GIST cells.

1. SAHA up-regulates *Notch1* mRNA and protein expression

To test our hypothesis that treatment with SAHA could up-regulate Notch genes in GIST cells, we treated these cells with SAHA by increasing concentrations for 72 hours. We found that *Notch1* mRNA expression was significantly upregulated in all three cell types (Figure.11A). *Notch1* mRNA level increase with treatment with 2 $\mu\text{mol/L}$ SAHA (6 and 1.5 fold increase in GIST-T1 and GIST882 cells, respectively, $P < 0.05$ in both cases). In

GIST48IM, treatment with 5 μ mol/L SAHA was required to up-regulate *Notch1* mRNA expression by a two-fold ($P<0.05$). To determine whether this increased of the *Notch1* transcript level translated to Notch1 protein expression on the surface of these cells, we performed flow cytometry analysis using a monoclonal antibody specific for Notch1 protein. The flow cytometry analysis indicated that, at the basal level, Notch1 receptor is absent from the surface of the GIST cells, and upregulated after treatment with SAHA (Figure.11B). A significant up-regulation of *Hes1* mRNA expression is also observed after SAHA treatment in GIST-T1 and GIST882 ($P<0.05$) although this up-regulation remains small (Figure.11C).

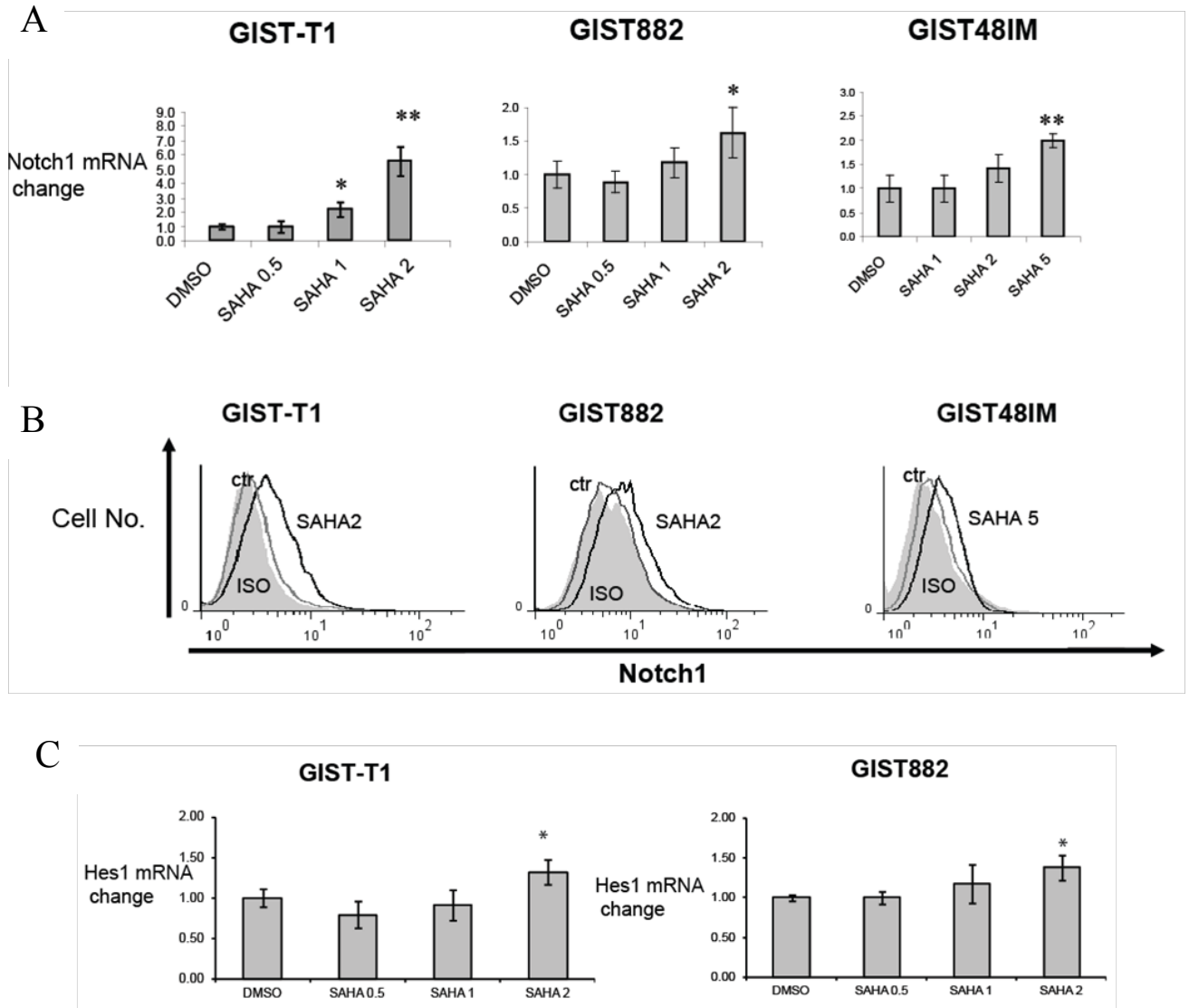


Figure 13. SAHA up-regulates Notch1 mRNA and protein expression

A. Quantitative RT-PCR analysis of GIST cells treated with increasing doses of SAHA for 24 hours showing *Notch1* mRNA expression. The data are presented as the relative expression levels using GAPDH as reference gene and normalized with untreated cells. *Notch1* mRNA increases after SAHA treatment. Experiments were performed in triplicate, and repeated 3 times, the error bars represent standard deviation, statistical differences : * $P < 0.05$, ** $P < 0.01$.

B. GIST-T1 and GIST882 cells were treated with 2 $\mu\text{mol/L}$ SAHA, and GIST48IM cells were treated with 5 $\mu\text{mol/L}$ SAHA. Cells were collected 48 hours after drug treatment and evaluated for cell-surface Notch1 expression using flow cytometry. Notch1 expression increases after SAHA treatment. Three independent experiments were performed with similar results; one representative result is shown.

C. Quantitative RT-PCR analysis of GIST cells treated with increasing doses of SAHA for 24 hours showing up-regulation of *hes1* mRNA expression. The data are presented as the relative expression levels using GAPDH as reference gene and normalized with untreated cells; the error bars represent standard deviation. From Dumont et al, Copyright 2012, with permission from Oxford University Press.

2. SAHA inhibits cell growth, activates apoptosis, and suppresses KIT expression in GIST cells

To determine the antiproliferative effects of SAHA, we performed colorimetric MTS assays to evaluate the GIST cell proliferation after treatment with this HDAC inhibitor. Specifically, we treated the GIST cell lines with SAHA at clinically-relevant concentrations for 72 hours (100nmol/L to 5 μ mol/L). Compared with the control group, we found a dose-dependent growth inhibition in IM-sensitive GIST-T1 and GIST882 cells, and in IM-resistant GIST48IM cells (Figure.12A).

