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Histone modifiers driving Notch context-dependent activity in cancer.

PhD in Molecular Medicine

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Abstract

Deregulated Notch signaling is linked to onset and progression in various cancers. Epigenetic machinery writing or erasing chromatin status of NOTCH genes has been vigorously studied but not fully described yet. In particular, methylation status of H3K27 and availability of corresponding DNA loci for transcription is based on balanced work of antagonizing chromatin modifiers including the EZH2 methyltransferase component of Polycomb-Repressive Complex 2 (PCR2) and demethylases like JMJD3 or UTX. In the current work, we aimed to contribute to the discovery of additional mechanisms regulating transcription of NOTCH1 and NOTCH3 genes in contrasting cancer contexts: T-cell acute lymphoblastic leukemia (T-ALL) where Notch is a well-known oncogene and cervical cancer where Notch proteins are supposed to act as oncosuppressors, concentrating on roles of demethylase JMJD3 and methyltransferase EZH2 favoring chromatin opening and condensation, respectively.

In case of T-ALL where low methylation status of H3K27 at NOTCH-gene corresponding loci favors transcription of NOTCH genes and their targets, we found that pharmacological inhibition of JMJD3 by GSKJ4 decreased the expression levels of NOTCH3, NOTCH1, the target gene DELTEX1, and c-Myc, and abrogated cell viability in both Notch1- and Notch3-dependent T-cell contexts, as confirmed with the accumulation of anti-proliferative factor p27 and apoptosis-associated cleaved form of PARP. Anti-growth effects of GSKJ4 were partially rescued by exogenous Notch1, Notch3, and c-Myc expression indicating a possible involvement of Notch/c-Myc regulatory axis.

Specularly, in cervical cancer, EZH2 is responsible for the epigenetic silencing of tumor-suppressor genes. Supporting the anti-cancerous role of Notch1 protein in this context, we found that EZH2 inhibition upregulated the levels of the Notch receptors, ligands, and target genes, potently suppressed the growth of cervical cancer cells in vitro, and was associated with upregulation of cell cycle blockers $p21^{cip}$, $p27^{kip}$, and p53 and reduced expression of c-Myc. Similar effects on cell viability and expression of cell cycle regulators were achieved through transient ectopic introduction of Notch1 in model cell lines. Confirming the partial dependence of observed anti-growth effects of EZH2 inhibition on Notch activation, combined treatment with GSK126 and γ-secretase

inhibitor (GSI) abrogated GSK126 effects on cell viability by restoring the number of viable cells and $p21^{cip}$, $p27^{kip}$, and p53 levels. Furthermore, EZH2 inhibition suppressed the motility of human cervical cancer cells and upregulated epithelial phenotype marker E-cadherin (E-Cadh) and/or reduced the expression of promesenchymal vimentin in cell-line dependent fashion. Proving the relevance of Notch signaling in the epithelial phenotype maintenance, the combination of GSK126 with GSI restored E-Cadh and/or vimentin levels and partially rescued cell migratory capacity.

The results of our work contribute to the increasing amount of evidence that pharmacological inhibition of histone methyltransferases and demethylases might be a promising strategy for controlling oncogene activation, malignant cell growth and metastatic capacity thus opening new roads for creation of novel targeted cancer therapies.

1. Introduction

1.1 Insights of the molecular signaling of Notch

1.1.1 General aspects of Notch signaling

Notch signaling is an evolutionarily conserved cell communication pathway regulating many biological processes in different tissues during embryonic and adult life. Several studies carried out in *Drosophila Melanogaster*, *Caenorhabditis Elegans* and in vertebrates have characterized the molecular functions of the main components and secondary factors of the Notch signaling pathway and it has been reported that its activation has varied outcomes depending on the tissue context as well as on the differentiative state of the cells. Proper functioning of Notch signaling determines cell fate in a tissue-dependent fashion by regulating cell proliferation apoptosis, stem cell self-renewal and differentiation. Notch signaling is a short-range intercellular communication system, wherein a Notch ligand expressed on the signal-sending cell interacts with a membrane-tethered Notch receptor on the juxtaposed signal-receiving cell. Notch receptors are highly conserved type I transmembrane multidomain proteins that are synthesized as single precursors and which function, besides the ligand interaction, depends on the activity of some central components of the pathway which drives their proper maturation and activation. The main components of the Notch pathway include:

- Furin convertase, that is involved in the first proteolytic cleavage (S1) of the immature Notch receptor which takes place at the endoplasmic reticulum (Logeat F, et al. 1998; Gordon WR, et al. 2009);
    - Fringe Glycosyl-transferases (Lunatic, manic, radical) that catalyze the Notch receptor glycosylation which occurs at Golgi level (Moloney DJ, et al. 2000);
- ADAM 10 and 17 Metalloproteases which are implicated in the second cleavage (S2) of the Notch receptor, which follows the ligand-receptor contact (Brou C, et al. 2000; Mumm JS, et al. 2000; Lieber T, et al. 2002);
- γ-Secretase Complex (Presenilin-Nicastrin-APH1-PEN2) that performs the final proteolytic cleavage (S3), which releases the Notch-activated intracellular domain that translocates into the nucleus in order to induce the transcription of its target genes (De Strooper et al. 1999; Fortini ME, 2002; Tagami S, et al. 2008).

Apart from the above mentioned central components of the pathway, other factors, are involved in the Notch signaling regulation by acting at several levels of the pathway activation, starting from the ligand interaction, to the subsequent receptor cleavages and Notch nuclear translocation and transcriptional activity as well as to its protein stability. Some of these proteins are E3 ubiquitin ligases that regulate the amount of the receptor available on the cell surface by controlling its trafficking towards degradation or recycling (Bray SJ. 2006). The E3-Ubiquitin Ligase NEDD4/Su(dx), Numb and Notchless are negative regulators of Notch, whereas Deltex, which is a direct target gene of Notch, is considered a Notch activator as antagonize NEDD4/Su(dx) activity (Xu T, et al. 1990). On the other hand, Strawberry Notch (Sbno in vertebrates, Sno in Drosophila) is a helicase-related nuclear factor that belongs to the SF2 superfamily. It is highly conserved among species and it is involved in the transcriptional control of Notch gene expression by acting as a chromatin remodeler. Although its role is still unclear studies suggest that it functions downstream of Notch acting through the Epstein-Barr virus latency C promoter binding factor 1, Suppressor of Hairless, and Lag1 (CSL) complex inhibition removal (Watanabe Y, et al. 2017). Notch signaling regulation may occur also by controlling ligands activity. Instead, Neutralized and Mindbomb are proteins containing multiple ankyrin repeats and *RING* finger domains that functions as an E3 ubiquitin ligase which interacts with the intracellular domain of the Notch ligand Delta to promote its ubiquitination and internalization, therefore their function in the signaling cell is essential for efficient activation of Notch in neighboring cells (Lai EC, et al. 2001). Finally, Notch signaling regulation occurs also subsequently to the ligand-binding activation step Indeed, the E3-ligase FBXW7/hCdc4 (FBXW7) negatively regulates Notch signaling by targeting the intracellular domain of Notch to the proteasomal degradation in the cytoplasm and therefore regulating the potency and the intensity of the signaling (Matsumoto A, et al. 2011). The following figure 1.1 shows a complete protein map that resumes the Notch signaling pathway since its maturation to the proper degradation after completing its function.

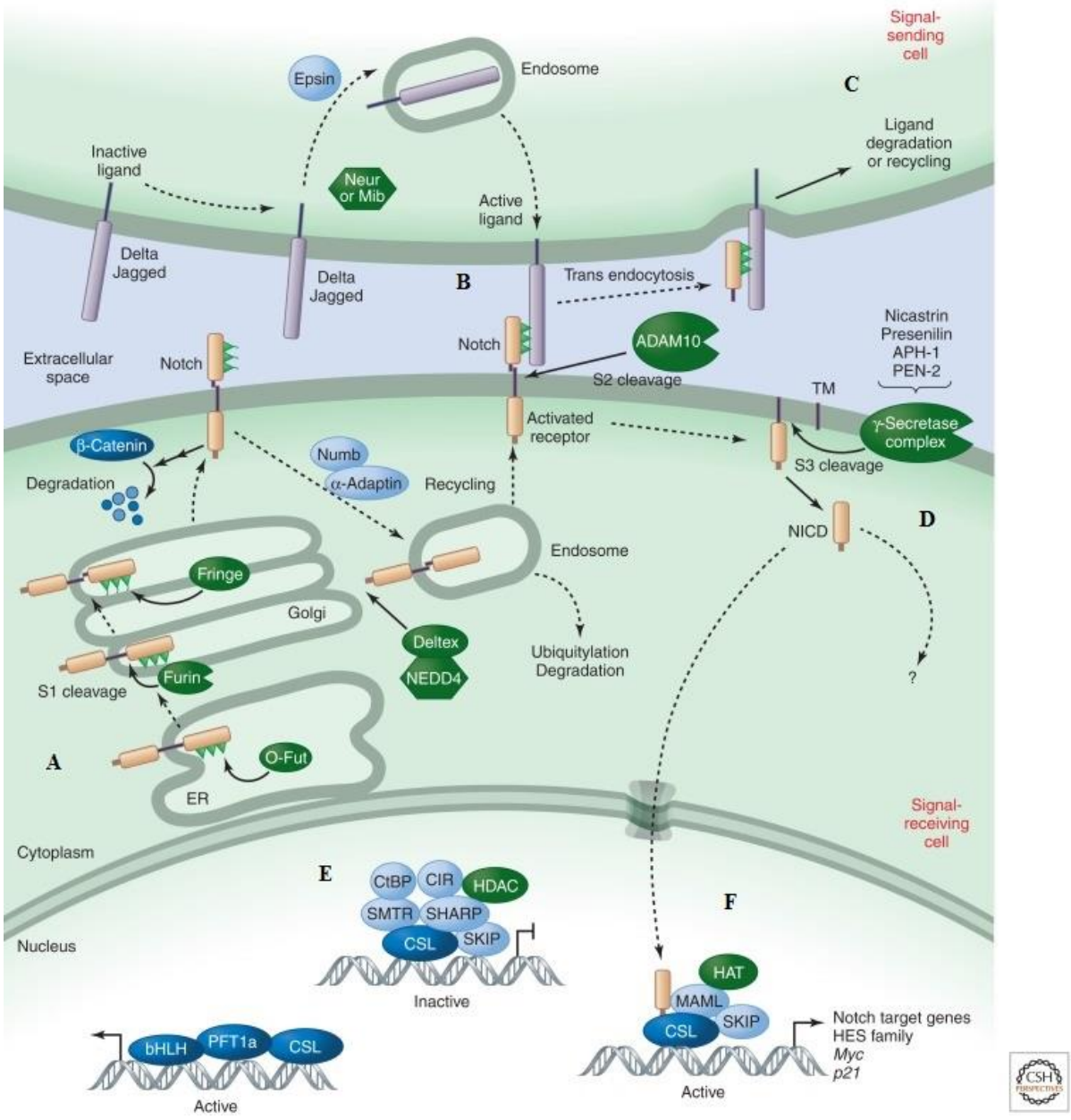


Figure 1.1: Complete cycle of the Notch signaling pathway. *(**A**) The Notch receptor becomes mature after its processing by Furin and Fringe (S1 cleavage). (**B**) Metalloproteases ADAM 10 and 17 perform the second cleavage (S2) of the Notch receptor, which follows the ligand-receptor contact and (**C**) the extracellular domain of Notch, which remains attached to the ligand, become endocytosed by the ligand presenting cell. (**D**)After the γ-Secretase Complex S3 cleavage, the intracellular domain of Notch (NICD) becomes translocated into the nucleus (**F**) where it develops the induction of the target genes transcription. (**E**) In the absence of activated NICD, the target region of the chromatin is silenced by several repressing factors. Adapted from: Kopan R, 2012.*

1.1.2 Ligands and receptors from the Notch family

There is just one characterized Notch receptor in *Drosophila* (dNotch), while four different Notch receptors (Notch1-4) in vertebrates. As it is faithfully described, mammalian Notch1 and Notch2 are pretty much similar between them under a structural point of view, while Notch3 and Notch4 differ from these two on both extracellular and intracellular domains. Serrate and Delta are the only Notch ligands that exist in *Drosophila*, while in vertebrates, have been described 5 different ligands: Jagged 1-2 (Jag1-2) and Delta-like 1/3-4 (Dll1/3-4). The output signal of the many different ligand-receptor interactions is context-dependent, further supporting the high functional complexity of the Notch-signaling pathway (Shimizu K, et al. 2002).

1.1.3 Notch receptor structure

The Notch gene codifies a 300KDa protein that undergoes post-translational modifications during its transport to the cell surface. In the t*rans*-Golgi apparatus Notch precursor is cleaved by a furin-like protease (S1 cleavage) in an unstructured region of the hetero-dimerization domain generating a heterodimeric receptor composed of an extracellular domain (NECD) non-covalently linked to a transmembrane fragment (NTM) that is followed by an intracellular cytoplasmic domain (NICD). The heterodimeric mature receptor is then transported into the membrane through a Golgi vesicle system in which it is glycosylated by Fringe glycosyl-transferase.

