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XXV ciclo

Post-translational modifications in the control of Notch3 protein signaling

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INTRODUCTION

1. The Notch gene family: structure and function

Notch receptors are large single-pass type I transmembrane proteins whose function is important for normal cell-fate determination in many multicellular organisms. The Notch signalling cascade represent an evolutionarily old and very well conserved system for signal transduction and can be found in species as diverse as flies, worms and humans.

The Notch family consists of one member in Drosophila, two receptors, LIN-12 and GLP-1, in Caenorhabditis elegans and four highly conserved transmembrane receptors (Notch-1, 2, 3 and 4) in mammals (Lardelli M. et al., 1995).

The structure

Notch is synthesized as an ≈300 kDa protein with a single-pass transmembrane domain harbouring a large extracellular domain involved in ligand binding, and a cytoplasmatic domain involved in signal transduction. As for the structural organization, all Notch proteins share similar basic structure (**Figure 1**).

The extracellular domain (≈1700 aa) includes 29-36 epidermal growth factor (EGF)-like repeats. EGF repeats are small molecular domain that typically contain three disulphide bonds and are primarily composed of β-strand, forming an extended rod-like structure. Some of EGF repeats (11-12 and 24-29) mediate interactions with the Notch ligand. Many of their EGF repeats bind calcium, which has been shown to be important for receptor-ligand complexation (Cordle J. et al., 2008; Raya A. et al., 2004) and can be modified by two forms of O-glycosylation, O-fucose and O-glucose (Haines and Irvine 2003). The effects of these modifications are complex and have

implications for Notch folding, sensitivity to the ligands and signaling efficiency.

Within the extracellular domain structure , the EGF repeats are followed by a unique negative regulatory region (NRR), The NRR functions to keep Notch in an off state until it interacts with a DSL ligand. NRR is composed of three cysteine-rich Lin12-Notch repeats (LNR) (each LNR domain is disulphide bonded and binds calcium) and a hydrophobic stretch of amino acids the heterodimerization domain (HD). The three LNR domains protect the S2 site contained within the HD from cleavage. The structure of the NRR suggests that some sort of conformational change or unfolding event occur to expose the S2 site for cleavage by ADAM.

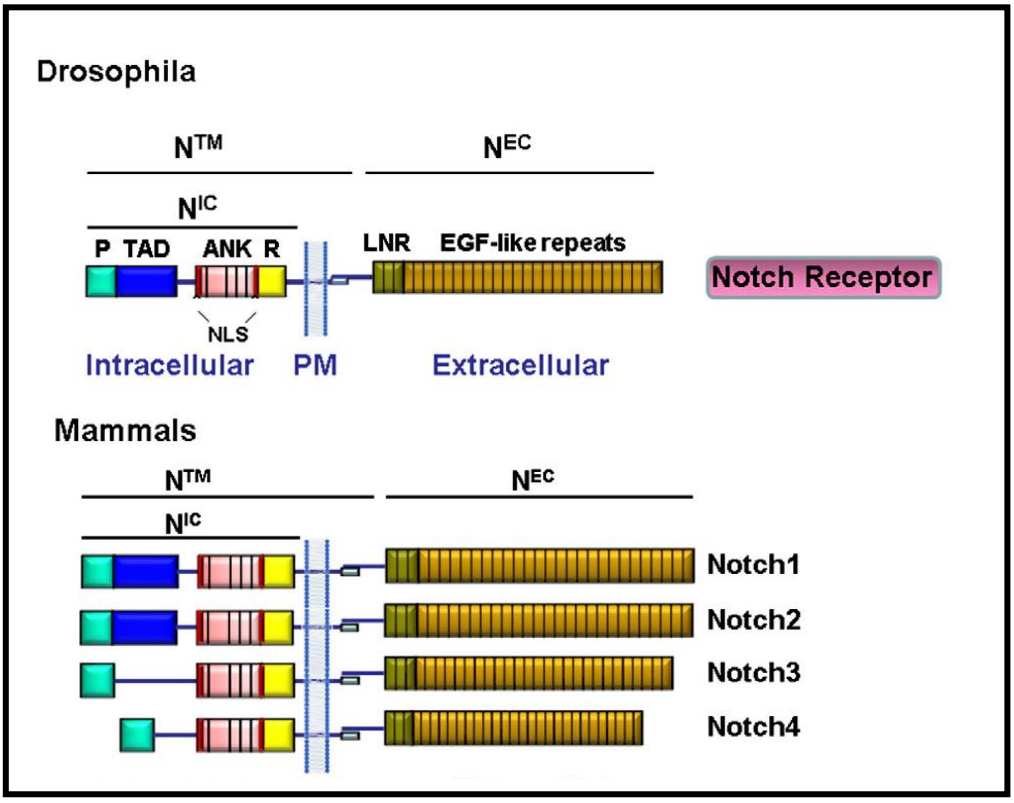
In contrast to *Drosophila* Notch, during the secretory pathway, mammalian Notch proteins are cleaved by furin-like convertases at site 1 (S1). This cleavage generates a Notch extracellular domain-Notch transmembrane and intracellular domain (NECD-NTMIC) heterodimer that is held together by noncovalent interactions between the N- and C- terminal halves of the heterodimerization domain. The single transmembrane domain of the Notch receptor contains 3-4 arginine/lysine (Arg/Lys) residues with a C-terminal "stop translocation" signal.

The intracellular region of all Notch orthologs (NIC) harbors multiple conserved elements. The membrane-proximal RAM (RBP-jK association module) domain contains a high-affinity binding module of 12-20 amino acids with a conserved "tryptophan- any amino acids-proline" (WxP)motif. Notch intracellular domain also contains two nuclear localization sequences (NLSs), a seven ankyrin repeats (ANK) domain and a loosely defined and evolutionarily divergent transactivation domain (TAD). While Notch1 and Notch2 also contain a transcriptional transactivation domain (TAD), such domains have not yet been described for Notch3 and Notch4. Finally, the C terminus of Notch receptor holds a glutamine-rich repeat (OPA) that is

present only in *Drodophila* and a conserved proline/glutamic acid/serine/threonine-rich (PEST) sequence motif that harbor degradation signals and negatively regulates protein stability.

In mammals, the different members of the Notch family have a variable structural homology. Despite a similar basic structure with respect to Notch1 and 2, Notch3 possesses a number of structural differences at the level of EGF-like repeats, and mostly in the intracellular domain (Lardelli M. et al., 1994). Notch4-Int3 is considered a Notch protein-related.

Figure 1: The structure of *Notch receptors*



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Notch Signaling Pathway

Notch signaling is initiated by dynamic interactions between membrane-bound Notch receptors and ligands during direct cell-cell contact (Cordle J. et al., 2008) (**Figure 2**). Most Notch ligands are themselves type I transmembrane proteins characterized by three related structural motifs: an N-terminal DSL (Delta/Serrate/LAG-2) motif, involved in Notch binding, a DOS (Delta and OMS-11-like proteins) domain that holds specialized tandem EGF repeats (Komatsu H. et al., 2008), and a variable number of EGF-like repeats.

Several genetic and molecular studies have identified a real family of Notch ligands structurally related: *Delta* and *Serrate* in *D. melanogaster* and *lag-2* and *apx-1* in *C. elegans*. In mammals, five differents ligands have been identified: three ortologs (Delta -1, -3, -4) are structurally related to *Drosophila* Delta (Dunwoodie S. et al., 1997), and two orthologs (Jagged -1 and -2) to *Drosophila* Serrate (Lindsell CE et al., 1995; Shawber C. et al., 1996; Luo B. et al., 1997).

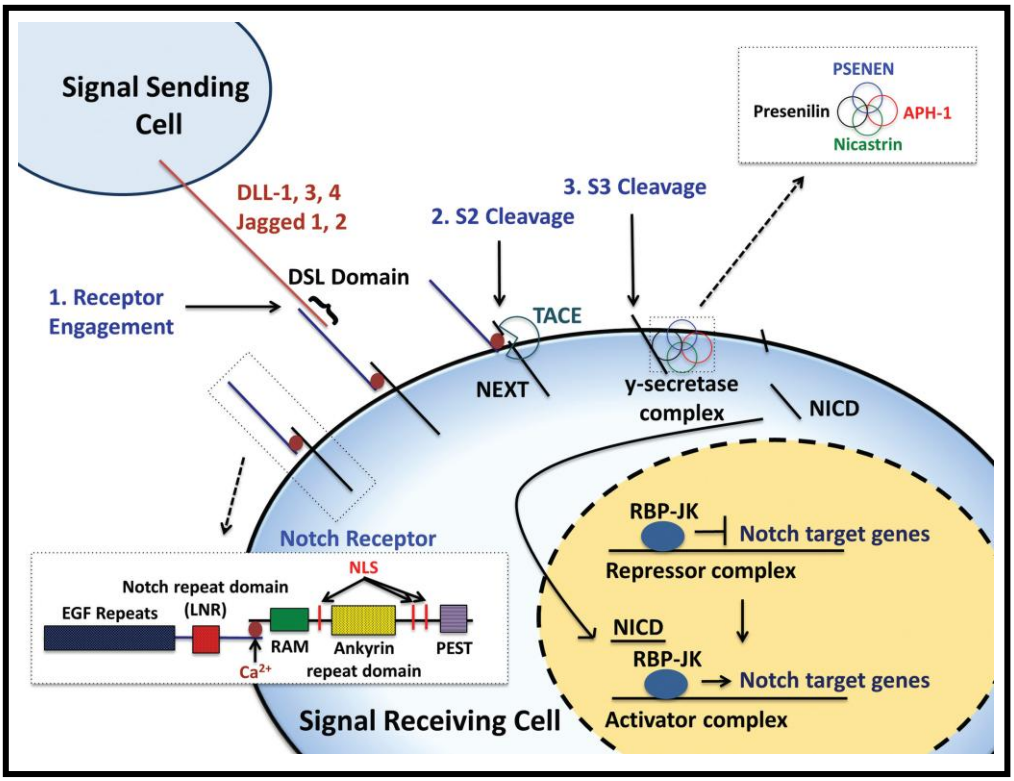
After ligand binding Notch receptor undergoes a conformational change, triggering two sequential proteolytic cleavages of Notch (Bray S.J. 2006). The first cleavage is catalyzed by the tumor necrosis factor α-converting enzyme (TACE), an ADAM-type metalloproteinase. This cleavage affects site 2 (S2), located nearly 12 amino acids before transmembrane domain and within the negative regulatory region and creates a short-lived transmembrane Notch domain that becomes a substrate for γ-secretase. γ-Secretase is a protein complex that consists of Presenilin, the catalytic subunit, and three other proteins that contribute to substrate recognition and stability: APH-2/Nicastrin, APH-1 and PEN-2. This complex cleaves the Notch transmembrane domain, from site 3 (S3) to site 4 (S4) releasing a soluble cytoplasmic domain of Notch (Fortini M.E. 2002), the Notch

intracellular domain (NICD). Then, liberated NICD translocates to the nucleus where it interacts (through its RAM domain) with a transcription factor, called CSL (CBF1 in *humans*, RBP-jk in *mice*, Suppressor of Hairless in *Drosophila*, Lag1 in *C. Elegans*), and a transcriptional coactivator of the Mistermind-like family (Wu L. et al., 2000). In the absence of NICD, CSL proteins bind to promoters of its target genes recruiting histone deacetylases and corepressor and are able to inhibit transcription (Oswald F. et al., 2005). The NICD/CSL interaction induces allosteric changes in CSL, that allow displacement of transcriptional repressor and recruitment of coactivators, thus inducing transcription of target genes.

A number of target genes whose expression appears to be transcriptionally regulated by Notch signalling in vertebrates have been identified. The best-characterized downstream targets of Notch/RBP-jk are members of the HES and HERP families of basic helix-loop-helix (bHLH) transcriptional repressors (Iso T. et al., 2003), NFkB (Oswald F. et al., 1998), the locus control region of the β-globin locus (Lam and Bresnick 1998), the cell cycle regulator p21 (Devgan V et al., 2005), Deltex (Ordentlich P. et al., 1998) and the pre-T cell receptor-α gene (Reizis B. et al., 2002).

The model of signal transduction, just described, is characterized by a remarkable immediacy. The intracellular domain of Notch is able itself, without intermediaries, to influence the gene expression of the target cell. This feature helps to understand the high degree of evolutionary conservation. The simplicity of this model is greatly complicated by the numerous levels of control that are exercised on it: Notch signalling can be regulated by several modulators that acts at extracellular, cytoplasmic or nuclear levels (Artavanis-Tsakonas S. et al., 1999).