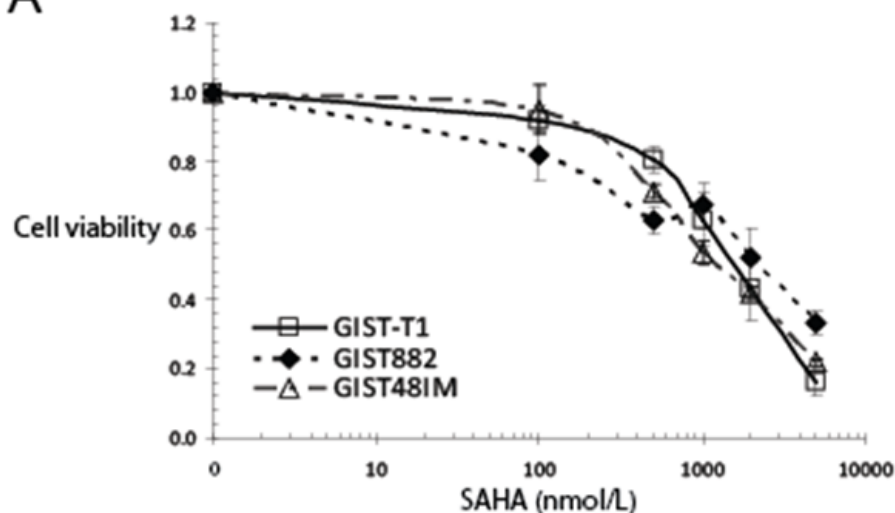
To confirm that the antiproliferative effect of SAHA on GIST cells is related to apoptosis, we performed cell cycle analysis of detached and attached cells treated with SAHA. We found a dose-dependent increase in the number hypodiploid sub-G₁ cells for all three cell lines (Figure.12B). Notably, in GIST-T1 cells, treatment with 2 μ mol/L SAHA resulted in a 25-fold increase in the sub-G₁ population over that in cells treated with control DMSO. In GIST48IM cells, the effect of treatment with 2 μ mol/L SAHA was minimal and treatment with 5 μ mol/L was required to achieve a 2.5-fold increase of the sub-G₁ population compared with control cells.

In addition, to determine whether SAHA affects KIT expression, we first investigated the effects of treatment with SAHA on *KIT* mRNA level in GIST cells. To prevent the confounding late effects of apoptosis on KIT expression, we performed quantitative RT-PCR analysis to measure the *KIT* mRNA expression in the three GIST cell line at an early

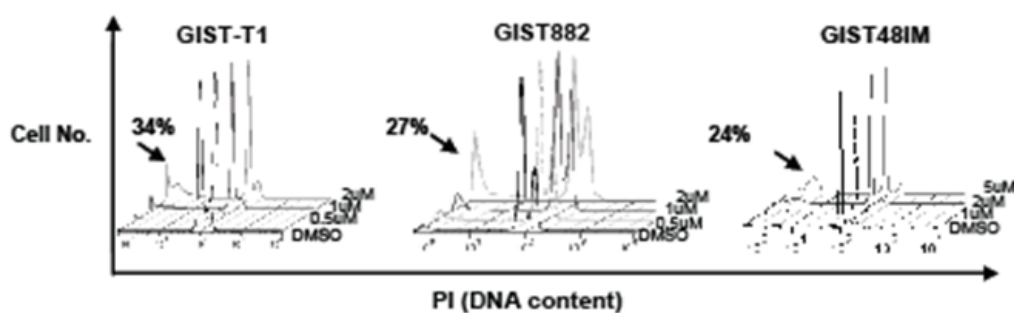
time point (24 hours). In agreement with published findings, *KIT* mRNA level decreased in a dose-dependent manner with treatment with 2 μ mol/L SAHA (85% and 62% in GIST-T1 and GIST882 cells, respectively). In the imatinib resistant GIST 48IM, treatment with 5 μ mol/L SAHA down-regulated *KIT* mRNA expression by 73% (Figure. 12C). In contrast, treatment with imatinib did not decrease *KIT* mRNA level compared with DMSO control.

Furthermore, to determine whether the loss of *KIT* mRNA expression affected *KIT* protein expression and activation, we performed Western blot and flow cytometric analysis of the three GIST cell lines after 48 hours of treatment with SAHA. The treatment resulted in a decrease of total *KIT* expression and a complete inhibition of phosphorylated *KIT* in the three cell lines (Figure.12C). As expected, we observed parallel dose-dependent hyperacetylation of histone H3 at lys9 in the three cell lines after HDAC inhibitor treatment (Figure.12C).

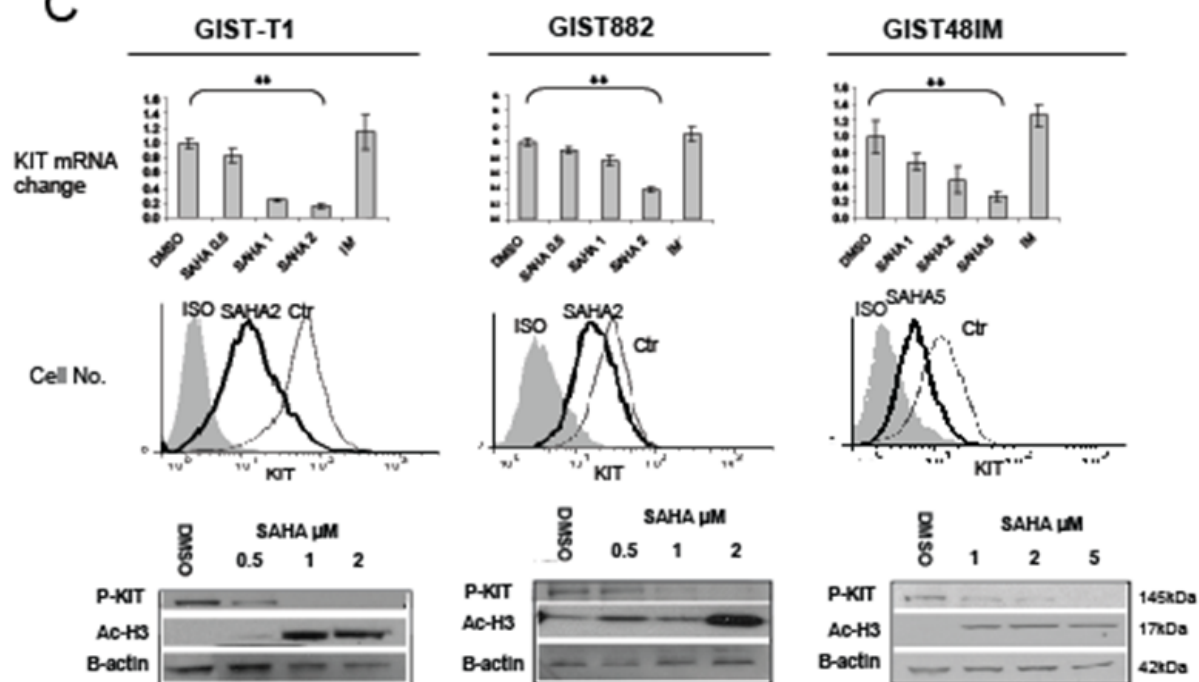
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Figure 14. Dose-dependent decrease in cell viability, increase in apoptosis, and downregulation of KIT expression in GIST cell lines following exposure to SAHA