Notch modular structure is highly conserved in all four Notch paralogues which have been identified in mammals; (Kopan R, et al. 2009; Aster JC, et al. 2017). The NECD comprises a series of epidermal growth factor-like (EGF) repeats (36 for Notch-1 and Notch-2, 34 for Notch-3, and 29 for Notch-4) ,which mediate the ligand-binding of the receptor (Gordon WR. et al. 2008) and the negative regulation region (NRR) consisting of three cysteine-enriched repeats (Notch/LIN12) and a heterodimerisation (HD) region which prevent the ligand-independent activation of the receptor. Indeed, mutations, occurring at the NRR module lead to the receptor destabilization and to the constitutive activation of the signaling (Sanchez-Irizarry C, et al. 2004). The NICD contains an RBP-jk- associated molecule domain (RAM) involved in the NICD/CLS complex formation (Tamura K, et al. 1995), and seven highly-conserved ankyrin/cdc10 repeats (ANK) that are required for protein/protein interactions. Flanking the ANK domain,

there are two nuclear localization signaling motifs (NLS) necessary for NICD nuclear translocation (Blank V, et al. 1992). In addition, Notch receptors 1 and 2 contain a trans-activation domain (TAD) that is required to properly assemble the Notch/Rbpj/Maml transcriptional complex. At the carboxyterminal region of Notch is located a glutamine-enriched (OPA) and a proline (P), glutamic acid (E), serine (S), threonine (T) enriched region. This aminoacid combination defines the NICD ubiquitination and degradation domain (PEST) (Rechsteiner M, 1988).

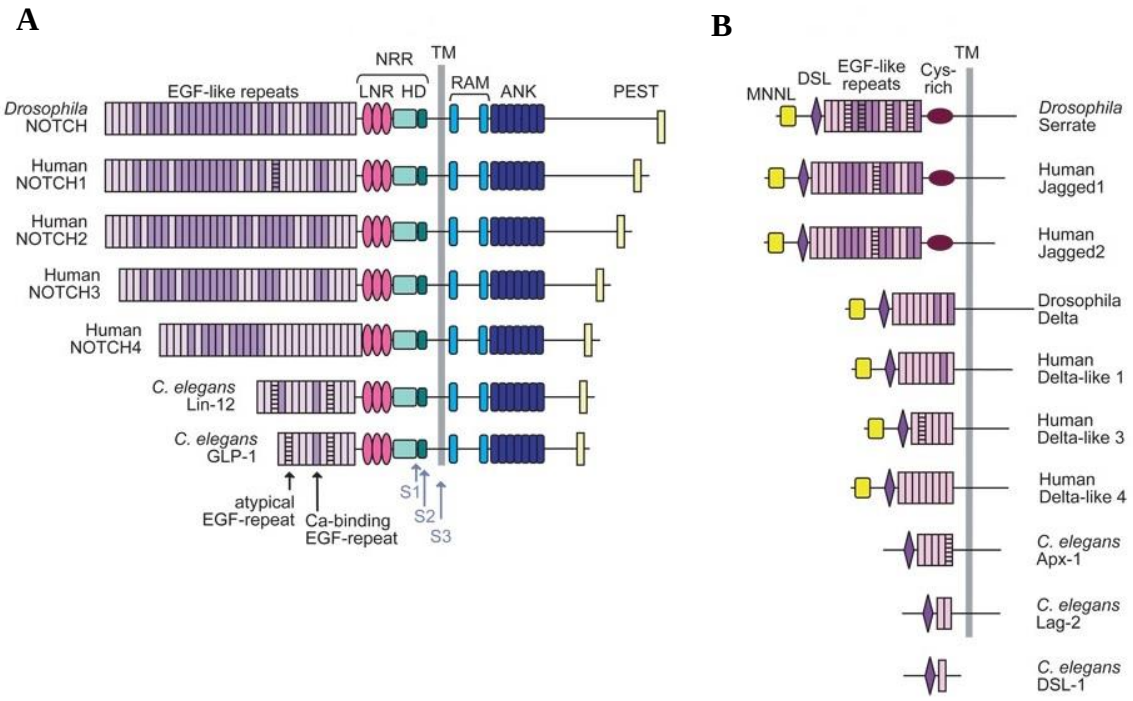


Figure 1.2: Characterized Notch receptors and ligands of Drosophila and mammals. *(**A**) The domain organization of Notch receptors including highly conserved EGF-like repeats, NRR, RAM, ANK and PEST domains. (**B**) The DSL-family ligands from fly, human, and worm. Adapted from: Gordon WR 2008.*

1.1.4 Molecular mechanisms of the Notch-signaling pathway

In the absence of ligand binding, the conformational integrity of the NRR holds the basal activation of Notch receptors. The Notch-signaling pathway is triggered by the binding of NECD expressed on the membrane of a signaling receiving cells and a ligand located on the surface of neighboring cell sending signal The ligand-interaction leads to NRR unfolding that allows two sequential proteolytic cleavages of the receptor (S2 and S3) (Bray SJ, et al. 2006).:

1- The ADAM metalloproteases mediate the S2 Cleavage of 12/13 aminoacids at the early transmembrane domain, allowing the generation of a membrane-anchored intermediate fragment called Notch Extracellular Truncated domain (NEXT).
2- The γ-Secretase Complex (Presenilin-Nicastrin-PEN2-APH1) catalyzes the S3 cleavage, releasing the activated intracellular domain of the receptor.

The NEC, released by the S2 cleavage, is endocytosed by the ligand-presenting cell. This process depends on the mono-ubiquitination of the cytoplasmic tail of the ligand. S3 cleavage allows the release of the NICD that it is driven to the intranuclear compartment thanks to its NLS motifs. In the nucleus, NICD promotes the transcriptional activation of its target genes (Bray SJ, et al. 2006). However, NICD does not bind directly the DNA, but, to drive target gene expression, it heterodimerizes with the Recombinant Signal Sequence-Binding protein jK (CSL, also known as CBF1, Su(H) or LAG-1 (Dou S, et al. 1994; Barrick D, et al. 2006). In the absence of nuclear NICD, CSL is bound to the regulatory regions of Notch target genes in association with a co-repressor complex, including the Histone Deacetylases 1 and 2 (HDAC1, HDAC2) (Morel V, et al. 2001), and several other co-repressors as SMRT/Ncor and SHARP/MINT/SPEN to keep Notch target genes expression switched off (Oswald F, et al. 2005). NICD interaction with CSL is crucial for the switch from repressed to the activated transcriptional state of Notch target genes. Although NICD carries a transcription activation domain (TAD), the incorporation of Mastermind Like (MAML) protein into the NICD/CSL complex is essential for the transcription initiation. Indeed, NICD/CSL complex formation recruits the co-activator (MAML), that promotes the displacement of the co-repressors and allows the engagement of additive co-activators (Kao HY, et al. 1998), including the SKI-interacting protein (SKIP) (Zhou S, et al. 2000), the acetyl-transferases p300 and PCAF (Wallberg AE, et al. 2002; Bray SJ. 2006), and the elongation factor CyclinT1:CDK9 (Chopra VS, et al. 2009). In the early

stage of the transcription activation, there is the formation of a multimeric complex; SKIP associates with the ANK domain of NICD and MAML interact with SKIP, forming the definitive transcription cluster which associates with the DNA-anchored CSL through the N-terminal domain of MAML. At this step, MAML protein is required for the recruitment of the epigenetic modulators CBP/p300 to the regulatory regions (Bray SJ. 2006). Complete complex promotes the transcription of Notch downstream genes, including the Enhancer of Split (ESpl) complex components in *Drosophila*, or the Hairy enhancer-of-split (*HES)* and Hairy/enhancer-of-split related with YRPW motif (*HEY)* in mammals (Davis RL, et al. 2001; Iso T, et al. 2003). The proper timing of Notch signaling is determined by mechanisms that control nuclear NICD levels by regulating its turn over. Indeed, MAML binds to CyclinC:CDK8, thus allowing NICD phosphorylation within the TAD and PEST domains and therefore promoting the NICD PEST-dependent degradation by the Fbw7/Sel10 ubiquitin ligase (Fryer CJ, et al. 2004). In addition, to PEST phosphorylation, NICD function is also regulated by other signaling pathways and several post-translational modifications, including arginine methylation and lysine acetylation (Palermo R, et al. 2014**).**

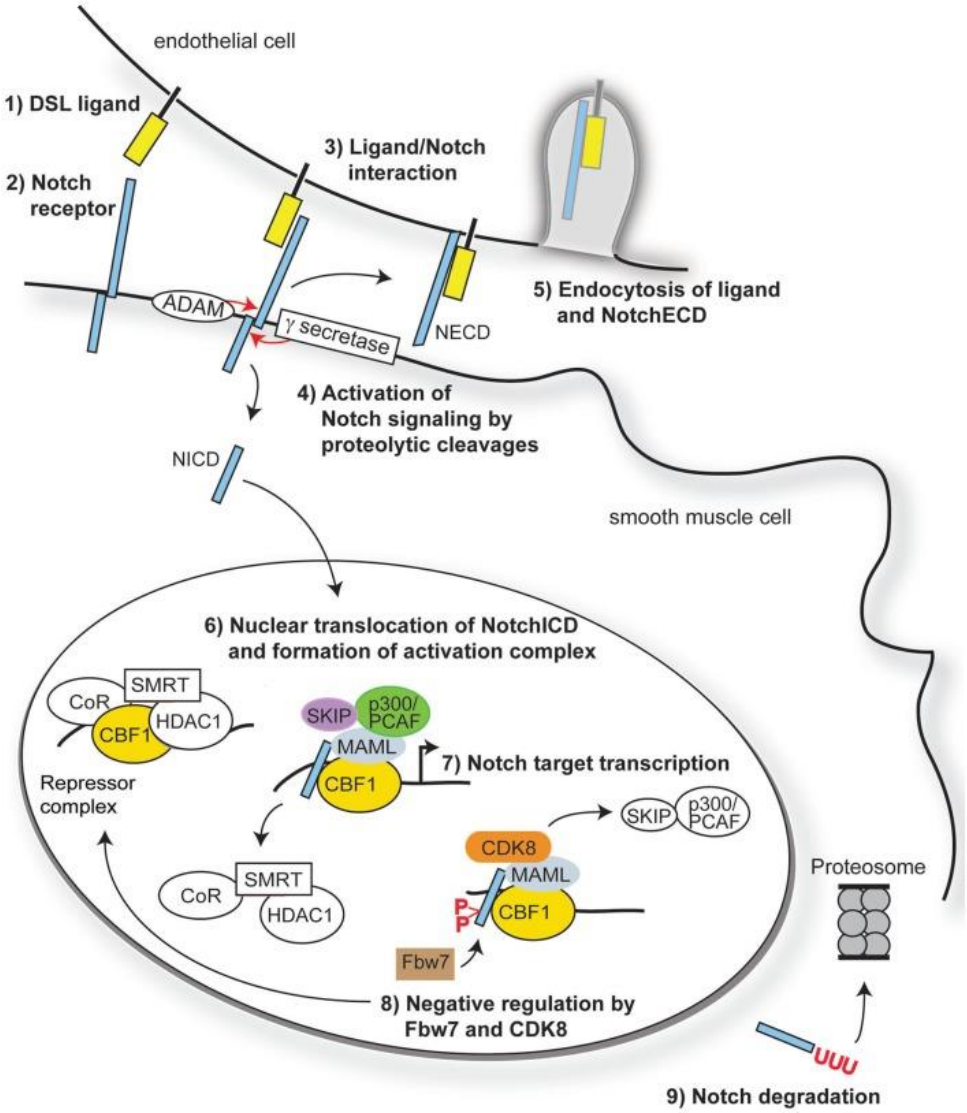


Figure 1.3. Overview of Notch signaling. Notch signaling is activated by the interaction of a transmembrane ligand of the Delta–Serrate Lag (DSL) family *(1) to the EGF-like repeats of the NECD* ***(2)*** *on a neighboring cell. Ligand/receptor interaction (3) induces a structural change in the receptor, exposing target sites for ADAM metalloproteases (S2) and γ-secretase (S3) cleavage of Notch* ***(4)****. Cleavage of Notch outcomes in the release of the intracellular domain (NICD) from the membrane and its translocation to the nucleus* ***(6)*** *while the NECD is endocytosed by the ligand-bearing cell* ***(5)****. In the absence of Notch signaling, CBF1 is associated with the regulatory regions of Notch target genes with the transcriptional co-repressors, such as SMRT and HDAC1, which actively keep gene expression switched off). Conversely, NICD binds CBF1, displaces the co-repressors and assembles an active transcriptional complex including the co-activators MAML, p300, and PCAF to promote the transcription of its responsive genes* ***(7)****. Finally, Notch signaling is turned off by the recruitment of CDK8 by MAML to the active complex that phosphorylates NICD at the PEST domain. Phosphorylated NICD is then ubiquitinated by theE3 ubiquitin ligase Fbw7 (8) and subsequently degradated by the proteasome (9). As a consequence of the activator complex destabilization, CBF1 re-associates with the co-repressors. Adapted from: Boucher J, et al. 2012.*

1.1.5 Notch mutations

Genomic sequencing of different human tumors has revealed three different patterns of Notch gene mutations altering the signaling activity. The first discovered type of mutation occurring on Notch genes was identified in T-ALL patients and it is a rare chromosomal translocation (7;9)(q34;q34.3) that generates a chimeric gene consisting of the 3 'end of Notch1 bound to the TCRβ gene enhancers (Ellisen LW, et al. 1991). This type of chromosomal rearrangement results in the deletion of the coding sequence of the Notch1 NRR that leads to the expression of a constitutively and ligand-independent active Notch1 ICD (N1ICD). Similar to the above-described translocation, point substitutions and in-frame insertion/deletion mutations that disrupt the structure of the Notch1 NRR result in the constitutive ligand-independent activation of the receptor in T-ALL and in triple-negative breast cancer (Weng AP, et al. 2004; Robinson DR, et al. 2011). Moreover, in these type of cancers have been described also nonsense and frameshift mutations occurring at the C-terminal sequence of Notch1, that, by deleting the C-terminal PEST degron domain, result in increased stability of the N1ICD (Weng AP, et al.2004; Wang K, et al. 2015). Rarely, NRR and PEST mutations occur in *cis* in the same allele leading to very high levels of constitutive Notch activation (Weng AP, et al. 2004).