Figure 2: ***Notch signaling***



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2. The role of Notch signalling in T-cell development

The first step of T-cell differentiation occur within the thymus, a specialized organ of the immune system, composed of a central medulla and a peripheral cortex, surrounded by an outer capsule. Proliferation, cell fate specification or death of T-cell progenitors are determined by instructive and selective signals, that are either cell autonomous or arise from interactions with the thymic stroma (Boyd and Hugo 1991) and lead the thymocyte progression from immature $CD4^-CD8^-$ double negative (DN) towards $CD4^+CD8^+$ double positive (DP) phenotype and their $CD4^+CD8^-$ versus $CD4^-CD8^+$ single positive (SP) and/or αβ versus γδ cell lineage choise. In addition to the signals generated by T cell specific differentiation products, e.g. the pre-T-cell receptor (pre-TCR) (von Boehmer H. et al., 1999) and mature TCR αβ (Jameson and Bevan 1998) other general biological regulators provide to control the intrathymic T cell differentiation. Multiple Notch receptors and their cognate ligands are described in distinct thymic cell compartments and play a major role during thymocyte differentiation (Felli MP et al., 1999). The first suggestion that Notch signaling could be an important regulator of haematopoietic progenitor commitment to the T-cell lineage came in the early 1990s, when human NOTCH1 was identified through the analysis of a chromosomal translocation t(7;9)(q34;q34.3) detected in a small number of T-ALL patients (Ellisen L.W. et al., 1991) suggesting that mutated "active" Notch1 could transform T-cell progenitors. Several years later multiple investigators demonstrated that Notch1 is required for early T cell-commitment (Radtke F. et al 1999), T cell lineage decision (αβ or γδ) and VDJβ rearrangement (Wolfer A. et al., 2002). More specifically, Notch1 expression is high in early DN thymocytes, low in DP cells and intermediate in CD4 and CD8 SP cells (Hasserjian R.P. et al., 1996). Unlike Notch1, Notch3 is highly expressed in double negative immature

thymocytes just before their transition through the β selection checkpoint and is subsequently downregulated across the DN to DP transition (Felli MP et al., 1999), that is controlled by the pre-TCR signaling pathway and characterized by an activated NF-kB (Voll RE et al., 2000), suggesting a specific role of Notch3 at this stage of thymocyte development. The ability of Notch3 to influence T-cell development was confirmed by generating Lck proximal promoter–driven Notch3-IC transgenic (*N3-IC-tg*) mice, in which the active intracellular domain of Notch3 (Notch3-IC) is selectively overexpressed in immature DN thymocytes (Bellavia et al., 2000). *N3-IC-tg* mice display a phenotype of dysregulated early T cell development, characterized by the impairment of the pre-TCR selection stage of T cell differentiation (e.g. expansion of stage II and III DN cells, retention of CD25 expression in post-DN cells and constitutively activated NFkB) and the outgrowth of aggressive T-cell lymphoblastic lymphomas (Bellavia D. et al., 2000). These data strongly suggest a specific role of Notch3 in controlling the crucial events occurring at the DN – DP transition, in agreement with the physiological expression profile of the receptor. The important role of both Notch1 and Notch3 in different stages of T cell development is also supported by the onset of lymphoproliferative diseases in murine models displaying constitutively active signaling of these receptors (Robey E. et al., 1996; Deftos ML et al., 2000; Bellavia et al., 2000). In other studies, introduction of constitutively active downstream components of the pre-TCR signaling machinery into RAG-deficient DN3 cells was insufficient to restore progression to the DP stage in the absence of Notch signaling (Ciofani M. et al., 2004). Furthermore, DN3 cells cultured in the absence of Notch signaling died, suggesting that Notch functions as a survival factor at this stage of development. Moreover, Notch3 overexpression in a *pTα knockout* background (obtained by the generation of *N3-IC/pTα*$^{-/-}$ double mutant mice),

is able to partially rescue the impaired T cell differentiation displayed by *pTα*$^{-}$ ⁄ mice (Bellavia et al., 2002).

3. Notch in molecular pathogenesis of T-ALL leukemia

In recent years aberrant Notch signaling has been linked to various forms of tumors, but the best-documented role of activated Notch signaling in human carcinogenesis is certainly T cell acute lymphoblastic leukemia (T-ALL).

T-ALL is an aggressive hematopoietic malignancy of developing thymocytes that represents 15% of pediatric and 25% of adult acute lymphoblastic leukemia (ALL) cases and is characteristically more prevalent in males than in females (male-to-female ratio 3:1). The disease is generally associated with more unfavourable clinical features such as a high white blood cell counts, increased numbers of blast cells, enlarged mediastinal lymph nodes and involvement of the central nervous system (Uckun FM et al., 1997). Transformation events occur in crucial steps in thymocytes development and involve various genetic alterations that give rise to abnormal cell-cycle control with uncontrolled growth and clonal expansion of T cells. The 50% of T-ALL cases show an abnormal kariotype. The chromosomal translocations that occur frequently in cases of T-ALL involve the juxtaposition of genes coding for transcription factor next to strong regulatory elements located in the vicinity of the T-cell receptor β (TCRβ) gene in chromosome 7q34 or the T-cell receptor α-δ (TCRαδ) locus in chromosome 14q11 (Ferrando and Look 2000). These T-ALL specific transcription factor onocogenes include basic helix-loop-helix transcriptions factors such as TAL1, TAL2, LYL1, bHLHB1; LIM-only domain (LMO) proteins such as LMO1 and LMO2; HOXA homeobox genes; the MYC and MYB oncogenes. About 10% of T-ALL cases harbor translocations resulting in the expression of fusion transcripts encoding chimerical proteins with oncogenic properties such as the

SIL (SCL-interrupting locus)-TAL1. Although some T-ALL tumors seem to result from chromosomal translocations or rearrangements that activate oncogenes or create oncogenic fusion genes, more than 50% of T-ALL patients have mutations leading to the hyperactivation of the Notch1 pathway, suggesting that this signaling cascade plays a central role in T-ALL pathogenesis. Generally, Notch1 mutations cluster in two general regions either at the PEST or the HD domain. Mutations about the C-terminus of the Notch1 receptor are frequently nonsense or frameshift mutation leading the deletion of the PEST domain, normally regulating Notch1 stability and degradation (Chiang MY et al., 2006). Whereas mutations (such as single amino acids changes, short insertions or deletions that maintain the reading frame) within the HD domain (exon 26 or 27) render Notch1 susceptible to ligand-indipendent S2 cleavage (Malecki MJ et al., 2006). Constitutive activation of Notch signaling affects the expression of specifics target genes and the downstream signaling pathways. The best-characterized direct target genes include the bHLH transcriptional repressor Hes1 (Dudley DD et al., 2009), the transcription factor c-Myc (Weng AP et al., 2006; Palomero T et al., 2006), the PI(3)-kinase/Akt (Palomero T et al 2007; Sade H et al 2004), and mTor (Chan SM et al., 2007) signaling cascade. Another important signaling pathway activated in response to the expression of NICD or human T-ALL *NOTCH1* mutations in hematopoietic progenitors is the NF-kB cascade. Notch1 signaling is able to promote G1/S cell cycle progression via upregulation of CDK4 and CDK6 (Joshi I. et al.,2009) and downregulation of p27/KIP1 and p18/INK4C cell cycle inhibitors (DohdaT. et al., 2007; Palomero T. et al., 2006).

Several experimental models of Notch receptor dysregulated expression have suggested critical and distinct roles for different Notch receptors in differents steps of T cell differentiation and in T cell leukomogenesis, giving rise the initial basis to analize for their oncological potential. Indeed, the oncogenic

potential of NOTCH1 was demonstrated in murine bone marrow (BM) reconstitution experiments. Mice reconstituted with BM cells expressing a truncated human form of NOTCH1 developed haematological malignancies characterized as T-ALL (Pear WS et al., 1996). The importance of Notch family member in the development of T-ALL has been further elucidated by a study in which Notch3 was shown to be expressed in all 30 human T cell acute leukemia samples examined, whereas Notch3 expression was dramatically reduced or absent in remission and in other types of T-ALL (Bellavia D. et al., 2002). These data together with the generation of Notch3 transgenic mice has confirmed and reinforced the involvement of other Notch receptors in T-ALL pathogenesis. The T cell lineage-targeted enforced expression of the constitutively active Notch3-IC leads to an aggressive T-cell leukemia, characterized by sustained expression of pTα, the invariant chain of pre-TCR, enhanced expression of specific Notch target genes, such as HES-1 and Deltex and the constitutive activation of NFkB in thymocytes and peripheral T cells (Bellavia D et al., 2000), consistent with improved survival and increased numbers of thymocytes in Notch3 tg mice.

The combined misexpression of the genes encoding Notch3, pTα and HES1 in human T-ALL suggests that a signaling defect at a specific step in T-cell development, the pre-TCR checkpoint, is responsible for T-cell leukemogenesis. Indeed, deletion of pTα in Notch3-transgenic mice prevents tumor development.

The important role of pTα was already observed in previous experiments that required the use of bone marrow precursors with rag2$^{-/-}$ genotype unable to successfully rearrange TCR. Consequently, thymocytes differentiation is blocked in an early stage due to inability to express the β chain of the TCR, an essential component of this receptor complex. Also in this case the development of the disease was completely abrogated. The subsequent re-introduction of a construct expressing the transgenic β chain of the TCR

instead was able to restore the tumor phenotype (Allmann D. et al., 2001). The result has been further confirmed using hematopoietic stem cells from mice SLP-76$^{-/-}$, carrying a deletion of an adapter protein, essential for the pre-TCR signal transduction (Allmann D. et al., 2001): also in this case it was observed the complete absence of malignant disease.

Ultimately, as suggested for the role of oncogenetic Notch1 (Allmann D. et al., 2001), the development of Notch3-induced T-cell leukemia appears to be closely linked to the presence of the pre-TCR functionally active complex. Conversely to *Notch1* for the *Notch3* gene were not described specific mutations or obvious gene rearrangements, while an increase was observed in all cases of T-ALL analyzed (Bellavia D. et al., 2002). Therefore, differently from what happens for Notch1, the mechanism that regulates and supports the overexpression of Notch3, in association with that of pTα, has yet to be characterized.

4. Proteins Post-translation Modifications

Posttranslational modifications (PTMs) of proteins represent fascinating extensions of the dynamic complexity of living cells' proteomes. PTMs have a significant physiological/biological impact, playing crucial roles in regulating signaling, protein-protein modifications, protein conformational stability and subcellular localization. They can be either transient or permanent and may result from either targeted, enzymatically catalyzed reactions or spontaneous chemical reactions in the cell. Modifications include phosphorylation, glycosylation, nitrosylation, methylation, acetylation, lipidation and proteolysis and can act alone and in combination to regulate nearly all aspects of protein function.

Notch signaling is used reiteratively for a vast variety of developmental processes as well as during the adult life of many organisms. To fulfill its multiple functions in distinct tissue, Notch signaling is tightly controlled both in time and space (Le Borgne R. 2006). Increasing number of reports have shown that Notch is subject to a variety of post-translational modifications which regulate its activity. These modifications include glycosylation, ubiquitylation, phosphorylation, acetylation and hydroxylation.

Glycosylation of Notch receptors by Fringe enzymes (N-acetylglucosaminidyltransferases) affects binding affinities between ligands and specific EGF-repeats (Okajima T. et al., 2003). Phosphorylation of Notch occurs at different residues and is caused by different kinases .

Ubiquitination is a main player in regulating a broad variety of cellular processes including cell division, differentiation, signal transduction, protein trafficking and quality control (Mukhopadhyay D et al., 2007). Therefore, ubiquitin is a versatile modification designed to shape cell-signaling pathways and is at the heart of the spatiotemporal control of Notch signaling. Several

studies have suggested that proteasomal degradation of activated forms of Notch (Notch-IC) may be required for the negative regulation of Notch signaling (Hubbard EJ. et al., 1997; Schweisguth F. 1999). c-Cbl, Itch and Sel-10 are all involved in the negative regulation of Notch signaling (Qiu L. et al., 2000; Oberg C. et al., 2001; Wu G. et al., 2001; Jehn BM et al., 2002). Indeed, C elegans and mammalian SEL-10/Fbw7 (a WD40-repeat containing F-Box protein component of an Skp1/Cul1/F-box protein-Rbx1-type ubiquitin ligase) were shown to ubiquitinate NICD to promote its proteosomal degradation (Hubbard EJ et al., 1997; Oberg C. et al., 2001; Wu G. et al., 2001). Although , c-Cbl has been shown to promote ubiquitin-dependent lysosomal degradation of membrane-associated Notch1 (Jehn BM. et al., 2002), recently we have demonstrated that c-Cbl is also able to target Notch3-IC protein to the proteosomal-degradative pathway only in the presence of pTα and that this depends on the cytoplasmic localization and tyrosine phosphorylation state of c-Cbl (Checquolo S. et al., 2010). In addiction to ubiquitination, other PTMs suchs as phosphorylation, glycosylation and acetylation have been identified in Notch. Different PTMs form a complex regulatory program with characteristics of a sophisticated language and such a program is fundamental to normal development and disease pathogenesis.

4.1 Acetylation and deacetylation: HATs and HDACs.

Epigenetic modifications are defined as heritable changes in gene expression that are not due to any alteration in the genetic information represented by the DNA sequence. Unlike genetic modifications, epigenetic modifications are reversible. Acetylation has emerged as a major posttranslational modification for histones. Cross-regulation between this and other modifications is crucial in modulating chromatin-based transcriptional control and shaping inheritable epigenetic programs. It has been nearly 40 years since Allfrey and co-workers

proposed that acetylation state of histones within chromatin is correlated with gene regulation. The acetylation neutralizes the positive charge of the histone lysine residues, relaxing the chromatin conformation and enabling greater accessibility of the transcription machinery (Haberland M. et al., 2009). In contrast, the removal of the acetyl groups from histones induces chromatin condensation and gene transcriptional repression (Haberland M. et al., 2009).
In addiction to transcription, the status of histone acetylation may influence cell growth and differentiation (Mizuguchi G. et al., 2001).
Like histones, many nonhistone proteins are subject to acetylation.. There is a growing body of evidence supporting the notion that acetylation, like phosphorylation, is an important regulatory protein modification. Indeed, the acetylation of several transcription factors and cytoplasmic proteins may regulate multiple mechanisms, such as modification of DNA binding ability, secondary protein-protein interactions, protein half-life and protein localization thus affecting DNA repair, cell cycle progression, apoptosis and various signaling pathways.

Acetylation is a reversible modification controlled by the antagonistic actions of two types of enzymes, histone acetylases (HATs) and histone deacetylases (HADCs).