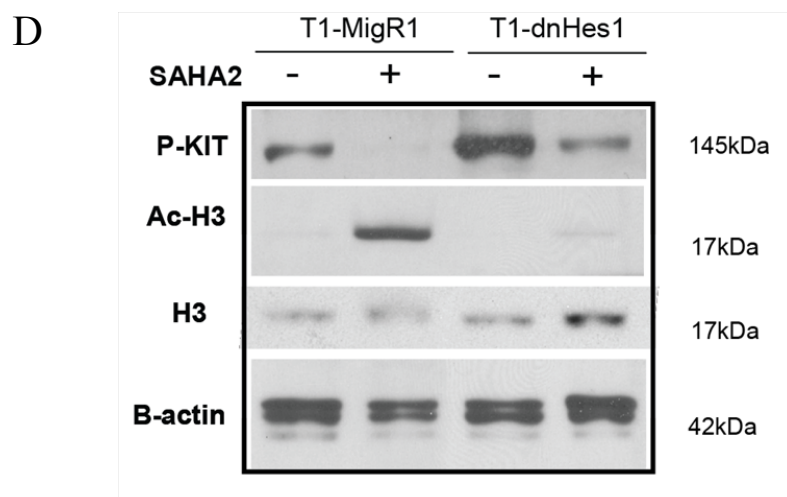
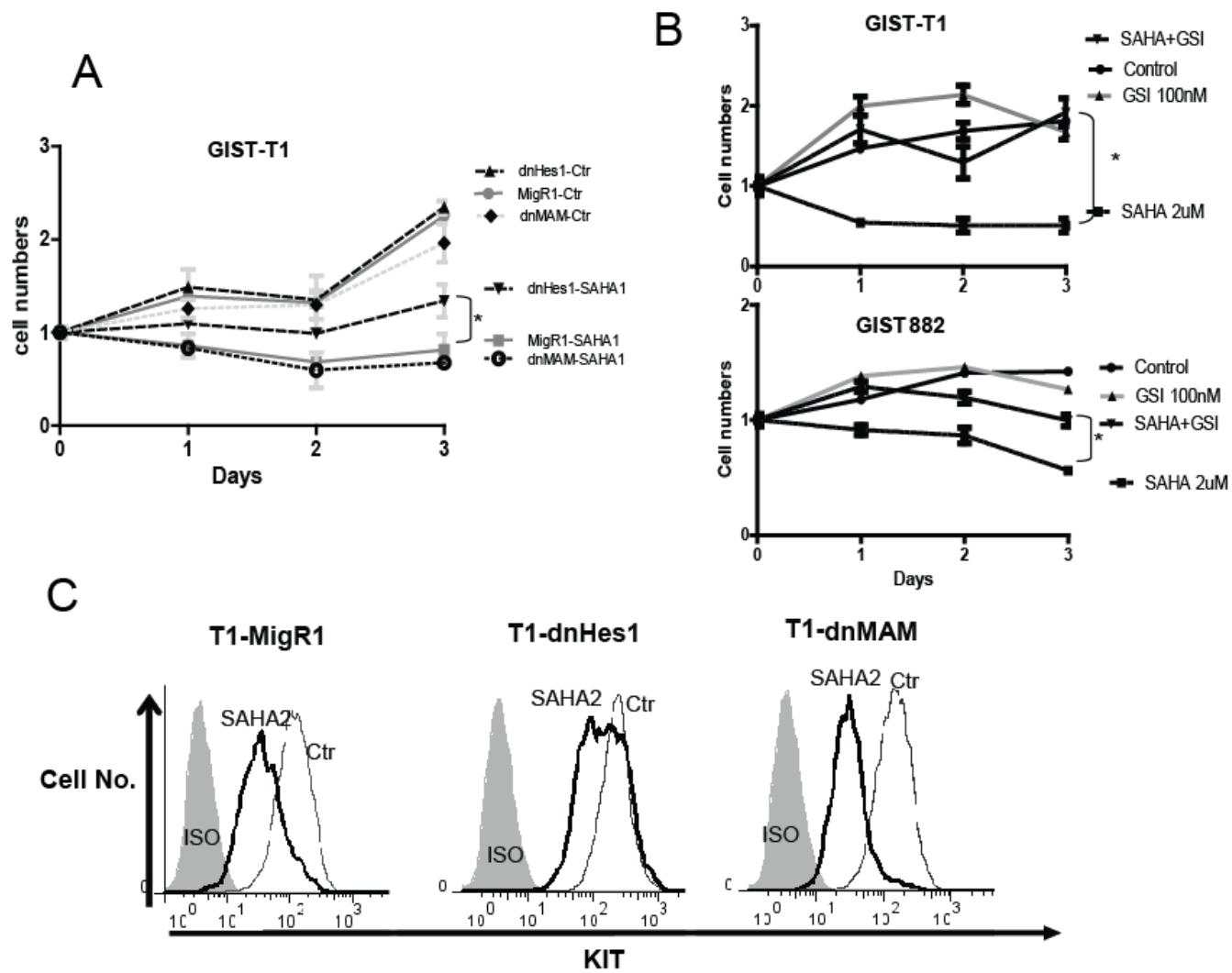
- A. Exponentially growing GIST cells were exposed to varying concentrations of SAHA. Cell proliferation was assessed using an MTS assay 3 days after drug exposure. Points represent mean absorbance of triplicate wells relative to DMSO vehicle controls; bars, standard deviation.
- B. Results of cell-cycle analysis using propidium iodide (PI) staining and flow cytometry: GIST cells were incubated with SAHA at the indicated concentrations for 72 hours. Proportion of apoptosis cells estimated by the amount of the hypodiploid DNA peak (sub-G1 populations) indicated by the arrows. The experiments were repeated on three separate occasions with similar results.
- C. Quantitative RT-PCR analysis of *KIT* mRNA expression in GIST-T1, GIST882, and GIST48IM cells 24hours after increasing doses of SAHA. The histograms show the changes in *KIT* mRNA expression after normalization with GAPDH expression in triplicate wells; Bars, standard deviation, statistical differences: $**P<0.01$. GIST cells were treated with SAHA 2 $\mu\text{mol/L}$ for 48 hours and evaluated for cell-surface KIT expression by flow cytometry. The Western blot analysis shows the level of phosphorylated KIT (P-KIT) and acetylated Histone H3 (Ac-H3) in GIST cells after 48hours of SAHA treatment. Actin served as control for equal protein loading. Three independent experiments were performed: one representative result is shown. IM, imatinib; ISO, isotype control; Ctr, DMSO control. . From Dumont et al, Copyright 2012, with permission from Oxford University Press.

3. Inhibition of Notch pathway partially rescues GIST cells from the effects of SAHA treatment

Given that the treatment with HDAC inhibitors potentially affects the expression of hundreds of genes, we performed a rescue assay to elucidate the relative contribution of upregulation of *Notch1* expression to the mechanism of action of SAHA in GIST cells. We transduced GIST-T1 cells with dominant negatives dnHes1 and dnMAM and examined cells' viability and KIT expression. To avoid counting dead or dying cells, we assessed cell growth by counting the nuclei after chemical lysis of the plasma membrane. After 72 hours of treatment with 1 $\mu\text{mol/L}$ SAHA, we observed a significant difference between growth of

cells transfected with dnHes1 and vector control ($P<0.001$) but not with the dnMAM construct (Figure.5A). Pharmacological inhibition of Notch activation by a γ -secretase inhibitor (GSI XXI) showed a partial rescue of the effect of SAHA in GIST-T1 and GIST882 cells. Indeed the addition of GSI XXI decreased the inhibitory effect of SAHA alone ($P<0.05$) (Figure 5B).

We then studied the KIT cell surface expression after SAHA treatment in cells transduced with these constructs. Down-regulation of KIT cell surface expression was partially abolished by dnHes1 expression. In contrast, dnMAM did not rescue the KIT protein expression after the treatment (Figure.5C). After SAHA treatment, in presence of dnHes1, a partial rescue of the KIT expression was found by flow cytometry and western blotting (Figure13.C and Figure.13D).