The second pattern of mutations is defined by tumors in which changes in the reading pattern, meaningless, or alternative splicing disrupt the PEST domain. These mutations are found in the absence of mutations affecting the NRR domain. PEST mutation only is mainly associated with B-cell tumors including chronic lymphocytic leukemia, marginal zone lymphoma of the spleen and mantle cell lymphoma (Fabbri G, et al. 2011; Trøen G, et al. 2008; Kiel MJ, et al. 2012; Kridel R, et al. 2012). Based on the logic of Notch receptor activation, it has been proposed that Notch activation in tumor cells bearing only PEST deletions could depend on ligands expressed in the tumor cells instead of the engagement with ligand-expressed by the microenvironment (Rosati E, et al. 2009). Although such interpretation is in disappoint with experimental observations indicating that coexpression of ligand and receptor in the same cell results to Notch inhibition rather than Notch activation, a phenomenon referred to as cis inhibition (del Alamo D, et al. 2011).

The third mutational pattern is characterized by nonsense or point substitutions mutations occurring at the N-terminal region of the Notch receptor, all of which are predicted to result in loss of function of Notch. Some of these mutations simply lead to

a failed production of the protein, while occasionally result in the expression of a negative dominant receptor with a deleted or useless intracellular domain. Mutations of loss of function in Notch receptors are particularly prevalent in squamous cell carcinomas of the skin (Wang NJ, et al., 2011).

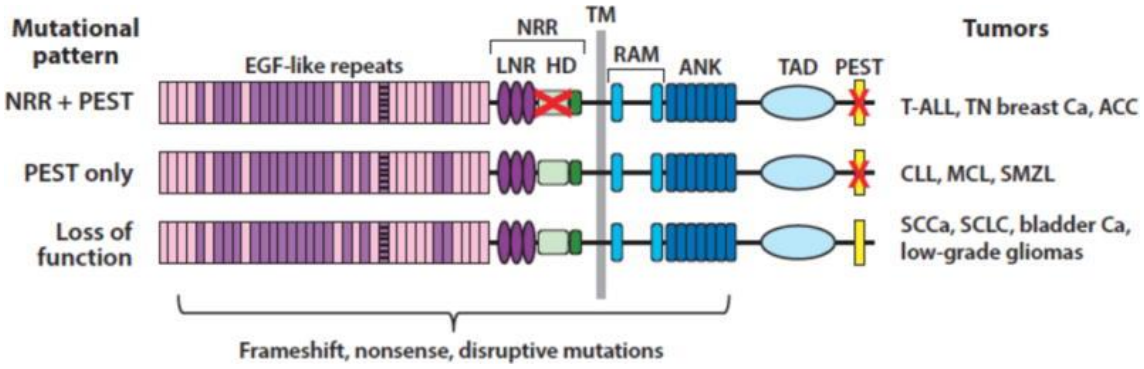


Figure 1.4: Patterns of Notch mutations in various cancers. *The red X in the negative regulatory region (NRR) corresponds to point substitutions, in-frame indels and rare deletions that remove the 5' coding exons of Notch receptors. The red X in the PEST domain corresponds to nonsense or frameshift mutations that lead to loss of the PEST domain. Adapted from: Aster JC, et al. 2017.*

1.2 Notch signaling in development and tissue homeostasis.

1.2.1 Pleiotropic roles of Notch

The Notch signaling pathway is a cellular mechanism highly conserved throughout the animal kingdom that regulates proliferation, fate decisions and differentiation of cells during embryonic and postnatal development and it takes part also in other functional processes such as cell adhesion, epithelium-mesenchymal transition, and angiogenesis. Despite the relative simplicity of the core of this route, the final representation of Notch activation is different in varied tissues and during the stages of development and adulthood. The simplistic explanation for its pleiotropic function is that Notch is able to activate groups of target genes which vary depending on the epigenetic context, making the outcome of Notch signaling strictly tissue-dependent (Aster JC, et al. 2017). Therefore, Notch context-dependent transcriptional output is likely driven by the chromatin states dictated by upstream transcription factors capable to regulate the chromatin state or conversely by the ability of Notch to turn on some downstream factors, which then modify chromatin landscapes so as to enable Notch to drive different transcriptional outputs. In addition, Notch signaling frequently is integrated with the output from other pathways, such as Hegegogh, NFkB and Wnt and also this kind of interplay differs according to the context (Du Q, et al 2010). Indeed, although the comparison of genes that rapidly respond to Notch activation in T cell acute lymphoblastic leukemia (T-ALL), mantle cell lymphoma and triple-negative breast cancer identified about 100 genes that were stimulates by Notch only 5 of them were upregulated across all three cell types, and among them, only Nrarp, Hey1, and Notch3 overlapped with a set of direct Notch1 target genes in murine myoblasts. Similarly, to cancer contexts, lineage-dependent variation in downstream transcriptional responses to Notch activation was observed also in different Drosophila cell types. (Stoeck A, et al. 2014; Krejcí A, et al. 2009).

Currently, there is no strong evidence to suggest that Notch is a general factor of stemness. However, the evidence demonstrated that the pathway activation favors the maintenance or expansion of stem cells to the detriment of differentiation, an activity with a more than obvious potential relevance in cancer. Confirming these observations, studies in murine models have demonstrated that the activation of the Notch signal is involved in the maintenance of the populations of neuronal stem cells in the fetal brain

(Imayoshi I, et al. 2010**).** Since its discovery, the role of Notch in the differentiation, morphogenesis, and function in the Central Nervous System has become highly valued in developmental biology research. Through his work on Drosophila, Poulson and colleagues were the first to associate the lack of function of Notch to a lethal embryonic phenotype (Poulson, 1939), referring to them as "a kind of hopeless monster". The main cause is the failure in the early neurogenic ectoderm when it comes to segregating epidermal cell lines from those destined to be neural. In vertebrates, Notch is necessary when the epidermal and neural lineages are segregated; its activation results in the "neurogenic phenotype", characterized by a premature differentiation of the neuronal progenitors, leading us to interpret the role of Notch in this context as an inhibitor of the differentiation that maintains the progenitor state of the cells (Poulson DF, 1968). The first gene of the Notch pathway to be disrupted by homologous recombination was Notch1; Mutant embryos died in the middle of gestation because of defective and deleterious somitogenesis and placentation (de la Pompa, JL, et al. 1997). Several conditional deletions of Notch1 support the idea that the activation of its signal inhibits neuronal differentiation and sustains the neural progenitor populations in the cerebellum and the telencephalon (Lütolf, et al. 2002; Yang X, et al. 2004; Yoon K, et al. 2004).

In contrast to its role in neuronal differentiation, Notch appears to have an instructive role in gliogenesis by directly promoting the differentiation of many glial subtypes. Activation of Notch signaling favors the generation of Müller glia cells at the expense of neurons, whereas reduced Notch signaling induces the production of ganglion cells, causing a reduction in the number of Müller glia (Furukawa T, et al. 2000; Del Debbio CB, et al. 2016). Notch signaling has been also linked to somitogenesis, as defects in somite morphology have been observed in mice with targeted mutations in the *Notch1*. These findings suggest that Notch is critical in the patterning process leading to somite boundary formation and the establishment of the Anterior-Posterior polarity of somites (Pourquié O, 2001).

Tissue interactions between the myocardium and endocardium in the atrioventricular canal and outflow tract regions lead to epithelial-mesenchyme transition (EMT) of endocardial cells, to participate in cardiac valves and membranous septa formation. Different Notch ligands and receptors, as well as their downstream effectors and target genes, are expressed in the vascular system. Evidence for a crucial function of Notch in

vascular development and homeostasis is the finding that the human disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarction and leukoencephalopathy), which involves the *NOTCH3* gene, causes stroke and vascular dementia (Joutel A, et al. 1996).

Specific Notch pathway elements and downstream effectors are expressed in the developing pancreas, suggesting a role for Notch in pancreatic development (Lammert E, et al. 2000). The physiological relevance of Notch in the exocrine pancreas has been shown in the mouse, where Notch is active in committed exocrine progenitor cells and whose ectopic activation in pancreatic bud explants represses acinar cell differentiation (Esni F, et al. 2004). The available data indicate that Notch regulates the progressive recruitment of endocrine and exocrine cell types from a common precursor pool in developing pancreas. The inhibitory effect of Notch signaling in exocrine differentiation has been well characterized in zebrafish, where endocrine and exocrine cells arise independently (Field HA, et al. 2003). Genetic data indicates that Notch-mediated *Hes1* expression regulates a binary cell fate decision between adsorptive and secretory cell fates in gut development. However, *Hes1*-deficient mice do not show a change in the proliferative status of the intestinal precursor pool (Jensen J, et al. 2000), whereas Notch activation profoundly affects the proliferation potential of intestinal progenitors (Fre S, et al. 2005), suggesting that other Notch targets may be responsible for the increased in proliferation.

The potential of Notch pathway in osteoclastogenesis and osteoblastogenesis has been investigated in several *in vitro* experimentations. Evidence indicates that Notch down-regulates osteoclastogenesis activation in osteoclast precursor cells by reducing the surface expression of c-Fms, which is a receptor for macrophage colony-stimulating factor, and enhances the expression of osteoprotegerin (OPG) / osteoclastogenesis inhibitory factor in stromal cells (Yamada T, et al. 2003). Continuous NICD expression inhibited bone morphogenetic protein 2 and induced osteoblast differentiation in osteoblast precursor cells (Sciaudone M, et al. 2003). In contrast, transient expression of N1ICD in osteoblast precursor cells leads to an enhanced bone mineral deposition (Regan J, Long F. 2013). Notch1 is expressed in the mesenchymal condensation area and subsequently in the hypertrophic chondrocytes during chondrogenesis (Watanabe N, et al. 2003). Another study shows that Notch1, Delta1, and Jagged1 are expressed in

cultured osteoblast precursor cells as well as in differentiating osteoblasts during bone regeneration and that Notch1 is activated in these cells. These results suggest that Notch signaling plays an important role in the commitment of mesenchymal cells to the osteoblastic cell lineage (Nobta M, et al. 2005). Overall, this evidence suggests the therapeutic potential for Notch in bone regeneration as well as in osteoporosis. Moreover, it is clearly demonstrated that Notch signaling is crucial for generating the marginal zone B-cell population located within the spleen and there are convincing proofs that Notch signaling controls multiple stages of B-cell differentiation and that it takes part in the shaping of the antibody repertoire (Cruickshank MN, et al. 2010).

1.2.2 Notch and Hematological Stem Cells (HSCs)

The first definitive Haematological Stem Cells (HSCs) are defined as those capable of generating adult erythrocytes, myeloid and lymphoid cells. In mice, HSCs arise at around embryonic day 9.5 and express Notch1, Notch2, and Notch 4, but among them only Notch1 seems to be necessary for the final HSCs development, as.. Notch1 $^{-/-}$ models (Kumano K, et al. 2003) and CSL$^{-/-}$ (Robert-Moreno A, et al. 2005) exhibited low level of expression of the transcription factors Scl, Gata2 and Runx1 required for the hematopoietic differentiation. However, the role of Notch in the maintenance of the adult hematopoietic stem component is still unclear as .controversial conclusions have been found by in vitro and in vivo experimentations. The in vitro overexpression of Notch ligands and receptors (Varnum-Finney B, et al. 2000; Stier S, et al. 2002; Varnum-Finney B, et al. 2003) or the constitutive expression of their direct transcriptional targets like HES1 (Kunisato A, et al. 2003; Shojaei F, et al. 2005), inhibit the hematopoietic progenitors differentiation, thus suggesting that Notch pathway contributes to the stemness maintenance. Consistently, genetic modifications leading to an increased expression of the Notch ligand Jagged1 at the bone marrow level results in increased self-renewal of adult HSCs (Calvi LM, et al. 2003). However, in vivo loss-of-function experiments still does not fully support the above observations. Therefore, inducible Cre-loxP-mediated inactivation of the Jagged1 gene in bone marrow progenitors and stromal cells does not impair HSC self-renewal or differentiation in blood lineages and, similarly, the simultaneous inactivation of Notch1 and Jagged1 in mice does not affect their stemness maintenance or survival capability (Mancini SJ, et al. 2005). Moreover, blocking Notch by CSL silencing in HSCs, does not corrupt

Myeloid and B lineage differentiation in the bone marrow (Han H, et al. 2002) and Notch pathway inhibition based on the expression of a mutated MAML incapable to bind CSL, revealed that Notch signaling is not necessary for adult HSCs maintenance (Duncan AW, et al. 2005).