The Histone Acetylases (HATs)

Histone acetyltransferase (HAT) enzymes are the catalytic subunits of multisubunit protein complexes that acts by transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to an ε-amino group of certain lysine side chains within a histone's basic N-terminal tail region. Acetylation of lysine residues at the ε-NH_2 is highly dynamic and neutralizes part of histone tail region's positive charge, weakening histone-DNA or nucleosome-nucleosome interactions (Nakatani Y. 2001), thereby destabilizing nucleosome structure

or arrangement. In this way, nuclear factors, such as the transcription complex, have more access to a genetic locus. HAT proteins, forming multiple complexes, are recruited to chromatin to acetylate histones and/or transcription factors. A large number of transcription factors are now known to have HAT activity. Sequence analysis of these proteins reveals that they fall into distinct families that show high sequence similarity within families but poor to no sequence similarity between families (Kuo M.H. et al., 1998).

HAT proteins generally belongs to one of two categories (Sterner E. and Berger S. L. 2000):

- type A, located in the nucleus, catalyze the postsynthetic acetylation of all four nucleosomal histones.
- type B, located in the cytoplasm, are responsible for the acetylation of newly synthesized histone proteins. This allows the transport of "de novo" translated histones through the nuclear membrane and the subsequent replacement into newly replicated DNA.

The A-type HATs are organized in five distinct groups based on structural homology in the primary sequence as well as on biochemical mechanism of acetyl transfer **(table 1)**:

- human Gnc5 (KAT2A), PCAF (KAT2B) and ELP3 (KAT9) belong to the ***GNAT family*** and are known as transcriptional activators;

- the ***p300/CBP family*** members are the most studied. Unlike the other families, they don't bind directly to DNA, but are recruited on the promoters of target genes by other transcription factors (capable themselves of binding DNA) , such as E1A and CREB, only in phosphorylated form (Roth SY et al., 2001). The HATs CBP and p300 are two ubiquitous transcriptional co-activators and structurally contain several protein domains, including a bromodomain (believed to facilitate interactions with acetylated lysine residues) , three regions of zinc-finger (Cys, ZZ and TAZ), a centrally located and highly conserved acetyltransferase (HAT) domain and two

independent regions that interact with different transcription factors (Janknecht R. and T. Hunter, 1996);

- the ***MYST family*** of HAT proteins are involved in divergent biological functions regulating transcriptional silencing, dosage compensation in Drosophila, HIV Tat interaction, DNA repair and includes Tip60 (KAT5), MOZ/MYST3 (KAT6A), MORF/MYST4 (KAT6B), HBO1/MYST2 (KAT7) and HMOF/MYST1 (KAT8);

- TAF1/TBP (KAT4) and TIFIIIC90 (KAT12) belong to transcriptional factor related HAT family (***TF-related HATs family***);

- different steroid receptor co-activators have been described to be catalytically active histone acetyltransferases, e.g. SRC1 (KAT13D), p600 (KAT13C) and other (***NR-coactivators***).

While the GNAT and MYST families have homologs from yeast to man, p300/CBP is metazoan-specific.

Table 1. Nuclear type A HAT families

Family	HAT	KAT	Histone substrate	Function
GNAT	Gcn5	KAT2A	H3*K9,14,18,36*	transcriptional activation,
	PCAF	KAT2B	H3*K9,14,18,36*	DNA repair
	ELP3	KAT9		
p300/CBP	CBP	KAT3A	H2A*K5*	transcriptional activation
			H2B*K12,15*	
			H3*K14,18*	
			H4*K5,8*	
	p300	KAT3B	H2A*K5*	
			H2B*K12,15*	
			H3*K14,18*	
			H4*K5,8*	
MYST	Tip60	KAT5	H4*K5,8,12,16*	transcriptional activation,
	MOZ/MYST3	KAT6A	H3*K14*	DNA repair, replication
	MORF/MYST4	KAT6B	H3*K14*	dosage compensation
	HBO1/MYST2	KAT7	H4*K5,8,12*	
	HMOF/MYST1	KAT8	H4*K16*	
TF-related HATs	TAF1/TBP	KAT4		transcriptional activation,
	TFIIIC90	KAT12	H3*K9,14,18*	Pol III transcription
NR co-activators	SRC1	KAT13A		transcriptional activation
	AIB1/ACTR/SRC3	KAT13B		
	P160	KAT13C		
	CLOCK	KAT13D		

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Despite the lack of significant sequence similarity between each HAT subfamilies, all HATs share a globular α/β fold in which the central αβ core region is surrounded by a bundle of alpha helices at opposite ends (N- and C-terminal) of the enzyme. This core region participates in acetyl CoA co-factor binding and templating the respective substrate protein for acetylation and appears structurally conserved among the various subfamilies of HATS. Unlike the core region, the various HAT sub-families contain structurally divergent regions, the N- and C- terminal regions (that flank the core region). The diversity in these regions could be correlated with their distinct substrate specificities, biological activities and with their different chemical strategies to acetylate their substrates (Yuan H. and Marmostein R. 2013).

HATs are increasingly being recognized as modifier of both histones and nonhistone proteins (Sterner D.E. et al., 2000). Acetylation of non-histone proteins, has been shown to modulate their functions by altering their stability, cellular localization or interactions, thus, contributing to several processes which are crucial for cellular fate. Protein acetylation patterns (which are often collectively termed the "acetylome") involve more than 60 transcription factors and many other proteins that regulate DNA repair and replication, metabolism, cytoskeletal dynamics, apoptosis, nuclear import, protein folding, and cellular cellular signaling **(Table 2)**, allowing to interfere with every step of regulatory processes from signaling to transcription to protein degradation. The tumor suppressor p53 was the first non-histone protein shown to be acetylated by HATs (Gu and Roeder 1997). p53 can be acetylated at multiple lysines by distinct acetyltransferases and enhancement of p53 acetylation levels strongly correlates with protein stabilization and activation in response to cellular stress (Luo J. et al., 2000; Ito A. et al., 2001). PCAF and p300/CBP catalyse acetylation of C-terminal p53 lysine residues which overlap with ubiquitination sites and abrogates complex

formation between p53 and Mdm2, preventing p53 proteasomal degradation (Ito A et al 2002). Numerous HATs (CBP, Tip60, Gcn5 and PCAF) were shown to be able to acetylate c-Myc at multiple lysine, preventing its ubiquitination and proteasomal degradation (Patel JH et al., 2004; Vervoorts J. et al., 2003). Posttranslation modifications of NF-kB dimers have been shown to alter their interactions with co-activators. Phopsphorylated p65 (a NF-kB subunit) preferentially interacts with p300/CBP resulting in p65 acetylation at multiple site and increased transcription of NF-kB target genes (Greene and Chen 2004).

Aberrant lysine acetylation has been reported in malignant cells (Yang XJ 2004) and HATs and HDACs are closely linked to severe disease such as cancer, neurodegeneration, cardiovascular disorders, inflammation and functional alterations in metabolic cascades. Hence, the HAT-HDAC interplay represents an important target system for regulatory mechanisms and for the development of potential therapeutical strategies. In the last ten years a limited number of compounds have been identified to address lysine acetyltransferase activities, inhibiting or activating these histone modifying enzymes: natural products, synthetic derivates, bisubstrate inhibitors and syntetic small molecules. Anacardic acid was the first non-competitive HAT inhibitor found with inhibitory activity against PCAF and p300 that show anticancer activity. This compound inhibits the acetylation of the p65 subunit of NF-kB blocking its activation and nuclear localization (Sung B.M. et al., 2008). Curcumin, an other natural product, show a certain selectivity within the HAT family. Indeed, it is unable to affect the enzymatic activity of PCAF but show specifity towards p300. Curcumin is able to repress p300-mediated p53 acetylation and inhibits histones H3 and H4 acetylation (Balasubramanyam K. et al., 2004). Other compounds, such as a derivative of quinoline, MC1626 (Smith et al., 2007) and the isothiazolones (Stimson L. et al., 2005), act instead as inhibitors of GCN5, PCAF and p300, respectively.

Table 2. Selected non-histones proteins and functional consequences of their acetylation

Biological implication	Proteins affected by acetylation	
Protein stability	Acetylation increases stability p53, p73, Smad7, c-Myc, Runx3, AR, H2A.z, E2F1, NF-E4, ER81, SREBP1a, HNF6, BACE1	Acetylation decreases stability GATA1, HIF-1α, pRb, SV40 T-Ag
DNA binding	Increased DNA binding p53, SRY, STAT3, GATA transcription factors, E2F1, p50 (NFκB), Erα, p65 (NFκB), c-Myb, MyoD, HNF-4, AML1, BETA2, NF-E2, KLF13, TAL1/SCL, TAF(I)68, AP endonuclease	Decreased DNA binding YY1, HMG-A1, HMG-N2, p65 (NFκB), DEK, KLF13, Fen-1
Gene expression	Transcriptional activation p53, HMG-A1, STAT3, AR, ERα (basal), GATA transcription factors, EKLF, MyoD, E2F1, p65(NFκB), GR, p73, PGC1α, MEF2D, GCMa, PLAG1, PLAGL2, Bcl-6, β-Catenin, KLF5, Sp1, BETA2, Cart1, RIP140, TAF(I)68	Transcriptional inactivation Erα (ligand-bound), HIF-1α, STAT1, FOXO1, FOXO4, RIP140
Protein interactions	Enhanced STAT3, AR, EKLF, Importin A, STAT1, TFIIB, α-Tubulin, actin, cortactin	Decreased p65(RelA), Ku70, Hsp90
Localisation	Ac→ nucleus PCAF, SRY, CtBP2, POP-1, HNF-4, PCNA Sub-nuclear WRN, PCNA	Ac→ cytosol c-Abl, p300, PAP
mRNA stability	Increased p21, Brm	Decreased Tyrosinhydrolase (Th), eNOS
Enzymatic activity	Enhanced p300, ATM	Decreased PTEN, HDAC1, Mdm2, ACS, Neil2, Polβ
Mitochondrial proteins	ACS (Ac-CoA-Synthetase), Sod1/2, Profilin I, Thioredoxin; multiple components of metabolic and oxidative phosphorylation machinery	
Viral proteins	E1A, S-HDAg, L-HDAg, HIV Tat, SV40 T-Ag	

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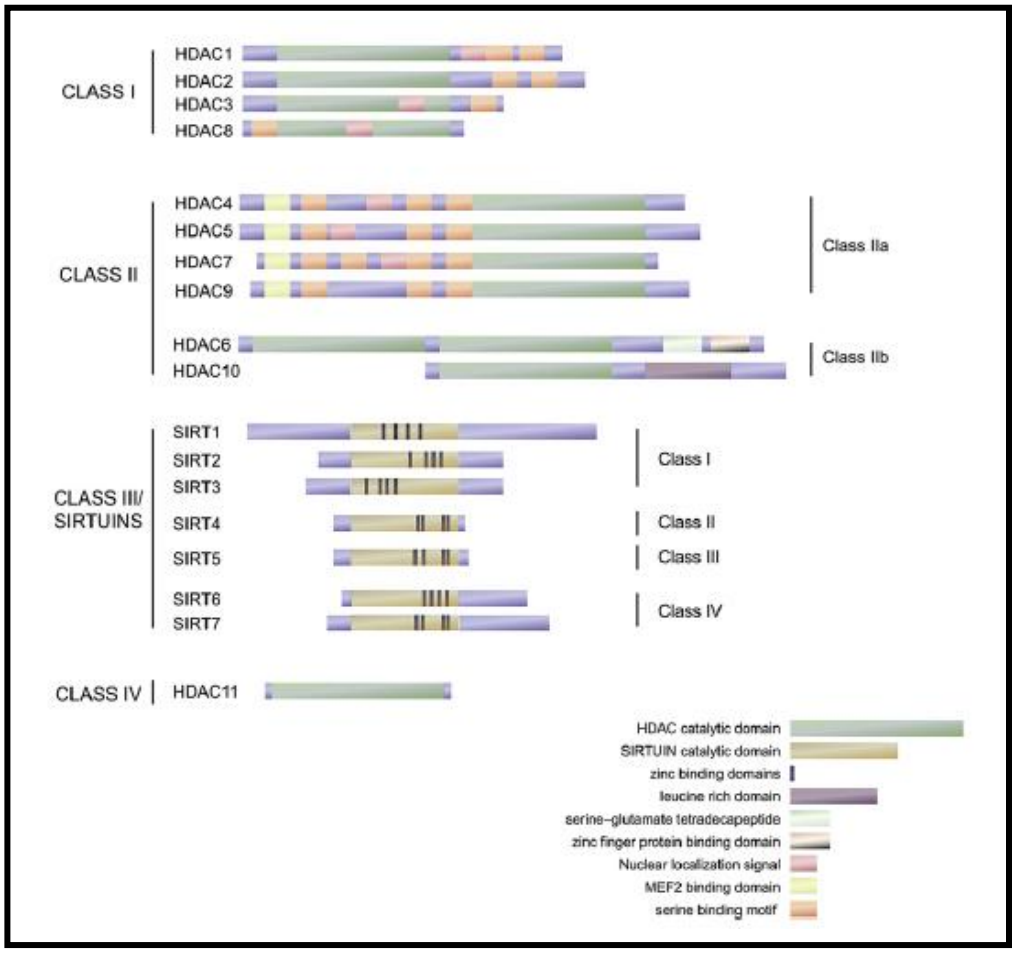
HDACs

HDACs have emerged as crucial transcriptional co-repressors in highly diverse physiological and pathological systems. In human, HDACs comprise a family of 18 genes sub-diveded into 4 classes on the basis of their sequence homology to ortholog yeast proteins, sub-cellular localization and enzymatic activities (Thiagalingam S. et al., 2003) **(Figure 3).**

Class I, II and IV HDACs are also referred to as "classical" HDACs and are Zn2+-dependent enzymes whereas class III HDACs, also called sirtuins require NAD+ as a cofactor.

Figure 3. Human HDACs superfamily



Barneda-Zahonero B. and Parra M. Molecular Oncology 2012.