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Figure 15. Dominant negative Hes1 and γ -secretase inhibitor treatment partially rescue the cell growth from SAHA treatment

A. Growth curve of GIST-T1 cells transduced with dnHes1, dnMAM, and vector alone after DMSO control and 1 μ mol/L SAHA. The numbers of cells were assessed by automated Vi-CELL Analyzer. DnHes1 partially rescues the cell growth, while dnMAM does not. Data are presented as the mean of three independent experiments \pm standard deviation, statistical differences: $*P < 0.05$.

B. Growth curve of GIST-T1 and GIST882 cells after treatment with SAHA 2 μ mol/L alone, GSI XXI 100nmol/L alone or in combination of the two drugs. The numbers of cells were assessed by Moxi Z cell counter. Data are presented as the mean of three independent experiments \pm standard deviation, statistical differences: $*P < 0.05$.

C. GIST-T1 cells transduced with a MigR1, dnHes1 and dnMAM cells were treated with 2 μ mol/L SAHA. Cells were collected 48 hours after drug treatment and evaluated for cell-surface KIT expression using flow cytometry. The KIT expression is rescued by dnHes1 but not dnMAM. Three independent experiments were performed with similar results.

D. Western blots analysis showing levels of phosphorylated KIT (P-KIT), total Histone H3 (H3), and acetylated Histone H3 (Ac-H3) in GIST-T1 cells after 48 hours of SAHA treatment. Actin served as control for equal protein loading. Modified from Dumont et al, Copyright 2012, with permission from Oxford University Press.

Chapter 5. Discussion, future directions and conclusion

Despite the major success of imatinib in the treatment of GIST, acquired resistances mainly caused by secondary mutations of *KIT* remain a major clinical challenge. Currently, second-generation tyrosine kinase inhibitors are used after failure of imatinib, but these drugs provide little clinical help prior to disease progression. For those patients, the need for new therapeutic strategies beyond KIT inhibition is critical.

1. Impact of Notch1 in GIST

Notch signaling pathway is an evolutionary highly conserved pathway that regulates fundamental cellular functions in multicellular organisms, particularly proliferation, stem cell maintenance and differentiation. Extensive studies have shown that Notch signaling regulates cell fate decisions in the intestinal epithelium as well as neural crest-derived tissue via Hes family member transcriptional repressors. In cancer, where cells fail to regulate their growth within normal homeostasis, studies have implicated the Notch pathway in the initiation and progression of different hematological malignancies and solid tumors. However, prior to our current work the role of Notch pathway in GIST has not been studied.

In the present study, it was established that GIST cell lines and patient samples had very low mRNA *Notch1* expression. By constitutively activated *Notch1*, by viral transduction of the intracellular domain of Notch1, in three human GIST cells we observed a growth inhibition in GIST cells with diverse *KIT* mutations and sensitivities to imatinib. It was reasonable to speculate that the growth inhibition caused by ICN1 in GIST cells resulted from the depletion of KIT. As a result, we demonstrated that KIT expression was down-regulated in response to *Notch1* activation. Interestingly, we found that transduction with a

dominant negative of *Hes1* increased KIT expression in GIST-T1 cells. Taken together, our work suggests a negative interaction between the Notch1 and KIT pathways in GIST. To determine the effect of downstream target genes of Notch signaling on GIST biology, we tested the effects of *Hes1* and *Hes5* on GIST-T1 cells. We found that *Hes1* or *Hes5* alone decreased cell proliferation but did not fully recapitulate the profound effect of ICN1 on growth inhibition in GIST-T1 cells. This finding may indicate that, although *Hes1* and *Hes5* have an inhibitory effect, they may have additive effects, or may not be the only downstream targets of ICN1 implicated in this observation.

To further investigate the mechanism by which ICN1 decreases KIT expression, we performed an *in silico* analysis of the KIT promoter sequence up to 1306bp. This analysis revealed the presence of two N-box consensus sequences (CACNAG) at -738,-733bp and -520,-515 bp that constitute binding site for Hes family members (Figure.14).

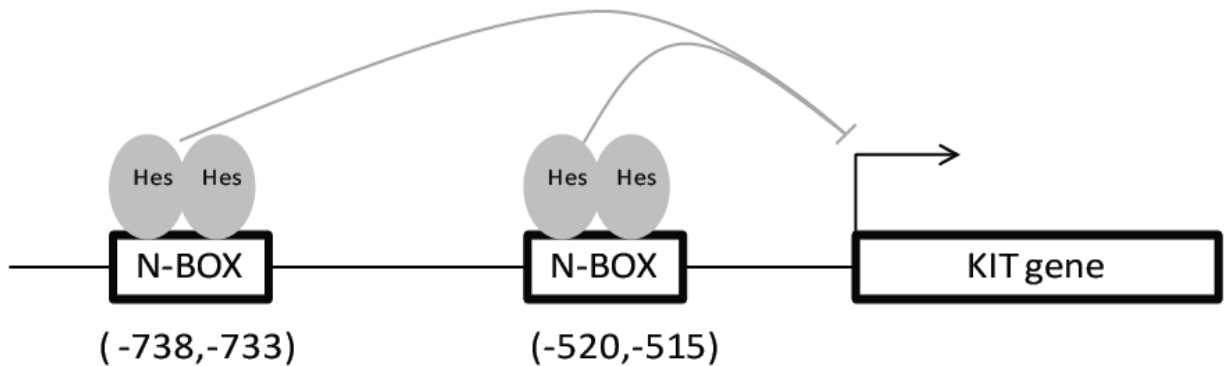


Figure 16. N-Box consensus sequences found in the KIT promoter region

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Additionally, epigenetic modifications and microRNA interactions may modulate the level and stability of *KIT* mRNA in a Notch-dependent manner. Further studies are needed to

clarify the mechanisms and effectors of Notch-mediated growth inhibition and downregulation of KIT expression in GIST cells.

As previously mentioned, one of the more common cytogenetic aberrations found in GIST is the loss of the chromosome 1p region (between 1p36 and 1p13). This deletion is associated with aggressiveness in GIST and is less frequently found in other sarcomas such as leiomyosarcomas. Interestingly, this region contains the *hes2*, *hes3*, *hes4* and *hes5* genes. It was proposed that the deletion of chromosome 1p plays an important role in GIST progression by the loss of unknown tumor suppressor genes. Considering our results, we envisage that these genes may include Hes family members.

Having found that *Notch1* activation induces growth arrest and downregulates *KIT* expression in GIST cells, we next examined whether difference expressions of Notch signaling members were associated with differences in clinical outcome in patients. Using 15 pre-treated GIST patient samples we found that the level of *Hes1* mRNA expression was a prognostic factor for relapse in our cohort. Larger studies with proper controls are needed to confirm the prognostic role of *Hes1* in GIST.

2. Activity of SAHA in GIST cells

SAHA is an HDAC inhibitor that has been shown to cause *in vitro* growth arrest, apoptosis, and to promote differentiation, as well as to exhibit antitumor activity in patients by re-inducing expression of tumor suppressor genes and downregulating expression of oncogenes. To use clinical relevant concentration of SAHA in our study, we performed a review of the pharmacokinetic data available. As we recently pointed out in the journal

Cancer Research, the utilization of correct doses for *in vivo* studies is critical [234]. In the case of SAHA, phase I studies have shown that the drug has a linear pharmacokinetic, an apparent half-life around 100 minutes and an acceptable bioavailability of 40 to 50%. The Cmax after multiple oral administrations ranged between 1.35 and 2.04 μM [235-237].