Although dispensable for HSCs maintenance, recent studies carried out *in vitro* and *in vivo* involved Notch in the early stages of myeloerithroid differentiation. Co-culture of HSCs and OP9 bone marrow stromal cells expressing Dll1 ligand undergoes megakaryocytic differentiation (Mercher T, et al. 2008) and Notch4, as well as many of Notch transcriptional targets, have been found expressed in *ex vivo* megacario-erythrocytic precursors However, *in vivo* studies have not yet identify the precise members of the Notch pathways involved in this process. Published evidence clearly linked the development of megakaryocytic leukemia to dysregulated Notch-CSL signaling activation (Mercher T, et al. 2008). In multipotent progenitors that have lost their erythro-megakaryocytic potential, the maintenance of Notch1 expression is part of a lymphoid specification program induced by specific transcription factors. The expression of Notch1 in lymphoid primed multipotent progenitors (LMPPs) appears to be specifically dependent on the transcription factors E2A (Dias S, et al. 2008; Ikawa T, et al. 2006) and Ikaros (Ng SY, et al. 2009).

1.2.3 Notch in T-cell development

As soon as the role of Notch in the development and differentiation of hematopoietic precursors remains doubtful, its implication during the intrathymic differentiation of T lymphocytes is clear (Pear WS, et al. 2003). T lymphocytes are a crucial cellular component of the adaptive immune system, actively engaged in the recognition and elimination of specific foreign antigens. T lymphocytes originate in the bone marrow but reach full specification ($CD4^+$ or $CD8^+$) in the thymus. Mature thymocytes express a unique receptor system capable of binding and recognizing the antigen, the T-cell receptor (TCR). This receptor contacts the major histocompatibility complex (MHC) expressed by a non-self antigen-presenting cell. During development, the reactivity of mature T lymphocytes to the subject's own antigens is examined. In case there is reactivity for self-molecules, the lymphocyte is pushed towards apoptotic programmed death according to a process known as *negative selection*. On the other hand, due to effective recognition of exogenous antigens, the activated lymphocyte proliferates, thus expanding the number of reactive lymphocytes; in this case, we speak of a *positive*

selection (Starr TK, et al. 2003). Thymocyte maturation consists of three fully described main phases:

1) **Double negative** state (DN): thymocytes in the early stages of differentiation do not have the classic markers CD4 and CD8 (CD4-CD8-). During the DN phase, the presence of other differentiation markers allows to distinguish 4 sub-phases (DN 1-4) depending on the expression of the adhesion molecule CD44 ($CD44^+$), or of the α-chain of the interleukin receptor 2 (IL2- R) CD25 ($CD25^+$). The cell that reaches the thymus to begin its maturation (DN1) is, in fact, capable of giving life to any lymphocyte subtype and phenotypically stands out from the combination $CD44^{hi}/CD25^-$. When the DN1 cell enters the thymic environment and makes contact with it, it begins to actively proliferate and express the CD25, becoming $CD44^{low}/CD25^+$. With the beginning of the TCR gene rearrangement, they actually enter the DN2 sub-phase. Moving from DN2 to DN3, the cell becomes $CD44^-$ and stops proliferating. The rearrangement of the TCR β-chain begins, rapidly bringing the cell from DN3 to DN4 and completely shutting off the CD25.
2) **Double positive** state (DP): in this phase the thymocytes present contextual expression of the CD4 and CD8 markers ($CD4^+CD8^+$). The cells divide rapidly, contributing to the thymocyte cell diversity increase. While the ability of T cells to recognize antigen-MHC complex is vital for their ability to fight pathogens and other foreign cells, it is equally important that these T cells do not recognize and attack our own cells. This is where *negative selection* comes into play. After the proliferative phase, the TCR α-chain rearrangement begins; cells that manage to properly develop their own TCR, or are activated by antigens of the developing subject, are eliminated by apoptosis, while a moderate degree of self-antigen binding leads to survival. One of the most intriguing aspects of negative selection is that it occurs mainly in the thymus, which means that T cells depend exclusively on thymus cells to present self-peptides in MHC molecules. Due to this fact, a protein called autoimmune regulator or AIRE in the thymus induces the production of many different proteins that are not classically expressed in thymic cells, such as proteins characteristic of other tissues. Thanks to this, developing T cells are exposed to many peptide-MHC complexes, not only those typically present in thymic cells, which avoids autoimmunity once the T cells leave the thymus.

3) **Single-positive** state (SP $CD4^+$ / SP $CD8^+$): the cells mature from DP to SP extinguishing one of the two markers and maintaining the expression of the single CD4 or CD8 depending on the specification in T-Helper lymphocyte ($CD4^+$), or T - cytotoxic ($CD8^+$). Terminally differentiated lymphocytes migrate from the cortical thymus gland to the medulla, where they meet the blood system of the thymus and enter the bloodstream.

Notch signaling is implicated in the development of intrathymic T lymphocyte precursors, while it is dispensable in that of B cells and myeloid bone marrow. For this reason, the expression of Notch1 in pre-thymic progenitors such as LMPPs represents one of the earliest events in thymocyte specification. The high expression of Notch ligands in bone marrow (Yan XQ, et al. 2001), indicate that it might subsist a mechanism by which LMPPs-mediated Notch activation is inhibited, permitting the generation of T cells prior to thymic implantation. This mechanism involves a so-called lymphoma-associated repressor factor (LRF). It has been shown, that the retroviral expression of the Dll4 ligand in medullary cells could elude this repression, inducing the development of double-positive CD4 / CD8 (DP) T cells in bone marrow (Yan XQ, et al. 2001). It is important to underline that these studies, as well as others (Koch U, et al. 2001; Wilson A, et al. 2001), demonstrated that the bone marrow represents a suitable environment for the differentiation of T cells. Similarly, Notch/CSL signaling promotes the extrathymic development of DP cells in the spleen and lymph nodes of irradiated mice, but this process appears to be suppressed in cases of lymphopenia (Maillard I, et al. 2006). T-cell extrathymic development may have therapeutic relevance in bone marrow transplants. Further studies are needed to identify additional mechanisms that prevent the activation of the Notch pathway outside the thymus.

Notch1 receptor is present at diverse levels during the intrathymic differentiation stages and it is highly expressed in the early phases of DN, less so in DP cells, and then returns to be quite expressed in SP thymocytes (Izon DJ, et al. 2002). Upregulation of Notch1 is critical for early stages of T cell development, whereas downregulation of Notch1 past the β-selection checkpoint at the DN3 stage of T cell development may be important in avoiding sustained Notch signaling, which is strongly transforming in this lineage. The latter event may occur via signals transduced by the pre-TCR which upregulate Id3 and inhibits Tcf3 (also known as E2A). Tcf3 is proposed to be an important positive

regulator of Notch1 expression at the DN3 stage of thymocyte development (Yashiro-Ohtani Y. et al, 2009). Notch3 is more expressed than Notch1 in the phases of transition from DN to DP, after this transition, it is down-regulated or turned off in SP cells (Felli MP, et al. 1999; Bellavia D, et al. 2002). At the thymic level, Notch1 also has a role in the selection of the SP $CD8^+$, favoring this T-cell specification at expense of the $CD4^+$. In the same way, it would allow the rearrangement of the αβ-TCR with respect to the γδ type (Robey E, et al. 1996; Washburn T, et al. 1997; Fowlkes BJ, et al. 2002), besides being necessary in gene rearrangement VDJk (Wolfer A, et al. 2002). This is a mechanism of genetic recombination that occurs in vertebrates by which amino acids are selected and assembled randomly from genes that encode specific proteins with important roles in the immune system. This location-specific recombination generates a diverse repertoire of TCR and immunoglobulin (Ig) molecules that are necessary for the recognition of various bacterial, viral and parasite antigens, as well as dysfunctional cells, such as tumor cells (Wolfer A, et al. 2002). Notch3, induced to over-expression by the intrathymic stromal cells in the DN-SP transition, plays a decisive role in the pre-TCR control phase (Bellavia D, et al. 2003).

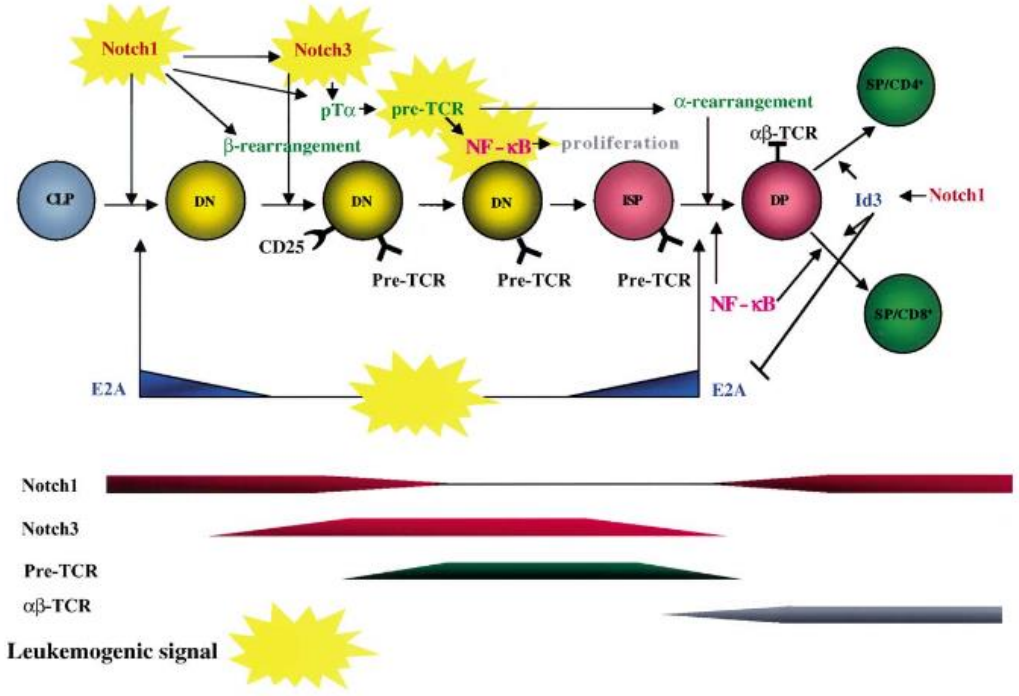


Figure 1.5: Schematic model of relationships between murine T cell development and leukemogenic events. *The sequence of developmental stages and the main signaling pathways involved are shown. Abbreviations: CLP, common lymphoid precursor; DN, CD4−CD8−double negative thymocytes; DP, CD4+CD8+double positive thymocytes; SP, CD4+and CD8+thymocytes. Adapted from: Campese AF, et al. 2003.*

1.2.4 Notch in epithelial tissue homeostasis

The epidermis is a finely organized and continuously renewing squamous epithelium that consists of four functionally different layers: basal, spinous, granular and cornified (in ascending order from the basal membrane). The stratum corneum is the epithelial barrier with the environment that limits water loss and prevents the invasion of microorganisms. Epidermal homeostasis depends on the balance between proliferation and differentiation of keratinocytes (Fuchs E, 1990). Tissue renewal is sustained by a small population of stem cells located at the basal layer that undergoes proliferation into transiently amplifying cells with limited proliferative capacity.

In the early phase of the differentiation, the cells stop proliferating, detach from the basement membrane and migrate to the spinous and granular upper layers., Keratinocytes start to express a specific protein pattern including keratin 1, 10 and involucrin in the spinous layer, as well as filaggrin and loricrin in the granular layer. Finally, a series of biochemical and morphological changes take place that results in the conformation of the stratum corneum. This complex process during the epidermal differentiation requires the coordination among the molecular mechanisms that drive the generation of the stratified epithelium and in which Notch and its ligands play an important role (Okuyama R, et al. 2008).

Epithelial cells make up the tissues through a paired structure with apicobasal polarity maintained stably thanks to extensive cell-cell contacts and adherence to the basement membrane (figure 1.6 A) (Cereijido M, et al. 2004; Shin K, et al. 2006). The cells of the epithelial tissues also have another type of axis, called planar cell polarity (PCP). This polarity presents an orthogonal structure between the apical and basal ends in the orientation of cell-cell and cell-extracellular matrix contact (figure 1.6 B, C) (Devenport D, et al. 2008). The signaling proteins of the PCP group include frizzled (fz), disheveled (dsh), Van Gogh (Vang), prickle (pk), diego (dg) and flamingo (fmi), which present different distribution to establish the proximal and distal sides of polarity (Wu J, et al. 2009). However, during the development stages, the epithelial cells show the ability to change to a mesenchymal phenotype in situations in which deep cellular readjustments are necessary. The mesenchymal cells are characterized by fusarium morphology due to the lack of cell-cell contacts; this confers the ability to migrate through the basement membrane. This process of phenotype change is called epithelial-mesenchymal

transition (EMT), and there is solid evidence that maintains its relevance in the progression of epithelial tumors since the transformed cells acquire the ability to escape from the extracellular matrix and invade the surrounding tissues (Moreno-Bueno G, et al. 2008; Onder TT, et al. 2008; Yang J, et al. 2008). The following figure shows the structure of an adherent tissue according to its vertical and horizontal polarity.