Class I HDACs, ubiquitously expressed in all tissue, are nuclear proteins (with homology to the yeast RPD3 protein) able to exert a strong catalytic effect on histone lysine. Phylogenetic analysis suggests that this class can be sub-divided in class IA (HDAC1 and 2), class 1B (HDAC3) and class 1C (HDAC8). The protein structure of this class of proteins is characterized by a highly conserved deacetylase domain flanked by short amino and carboxy-terminal extensions (Yang and Seto 2008). HDAC1 and HDAC2 are highly similar and play a critical role in proliferation, cell cycle and apoptosis processes (Segre and Chiocca 2011); HDAC3 is involved in cell cycle

control and DNA damage response (Reichert N. et al., 2012); HDAC8, predominantly found in the cytosol, associates with smooth muscle alpha-actin playing an important role in smooth muscle cell contractility (Waltregny D. et al., 2005). HDAC class II is divided in class IIA (HDAC4,5,7 and 9) and class IIB (HDAC6 and 10) on the basis of the presence of a double deacetylase domain typical of HDAC6 and 10 (Verdin E. et al., 2003). This class of proteins is constituted by large proteins shuttling between cytoplasm and nucleus with homology to the Hda1 yeast protein (Verdin E. et al., 2003). HDACs class IIA are expressed in a tissue-specific manner and are involved in differentiation and development. Signal-dependent phosphorylation of class IIA is responsible whether they are localized in the nucleus or cytoplasm affecting their ability to act as transcriptional co-repressors in the nuclear compartment (Yang X.J. and Seto E. 2008). HDAC6 is the only HDAC that has substrate specificity versus α-tubulin, due to the presence of a α-tubulin deacetylase domain. HDAC10 is found in the nucleus and cytoplasm but its specific substrates remain unknown. Class III HDACs (SIRT1-7) homologues of the yeast SIR2 protein, are widely expressed and localized in different cellular compartments. Sirtuins have a critical role in regulation of oxidative stress, DNA repair, regulation of metabolism and aging (Saunders L.R. and Verdin E. 2007). HDAC11 is currently the only member of the class IV HDAC subfamily and is characterized by a deacetylase domain sharing homology with both HDAC class I and class II domains (Gao L. et al., 2002). HDACs are found as part of multi-protein complexes with differents proteins such as Silencing mediator for retinoid and thyroid receptors (SMRT), Nuclear co-repressor (N-Cor), Sin3 and Nuclear remodeling complex (NURD) (Yang X.J. and Seto E. 2008). Although histone deacetylases catalyse the removal of acetyl groups from lysine residues in histone amino termini, leading to chromatin condensation and transcriptional repression (Roth S. et al., 2001)

the activity of the different HDACs is not limited to histones and consequent chromatin modification, but also effects several addictional substrates (Glozak MA. et al., 2006). Transcription factors, signal transcription mediators, DNA repair enzymes, chaperones, structural proteins have been described to be subjected to reversible acetylation by HATs and HDACs.The identification as HDAC substrates of proteins, such as p53, HSP90, E2F, pRb and BCL6 (Juan LJ et al., 2000; Kovacs JJ et al., 2005; Bali P. et al., 2005; Martinez-Balbas MA. et al., 2000; Nguyen DX et al., 2004; Bereshchenko OR et al., 2002) , clearly involved in oncogenesis and cancer progression, suggests that the aberrant pattern of acetylation occurring in cancer cells, is not limited to the histone proteins but could be extended to some or all HDAC possible substrates.

HDACs and cancer

Cancer has traditionally been considered a disease of genetic defects (gene mutations, deletions and chromosomal abnormalitities), resulting in the loss of function of tumor suppressor genes and/or gain of function or hyperactivation of oncogenes (Hanahan D. and Weinberg R.A. 2000). However, there is growing evidence that gene expression governed by epigenetic changes is also crucial to the onset and progression of cancer (Lund A.H. and van Lohuizen M. 2004). Most studies show the contribution of HDACs to cancer.

- HDACs can be aberrantly recruited to target genes via their interaction with oncogenic DNA-binding fusion proteins (that result from chromosomal translocations). Is the case of the oncogenic PML-RARα, PLZF-RARα and AML1-ETO fusion proteins that induce acute promyelocytic leukemia (APL) and acute myeloid leukemia (AML) by recruiting HDAC-containing repressor complexes to constitutively repress expression of specific target genes.

- HDACs can physical interact with overexpressed repressive transcription factors. For example, B-cell lymphoma 6 (BCL6) is a transcription factor overexpressed in ≈40% of diffuse large B-cell lymphomas (DLBCLs) that results hypo-acetylated by HDACs and specifically recruits HDAC2 to repress growth-regulatory target genes such as CDKN1A (encoding $p21^{WAF1/CIP1}$) (Pasqualucci L et al., 2003).
- Mutations and/or aberrant expression of various HDACs have often been observed in human disease, in particular cancer. HDAC1 is overexpressed in prostate, gastric, colon and breast carcinoma (Halkidou K et al.2004; Choi J.H. et al 2001; Wilson A. et al 2006; Zhang Z et al., 2005). Somatic mutations of HDAC2 gene have been identified in human epithelial cancers with microsatellite instability (Ropero S. et al., 2006).

4.2 The role of acetylation on Notch signaling pathway

Notch is a vitally important signaling receptor which modulates cell fate determination and pattern formation in a number of ways during the development of both invertebrate and vertebrate species. Because of its important function, the mechanism of Notch receptor signaling uses numerous control points. Ligand activation of Notch receptors leads to release of the intracellular receptor domain (NotchIC) which translocates to the nucleus and interacts with the DNA-binding protein RBPjk and coactivators to control expression of specific target genes. In addiction to ligand-mediated activation, Notch signaling can be further modulated by interactions of NotchIC with a number of other proteins. These include p300 and PCAF, suggesting that acetylation may be involved in Notch activity regulation. p300 plays a key role in facilitating the ability of MAML1 (Notch coactivator) and PCAF to potentiate NotchIC-mediated transcriptional

activation from chromatin templates (Wallberg A.E. et al., 2002). Both p300 and MAML1 can physically interact with NotchIC: MAML1 binds to the ankyrin repeats, and p300 binds to a three-amino-acid motif in a region that has been shown to be a transactivating domain (Oswald F. B. et al., 2001; Beatus P.J. et al., 2001). The primary function of p300 is to act as a histone acetyltransferase. This was confirmed by experimentally altering the levels of acetyl-CoA in the in vitro transcription experiments. Furthermore p300 is able to interact with MAML1 leading to p300 recruitment for subsequent chromatin modification. This interaction potentiates p300 autoacetylation and thereby p300 coactivator function. MAML1 enhances p300 HAT activity directly, and this coincides with the translocation of MAML1, p300 and acetylated histones to nuclear bodies (Hansson A.E. et al., 2009). The function of PCAF as a coactivator of Notch1 and Notch3 appears to be dependent on the same p300 and MAML1: PCAF shows histone acetyltransferase activity and enhances Notch1 and Notch3 transcriptional activation (Kurooka H., T. Honjo 2000) only in the presence of p300. This may relate to the fact that PCAF does not interact cooperatively with MAML1 but directly with the same p300 protein (Yang XJ et al., 1996). There is therefore a hierarchical order for co-activators function, in which p300 plays a critical role by facilitating the action of both MAML1 and PCAF in enhancing Notch-IC- mediated transcriptional activation. Tip60 is another HAT enzyme involved in regulation of Notch1 signaling pathway. Tip60 harbors a chromodomain, a zinc finger motif, and an acetyl-CoA binding domain. The zinc finger motif and acetyl-CoA binding domain play pivotal roles in the HAT activity and are essential for the binding of Tip60 to Notch1. Tip60-Notch1 binding negatively regulates the transactivation of Notch1-IC target genes via the suppression of the interaction between Notch1-IC and CSL.

4.3 Histone deacetylase inhibitors

The initiation and progression of cancer is controlled by both genetic and epigenetic events. Unlike genetic alterations, which are almost impossible to reverse, epigenetic aberrations are potentially reversible, whereby specific drugs can be used to return to a physiological condition. The use of inhibitors of histone deacetylases (HDACi) allows the re-activation of the expression of tumor suppressor genes silenced in cancer cells. A relatively wide range of structures have been identified that are able to inhibit the activity of class I, class II and class IV HDACs (Johnstone R.W. et al., 2002). They derive from both natural sources and from synthetic routes and with a few exceptions, they can all be divided into chemical classes including hydroxamic acid derivates, carboxylates, benzamides, electrophilic ketones and cyclic peptides (Mai A. et al., 2005) and inhibit the enzymatic activity of HDACs with varying efficiency (**Table 3**). Inhibition of deacetylase activity involves not only chromatin remodeling but also hyperacetylation of the non-histone proteins that can lead to changes in the interactions, localization and stability of the same proteins (Caron et al., 2005; Glozak et al., 2005). To date, the HDACi anticancer drugs are considered very promising: at the cellular level, in fact, are able to induce differentiation, cell cycle arrest, senescence, apoptosis (activation of both death-receptor and intrinsic apoptotic pathways), and mitotic cells death. In "in vivo" experiments, inhibitors of HDACs are able to reduce the tumor invasiveness, angiogenesis and metastasis formation: their key feature in the therapy against cancer is represented by the selective toxicity to tumor cells compared to normal cells (Bolden et al., 2006; Minucci and Pelicci, 2006; Xu et al., 2007) . Despite the highly varied structure, all of HDACi compounds show the same mechanism of action: the ability to block the enzymatic activity of HDAC proteins by binding to the zinc ion (Zn^{2+}) located in its catalytic site.

Butyrate, first synthesized in 1949, and valproic acid (VPA), an anti-epileptic drug, were the first known HDAC inhibitors that can inhibit cell growth and induce apoptosis both *in vitro* and *in vivo* (Candido, E. P et al.,1978; Sealy L et al., 1978). Both butyrate and valproic acid are not specific and require high concentrations to inhibit HDAC, due to their short-chain that restricts the contacts with the catalytic site of HDAC (Yoo and Jones, 2006); VPA selectively induces the degradation of HDAC2 *in vitro* and *in vivo* and can be used in combination with other anticancer agents; it has been successfully used in combination with all-*trans* retinoic acid in elderly patients with acute myelogenous leukaemia (Raffoux, E. et al., 2005). Hydroxamates includes Trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA), PXD101, LBH589 and NVP-LAQ824. All these compounds are potent inhibitors of HDACs, and are active at micromolar to subnanomolar concentrations. TSA, derived from *Streptomyces,* was first shown to be a potent inducer of differentiation and cell-cycle arrest, and later reported to possess anti-HDAC activity (Yoshida, M., et al., 1990). The benzamides MS-275 and CI-994 are two of the most well-known synthetically derived inhibitors of HDACs.

The HDACi are used in the treatment of many solid tumors, as well as in models of leukemia and lymphomas. In HT-29 cells (human colon carcinoma) treated with sodium butyrate, inhibition of cell growth is associated with the decrease in the levels of cyclin B1 mRNA: the mechanism involves the histones hyperacetylation and is dependent from p21 expression , which directly represses the transcription of cyclin B1 (Archer SY et al., 2005). Even valproic acid is able to induce the expression of p21, as well as apoptosis and cell cycle arrest in leukemic cell lines and in cell cultures from leukemic patients (MR Trus et al., 2005). The use of HDACi induces apoptosis in three cell lines of pancreatic adenocarcinoma by increasing the levels of Bax protein and subsequently the release of apoptosis-inducing factor (AIF), which induces the intrinsic mitochondrial

program of apoptosis (Garcia-Morales P et al., 2005). The treatment of human lung carcinoma with TSA is able to induce apoptosis of the same cells associated with the decrease of expression of anti-apoptotic proteins Bcl-2 and with the increase of expression of pro-apoptotic protein Bax, with consequent activation of the proteolytic activity of caspase 3, and 9 (ChoiY.H., 2005). In chronic myeloid leukemia (CML), the activity of tyrosine kinase bcr-abl activates several molecular mechanisms responsible for the inhibition of apoptosis: the use of the deacetylase inhibitor SAHA is able to induce apoptosis of leukemia cells by decreasing levels of bcr-abl, c-myc and HDAC3 (Y. Xu et al., 2005).

To date, many "Clinical Trials" conducted with HDAC inhibitors are showing a significant anti-proliferative activity in solid and haematological tumors.