Our current study demonstrated that the HDAC inhibitor SAHA can upregulate *Notch1* expression in GIST cells as it was previously observed in neuroendocrine tumors. In our work, we established that treatment with SAHA at a concentration of 2 μM decreased viability, induced apoptosis, increased Notch1 expression and reduced KIT expression in two cell GIST lines. Interestingly, the third cell line GIST48IM that is resistant to IM appeared also to be more resistant to SAHA. This apparent cross resistance may reveal drug efflux, epigenetic alterations, resistance to apoptosis or pro-survival mechanisms. In this case, combination therapy with DNA demethylating agents or Bcl-2 inhibitors, for example, could be important in achieving efficacy in GIST.

The mechanism of action of HDAC inhibitors in GIST cells remained controversial. A previous study demonstrated that HDAC inhibitors could deregulated the heat shock protein 90 (Hsp90), a KIT protein chaperone, and proposed that HDAC inhibitors may silence KIT expression via increase of acetylation of HSP90 and a subsequent disruption of KIT at the protein level. However, we found in the current study that downregulation of KIT protein expression could be the result of the strong decrease of the *KIT* mRNA level observed by Q-RT-PCR. Although both events,(the decrease of *KIT* mRNA level and the loss of HSP90 chaperone activity) may contribute to the effects of SAHA on GIST cells, we did not quantify the respective contributions of these mechanisms. Nevertheless, the profound decrease in *KIT* mRNA transcript level that we observed seems to point out a major

transcriptional effect rather than a post-translational regulation. To confirm that SAHA's effect is partially due to its upregulation on the *Notch1* pathway, we showed a partial rescue of cell growth and KIT expression after Notch inhibition with dnHes1 and the γ -secretase inhibitor XXI.

3. Future directions

In Chapter 3, we have demonstrated that Notch 1 activation decreases cell growth. However additional work is required to better understand this observation. The decrease in cell growth can be the result of apoptosis, necrosis, quiescence or senescence. To answer this point a deeper analyze of apoptosis, senescence and differentiation of the GIST cells after forced expression of Notch1 would be important. In particular, a recent study from Kannan et al, demonstrated that Hes1 interacted with the Poly ADP-ribose polymerase-1 (PARP1) in a cell type-specific manner, ultimately resulting in apoptosis [186]. It would be interesting to observe if such a PARP1/Hes1 interaction exists in GIST cells.

Our gene expression analysis on 37 GIST human samples may indicate that Hes1 is not under the strict control of Notch1 as they are not in the same cluster in the Heatmap figure 12. Nevertheless, it is important to mention that gene expression and protein level rarely correlate. Only an immunohistochemistry analysis on tumors samples would address this point. The direct demonstration of the activation of the Notch receptors by showing an increase of the intracellular domains of those receptors would be critical.

Our study established that Notch 1 expression results in decreased expression of KIT receptor. However, the mechanism is not completely understood. Our data suggest that the downregulation of KIT protein is the result of a decrease of KIT mRNA level, but a rigorous

demonstration would be required. The decrease of the mRNA level could be the result of a reduction in transcription rate or mRNA stability. To evaluate the transcription rate, a nuclear run-assay with radioactive nucleotides would be the best method. To test the degradation rate, a study of the mRNA half-life in presence of a transcription inhibitor such as actinomycin D could be proposed.

We found two consensus sequences for Hes family members in the promotor region of *KIT*. To establish definitely whether Hes family members binds these two putative sites, the gold standard would be a chromatine immunoprecipitation (chip) assay using specific primers for these binding sites. Alternatively, a mutagenesis site directed assay and reporter luciferase assay could be pertinent.

In the current work, we studied the cross-talk between KIT and Notch exclusively in GIST cells. Investigating whether other tumors have similar negative relationship in tissues where KIT is highly expressed, such as hematopoietic stem cells, melanocytes, mast cells, and germ cells, would be relevant. Growing evidence points to the limitations of in vitro work, in particular, in conventional cell line culture. To address this concern, the impact of Notch pathway in GIST could be evaluated with an in vivo model that includes the tumor microenvironment. During the time of this study, such model was not available. Consequently, we tried to establish a clinically relevant animal model for GIST. We generated an orthotopic xenograft model by a surgical inoculation of human GIST-T1 cells into the gastric wall of NOD/SCID Il-2-R-gamma-/- mice. GIST-T1 cells were stably transduced to express the luciferase gene in order to allow non-invasive bioluminescence imaging. The xenografts were established in twelve mice by injecting 1×10^6 cells and resulted in a tumor take in nine mice (75%) without dissemination of cells into the abdomen

due to the procedure (Figure 17). The luciferase signal increased until the week 5 after injection and reached a plateau. Paradoxically, the cells were not able to growth further, and invasion was not observed. As a resulted, this model did not completely mimic aggressive human GIST and has limited interest. An evaluation of other GIST cell lines available would be relevant. The utilization of a knock-in mouse GIST model harboring KIT mutation could be an alternative to the xenograft model.