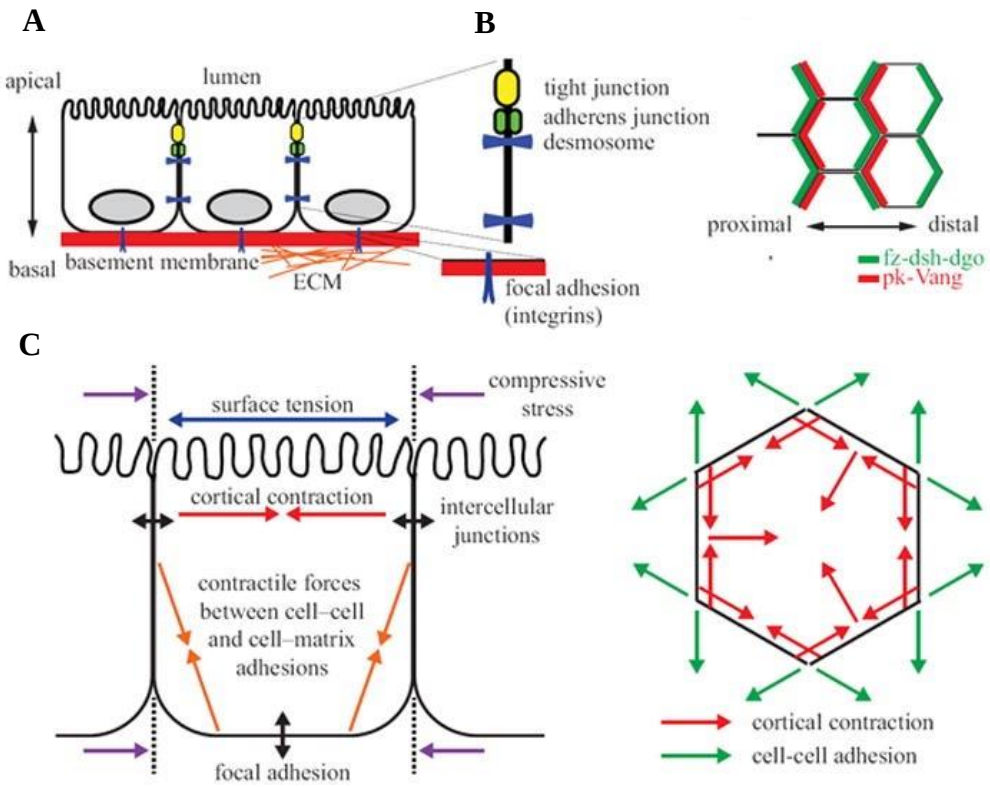


Figure 1.6: Detail of the morphology of a polarized epithelial monolayer. *(**A**) Vertical epithelial polarization towards the apical, basal and lateral domains of the plasma membrane. The apical surface presents specialized microvilli in the absorption and secretion. The basal surface adheres to the proteins of the extracellular matrix through integrins for the formation of focal adhesions. Lateral membranes contain complexes such as tight junctions, adherents, and desmosomes that maintain the cohesion of the tissue and favor diffusion barriers. (**B**) Horizontal polarization of the core planar cell polarity proteins. In the wings of Drosophila, the wing, fz, dsh, and dgo proteins are found in the distal chord domains, while pk and Vang are located in the proximal cortical domains. (**C**) The intracellular and intercellular tension maintains the shape of the cells. The constriction promotes forces of linear tension in the area of contact between cells and serves as an energy barrier for cell readjustments. Adapted from: Wang CC, et al 2012.*

The information is transmitted directly between adjacent cells through signaling receptors. The major homotypic receptor in epithelial cells is E-cadherin, a calcium-dependent receptor that is critical in cell-cell recognition and adhesion (Gumbiner BM, et al. 2005). The cytoplasmic domain of E-cadherin connects to the actin cytoskeleton via α and β-catenin to preserve cell shape and polarity. In Madin-Darby Canine Kidney (MDCK) cells, the cadherin-catenin complexes affect signaling predominantly through small GTPases. Rac-induced lamellipodia activity is repressed as E-cadherin amasses, and Rho-dependent actomyosin rises during the establishment of new contacts once the cytoskeletal networks are formed (Yamada S, et al. 2007) (figure 1.7 A). Computational models were used to investigate whether Delta-Notch negative feedback loops are sufficient to generate long-range signaling patterns during development. In some contexts, high expression of the Delta ligand in a cell inhibits the expression of the same ligand in the neighboring cell by a process called lateral inhibition (Kimble J, Simpson P. 1997). In other contexts, a lateral induction process may occur (de Celis JF, Bray S. 1997). Interestingly, Webb and Owen found evidence that both mechanisms were capable of generating long-range patterns for certain feedback forces. It is possible that these computational studies in the Delta-Notch signaling could be useful to identify disturbances in the signal or feedback that could distinguish whether the inhibition or lateral induction was the dominant non-cell autonomous mechanism (Webb SD, et al. 2004) (figure 1.7 B).

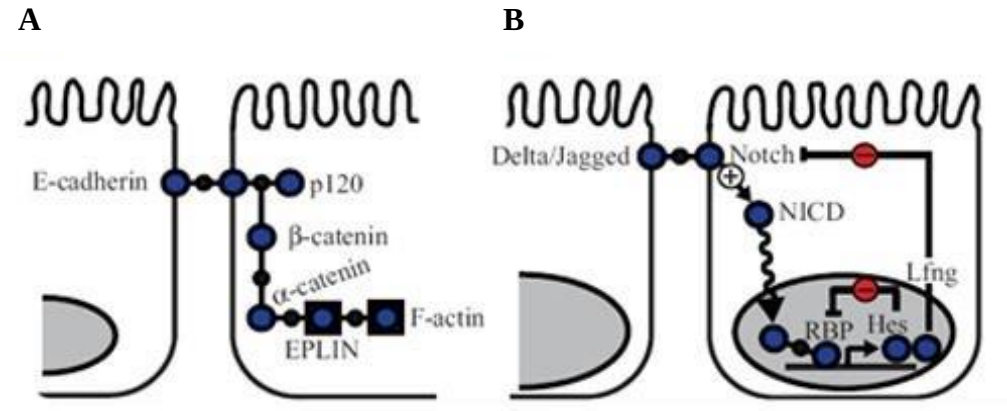


Figure 1.7: Detail of the intercellular communication between adjacent epithelial cells by cell-cell signaling receptors. (A) *E-cadherin forms homophilic complexes between neighboring cells. Catenin p120 binds to the juxtamembrane domain of E-cadherin and stabilizes it by preventing its clathrin-mediated endocytosis. B-catenin is bound to E-cadherin. The alpha-catenin acts in connection with the epithelial protein lost in neoplasm (EPLIN), finally connecting it with the F-actin.* ***(B)*** *Notch binds to its ligand (Delta or Jagged) by contact with a neighboring cell, releasing NICD by proteolysis that translocates to the nucleus and interacts with the RBPkJ DNA binding protein together with various coactivators for the regulation of the expression of Notch's target genes, such as Hes and lunatic fringe (Lfng), which could negatively regulate Notch. Adapted from: Wang CC, et al. 2012.*

In normal skin, Notch receptors and ligands are expressed in keratinocytes within the epidermis as well as in adnexal structures. Notch1 is expressed in all epidermal layers, while Notch2 is expressed only in the basal layer (Rangarajan A, et al. 2001; Okuyama R, et al. 2004) and the Notch ligands Jagged and Delta-like family members are expressed in overlapping patterns with Notch in the epidermis. Former experimentation indicated that Notch signaling induces differentiation in human and murine keratinocytes (Rangarajan A, et al. 2001; Lowell S, et al. 2000; Nickoloff BJ, et al. 2002) (figure 1.8). Indeed, Notch1 signaling stimulates the expression of early differentiation markers such as keratin1 and involucrin in mouse keratinocytes. These results correlate with the epidermal phenotype observed in mice models of constitutive activation of Notch1 in the epidermis (Blanpain C. et al 2006). On the other hand, Notch1 deletion in mice results in decreased epithelial cell differentiation and increased proliferation of cell layers (Rangarajan A, et al. 2001).

In addition, it has been demonstrated that Notch1 signaling induces the expression of the cell cycle regulator p21Waf1 / Cip1 (Rangarajan A, et al. 2001) and promotes cell cycle arrest in proliferating keratinocytes thus allowing their terminal differentiation.

Conversely, it has been shown that Notch1 signaling activates caspase 3 in embryonic keratinocytes, thereby producing a decrease in proliferation and an increased differentiation by activating PKC-delta (Okuyama R, et al. 2004).

The different functions of Notch are complex and involve a cross-talk with other signaling molecules. Notch is linked to important factors controlling cell behavior such as p63 (also known as p51 or KET). The p63 gene was originally identified as a member of the p53 family (Osada M, et al. 1998; Yang A, et al. 1999) that codifies for a protein with high structural homology with p53. Furthermore, p63 binds to responsive elements of p53 and it acts as a positive regulator of p53 target genes, suggesting a functional analogy between these proteins. Mice lacking p63 show developmental deficiency, especially in the formation of the stratified epithelium (Mills AA, et al. 1999; Yang A, et al. 1999). Interestingly, the activation of Notch signaling suppresses the expression of p63, while, on the contrary, p63 inhibits Notch-induced p21 expression, cell cycle arrest, and epidermal differentiation (Nguyen BC, et al. 2006; Okuyama R, et al. 2007). Therefore, the proliferation or differentiation of keratinocytes is determined by a fine balance between the intracellular levels of Notch and p63. In addition to the p53 family members, it has widely demonstrated the existence of fine crosstalk between Notch signaling and the Wnt and Shh pathways. Both Wnt and Shh signaling pathways are known to be tumorigenesis regulators and abundant evidence indicates that are repressed by Notch1. Indeed, the deletion of Notch1 in skin of mice results in the constitutive activation of Wnt and Shh signaling and combined with the development of basal cell carcinoma and squamous cell carcinoma (Nicolas M, et al. 2003). The following figure 1.8 represents a diagram of the Notch interactions among the epidermal layers and its relevant crosstalk with other signaling pathways.

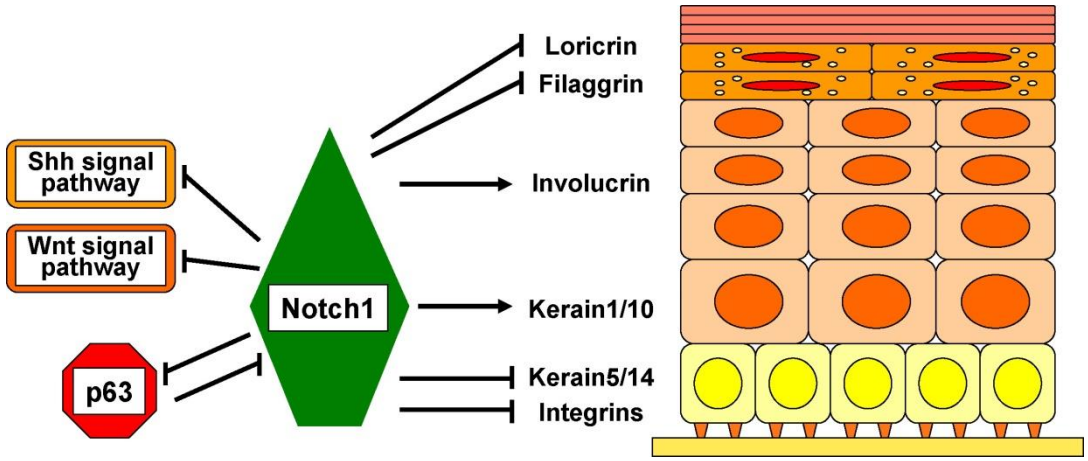


Figure 1.8: The Notch1signaling interplay within the epidermis. *Each epidermal layer shows a different state of differentiation and the expression of specific proteins. Notch1 enhances the expression of keratin1 and involucrin and prevents the induction of filaggrin and loricrin that are induced in later stages. Sonic hedgehog (Shh) and Wnt signaling are normally repressed by Notch1. In addition, p63 and Notch repress each other. Adapted from: Okuyama R, et al. 2008.*

1.3 Notch pathway dualism in tumor fate

The biological function of the Notch signaling pathway is considered context-dependent as activated Notch exerts opposing effects like growth and differentiation in diverse cell types and in the same cell context in physiological versus pathological condition (Lefort K, et al. 2016; Ranganathan P, et al. 2011). Notch receptors can function as cell-autonomous oncoproteins, cell-autonomous tumor suppressors, or microenvironment-dependent oncoproteins in different cellular contexts.