Table 3. Molecular characteristics and clinical trial status of HDACi

Class	Compound	[range]	HDAC specificity	Clinical trials
Short-chain fatty acid	Butyrate	mM	Class I, IIa[121]	Phase I, II
	Valproic acid (VPA)	mM	Class I, IIa[121]	Phase I, II
	AN-9 (prodrug)	μM	N/A	Phase I, II
Hydroxamate	Trichostatin A (TSA)	nM	Class I, II[121]	N/A
	Suberoylanilide hydroxamic acid (SAHA, Vorinostat)	μM	Class I, II[121]	Phase I, II, III (pre -registration)
	PXD101	μM	Class I, II[121]	Phase I
	Oxamflatin	μM	N/A	N/A
	LAQ824	nM	Class I, II[121]	Phase I
	LBH589	nM	Class I, II[121]	Phase I
	m-carboxycinnamic acid bis-hydroxamide (CBHA)	μM	N/A	N/A
	Scriptaid	μM	N/A	N/A
	Pyroxamide	μM	Class I, unknown effect on class II[176]	Phase I
	Suberic bishydroxamic acid (SBHA)	μM	N/A	N/A
	Azelaic bishydroxamic acid (ABHA)	μM	N/A	N/A
	SK-7041	nM	HDACs 1 and 2[177]	N/A
	SK-7068	nM	HDACs 1 and 2[177]	N/A
	CG-1521	μM	N/A	N/A
	Tubacin	μM	Class IIb[39]	N/A
Benzamide	MS-275	μM	HDACs 1, 2,3, 8 (marginally)[34]	Phase I, II
	CI-994 (tacedinaline)	μM	N/A	Phase I, II, III
Cyclic tetrapeptide	Depsipeptide	nM	Class I[36]	Phase I, II
	Trapoxin A	nM	Class I, IIa[121]	N/A
	Apicidin	nM	HDACs 1 and 3, not HDAC8[170]	N/A
	CHAPs	nM	Class I[178]	N/A
Electrophilic ketone	Trifluoromethylketone	μM	N/A	N/A
Miscellaneous	Depudecin	μM	Class I, unknown effect on class II[179]	N/A
	MGCD-0103		Class 1[205]	Phase I

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EXPERIMENTAL DATA

5. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is characterized by aberrant activation of Notch1 in over 60% of T-ALL cases. The high prevalence of activating *NOTCH1* mutations highlights the critical role of Notch signaling in the pathogenesis of this disease. We previously showed that Notch3 intracellular domain (Notch3IC) transgenic (tg) mice develops a very aggressive T-ALL with high penetrance, representing a suitable model of the human disease (Bellavia et al., 2000). Accordingly, Notch3 is overexpressed in virtually all human T-ALL cases, independently of the genetic mutations. Although the role of NotchIC as a transcriptional activator is widely known, the molecular mechanisms priming and/or regulating Notch signaling remain undefined. In recent years, it has become increasingly evident that Notch signaling pathway is highly regulated by posttranslation modification: glycosylation, ubiquitylation, phoshorylation. Ubiquitylation and subsequent proteasomal degradation of NotchIC protein plays a key role in the negative regulation of Notch signaling. Indeed, several data support the involvement of numerous ligase, such as c-Cbl, Itch and Sel-10 in the degradation of Notch1 (Jehn et al., 2002; Oberg et al., 2001). Recently, we have also demonstrated that c-Cbl ubiquitin ligase may represent a hypothetical common regulator of both proteins Notch3 and pTα (Checquolo et al., 2010). c-Cbl targets Notch3IC to the proteasomal degradation pathway only in the absence of pTα/pre-TCR complex.

Protein acetylation has been shown to be a reversible process regulated by different families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Grozinger and Schreiber, 2002).

Although HATs and HDACs are crucial regulators of development and tumorigenesis and recently data suggest their involvement in the development

and progression of T-cell acute lymphoblastic leukemia (T-ALL), their role in the control of Notch signaling is poorly understood.

We report here that Notch3 is an acetylated protein and that acetylation specifically influences Notch3 protein stability. Indeed, acetylated Notch3 is prone to ubiquitination and proteasomal-mediated degradation of the protein. Consistent with this, HDACi trichostatin (TSA) treatment promotes Notch3IC protein acetylation and its subsequent ubiquitination and proteasomal degradation in N3-232T-lymphoma cells and in thymocytes from Notch3IC tg mice. As a consequence, Notch3 protein expression and its transcriptional activity are decreased. TSA treatment is also able to inhibit both the development and progression of T-ALL in N3IC tg mice. Together, our findings demonstrate that acetylation process is involved in Notch3 protein expression and signaling regulation and ensure the fundamental prerequisites for a therapeutic approach by use of HDACi in the treatment of T cell leukemia.

6. Materials and Methods

Mice

The generation and typing of N3IC tg mice have been described (Bellavia et al., 2000). The studies involving animals have been conducted following the Italian National Guidelines for Animal Care established in Decree number 116 of 27 January 1992, in accord to the directive CEE 86/609, as well as in Circular number 8 of the Italian Ministry of Health, 23 April 1994.

Cell cultures and drugs treatments

HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS. N3-232T cells (Bellavia et al., 2000) were maintained in RPMI containing 10% FBS and 0.1% 2-β-mercaptoethanol. Freshly isolated thymocytes, preT 2017 and Molt-3T cells were cultured in RPMI containing 10% FBS. Cells were treated with TSA (Sigma-Aldrich, Poole, UK), MG132 (Sigma-Aldrich), cycloheximide (Sigma-Aldrich), Vorinostat (Sellek-Chemicals, Houston, TX, USA) and Chloroquine (Sigma-Aldrich) for the times indicated. To trigger Notch3 signaling, preT 2017 cells were co-cultured on a monolayer of the Notch ligand-expressing murine microvascular endothelial cell line SIEC, as previously described (Barbarulo et al., 2011). For in vivo treatement, TSA was administered at 10mg/kg intraperitoneally once (for 12 h treatment) or daily at 1mg/kg for 3 weeks, unless otherwise specified.

Cell proliferation assay

For proliferation analysis, N3-232T cells treated with TSA were treated with 1 μl of Ci/ml [^{3}H]-thymidine (Amersham-Pharmacia-Biotech, Piscataway, NJ, USA), and [^{3}H]-thymidine incorporation was measured by automated scintillation counter (Packard Instrument Company, Meriden, CT, USA). Cell

proliferation of preT 2017 cells, transfected with Flag-N3IC wt or Flag-N3IC K/R C vectors, was evaluated by BrdU incorporation (3 h pulse) followed by BrdU detection (Roche Diagnostics, Penzberg, Germany), performed according to the manufacturer's instructions. Transfected cells were counterstained with Hoechst and Flag antibody (F7425; Sigma-Aldrich). At least 600 transfected cells were counted in triplicate and the number of BrdU/Flag-positive cells were recorded. The preT 2017 cell proliferation was measured by using a CellTiter 96 AQ non-radioactive cell proliferation assay (Promega, Madison, WI, USA). The preT 2017 cells transfected with Flag N3IC wt or Flag N3IC K/R C vectors were plated in 96-well plates at a density of 10^4 cells/well in 100 μl of medium.

Cells were allowed to grow up to 12 h and then combined MTS (3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)-phenozinmethosulfate solution (20 μl/well) was added. After incubation for 2h at 37°C in a humidified 5%CO_2 atmosphere, the absorbance was measured at 490nm by using GloMax Multidetction System (Promega).

Cell transfections, plasmids and mutagenesis

Transient transfections were performed by Lipofectamine-2000 Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Luciferase and renilla activity were assayed with a dual-luciferase assay system (Promega). Expression vectors were as follows: Flag-Tip60, HA-p300 and GST–HAT/$_{domain}$ were kindly provided by M Fanciulli (Regina Elena Cancer Institute, Rome, Italy); p300 mutant D1472–1522 was kindly provided by M Levrero (University Sapienza, Rome, Italy); HA-HDAC1 was kindly provided by PL Puri (The Burnham Institute, La Jolla, CA, USA); Flag- N3IC (Checquolo et al., 2010), HA- N3IC (Bellavia et al., 2000), RBP-Jk (Talora et al., 2002), MAM and ptα promoter (Bellavia et al.,

2007) were previously described. GST-N3IC expression plasmid was created by insertion of Notch3 nucleotides encoding the intracellular region (amino acids 1664–2318) into the PGEX-4T vector (Pharmacia, Stockholm, Sweden). Single or multiple residues were mutated by the Quickchange site-directed or multi-site mutagenesis kit (Stratagene, La Jolla, CA, USA).

Protein extracts, immunoprecipitations and immunoblottings

Protein extracts preparation and immunoprecipitation assays were described elsewhere (Felli et al., 2005; Canettieri et al., 2010) and were performed using the following antibodies: anti-Flag (F7425), anti-Flag (A2220), anti-Flag-HRP (A8592) and anti-HDAC1 (H3284), purchased from Sigma-Aldrich; anti-Acetyl-Lysine (06-933) and anti-Acetyl-Histone H3 (06-599) were purchased from Upstate, Temecula, CA, USA; anti-HA (sc-7392), anti-HA-HRP (sc-7392), anti-Notch3 (sc-7424), anti-p300 (sc-584), anti-β-actin (sc-1616) and anti-Ub (sc-8017) were purchased from Santa-Cruz Biotechnology, Santa-Cruz, CA, USA.

Fluorescence-activated cell sorting analysis

Freshly isolated cells from thymi, spleens, blood and lymph nodes were prepared and stained, as previously described (Anastasi et al., 2003), and analyzed on a FACS-Calibur with CellQuest software (BD-Biosciences, San Jose, CA, USA). Cells were stained with APC-, PE-, PerCP-Cy5.5 or FITC-conjugated mAbs against: CD4 (553051), CD8 (553033), CD45R/B220 (561101) and Thy1 (553004) (BD-PharMingen, San Diego, CA, USA). For Notch3 intracellular staining cells were incubated with anti-Notch3 (sc-7424; Santa-Cruz Biotechnology). Apoptosis was detected by using Annexin V Apoptosis Detection Kit (00-6990; eBioscience, San Diego, CA, USA).

mRNA expression analysis

Total RNA was isolated with Trizol (Invitrogen) and reverse transcribed with Superscript II reverse transcriptase and Oligo(dT) 12–18 Primer (Invitrogen). Mouse ptα gene-specific primer sequences were as follows:

- forward 5'-CTACCATCAGGCATCGCT-3';
- reverse 5'-CTATGTCCAAATTCTGTGGGTG-3'.

Recombinant pGEX plasmids generation and GST-tagged proteins purification

GST-N3IC expression plasmid was created by insertion of Notch3 nucleotides encoding the intracellular region (amino acids 1664–2318) into the PGEX-4T vector (Pharmacia). GST–HAT/$_{domain}$ plasmid was kindly provided by M Fanciulli (Regina Elena Cancer Institute). GST-N3IC and GST–HAT/$_{domain}$ were transformed into Escherichia coli BL21 (DE3) (Stratagene) and the GST fusion proteins expression was induced for 4 h with 1mM IPTG (Sigma-Aldrich) at OD 0.5–0.6 nm. Bacterial cells were lysed in NTEN buffer (20mM Tris–HCl, pH 8, 100mM NaCl, 1mM EDTA, 0.5% NP-40) and the recombinat polypeptides were purified by affinity chromatography using glutathione-Sepharose-4B (GE-Healthcare, Pollards Wood, UK).

In vitro acetylation assays

In vitro acetylation assays have been performed using the HA-N3IC (purified from N3-232T cells) and GST-N3IC (purified from E. coli) as acetylation substrates. His-IF2 (purified from E. coli) and a pool of purified histones have been used as a negative and positive acetylation controls, respectively. Acetylation reactions have been performed incubating the acetylation substrates for 1 h at 30 °C in a reaction buffer (250mM Tris–HCl, pH 8, 50% glycerol, 0.5mM EDTA, 5mM DTT, 10mM sodium butyrate) containing 1 μg

of recombinant GST-HAT/$_{domain}$ (purified from E. coli) and 1 μl [14C]-acetyl-CoA (55 mci/mmol, Amersham-Pharmacia-Biotech). The reaction mixture was subjected to SDS–PAGE followed by autoradiography.

Mass spectrometry analysis

To perform mass spectrometry analysis of *in vivo* acetylated N3IC, Flag-N3IC wt and Flag-N3IC K/R C were expressed in HEK293T cells with HA-p300 and purified by Flag immunoaffinity chromatography and run on SDS–PAGE. The corresponding bands were cut from the gel and were processed via tryptic proteolysis, after reduction and alkylation steps. The peptide mixtures were analysed by MALDI-ToF mass spectrometry and the resulting peptide mass fingerprints used to identify proteins and determine their possible post-translational modifications by Mascot search engine (Canettieri et al., 2010).

Statistical analysis

All results were expressed as the mean ± s.d. of *n* experiments as indicated in the figure legends. Statistical analysis was performed using Student's t-test, analysis of variance (ANOVA) and Fisher's PLSD test at a significance level of 0.05.

Results

TSA treatment regulates Notch3 signaling

To study if the acetylation/deacetylation balance influences the growth of Notch3-dependent lymphoma cells, we studied the effect of the class I and II HDAC inhibitor, TSA, on the survival and proliferation of N3-232T cells, a T-lymphoma cell line previously established from the N3IC tg mouse model of T-ALL (Bellavia et al., 2000). Following a 6-h exposure to TSA, N3-232T-lymphoma cells exhibited a significant decrease of proliferation rate (**Figure 1a**), whereas fluorescence-activated cell sorting analysis, by Annexin V staining, revealed that the treatment does not induce significant increase in apoptosis (**Figure 1b**). Remarkably, TSA treatment of N3-232T cells leads to a significant reduction of N3IC protein expression levels, as revealed by western blot with either anti-N3 or anti-HA antibodies, against the HA-tagged N3IC transgene (**Figure 1c**). The efficacy of the HDACi treatment was documented by histone H3 hyperacetylation in TSAtreated cells (**Figure 1c**). TSA treatment also induced downregulation of N3IC protein levels in the human T-ALL cell line Molt-3 (**Figure 1d**) and in the previously described preT 2017 cell line, in which the triggering of endogenous N3IC was induced after 24 h of co-culture on a monolayer of the Notch ligand-expressing murine microvascular endothelial cell line SIEC (Barbarulo et al., 2011) (**Figure 1e**). Consistently with these data, we observed that TSA treatment also reduces Notch3 signaling, as the mRNA levels of endogenous pTalpha (ptα), a N3IC target gene (Talora et al., 2003), are downregulated in both N3-232T-lymphoma cells and in thymocytes, freshly isolated from N3IC tg mice (**Figure 1f**). Accordingly, TSA suppressed the activity of a luciferase reporter, whose transcription is driven by the ptα promoter (**Figure 1g**). Overall, these data indicate that the inhibition of HDACs suppresses Notch3

signaling by reducing N3IC protein levels, thereby inhibiting Notch3-dependent lymphoma cell proliferation.