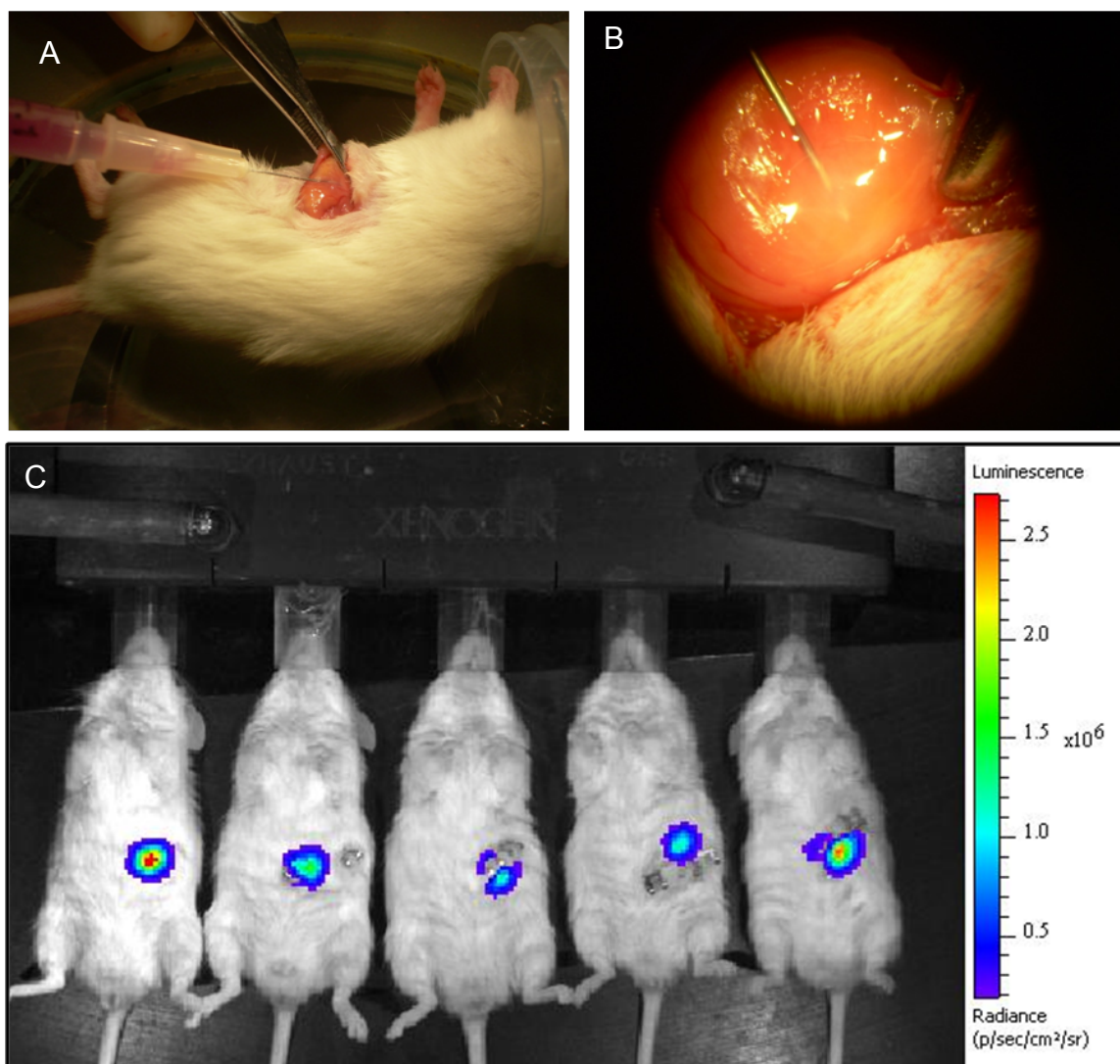


Figure 17. Orthotopic mouse model of GIST

A. Mice were anesthetized and a sub-line of GIST-T1 that express firefly Luciferase was implanted into the gastric wall. B. Representative image with stereo microscope. C. Mice were monitored using bioluminescence imaging (IVIS) following intraperitoneal injection with D-luciferin. Representative images 5 weeks after implantation of luciferase-expressing GIST-T1 cells into the gastric wall.

We have demonstrated in the chapter 4 that SAHA treatment suppresses oncogenic KIT and decreased the viability of the cells. Mechanistically, treatment with SAHA reduced KIT mRNA levels and up-regulated Notch1 mRNA levels. To confirm the functional activity of

SAHA-induced Notch1 a luciferase reporter assay incorporating binding sites of downstream effectors of Notch1 would be required.

A more comprehensive evaluation of other HDAC inhibitors in GIST, especially more selective HDAC inhibitors, would be important to determine if the phenomena described in the current study is also valid for other members of this therapeutic class.

Although HDAC inhibitor treatment resulted in promising *in vitro* data, results from clinical trials in solid tumors have been disappointing. In GIST, the result from a Phase 1 trial of imatinib plus panobinostat (LBH589) in third line therapy has been recently presented during the American Society of Clinical Oncology annual meeting [238]. In the 12 extensively pretreated patients receiving the treatment, limited activity on PET scan were observed with one partial response and eight stable disease.

4. Conclusion

In conclusion, in this study, we demonstrated that active form of Notch1 has a growth-inhibitory effect on GIST cells. Furthermore, in our small cohort, high mRNA *Hes1* expression levels are associated with improved relapse-free survival in patients with resected GIST and may be important prognostic markers for GIST. The up-regulation of *Notch1* may be a therapeutic opportunity for GIST patients as our data suggest that treatment with SAHA could increase the mRNA and protein Notch1 expression. Notch1 pathway activation with HDAC inhibitors may represent a novel strategy for treatment of GIST, especially after tyrosine kinase inhibitor failure. Altogether, our findings provide evidence for a direct negative regulation of KIT by Notch1 pathways and support a tumor suppressor of Notch1 pathway in GIST. Future studies are needed to characterize the mechanism underlying the

crosstalk between KIT and Notch pathways. Nevertheless, our study suggests that the Notch pathway represents a potential therapeutic target in GIST. In the last decade, despite its rarity, GIST has been at the center of a paradigm shift in the treatment of solid tumors. We hope that discoveries derived from GIST studies will persist to extend beyond this disease to influence the oncology field in general.

APPENDIX

A non-random association of gastrointestinal stromal tumor (GIST) and desmoid tumor (deep fibromatosis)

This appendix describes a supplementary work published in the Annals of Oncology [93]. As patients with GIST are living longer, their risk of developing other malignancies is evaluated and reported in the literature. However, the associations between GIST and other tumors remain unclear. Most reports described single cases or very small series of patients from single institution. GIST have been reported to coexist with gastric, breast, prostate, renal, esophagus, colorectal, lung and pancreatic carcinomas; carcinoids; lymphomas and melanomas [239, 240]. Additionally, two case reports of a patient with desmoid tumors (DT, also known as deep or aggressive fibromatosis) and GIST in the same anatomic location were recently reported [241, 242]. Interestingly, DT are very rare sarcomas with a tendency for slow, local infiltrative growth where an embryonic conserved signaling pathway, the Wnt/ β -catenin signaling pathway appears to play a central role in the tumor formation. Like the Notch pathway, development studies on *Drosophila* have described the phenotype ‘wingless’ and demonstrated that this pathway was involved in the establishment of the body axis as well as the development of many organs.

In DT nuclear expression of β -catenin has increasingly been used in the differential diagnosis of spindle cell neoplasms, particularly in the abdomen [243]. Mutations in the *CTNNB1* gene, which codes for β -catenin, have been found in the majority of DT patients, with most mutations occurring in exon 3 [244-246].

DT do not metastasize but can cause significant morbidity through their locally destructive effects. Prior trauma, such as previous surgery, is known to increase the risk of sporadic and

FAP-associated DT [247, 248]. Complete surgical resection with a wide margin and/or radiation therapy constitute the mainstay of resectable DT therapy, while chemotherapy, anti-inflammatory agents, tyrosine kinase inhibitors are all treatment options for locally advanced DT.

Anecdotal reports of individuals with GIST and DT led us to evaluate a larger cohort to find patients with both tumors, in order to gain insight into whether their simultaneous occurrence is a coincidental event.

Material and Methods

Patients and tumor tissues

A retrospective analysis was performed from the patient records between 1978 and 2008 at The University of Texas - MD Anderson Cancer Center, Helsinki University Central Hospital, Fox Chase Cancer Center, Oregon Health & Science University Knight Cancer Institute, Cleveland Clinic, Istituto Nazionale dei Tumori, Prince of Wales Hospital, H. Lee Moffit Cancer Center, the Armed Forces Institute of Pathology, and Memorial Sloan-Kettering Cancer Center using a protocol approved by the respective Institutional Review Boards. We founded 28 patients with both GIST and DT. Clinical characteristics and formalin-fixed, paraffin-embedded GIST and DT specimens accrued were retrieved. Specimens were further screened and evaluated by experienced soft-tissue pathologists at MD Anderson or Fox Chase who confirmed DT and GIST histology. Demographic and clinical information, including treatment, histology and outcome related variables were tabulated for analyses.

Genomic DNA isolation

When tissues were available genomic DNA was extracted from 10- μ m-thick formalin-fixed, paraffin-embedded tissue sections cut from blocks with at least 80% tumor using the QIAamp DNA mini kit (Qiagen, Valencia, CA) or the Easy-DNA kit (Invitrogen, Carlsbad, CA).