1.3.1 Notch and T cell acute lymphoblastic leukemia (T-ALL)

As mentioned in the previous section 1.2.3, the role of Notch in correct thymocyte development and differentiation has been partially clarified, making even more complex to describe the contribution of this signaling pathway in the pathogenesis of T-ALL. This is a hematopoietic malignancy characterized by the accumulation of T lymphoblasts in blood and primary lymphoid tissue that represents the 15% of pediatric acute leukemia and almost 25% in adult cases (Bongiovanni D, et al. 2017). This hematological cancer is characterized by a less favorable prognosis than B Cell Acute Lymphoblastic leukemia (B-ALL). The clinical evidence regards a high number of leucocytes in blood, adenopathy and marrow infiltration of immature lymphocytes, frequently followed by central nervous system colonization of these cells (Van Vlierberghe P, et al. 2012). The tumor etiopathogenesis is due to the malignant transformation of thymic lymphocitary precursors during their differentiation process. The crucial mechanisms causing this outcome have been demonstrated to be related to an expression and/or activation deregulation of molecular pathways involved in thymic cells differentiation channeling. The T-ALL is characterized by genetic modifications as chromosomic aberrations, altered gene expression profiles, mutations or immunoglobulin (Ig) genes rearrangement, and T cell receptors (TCR) (Kraszewska MD, et al. 2012). In fact, the first scientific evidence of the involvement of notch1 in leukemic pathology dates back to 1991, when chromosomal translocation with function gain t (7; 9) (q34; q34.3) was identified in some patients with T-ALL. This gene rearrangement involves the dosing of the N1ICD domain with elements of the promoter of the TCR gene, resulting in the expression of a constitutively active form of Notch1 (Ellisen LW, et al. 1991). Murine models investigations involving the overexpression of the intracellular domains of Notch1 and Notch3, placed them under the control of the specific promoter of the immature thymic stages Lck (Robey E, et al. 1996; Bellavia D,

et al. 2000). Furthermore, transgenic mice for incomplete TAD domain of N1ICD only occasionally present the disease, whereas those with a complete region develope T leukemia (Weng AP, et al. 2006). Bellavia and colleagues generated a transgenic mouse that overexpresses the intracellular domain of Notch3 (N3ICD), again located under the control of the Lck promoter. Contrary to what happens in the murine Lck-N1ICD models, the Lck-N3ICD transgenic mice develop a particularly early and aggressive form of T-cell leukemia, suggesting the oncogenic role of Notch3 which takes place in the thymus (Bellavia D, et al. 2000). Subsequent investigations have shown that, at a phenotypic level, leukemic cells present characteristics similar to those of immature thymocytes such as $CD25^{+}$ and presenting the pTα chain. The Notch3-pTα axis would promote the constitutive activation of NF-kB, favoring the proliferation of these cells bypassing the apoptotic systems, and thus contributing to the neoplastic transformation of immature thymocytes (Bellavia D, et al. 2002). From this work, it has been shown how the activation of Notch3 and Notch1, together with NF-KB, is capable of inducing the transcription of several microRNAs, particularly of miR-223 (Kumar V, et al. 2014). This microRNA has been demonstrated to be an oncogene among whose targets it is the E3-ligase FBXW7, responsible for the ubiquitination and consequent degradation of the intracellular domain of Notch1 increasing its half-life and cytoplasmatic stability, together with its potential oncogenicity (Mansour MR, et al. 2013).

Recent experimental evidence stands out that these genetic aberrations join the effects of a deregulated expression of transcriptional oncogenic factors and their direct targets with a tumorigenic outcome, among which it has been described c-Myc and Cyclin-E (Szczepanski T, et al. 2011; Kumar V, et al. 2014). In murine models in which a stable viral insertion of a Notch1 allele has been performed, transcriptional induction of helix-loop-helix domain (bHLH) factors of the Hes family is observed as a result. Among these, Hes1 seems to be a potential inducer of leukemogenesis (Lee JS, et al. 1999). Similarly, the DELTEX oncogene is overexpressed in murine transgenic models for both Notch1 and Notch3, emphasizing the oncogenic role of this protein in T-ALL. The combined expression of Notch3, HES1, and DELTEX is characteristic of T-ALL since, interestingly, the isolated expression of these oncogenic mediators is not sufficient to induce the disease (Zúñiga-Pflücker JC, 2004), thus showing that Notch expression is an important requirement for the development of leukemia.

Another oncogenic mechanism dependent on Notch lies in the ability of the activated receptor to promote the G1 / S cell cycle progression by direct or indirect regulation of crucial proteins for this checkpoint, such as the CDK4 or CDK8 kinases or the cyclin-dependent kinase inhibitors $p27^{KIP}$ and $p18^{INK4C}$ (Dohda T, et al. 2007). All these deregulatory processes act in a synergistic manner with those Notch mutations mentioned in point 1.1.5 thus suggesting that an aberrant regulation that induces a constitutive and/or uncontrolled activation of the Notch pathway is decisive and necessary for the full development of the T-ALL.

1.3.2 Notch signaling in cervical cancer

Cervical cancer is one of the leading causes of cancer-related deaths among women in the developing world accounting for the fourth most common cause of cancer deaths in the world. However, in developed nations, cervical cancer deaths do not exceed 2% per year; this is due to the systematic use of the Pap test to detect cervical abnormalities and proceed to early treatment. Most cervical cancers (80 to 90 percent) are squamous cell cancers, while adenocarcinoma is the second most common type of cervical cancer, accounting for the remaining 10 to 20 percent of cases. Adenocarcinoma types develop from the glands that produce mucus in the endocervix. While less common than squamous cell carcinoma, the incidence of adenocarcinoma is on the rise, particularly in younger women. Solid evidence associates cervical cancer with the infection of squamous and columnar cells of the epithelium of the ectocervix and endocervix with high-risk human papillomavirus (HPVs) (Zur Hausen H, 2002). In the so-called transition zone located between squamous and columnar cells, there is a population of cells with differentiation capacity in one or another subtype (figure 1.9). The integration of the HPV genome, as well as the neoplastic transformation process, is believed to begin in this region (Reid R, 1983).

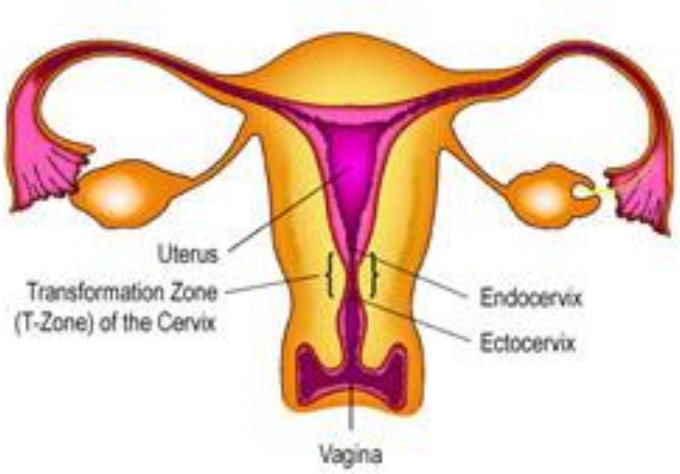


Figure 1.9: Cervical cancer initiates in the cervix, the narrow opening into the uterus from the vagina. *The region known as ectocervix is the portion of the uterus extending into the vagina. In a healthy state, it shows itself covered by the flat, thin squamous cells. The endocervix or cervical canal is built by columnar cells. The area between them is called the transformation zone (T-zone), considered most likely the location for abnormal or precancerous lesions. Adapted from: National Cervical Cancer Coalition (NCCC).*

Decades of study have unveiled the molecular insights of the virus. It has been well described that the integration of the virus DNA results in the disruption of the viral open reading frame that encodes for the viral E2 inhibitory subunit. This erases the repression of the rest of the viral genome transcription which encodes for oncogenes E6 and E7 (Goodwin and DiMaio, 2000; Zur Hausen H, 2000; Parish JL, et al. 2006). The best-known function of these two oncogenes is to transcribe for the viral subunits E6 and E7 that interact and block host proteins p53 and Retinoblastoma (Rb), respectively. This causes deregulation of the control of the cell cycle and the response to DNA damage, which contributes to tumorigenesis. The different pleiotropic roles of E6 and E7 continue to be identified and documented extensively (Oh ST, et al. 2004; Yim EK, et al. 2005; Jones and Wells, 2006; Tomaić V, 2016). The detection of the intracellular form of Notch in certain types of cervical cancers aroused great interest since such forms of Notch are really difficult to detect in the context of neoplasms. There is currently a large and controversial bibliography around the role of Notch in the development of cervical cancer (figure 1.10). The first evidence of the relationship between Notch signaling and solid tumor development arose from the studies on the integration of the murine mammary tumor virus (MMTV) by Robbins and colleagues in

1992 (Robbins J, et al. 1992). Up to four genes were detected and analyzed in the context of the murine mammary tumor, of which two were members of the Wingless family. The other two genes characterized were members of the fibroblast growth factor gene family and the Notch family (Robbins J, et al. 1992).

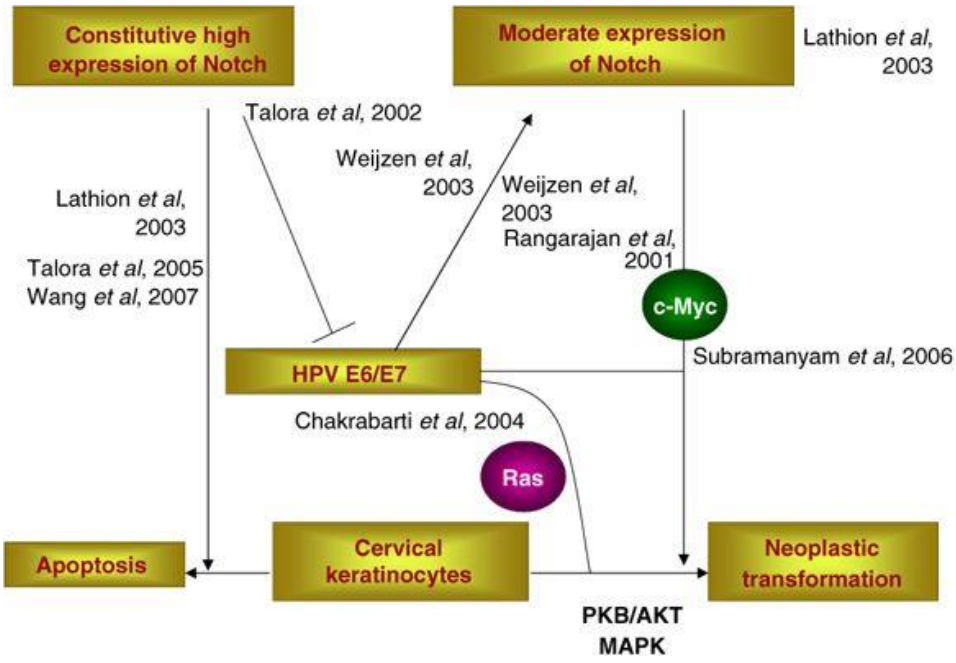


Figure 1.10: The role of Notch signaling in the neoplasic transformation of the cervix epithelium associated with the authors who have collaborated to unveil it. *The neoplasic transformation takes place by the action of the human papillomavirus (HPV) oncoproteins that cooperate with Ras and Notch1. The moderate expression of Notch1 results in the positive regulation of c-Myc and the activation of PKB / Akt, causing neoplasia. On the other hand, it has been demonstrated that the high constitutive expression of Notch1 can induce apoptosis in keratinocytes transformed by HPV. Adapted from: Maliekal TT, et al. 2008.*

Different to what observed in T-ALL in which activating mutations occur on Notch1 locus in about 50% of patients (Weng AP, et al. 2004), Notch1 mutations are very rare in human cervical cancers samples and Notch signaling activation is driven by a ligand-dependent mechanism sustained by elevated expression of its ligand Jagged1 and low levels of its negative regulator Manic Fringe (Veeraraghavalu K, et al. 2004). However, the role of Notch in cervical cancer is not clearly determined and several findings are controversial. It has been demonstrated that different levels of Notch activation are specific of the tumor phases and that Notch activation acts as oncogene by inducing cell growth, drug resistance and invasiveness through Epithelial-to-Mesenchymal transition (EMT) in some cervical cell lines (Yu H, et al. 2007; Wang L, et al. 2018). Conversely, it has been also shown that high levels of activated Notch can induce apoptosis and cell cycle arrest by stimulating the expression of markers such as p53 and p21 (Yousif NG, et al. 2015; Yun J, et al. 2015; Talora C, et al. 2005; Wang L, et al. 2007; Franko-Tobin LG, et al. 2012). For this reason, it is important to consider that a better and deeper understanding of the molecular mechanisms of the Notch pathway in cervical cancer could lead to the development of novel therapeutic strategies.

1.3.3 Notch and Epithelial to Mesenchymal Transition (EMT)

EMT is a highly conserved cellular process that permits polarized and fixed cells to lose their epithelial characteristics and gain motile attributes of mesenchymal cells. EMT occurs in a physiological way during embryonic development and wound healing, but it is also associated with cell migration and invasion and considered a critical step for cancer progression in solid tumors. This process alters the morphology of the healthy epithelium occupying the lumen and invading the basement membrane and neighboring vessels (figure 1.11) (Birchmeier C. et al, 1996; Gotzmann J. et al, 2004; Thiery JP. et al 2009). Not surprisingly, it has been widely described that the EMT program in epithelial cells is not only coordinated by multiple signaling pathways but also by epigenetic and post-translational modification (Serrano-Gómez, SJ. et al. 2016).

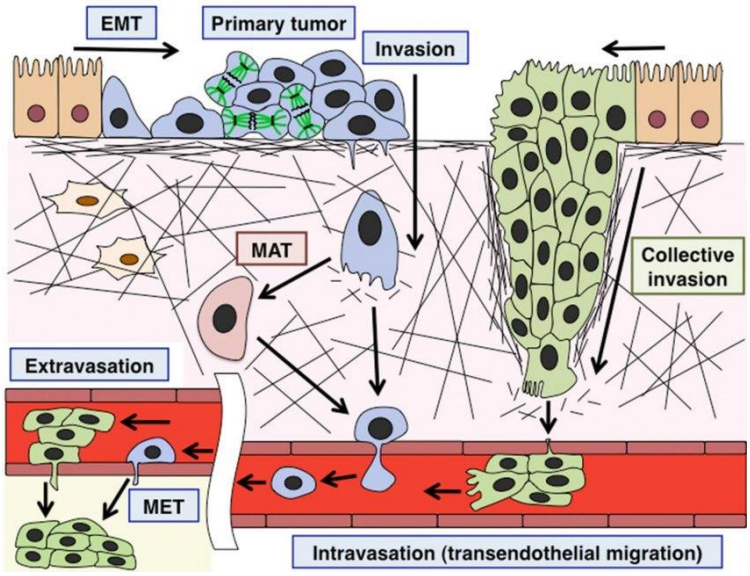


Figure 1.11: Invasion mediated by EMT and collective invasion in metastasis. *Healthy epithelial cells (orange) undergo a process of epithelial-mesenchymal transition (EMT) and give rise to the primary tumor (blue). Some primary tumors develop the ability to invade and migrate to nearby vessels as a multicellular strand (green). After filtering into circulating blood, these tumor cells can colonize other tissues where they undergo an inverse process of extravasation and settlement called mesenchymal-epithelial transition (MET). MAT: mesenchymal-ameboid transition. Adapted from Kawauchi T, 2012.*

During the last decade, it has been reported increased expression of Notch signaling in certain pathological frames of cervical cancer cells and cervical carcinomas with high grade, lymph node involvement, and parametrial invasion, frequently accompanied by activation of the NF-κB pathway (Weijzen S, et al 2003; Ramdass B. et al 2007; Sun XM, et al. 2009). Song, *et al*. reported that the over-expression of Notch-1 stimulates NF-κB activity in CaSki metastatic cervical cancer cells by associating with the IKK signalosome through IKKα (Song LL, et al. 2008). Conversely, several researches demonstrated that Notch-1 activation has a dose-dependent effect and it leads to cell cycle arrest and growth repression in cervical cancer cells (Talora C, et al. 2002; Talora C, et al. 2005; Wang L, et al. 2007; Yao J, et al. 2007; Maliekal TT, et al. 2008). Overall these findings unveil the complexity of Notch's role in different contexts of human cervical cancers.