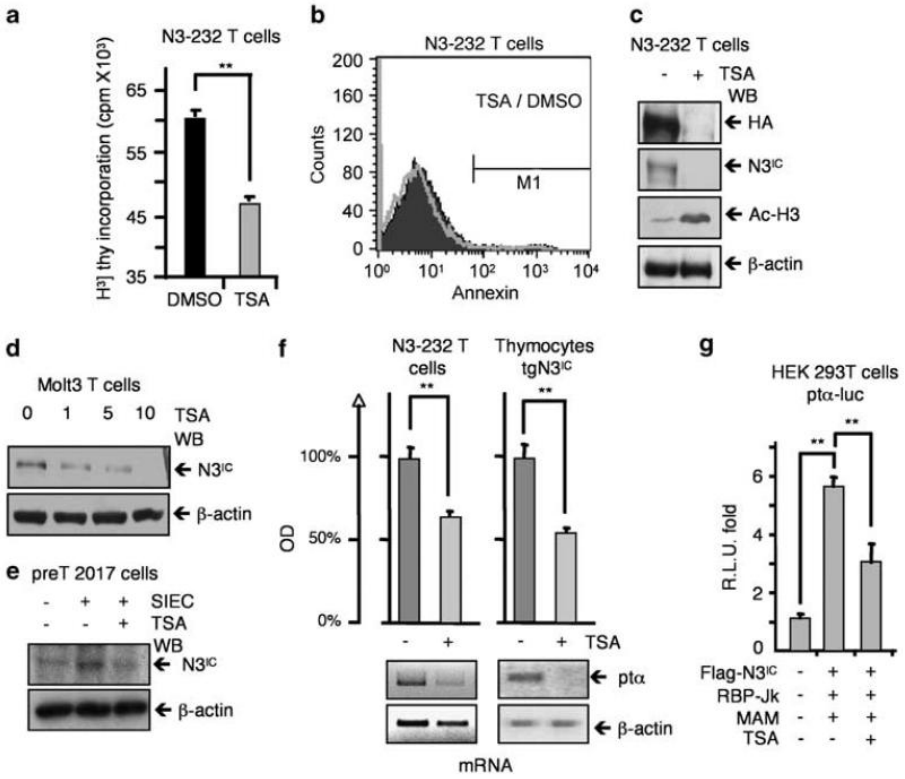

Figure 1 TSA treatment regulates Notch3 expression and signaling. N3-232T cells were treated with 1 μM TSA or with the vehicle alone (DMSO) for 6 h. **(a)** Proliferation was determined by measuring the [^{3}H]-thymidine incorporation (cpm) into DNA, and **(b)** apoptosis was evaluated by Annexin V-binding, analysed by flow cytometry. **(c)** Whole-cell extracts from TSA (6 h, 1 μM) treated or treated with the vehicle alone N3-232T cells were analyzed by immunoblotting (WB) with anti-HA, anti-Notch3 (N3IC), anti-acetyl histone H3 (Ac-H3) and anti-β-actin antibodies. Total lysates from Molt-3T cells treated for 12 h with increasing dose of TSA or with the vehicle alone **(d)** and from preT 2017 cells after 24 h of co-culture on SIEC cells plus 12 h of 1 μM TSA treatment or treated with the vehicle alone **(e)** were analysed by western blotting using antibodies against Notch3 and β-actin. **(f)** Semiquantitative RT–PCR expression analysis of murine pTα mRNA in N3-232T cells (left panel) or in thymocytes from tg N3IC mice (right panel) both treated with 1 μM TSA or with the vehicle alone for 6 h. Results were normalized by β-actin mRNA expression. The upper panels show the relative quantification as determined by optical densitometry (OD). **(g)** Luciferase assay performed on HEK 293T cells after the cotransfection with a luciferase reporter construct containing pre-TCR alpha chain (pTα) promoter, Flag-N3IC, MAM and RBP-Jk vectors, treated with 1 μM TSA or with the vehicle alone for 24 h. RLU, relative luciferase units were normalized to renilla. All the results showed in the figure are expressed as the means average deviations of three separate experiments and bars indicate s.d. **P<0.01.

N3IC is a substrate for p300- and HDAC1-dependent acetylation/deacetylation

The effect of TSA on N3IC expression and function suggested a direct relationship between Notch3 and HDAC. Indeed, we found that N3IC was bound to HDAC1 (**Figure 2a**) in immunoprecipitation experiments performed in HEK 293T cells co-transfected with N3IC and HDAC1 expression vectors. The protein complex formed by endogenous Notch3 and HDAC1 was also revealed by immunoprecipitation in whole extracts from N3-232T cells (**Figure 2b**). Previous reports have shown that acetylation of nonhistone proteins may be linked to regulation of their stability (Hernandez-Hernandez et al., 2006; Leduc et al., 2006; Mateo et al., 2009). To investigate whether the effect of HDAC inhibition on N3IC expression could be related to Notch3 acetylation, we first tested the effect of TSA treatment in HEK 293T cells transfected with a N3IC expression vector. TSA induced N3IC acetylation (**Figure 2c**). This observation implies that N3IC is a substrate of HAT-dependent acetylation. Notably, although both the acetyltransferases p300 and Tip60 have been reported to acetylate N1IC (Kim et al., 2007; Guarani et al., 2011), we found instead that only HAT p300, but not TIP60, induced a robust acetylation of N3IC (**Figure 2d**), whereas the p300 mutant Δ1472–1522, lacking acetyltransferase activity (Puri et al., 1997), failed to acetylate N3IC (**Figure 2e**). Moreover, we observed increased p300-N3IC proteins interaction in TSA-treated 232T cell (**Figure 2f**). To verify that the observed effect was not related to cross reactivity of anti-acetyl K antibody, N3IC acetylation was analyzed by in vitro acetylation assay, performed by incubating recombinant GST-N3IC with labelled [^{14}C] acetyl-CoA in the presence of GST-HAT/$_{domain}$. This assay showed that N3IC is acetylated in vitro (**Figure 2g**). A similar acetylation was observed in endogenous N3IC, constitutively expressed in N3-232T cells, after protein purification and incubation with labelled [^{14}C] acetyl-CoA in presence of GST-HAT/$_{domain}$

(**Figure 2h**). To map the acetylation sites in N3IC, we searched for the putative lysine targets of acetylation by a softwarebased sequence analyser. We found 11 evolutionarily conserved lysines in Notch3 of different species (**Figure 3a, upper panel**). Then, we performed two multisites lysines-to-arginine mutations from the Flag- N3IC wild-type (wt) construct. Flag-N3IC wt, containing all 11 lysines, was mutated to obtain two different mutants: Flag-N3IC K/R A (K1692-K1731-K1857-K1961-K1990) and Flag-N3IC K/R B (K2062-K2063-K2070-K2083-K2084-K2232) (**Figure 3a, lower panel**). When transfected with p300 vector, Flag-N3IC K/R A mutant was no longer acetylated when compared with N3IC wt and Flag-N3IC K/R B mutant, after transfection in HEK 293T cells and western blot against acetyl-lysine (**Figure 3b, lanes 2, 4 and 6**). To identify the acetylatable lysines, we constructed two additional mutants, by separately mutating the five lysines previously mutated in Flag-N3IC K/R A (that is, Flag-N3IC K/R C (K1692-K1731) and Flag-N3IC K/R D (K1857-K1961-K1990)) (**Figure 3a, lower panel**). Acetylation assay demonstrated that the Flag-N3IC K/R C mutant contained the specific acetylated residues (**Figure 3b, lanes 7–8**). Finally, we constructed two single K/R mutants, by separately mutating the two lysines previously mutated in Flag-N3IC K/R C (that is, Flag-N3IC K/R 1692 and Flag-N3IC K/R 1731). As shown in **Figure 3c**, when transfected with HEK 293T cells along with p300 expression vector, both the mutants were acetylated. Mass spectrometry analysis in HEK 293T co-transfected with Flag-N3IC wt, Flag- N3IC K/R C and HA-p300 expression vectors identified the tryptic peptide encompassing the region 1686–1694 as acetylated in wt protein but not in the K/R C mutant (**Figure 3d**). Mass spectrometry detection of tryptic peptide containing K1731 was indeed performed, but the presence of methionine and cysteine residues caused a severe fall in mass spectrometric sensitivity.

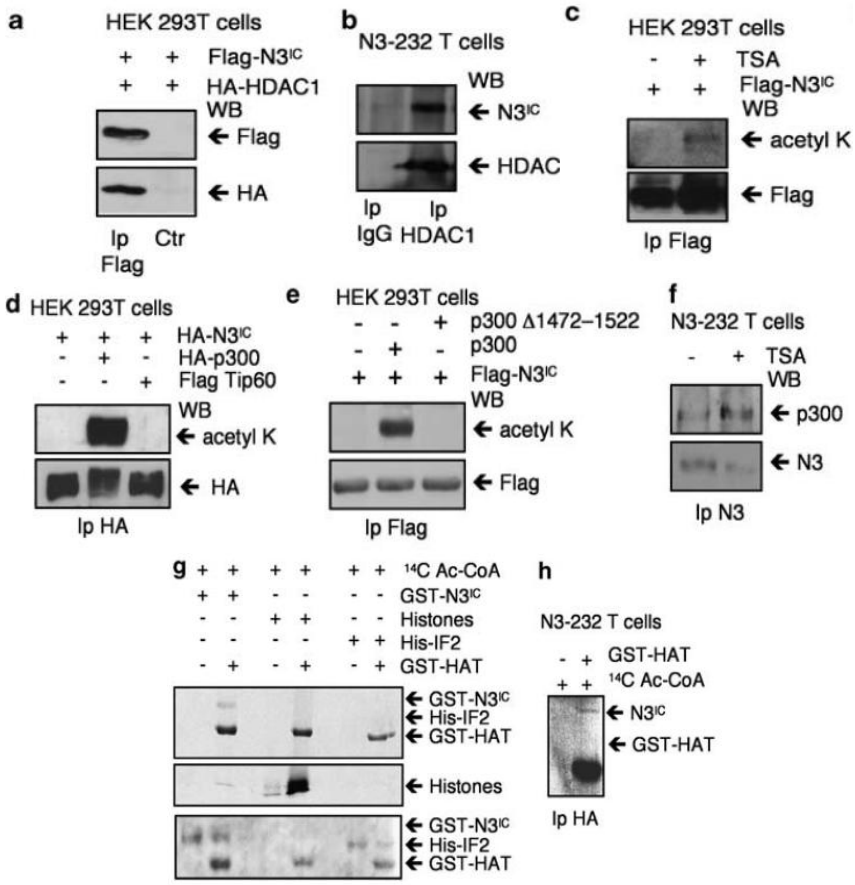

Figure 2 N3IC is a substrate for acetylation. **(a)** Total protein extracts from HEK 293T cells, transfected with Flag-N3IC and HA-HDAC1 expressing vectors, were subjected to immunoprecipitation with anti-Flag (Ip Flag) followed by western blotting with anti-HA and anti-Flag antibodies. For negative controls (Ctr), beads were pre-blocked with Flag peptide (0.1 mg/ml). **(b)** Total protein extracts from N3-232T cells were immunoprecipitated with anti-HDAC1 antibody (Ip HDAC1) and IgG (Ip IgG) followed by immunoblotting with anti-Notch3 and anti-HDAC1 antibodies. **(c)** HEK 293T cells were transfected with Flag-N3IC vector and treated for 24 h with 1 μM TSA or with the vehicle alone. Total protein extracts were subjected to immunoprecipitation with anti- Flag followed by immunoblotting with anti-acetyl K and anti-Flag antibodies. **(d)** Protein extracts, from HEK 293T cells transfected with HA-N3IC and HA-p300 or Flag-Tip60 expressing vectors, were subjected to immunoprecipitation with anti-HA (Ip HA) followed by imunoblotting with anti-acetyl-Lysine (acetyl K) and anti-HA antibodies**. (e)** Protein extracts, from HEK 293T cells transfected with Flag-N3IC and p300 or p300 Δ1472–1522 expression vectors, were subjected to immunoprecipitation with anti-Flag followed by imunoblotting with anti-acetyl-Lysine (acetyl K) and anti-Flag antibodies. **(f)**Whole-cell extracts, from N3-232T cells treated with TSA (6 h, 1 μM) or with the vehicle alone, were immunoprecipitated with anti-Notch3 antibody (Ip N3) followed by western blotting against anti-p300 and anti- Notch3. **(g)** Purified glutathione S-transferase-N3IC (GST-N3IC), Histidine-IF2 (His-IF2) and histones were incubated with ^{14}C-acetylCoA and with recombinant acetyl-transferase domain (GSTHAT/$_{domain}$). Acetylated proteins were revealed by SDS-PAGE followed by autoradiography (upper panel). A loading control gel was stained with Coomassie blue (lower panel). **(h)** Total protein extracts from N3-232T cells were subjected to anti-HA immunoprecipitation and analysed by in vitro acetylation assay using GST-HAT/$_{domain}$ in the presence of ^{14}C acetylCoA. All results showed in figure are representative of triplicate experiments.