Analysis of *KIT*, *PDGFRA* and *CTNNB1* mutations

Polymerase chain reaction (PCR) amplification of DNA for *KIT* exons 9, 11, 13 and 17; *PDGFRA* exons 12, 14 and 18 and *CTNNB1* (β -catenin) exon 3 was performed.. Each PCR amplification was performed in a 50- μ l volume containing 10 ng of genomic DNA, 15 μ M concentrations of each primer, 0.2 mM deoxyribonucleotide triphosphate, 5 μ l of 10 x reaction buffer, 2.5 mM MgCl₂ and 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems). The *KIT* and *PDGFRA* primers were previously published [108, 249]. The PCR conditions were as follows: 30 sec at 94°C; 30 sec at 52°C and 1 min at 68°C for 36 cycles, followed by 10 min extension at 68°C. The primers for *CTNNB1* exon 3 primers were as follows: forward: 5'-TTTGATGGAGTTGGACATGG-3' and reverse: 5'-CTGAGAAAATCCCTGTTCCC-3'. The PCR conditions for *CTNNB1*exon 3 were as follows: 30 sec at 94°C; 30 sec at 55°C and 1 min at 68°C for 36 cycles, followed by 10 min extension at 68°C. PCR products were analyzed in by gel electrophoresis and purified using a Qiaquick PCR purification kit (Qiagen). Direct sequencing was carried out from both directions using a BigDye Terminator v3.1 cycle sequencing kit on an ABI PRISM 3100 genetic analyzer (Applied Biosystems). The National Center for Biotechnology Information Basic Local Alignment Search Tool was used to analyze both strands to identify mutations.

Statistical analysis

A standardized incidence ratio (SIR) represents a comparison between observations and expectations. This ratio is calculated by the number of events observed divided by the number of events expected. In this study, SIR was calculated to compare the incidence of DT in patients with GIST to the incidence of DT in the general U.S. population from 2000 to 2008 [250]. We calculated the 95% CIs of SIRs using the formula proposed by Vandenbroucke [251]. Overall survival was calculated as the time between the date the first neoplasm was diagnosed and the date of death or the last known date at which the patient was alive. Survival curves were estimated using the method of Kaplan and Meier method and the log-rank test.

Results

The 28 patients harboring GIST associated with DT were identified as follows: MD Anderson Cancer Center (cases 1-4), Helsinki University Central Hospital (cases 5-6), Fox Case Cancer Center (cases 7-9), Oregon Health & Science University Knight Cancer Institute (case 10), Cleveland Clinic (case 11), Istituto Nazionale dei Tumori (case 12), Prince of Wales Hospital (case 13), H. Lee Moffit Cancer Center (cases 14-16), the Armed Forces Institute of Pathology (cases 17-23) and Memorial Sloan-Kettering Cancer Center (cases 24-28). Table 5 shows the clinical characteristics of this patient population, which included 19 men (68%) and 9 women (32%) whose ages ranged from 34 to 88 years.

Case no.	Age	Sex	Primary location of GIST	Imatinib	KIT or PDGFRA Mutations (GIST)	Site of DT	B-Catenine Mutations (Desmoid Tumor) CTNNB1	Interval between GIST and DT (months)	Outcome
1	62	M	Gastric	yes	KIT mutation exon 11: V560D	Perigastric - peripancreatic	exon 3:Wt	39	NED: August 2009
2	67	M	Gastric	yes	KIT exon 11 mutation : deletion	Gastric	exon 3:T41A	35	NED: February 2009
3	50	M	Gastric	no	ND	Retrogastric		0	NED: December 2007
4	45	F	Omentum	yes	KIT mutation exon 11: D579del	Colon	exon 3 : Wt	39	AWD: January 2008
5	39	F	Jejunum	yes	WT	Small bowel	exon 3 : Wt	32	Died on January 2009
6	62	F	Gastric	yes	KIT exon 11mutation: del Lys 550-Glu556+ insLeu	Right rectus abdominis	exon 3 : Wt	30	Alive: May 2010
7	59	F	Gastric	yes	KIT exon 11mutation: W557-K558del	Small bowel	exon 3: T41A missense mutation	42	Alive: May 2010
8	62	F	Jejunum	yes	KIT exon 11 mutation : del 556-573	Small bowel	exon 3: S45P	16	Alive: May 2010
9	74	M	Gastric	yes	ND	Omentum	exon 3: T41A	25	Alive: May 2010
10	75	M	Gastric	no	PDGFRA exon 18 mutation: D842V	Peripancreatic	ND	0	NED: January 2005
11	88	F	Small intestine	no	WT	Abdominal wall	exon 3: Wt	-7	NED: January 2003
12	61	M	Gastric	yes	ND	Abdominal wall	ND	28	Died: July 2004
13	62	M	Gastroduodenal	yes	KIT exon 11mutation: deletion	Infrapyloric mesenteric	exon 3: T41A	36	Died: March 2010
14	42	F	Jejunal	yes	ND	Pelvic	ND	21	NED : October 2006
15	71	F	Gastric	yes	ND	Caudate lobe liver	ND	24	Alive : May 2010
16	52	M	Pelvic	yes	KIT exon 9 mutation	Mesenteric	ND	30	Alive : May 2010
17	53	M	Gastric	no	ND	Spleen	ND	33	ND
18	75	M	Gastric	no	ND	Mesenteric	ND	0	ND
19	65	M	Gastric	no	ND	Mesenteric	ND	0	Died: July 1988
20	70	M	Gastric	no	ND	Thigh	ND	191	NED: March 2003
21	66	M	Gastric	no	ND	Mesenteric	ND	0	NED: January 2003
22	42	F	Small intestine	no	ND	Mesenteric	ND	0	Alive: January 2004
23	40	M	Small intestine	no	ND	Mesenteric	ND	12	No follow up since 1986
24	34	M	Proximal Jejunum	no	KIT exon 9 mutation : 502-3 AY duplication	Terminal ileum	ND	19	NED: Oct 2009
25	58	M	Gastric	yes	ND	Mesenteric	ND	62	AWD: May 2010
26	58	M	Gastric	yes	KIT exon 11 mutation : Del (6aaDEL 552-7 MYEVQW)	Mesenteric	ND	30	AWD: May 2010
27	54	M	Gastric	yes	KIT exon 11 mutation : Del (557-8 WK del)	Small bowel/mesentery	ND	33	NED: April 2010
28	68	M	Small intestine	yes	ND	Proximal jejunum	ND	6	Alive: April 2010

Table 5. Patient and tumor characteristics

Summary of the 28 patients who developed GIST and DT. GIST, gastrointestinal stromal tumor; DT, desmoid tumor; Wt, wild type; ND, no data; NED, no evidence of disease; AWD, alive with disease. Modified from Dumont et al, Copyright 2012, with permission from Oxford University Press.

The majority of patients had sought medical advice because of vague symptoms such as epigastric pain, nausea or abdominal discomfort. Interestingly, no cases with a family history of FAP were observed.

In 21 cases (75%), GIST was diagnosed before DT. The average time between these diagnoses was 30 months (range, 6 months-16 years). In 6 cases (21%), both tumors presented synchronously and in 1 case (case 11; 4%), GIST was diagnosed after DT. The primary GIST tumor site was gastric in 17 cases (60%), small intestinal in 9 cases (32%) and pelvic or mesenteric in 1 case each (4%). DT involved the extremity in only 1 patient, who developed DT of the thigh 16 years after treatment for gastric GIST. In 3 patients (10%), DT developed in the surgical incision site and mimicked a recurrence of GIST. 17 patients (60%) were treated with imatinib. Ten cases (10, 11, 16-23; (36%)) developed a GIST before imatinib therapy was available. A strong family history of GIST (father, brother and aunts) was found in case 4, caused by a rare germline mutation of *KIT* that was previously described [252].

Interestingly, there are reports in the literature investigating the use of imatinib for treating DT. Case reports have described responses to imatinib, and a series of 19 patients with DT showed a partial response in 3 patients [253-255]. In our series, patient number 2, a 67-year-old man, received imatinib at 400 mg/day for gastric GIST and developed a DT on the posterior wall of the gastric antrum 35 months after the first diagnosis. His dose of imatinib was increased to 800 mg/day, resulting in a partial response in both tumors (Figure 18).