1.4 Epigenetics

The term epigenetics was proposed by Conrad Waddington at the beginning of the 1940s, to define "the branch of the biology focused on study of the causal interactions between genes and their products which bring the phenotype into being" (Waddington CH, 1956; Noble D, 2015) including all molecular pathways regulating the expression of a genotype into a defined phenotype. Subsequently, thanks to the rapid growth of the genetic field, the meaning of the word has modified. Today, epigenetics has been defined as ''the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in the DNA sequence" (Wu CT, et al. 2001). This discipline investigates the modifications of the transcriptional potential of a cell in a different way with regard to the classical genetics that studies the alterations within the genomic sequence. Currently, it is well known that the mechanisms of epigenetic regulation are fundamental for the physiological development of a cell that can be extrapolated to the stability of the tissue-dependent genetic expression. In this way, aberrations in the fine control of this system can lead to genetic dysfunctions that are the basis of many diseases (Rodenhiser D, et al. 2006). The epigenetic modifications comprise histone variants, posttranslational modifications of histones tails, covalent modifications of DNA bases and RNA-associated silencing.

1.4.1 Eukaryotic organization of the chromatin

Within the eukaryotic cell, the DNA is in a form of complex nucleoprotein ultrastructure defined as chromatin. Its organization is functional as soon as the different nuclear processes take place during the life of the eukaryotic cell, such as the transcription, repair or replication of DNA (Felsenfeld G, et al., 2003). The nucleosome is defined as the fundamental unit of chromatin, comprising 146 base pairs (bp) of DNA around a complex of 8 nuclear proteins called histones organized in pairs of histone subunits H2A, H2B, H3, and H4 (Khorasanizadeh S. 2004). The histones are basic proteins of low molecular weight, constituted by a compact globular domain and an N-terminal segment of 20-30 residues rich in amino acids of positive charge that favors the interaction with the DNA. This segment, defined as histonical tail, emerges from the chromatin nucleus, being susceptible to modifications and accessible to the DNA involved in the nucleosome (Peterson CL, et al., 2004).

The structural organization of these components begins with the hetero-dimerization of a histone H3 and an H4, directly associating with another hetero-dimer forming a hetero-tetramer. In the same way the histones H2A and H2B form complex with the difference that they do not hetero-tetramerize between them. The assembly of this articulated protein complex with the DNA takes place when the tetramer H3-H4 makes contact with the nucleotide double helix and, immediately afterward, the two H2A-H2B dimers are associated (Kornberg, RD 1974, Luger K, et al. 1997). The globular domain of the nucleosome is composed of 3 alpha-helices involved in histone-histone / histone-DNA contact (Arents G, et al., 1991). The protein-DNA association takes place through hydrogen bonds that are established between the phosphate groups present in the nucleotide double helix and the above-mentioned basic residues of the surface of the histones. In the same way, other electrostatic interactions take place between the lateral base chain and the DNA that contribute to stabilizing the hydrogen bonds.

From the structural point of view, the histones H3 and H4 are evolutionarily preservative, suggesting that their morphology is functionally fundamental in the dynamic organization of the genetic material and its subsequent compact ultrastructure. The histones H2A and H2B could be considered less preservative since they present variants with specific properties depending on the species. DNA between two histone complexes is not free but associated with a fifth type of histone called H1 that is defined as "linker". Its function is not especially necessary for the association of nucleosomal components, but it is crucial for the establishment of a higher-order organization. H1 histones stabilize chromatin by neutralizing the electrostatic charges of the binding DNA through its C-terminal domain rich in positively charged amino acids (Szerlong HJ, et al. 2011).

A **B**

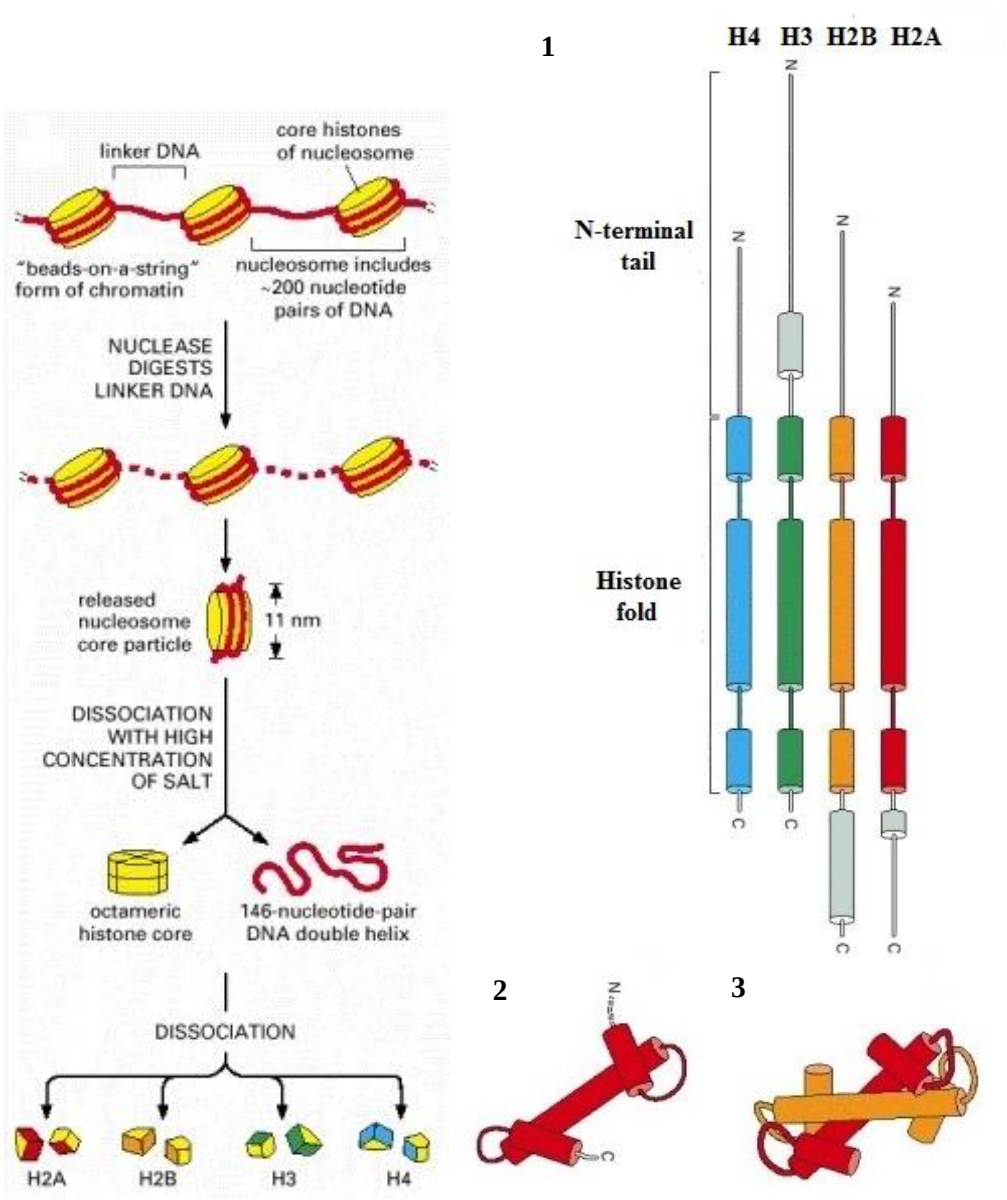


Figure 1.12: The different organization states of histones since their clustering to their association with the chromatin. (A) *Structural organization of the nucleosome. Each complex contains a nucleus formed by 8 histone subunits stabilized with the DNA by means of electrostatic interactions and hydrogen bonds susceptible to neutralization in the presence of a high concentration of salt.* ***(B)*** ***(1)*** *Each histone contains an N-terminal tail, a target of different forms of covalent modifications, and a histone fold region.* ***(2)*** *The fold histone structure, present in the 4 core histone types.* ***(3)*** *Histones H2A and H2B form a dimer through an interaction known as "handshake". Histones H3 and H4 dimerize through the same type of interaction. The assembly of the histone heterodimers takes place by the scaffolding between the tetramer H3-H4 with the two H2A-H2B dimers. Adapted from: Molecular Biology of the Cell. 4th edition.*

1.4.2 Post-traductional modifications of the histones

Chromatin aggregation is dynamically organized through a series of processes of post-translational modification of histones. The molecular modifications that affect these proteins are the following:

- Acetylation / deacetylation of lysines (Kxac);
- Methylation / demethylation of lysines (Kxmex) and arginines (Rxmex);
- Phosphorylation / dephosphorylation of serines (Sxp) and threonines (Txp);
- Ubiquitination and sumoylation of lysines (KxUb) (KxSu);
- ADP-ribosylation.

Most of these modifications affect the N-terminal histological tails, with the exception of ubiquitination, which takes place in the C-terminal tail of histones H2A and H2B (Bradbury EM, 1992). As will be discussed later, the alternation of these modifications will allow greater or lesser accessibility of the replicative machinery to the double helix thanks to the alteration of the epigenetic landscape of the chromatin. The compact DNA fiber associated with histones is defined as "dense" since it prevents the binding of transcription factors in order to maintain accurate control of the expression of the genes. This ultrastructure must be, therefore, highly removable and ready to be opened, in order to allow the genetic transcription. At a specific level, the modifications that regulate accessibility to genes are the following:

- **Acetylation**: consists of the transfer of an acetyl group from an acetyl-CoA to ε-$NH3^{+}$ of a histone lysine. This type of modification allows the neutralization of the positive charges of the histological tails, weakening their interactions with the phosphate groups of the DNA. The result is a relaxation of the structure of the nucleosome that allows the access of the transcription factors to the loci of genetic regulation. The acetylation is carried out by enzymes with histone acetyltransferase activity (HAT), located both in the nucleus and in the cytoplasm. Depending on their location, the HAT present different targets; the nuclear ones intervene in the dynamic remodeling of the chromatin and, consequently, in the transcriptional processes, while the cytoplasmic ones promote the acetylation of the newly synthesized histones that allows their translocation to the nucleus (Ehrenhofer-Murray AE, 2004). Among HAT proteins, those best characterized are p300 and GCN5 (Schiltz RL, et al. 1999). The acetylation of K5 of the histones H2A,

K12/15/20 of the histones H2B, in K4/9/14/27 of the H3 and in K5/8 of the H4 are, as a rule, considered prone to allow the genetic transcription. It is a reversible process catalyzed by the enzymatic activity of a family of proteins called Histone Deacetylases (HDAC) capable of eliminating the acetylation label and promoting the consequent chromatin compaction (Dokmanovic M, et al. 2007).

- **Methylation**: it consists of the transfer of a methyl group in the ε-$NH3^{+}$ of conserved residues of the histones. In terms of control of gene transcription, this type of epigenetic marks can trigger both activating and inhibitory changes, depending on the specific residue that is involved as well as the number of methyl groups transferred to it. The result of histological methylation can lead to a relaxation of the DNA-histone interaction, favoring the genetic expression. On the other hand, the histological polymethylation of certain amino acids implies the increase of hydrophobic type interactions between them, thus causing chromatin aggregation. This is the specific case of lysines, where the trimethylation of H3K9, H3K27, H3K29, and H4K20 induces the formation of heterochromatin, thus classifying them as transcriptional repression marks. On the other hand trimethylation of H3K4 and H3K36 are, classified as markers of transcriptional activity (Martin C, et al. 2005). The methylation in the arginine residues (Rxme) is undoubtedly classified as a pro-transcriptional marker (Barski A, et al. 2007). Each addition of a methyl group requires enzymes and specific cofactors; in the case of arginine methylation, it requires the methyltransferase action of PRMT family proteins while in the methylation of lysines the HMT class intervenes, characterized by having a specific SET domain (Zhang Y, et al. 2001). As happens in the acetylation process, methylation is also reversible and carried out by the action of HDM demethylases (Ehrenhofer-Murray AE, 2004).
- **Phosphorylation**: it is a transfer process of a PO_4^{3-} group, with an activating effect on the genetic transcription. It is a generally observable phenomenon in response to DNA damage so that histone H2A phosphorylation allows access to damaged genetic sequences (Rossetto D, et al. 2012). This mechanism is also stimulated in the phases of replication during mitotic division (Sauvé DM, et al. 1999). Histones H3 are phosphorylated at K10, in association with the acetylation label at K14 stimulates the initiation of transcription (Lo WS, et al. 2001). On the other hand, this phosphorylation in K10, either alone or in combination with another phosphate

signal, induces chromatin condensation and chromosomal segregation (Wei Y, et al. 1999).