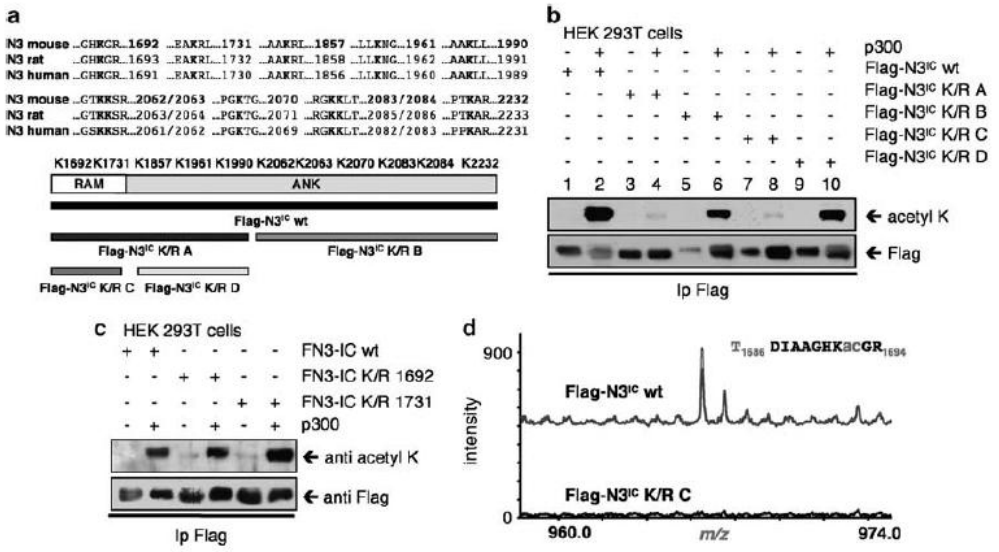

Figure 3 N3IC is acetylated at 1692 Lys and 1731Lys. (**a**) CLC Viewer protein sequence alignment of mouse, rat and human in the regions surrounding Notch3 murine putative acetylated lysines (**upper panel**). Schematic representation of N3IC wt and K/R mutants (**lower panel**). (**b**) Flag-N3IC wt vector and four different clusters (Flag-N3IC K/R A; B; C; D) were expressed in HEK 293T cells. Whole-cell extracts were immunoprecipitated with anti-Flag antibody (Ip Flag) followed by immunoblotting with anti-acetyl K and anti-Flag antibodies. (**c**) Flag-N3IC wt vector and two different mutants (Flag-N3IC K/R 1692; Flag-N3IC K/R 1731), together with p300 expression vector, were transfected in HEK 293T cells. Whole-cell extracts were immunoprecipitated with anti-Flag antibody followed by immunoblotting with anti-acetyl K and anti-Flag antibodies. (**d**) A particular of the MALDI-ToF spectra of N3IC wt and N3IC K/R C tryptic mixtures focused in the range 960–975 to highlight $_{T1686-1694}$ peptide (DIAAGHKacGR, MH^+ at m/z 966.5083; theoretical molecular mass = 924.5009). All results showed in figure are representative of triplicate experiments.

Acetylation regulates Notch3 ubiquitination, proteasomal degradation and function.

The data generated so far clearly indicate that HDAC inhibition induces both N3IC acetylation as well as a decrease of protein levels and signaling activity. Protein acetylation has been reported to prime subsequent ubiquitin-dependent stability of target proteins (Hernandez- Hernandez et al., 2006; Leduc et al., 2006). Although we and others suggested that proteasomal degradation of Notch intracellular domain (NIC) may be required for

repressing Notch signaling, the underlying mechanisms are still to be clarified. Therefore, we studied N3IC protein stability in N3-232T-lymphoma cells treated with TSA in the presence of the protein synthesis inhibitor, cycloheximide. As shown in **Figure 4a**, the half-life of N3IC in TSA-treated cells was reduced when compared with cells treated with the vehicle alone. In keeping with this observation, the addition of the proteasome inhibitor MG132, but not the lysosome inhibitor Chloroquine, reverted the reduction of N3IC protein levels in TSA-treated N3-232T cells (**Figure 4b**). Similar data were observed using vorinostat, another HDACi (**Figure 4c**) and in TSA-treated thymocytes freshly obtained from N3IC tg mice (**Figure 4d**). We have previously demonstrated that N3IC protein stability is regulated by an ubiquitin-proteasome system (Checquolo et al., 2010). Thus, we performed an ubiquitination assay, to assess whether TSA-induced N3IC acetylation was followed by its ubiquitination dependent proteasomal degradation. Whole-cell protein extracts from N3-232T cells, treated with TSA or vehicle alone, were subjected to Notch3 immunoprecipitation followed by western blotting with anti-ubiquitin. Treatment with TSA resulted in a strong increase of N3IC ubiquitination (**Figure 4e**) that was accompanied by a strong increase of N3IC acetylation (**Figure 4f**). To specifically address if the acetylation of the lysines 1692 and 1731 affects N3IC stability, we analyzed the half-life of both N3IC wt and the non-acetylatable N3IC K/R C mutant. HEK 293T cells, transfected with Flag-N3IC wt or Flag-N3IC K/R C mutant, were treated with cycloheximide in a time course assay. As shown in Figure 5a, the half-life of the Flag-N3IC wt was reduced when compared with the K/R mutant protein, which also displays an increased transcriptional activity toward pTa promoter (**Figure 5b**).

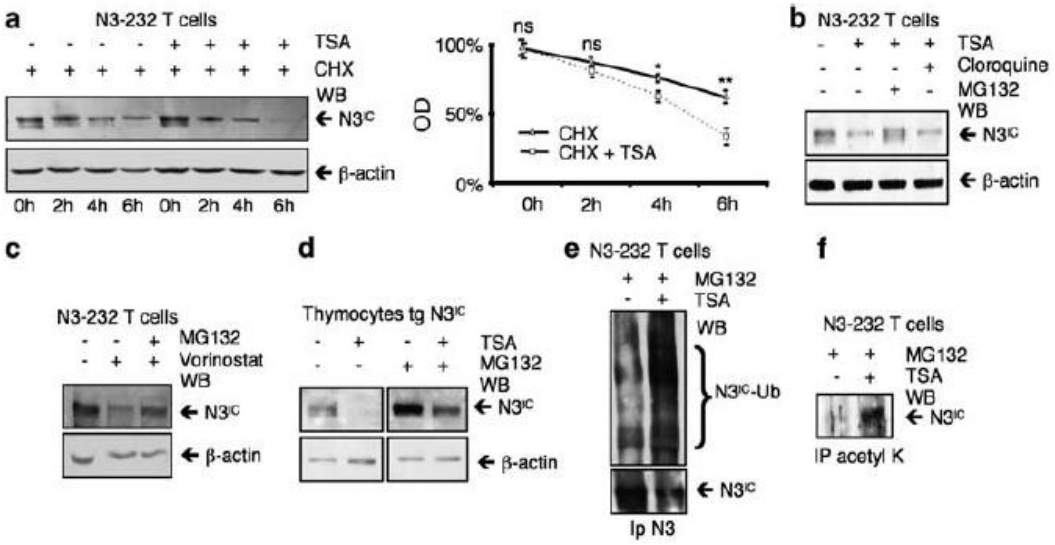

Figure 4. N3IC acetylation controls its ubiquitination and degradation. (**a**) Total protein extracts, from N3-232T cells treated with 1 μM TSA or with the vehicle alone in a time course assay with 10 μg/ml CHX, were revealed by immunoblotting with anti-Notch3 (N3IC) and anti-β-actin antibodies. The right panel shows the relative quantification as determined by optical densitometry (OD) and results are expressed as the means average deviations of three separate experiments and bars indicate s.d. *P<0.05; **P<0.01; ns, not significant. (**b**) Total lysates from N3-232T cells, treated with 1 μM TSA or with the vehicle alone for 6 h in the presence of 25 μM MG132 or 25 μM chloroquine, were analysed by western blotting using antibodies against Notch3 and β-actin. (**c**) N3-232T cells were treated with 1 μM Vorinostat or with the vehicle alone for 24 h in the presence or absence of 25 μM MG132 before extract preparation. Whole-cell extracts were revealed with anti-Notch3 and anti-β -actin antibodies by western blotting. (**d**) Total cell extracts, from thymocytes of tg N3IC mice treated for 6 h with 1 μM TSA or with the vehicle alone in the presence or absence of 25 μM MG132, were revealed by immunoblotting with anti-Notch3 and anti-β-actin antibodies. Whole-cell extracts, from N3-232T cells treated with TSA (6 h, 1 μM) or with the vehicle alone, were immunoprecipitated with anti-Notch3 (Ip N3) (**e**) or with anti-acetyl K (Ip acetyl K) (**f**) antibodies followed by western blotting against anti-Ubiquitin (**e**) and anti-Notch3 antibodies (**f**). All results showed in figure are representative of triplicate experiments.

Notch3 acetylation impairs T-cell proliferation

To investigate whether N3IC acetylation was related to the regulation of the proliferative output of Notch3 signaling, we transfected preT 2017 cells (that display no or very low N3IC expression) with wt N3IC or the nonacetylatable N3ICK/R C mutant. While being insensitive to TSA treatment, with respect to both Notch3 expression levels (**Figure 5c**) and cell proliferation (**Figure 5d**), preT 2017 cells transfected with the N3IC K/R C mutant display a significant increase of proliferation rate, as revealed by BrdU assay, when compared with cells transfected with Flag- N3IC wt construct (**Figure 5d**). Together these results suggest that N3IC acetylation at lysines 1692 and 1731 regulates proteasomal degradation-mediated protein stability and function, thus impairing the proliferative effect of Notch3 signaling.

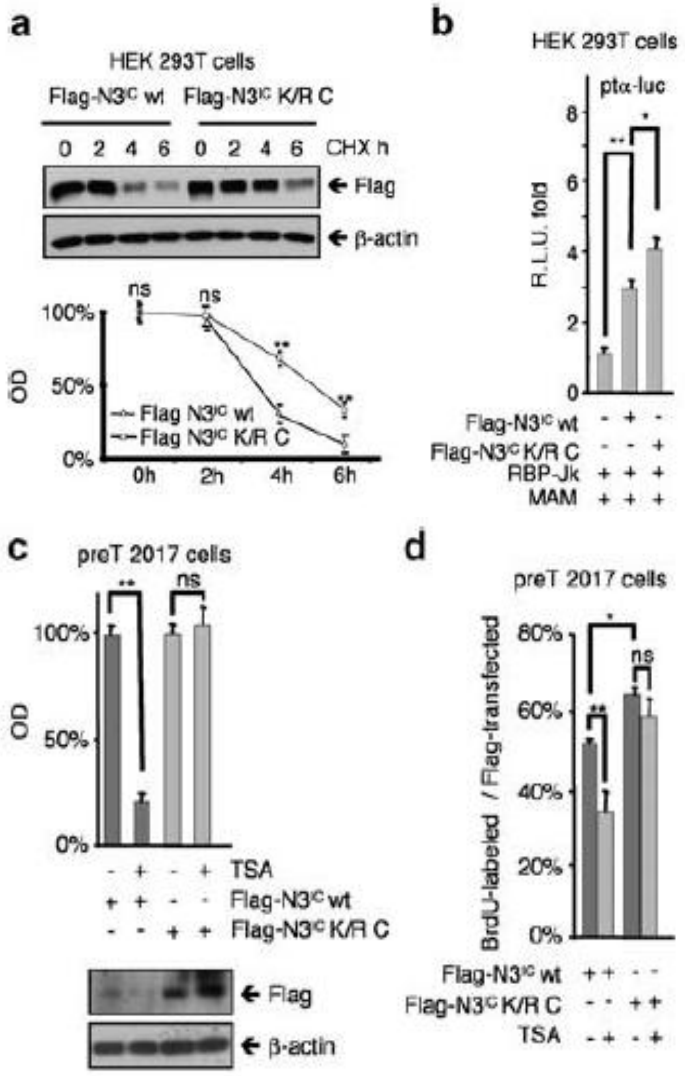

Figure 5 1692Lys and 1731Lys acetylation regulates N3IC protein stability and function. (**a**) HEK 293T cells were co-transfected with HA-p300 and Flag-N3IC wt or Flag-N3IC K/R C vectors and treated in a time course manner with 10 μg/ml CHX. Whole-cell extracts were revealed with anti-Flag and anti-β-actin antibodies. The lower panel shows the relative quantification as determined by optical densitometry (OD). (**b**) Luciferase assay performed on HEK 293T cells after the co-transfection with a luciferase reporter construct containing pre-TCR alpha chain (pTα) promoter, MAM, RBP-Jk and Flag-N3IC wt or Flag-N3IC K/R C vectors. RLU, relative luciferase units, were normalized to renilla. Results are expressed as the means average deviations of three separate experiments and bars indicate s.d. *P<0.05; **P<0.01. (**c**) Whole-cell extracts from preT 2017 cells transfected with Flag-N3IC wt or Flag-N3IC K/R C vectors and treated with 1 μM TSA for 12 h were revealed with anti-Flag and anti-β-actin antibodies. The upper panel shows the relative quantification as determined by optical densitometry (OD). (**d**) preT 2017 cells were transfected with Flag-N3IC wt or Flag-N3IC K/R C vectors for 12 h and then treated with 1 μM of TSA or with the vehicle alone for 12 h. BrdU was then added to the cells for 3 h and the percent of BrdU incorporation was measured in the population of transfected cells to monitor cell proliferation. All results showed in the figure are expressed as the means average deviations of three separate experiments and bars indicate s.d. *P<0.05; **P<0.01, ns, not significant.