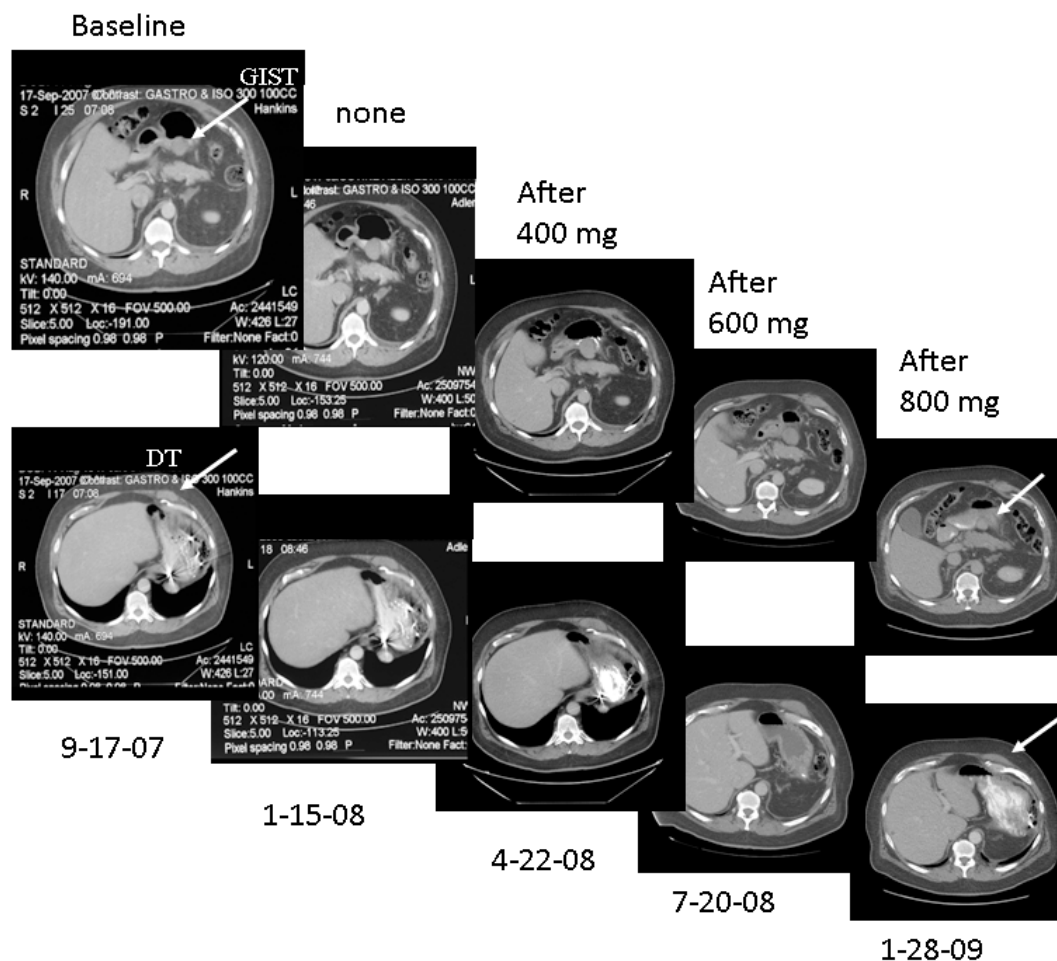


Figure 18. Partial response observed by Computed Tomography in GIST and DT after treatment with Imatinib at 800 mg/day.

Arrows indicate tumor localization. From Dumont et al, Copyright 2012, with permission from Oxford University Press.

The frequency of mutations in *KIT* and *PDGFRA* in GIST was similar to that previously observed in large cohorts of patients with GIST [256]. *KIT* was mutated in 11 cases of 14 (79%), of which 9 cases (64%) were in exon 11 and 2 cases (14%) in exon 9. *PDGFRA* was mutated in 1 case (7%). Two cases (14%) lacked mutations in *KIT* or *PDGFRA* (wild type).

β -catenin is commonly deregulated in DT indeed mutations in the *CTNNB1* gene have been identified with a prevalence of 85% in a large cohort of patients with DT [245]. In our

samples available for genotyping, *CTNNB1* was mutated in 5 cases (50%) in exon 3 (4 type 41A and 1 type 45F; Table A1).

The annual incidence of GIST in the United States is estimated to be 10-15 cases per million [257], while the annual incidence of DT is 2-4 cases per million [258]. The expected number of DT events in patients with GIST was calculated by applying the DT rate for theoretical GIST population in the US. To assess the SIR, only cases from the US from 2000 to 2008 were used. In this case series, 13 patients were diagnosed with GIST and DT in the US between 2000 and 2008. This number was compared to 0.16, an estimated number of expected cases assuming the independence of events. The estimated risk of developing DT was significantly higher (SIR = 82; 95% CI, 44-133) in GIST patients than in the general population.

Discussion

To our knowledge, this study was the first to report a series of patients with coexisting GIST and DT. This relationship raises the questions about of a potential link between these two neoplasms and the possibility of a cancer predisposition syndrome. In our patients who developed both tumors there were more men than women (19 vs 9). Accepting the possibility of a selection bias in this population, this gender distribution differs from that reported in previous GIST and DT studies. The incidence of GIST is equal among men and women GIST, and in DT a female predilection is suggested; with a female to male ratios commonly ranged between 1.4 and 1.8 [259, 260]. Furthermore, the peak incidence of DT has been reported as 25-35 years; however, in our case series, DT occurred at an average of 59 (range 34-88 years) [258, 260]. Statistical study on this limited population need to be interpreted carefully but because these tumors are very rare, the size of this series and the

SIR suggest a strong nonrandom association between the distinct tumors. At this stage, we can only speculate about this association.

Although the risk of developing both tumors appears extremely rare, this possibility should be considered when the patient has an occurrence of either of these tumors. There are no data to support a genetic predisposition syndrome, nevertheless, a germline mutation might underlie predisposition for these 2 tumors. We noted that most of the patients had abdominal surgery to remove their GIST and then developed a DT. It is possible that in some of the cases, the surgical trauma latter provokes the desmoid growth. No history of FAP was observed in our patients that could explain the increase of risk for developing a postoperative DT. The treatment with imatinib does not appear to play a role in the development of DT, because several cases in our series were not treated with imatinib. Interestingly, there are reports in the literature investigating the use of imatinib for treating DT. Case reports have described responses to imatinib, and a series of 19 patients with DT showed a partial response in 3 patients.[253-255] Another possibility is that GIST patients under physical and radiological surveillance are more likely to have DT detected compared to “normal” population. Finally, it is interesting to speculate that circulating stem cell factor (KIT ligand) or platelet-derived growth factor in patients with GIST could stimulate the growth of DT. Indeed, a cross-talk between the KIT and Wnt signaling pathways has been recently described in mast cell leukemia [263]. In this model, nuclear accumulation of β -catenin was increased by a gain-of-function mutation of *KIT* [261].

In this clinical and translational study, we show statistical evidence that there is a nonrandom relationship between the concomitant development of GIST and DT. Excluding a recurrence of GIST is fundamental for the clinical management of a patient that could

potentially still benefit from targeted therapy, such as imatinib. Further investigation is needed to establish the link between these tumors and to evaluate possible risk factors for their association.

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