TSA-induced downregulation of Notch3 prevents T-ALL development and progression in N3IC tg mice

The role of Notch3 acetylation in enhancing protein degradation would be consistent with the subsequent suppression of Notch3-dependent transcriptional activity and activation of a number of proliferative or oncogenic pathways (Talora et al., 2003, 2006; Vacca et al., 2006; Bellavia et al., 2007). Overall, these findings suggest that this acetylation mechanism might be exploited for controlling Notch3-dependent leukemia. Indeed, blocking acetylation by specific lysines mutation ($K/R^{1692-1731}$ mutant) increases proliferation rate of T cells. Therefore, to better address the role of acetylation/deacetylation mechanism in N3IC-dependent T-ALL, we studied the effect of TSA in vivo by treating T-ALL developing N3IC tg mice (Bellavia et al., 2000). We first investigated the *in vivo* effect of TSA on N3IC protein levels. To this end, 8-week-old N3IC tg mice were treated with one single intraperitoneal injection of 10 mg/kg TSA and after 12 h they were killed. Consistently with the *ex vivo* assays, *in vivo* acute TSA treatment, while being unable to modify thymocyte subset distribution with respect to CD4 and/or CD8 expression (**Figure 6a**), did cause a reduction of N3IC protein expression levels in whole thymocyte extracts of TSA-treated mice when compared with vehicle-injected littermates (**Figure 6b**). We next injected intraperitoneally TSA (1 mg/kg/day) for 3–12-week-old N3IC tg mice, displaying an overt aggressive leukemia (that is, splenomegaly and enlarged peripheral lymph nodes). Notably, as shown in **Figure 6c**, spleens and mesenteric lymph nodes, from TSA-treated tg N3IC mice, showed a significant reduction in size when compared with littermates injected with vehicle alone. Moreover, **Figure 6d** shows a reduction of the N3IC protein levels in whole-cell extracts from spleen of TSA treated with respect to untreated mice. We and others previously showed that accumulation of $CD4^+CD8^+$ DP cells in spleen, lymph nodes and ,peripheral blood represents

a pathognomonic feature of T-cell leukemias sustained by enforced expression of NIC in pre-T-cells or in bone marrow of mice (Pear et al., 1996; Bellavia et al., 2000, 2007). **Figures 6e** and **7a**, respectively, show that the cell population most importantly affected by the TSA treatments is represented by the $CD4^+CD8^+$ DP T cells in both spleen and lymph nodes when compared with vehicle-injected littermates, either when the percent distribution or the absolute number is considered. **Figure 6e** also show that the percent distribution of $B220^+$ cells (putative B cells) and $B220^-Thy^-$ cells (non-B, non T-cells) is unchanged or increased. Notably, the percent distribution of peripheral blood T cells, illustrated in the upper right panel of **Figure 7a**, also shows the specific decrease of DP cells. Finally, fluorescence-activated cell sorting analysis shows a decrease of Notch3 intracellular expression specifically in the cells of the spleens characterized by the $CD4^+CD8^+$ phenotype (**Figure 7b**). Taken together, these results suggest that the TSA administration in N3IC tg mice induces N3IC-dependent tumor regression, by promoting N3IC protein acetylation and subsequent degradation.

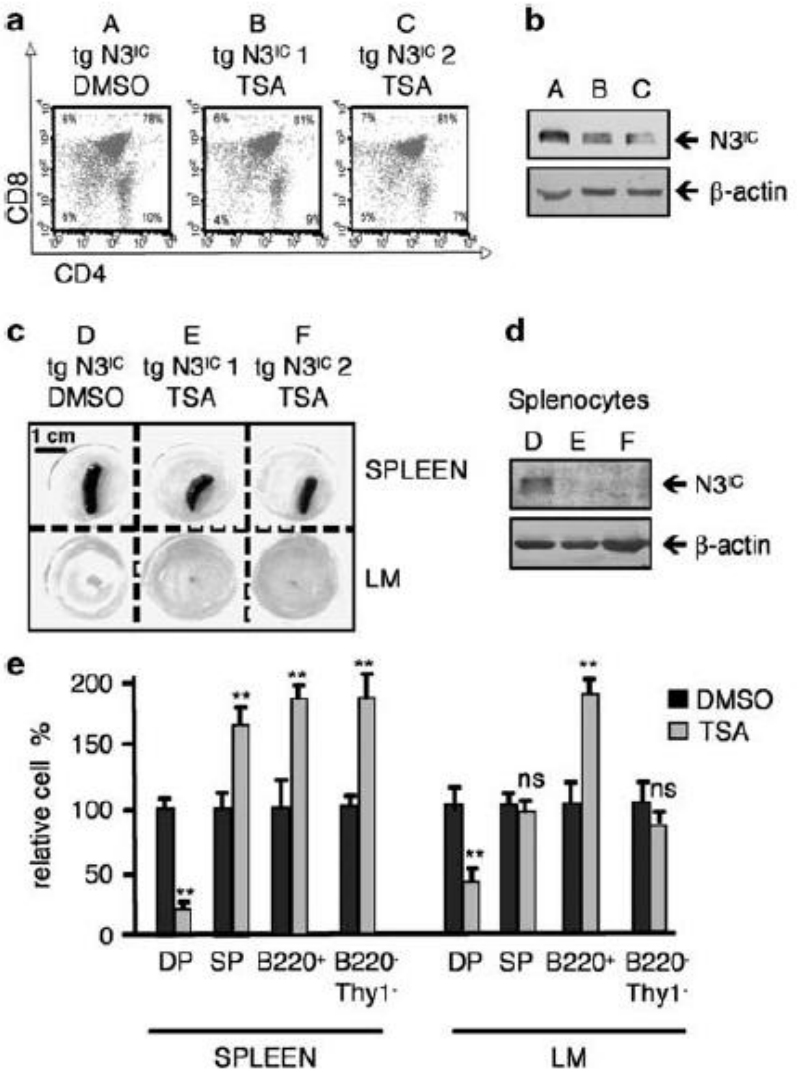

Figure 6 TSA-induced N3IC downregulation prevents T-ALL development in N3IC transgenic mice. (a) $CD4^+$ and/or $CD8^+$ different subset distribution of thymocytes from tg N3IC mice treated for 12 h with one single intraperitoneal injection with 10 mg/kg of TSA (B, C) or with the vehicle alone (DMSO) (A). (B) Whole-cell extracts from thymocytes of the same mice illustrated in panel (a) were revealed with anti-Notch3 (N3IC) and β-actin antibodies. (c) Macroscopic aspect of spleens and mesenteric lymph nodes isolated from tg N3IC mice treated for 3 weeks with TSA (1 mg/kg/day) (E, F) or with the vehicle alone (D). (d) Total protein extracts from splenocytes were revealed with anti-Notch3 and β -actin antibodies. (e) Bar graphs represent the percentages of $CD4^+CD8^+$ DP, the sum of $CD4^+$ and $CD8^+$ SP, $B220^+$ and $B220^-Thy^-$ cells from spleens and lymph nodes from tg N3IC mice treated with TSA, expressed as percent variation relative to those of mice treated with vehicle alone (DMSO). Results are expressed as the means average deviations of three separate experiments (n=3 mice per group) and bars indicate s.d. **P<0.01, ns, not significant.

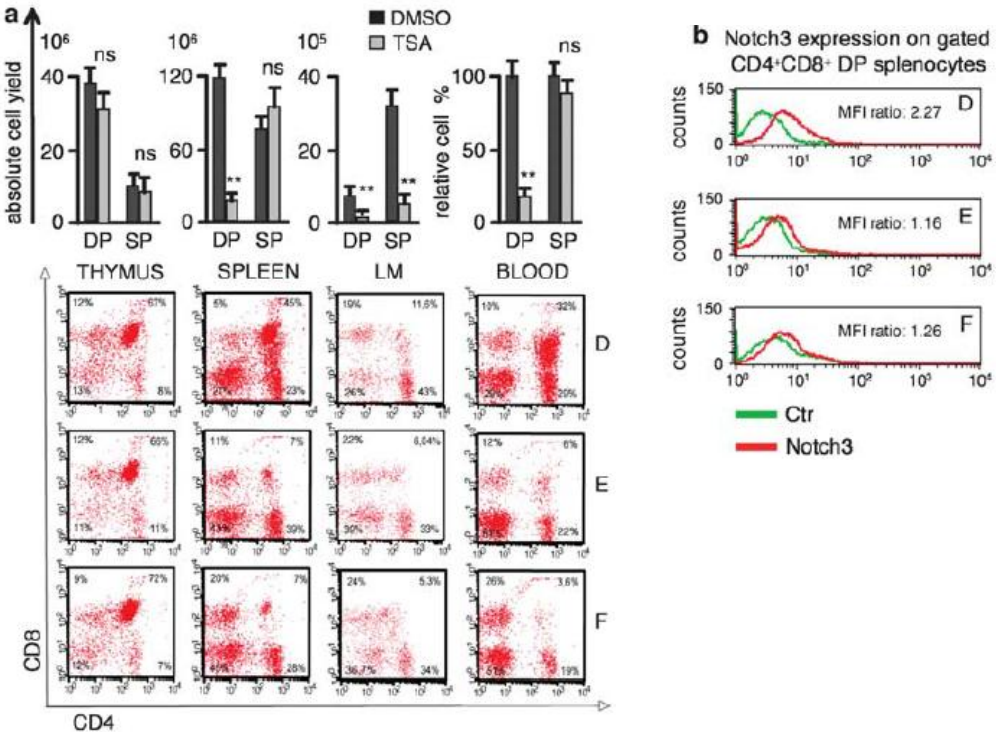

Figure 7 TSA treatment impairs the expansion of $CD4^+CD8^+$ DP cells in spleen and lymph nodes of N3IC transgenic mice. (**a**) $CD4^+$ and/or $CD8^+$ subset distribution of lymphocytes derived from thymi, spleens, blood and mesenteric lymph nodes of tg N3IC mice treated for 3 weeks daily with TSA (E; F) or with vehicle alone (D) (lower panels). Bar graphs represent the absolute cell number from thymi, spleens, mesenteric lymph nodes and the relative percentage from peripheral blood of $CD4^+CD8^+$ DP and the sum of $CD4^+$ and $CD8^+$ SP cells (upper panels). Results are expressed as the means average deviations of three separate experiments and bars indicate s.d. **P<0.01, ns, not significant (**b**) Notch3 expression by electronically gated $CD4^+CD8^+$ DP subsets from spleens of mice injected with TSA (E, F) or with the vehicle alone (D). Red curves represent the staining with anti-Notch3 antibody. Green curves represent the negative isotype control. The mean fluorescence intensity (MFI) ratio between Notch3 and isotypic control staining is indicated. The results showed in the figure are representative of three independent experiments. N=3 mice for group**.**

Discussion

Notch is a vitally important signalling receptor controlling cell fate determination in a broad spectrum of tissues and in both invertebrate and vertebrate species. To permit the Notch signal to be deployed in numerous contexts, many different mechanisms have evolved to regulate the level, duration and spatiotemporal distribution of Notch activity. Regulation involves multiple levels such as ligand and receptor expression , Notch-ligand interactions, trafficking of the receptor and ligands, and covalent modifications. Development and progression of T-ALL have been linked to mis-regulation of Notch signaling (Aifantis I. et al., 2008; Clappier E. et al., 2010). Especially, we demonstrated an overexpression of Notch3 in virtually all patients with T-ALL analyzed, despite is not due to *Notch3* gene mutations or gene rearrangements (Bellavia et al., 2002). Although, we have demonstrated that an altered ubiquitin-dependent proteolysis process may be responsible for Notch3-IC protein overexpression and sustenance of Notch3-induced T cells leukemia (Checquolo S. et al., 2010), post-translational modifications of Notch3 and their functional role with respect to Notch3 overexpression in T-cell leukemia are still poorly understood. For this purpose, we analized here the role of acetylation in the control of Notch3 protein stability. We initially demonstrated by *in vitro* and *ex vivo* experiments a novel mechanism that links acetylation and ubiquitination, in which the association of Notch3-IC with p300 determines its acetylation and subsequent ubiquitination with proteasomal degradation. Acetylation occours at two specific lysine (1962^{Lys} and 1731^{Lys}) located in the RBP-Jkappa associated (RAM) domain of Notch3-IC and require the integrity of the HAT domain of p300. This acetylation is completely reverted by the direct interaction of Notch3-IC protein with the histone deacetylase HDAC1.

Although the acetylatable lysine residues in the RAM domain are evolutionarily conserved in all Notch proteins, the role of acetylation seems

to be different among other members of Notch family and may depend on the different cellular contexts. Indeed, conversely with our data, it has been shown that Sirt1 loss of function-induced Notch1 acetylation sustains Notch1 stabilization, playing a positive role in endothelial cells and in in vivo models of vascular maturation/degradation (Guarani et al., 2011).

Recently, accumulating evidence sustains a novel specific role of HDACi in growth arrest, differentiation or apoptosis *in vitro* and *in vivo* (Johnstone RW 2002; Marks P. et al., 2001) and several classes of HDACi have been demonstrated to have promising therapeutic potential in both haematological and solid malignancies (Bolden et al., 2006; Piekark et al., 2007; Bots 2009; Mercurio et al., 2010). In this thesis we analized the effect of TSA HDACi in the regulation of Notch3 signaling and in Notch3-induced T cells leukemia development. HDACi treatment promotes Notch3-IC acetylation, affecting protein stability and thus enhancing its degradation in human and mouse T-ALL cell lines and in a *in vivo* experiments on T-ALL developing Notch3-IC tg mice. As a consequence, Notch3 transcriptional activity is decreased, thus resulting in the impairment of downstream signaling. We demonstrated that HDACi treatment inhibits Notch3-IC-enhanced pTα promoter activity and *in vivo* expression of endogenous pTα in Notch3-IC tg mice as well as *in vitro* T-cell proliferation and *in vivo* growth of Notch3-induced T-cell leukemia/lymphoma in tg mice. Our previous studies showed the importance of constitutive activation of pre-TCR signaling in tg Notch3-IC mice in sustaining a number of oncogenic pathways responsible for T-cell leukemogenesis (Bellavia D. et al., 2002; Talora et al., 2006). Therefore, the impaired ability of acetylated Notch3 to enhance pTα expression implies its control of T cell differentiation and tumorigenesis. We showed also that TSA-induced withdrawal of HDAC function in mice is able to decrese Notch3-IC protein level (and probably impairs its signaling) specifically in splenic $CD4^+CD8^+$ DP cells, possibly representing the precursors of leukemic cells in

circulating blood and in peripheral lymphoid organs (Pear et al., 1996; Beverly et al., 2005; Bellavia et al., 2007), thus blocking their expansion or migration. HDACi may interfere with critical step in T cell differentiation, impairing the development and progression of the lymphoproliferative disease. This highlights the relevance of physiological mechanisms that, by repressing HDAC function and subsequent Notch3 activity, can prevent pathological consequences. In conclusion, the central role of acetylation/deacetylation balance as a switch required for the fine-tuning on Notch3 signaling reveals the molecular basis for the use of HDACi as promising agents for treatment of human T-ALL.

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