- **Ubiquitination**: The process of ubiquitin (Ub) transfer can be simple or can form a chain of Ub molecules on a residue of a target protein. Ub is a protein of 76 amino acids unlike the rest of the small molecules that intervene in the processes of histone modification. This modification, as a general rule, has the specific function of inducing histone degradation through the proteasome. Histones H2B can be modified by a single molecule of ubiquitin, while H2A can be poly-ubiquitinated. Experimental evidence suggests that histone ubiquitination may have a direct role in transcriptional regulation if they are found in association with methylation marks (Alberts B, et al. 2002).
- **Sumoylation**: binding of a small ubiquitin-related modifier protein (SUMO) to a histone lysine residue. In the nucleosome, sumoylation would oppose acetylation, classifying this modification between those inhibitors in transcriptional terms (Iñiguez-Lluhí JA, 2006).
- **ADP-ribosylation**: addition of one or more ADP-ribose molecules to an amino acid residue. This reaction is catalyzed by the enzyme poly-ADP-ribose-polymerase (PARP), mainly affecting histones H1 and, to a lesser extent, H2Bs promoting the decondensation of chromatin. The purpose of this modification is, therefore, to impose an accessible transcriptional structure on chromatin (Dantzer F, et al. 1998; Kraus WL, et al. 2003).

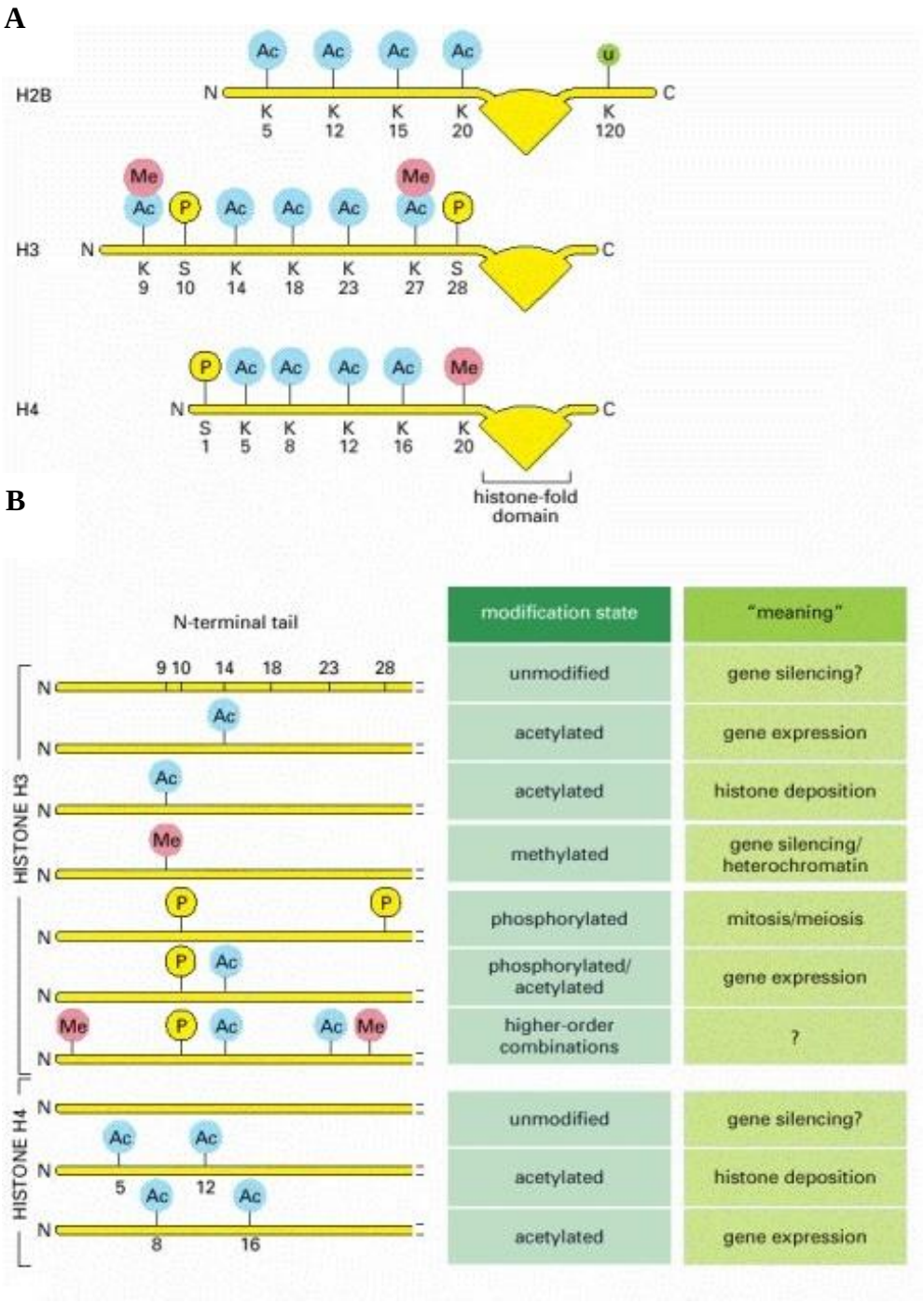


Figure 1.13: Histone modifications that modulate the chromatin condensation state and their effect on gene expression. *(**A**) The image shows the possible modifications of the four core histones with the following nomenclature: Me = methyl group, Ac = acetyl group, P = phosphate, u = ubiquitin. (**B**) The hypothesis of the histone code. Histone tails can be marked by different combinations of modifications. According to this hypothesis, each label confers a specific meaning to the piece of chromatin in which it takes place. The local structure of the chromatin also determines the different effects of these modifications. For example, the phosphorylation in position 10 of histone H3 is associated not only with the condensation of the chromosomes during mitosis or meiosis but also with the expression of certain genes too. Adapted from: Molecular Biology of the Cell. 4th edition.*

1.4.3 The Polycomb repressor Complex (PRC)

Polycomb group (PcG) proteins were originally identified in *Drosophila* as important regulators of fly development. Indeed, these proteins drive the spatiotemporal expression pattern regulation of important transcription factors, including *Hox* genes. *Drosophila* PcG mutants showed characteristic phenotypes with body segmentation defects (Laugesen and Helin, 2014) and murine models PcG - / - are characterized by high embryonic mortality (Aloia L, et al. 2013). PcG proteins assemble into large multimeric protein complexes, of which the best-described ones are Polycomb repressive complexes 1 and 2 (PRC1 and PRC2). PRC1 is a ubiquitin Ring Finger E3 ligase complex which includes several subunit proteins, BMI1, RING1 A/B, RING2, and CBX proteins, and catalyzes the monoubiquitylation toward lysine 119 of histone H2A (H2AK119ub1) (Margueron and Reinberg, 2011; Gao Z, et al. 2012). On the other hand, PRC2 is a methyltransferase with activity toward the lysine 27 of histone H3 (H3K27)., The SET-domain-containing components of PRC2 *enhancer of zeste homolog* 1 or 2 (EZH1 or EZH2) are closely associated with several other subunits. The catalytic core of this complex consists of EZH1/2, the Zinc-finger protein SUZ12, and the WD40 protein EED (Cao R, et al. 2002; Kuzmichev, et al. 2002; Pasini D, et al. 2004; Smits, et al. 2013). This core complex is also associated with additional proteins (RBBP4/7, JARID2, AEBP2, PCL1-3, C17orf96, and C10or12) (Smits AH, et al. 2013) as well as with ncRNAs (Blackledge NP, et al. 2015). Different interaction partners are thought to play roles in the regulation of PRC2 activity or the recruitment to target genes (Di Croce L and Helin K, 2013). Even though the mechanisms governing the interplay of mammalian PRCs are not fully delineated, it has been demonstrated that they preferentially bind CpG-rich sequences (Mendenhall EM, et al. 2010; Wachter E, et al. 2014).

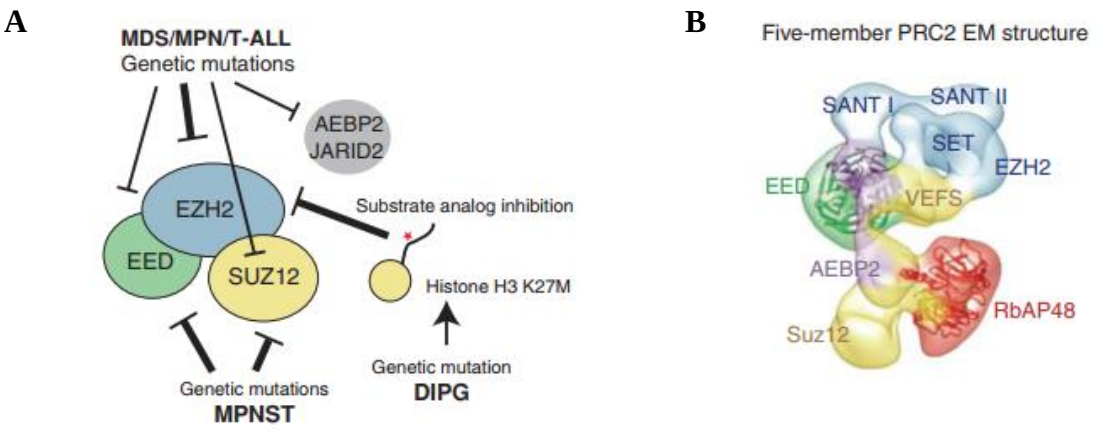


Figure 1.14: The Polycomb 2 repressive complex. (A) *Mutations conferring loss of PRC2 function are recurrent in several cancer types. The mutations are found in the genes coding for PRC2 core subunits or substoichiometric interaction partners, as well as in the genes coding for H3.1 and H3.3. Adapted from: Laugesen A, et al. 2016.* ***(B)*** *Electron micrograph (EM) structure of the PRC2 complex with RBBP4 (RBAP48) and AEBP2 (EMDataBank 2236, deposited image). Adapted from: Ciferri C, et al. 2012.*

Given that both PRC1 and PRC2 act by condensing the chromatin, they are considered transcriptional repressors. Most of the binding patterns of these complexes overlap throughout the genome and their functions are strictly related, as soon as the enzymatic activity of each of them influences the binding capacity to the chromatin of the other. This tight relation between PRC1 and PRC2 establishes a transcriptionally repressed status of the genes (Boyer LA, et al. 2006; Bracken AP, et al. 2006). The mechanism involved in the recruitment of the Polycomb chromatin complex formation is largely attributed to a "hierarchical model". This model asserts that the recruitment of PRC2 to the target sites promotes the trimethylation of the H3K27, which is then recognized by the PRC1 complex that subsequently promotes the H2AK119 monoubiquitination. However, recently, it has been proposed an alternative route of the "hierarchical model" by which PRC1 recruits PRC2 to the chromatin. Despite the mechanism by which PRC1 monoubiquitylates de novo the H2AK119 is not clear, this model proposes that PRC2 is recruited to the chromatin by the recognition of H2AK119ub1. Consistent with this mechanism, perturbations of PRC1 result in loss of H2AK119ub1, decreased binding of PRC2 and lower levels of H3K27me3 accumulation. However, the observations that some PRC2 target genes lack the

H2AK119Ub, and conversely, some genes have been found targeted by PRC1 in the absence of PRC2 (Schoeftner S, et al. 2006) suggest that relationship between PRC1 and PRC2 is regulated by more complex mechanisms than the above-described models (Blackledge NP, et al. 2014).

The lysine 27 on histone H3 methyltransferase EZH2 is crucial in the regulation of gene expression, and its aberrant activity is linked to the onset and progression of cancer (Gan L, et al. 2018) as well as increased risk of metastasis and recurrence (Alford SH, et al. 2012; Min J, et al. 2010). Diverse compounds have been developed in order to target this enzyme, among them, GSK126 is a cofactor S-(S′-adenosyl)-l-methionine (SAM)-competitive EZH2 methyltransferase inhibitor. SAM is a universal methyl donor for catalytic reactions of histone methyltransferases. Notably, GSK126 has a specific effect over EZH2, with a Ki value of ~0.5 nM which is > 150 folds smaller than that for EZH1 (McCabe MT, et al. 2012). Supported by these in vitro data, GSK126 is now being tested in phase I clinical trial for relapsed/refractory diffuse large B cell lymphoma, transformed follicular lymphoma, other non-Hodgkin's lymphomas, solid tumors and multiple myeloma (NCT02082977, https://clinicaltrials.gov/). The knockdown of EZH2 in prostate cancer cell lines results in decreased cell growth and invasion (Chinaranagari S, et al. 2014). EZH2-mediated transcriptional repression of putative tumor suppressors, such as E-cadherin (Tiwari N, et al. 2013), via an increase in H3K27me3 levels depends on the SET domain, methyltransferase activity, and histone deacetylase activity (Cao Q, et al. 2008). Augmented EZH2-dependent histone methylation is concomitant with decreased histone acetylation, further contributing to increased repressive histone marks, such as the H3K27me3 (Yuan W, et al. 2012).

Current evidence demonstrates that the PCR2 component EZH2 takes part in diverse biological processes and displays different mechanisms of action. It is considered a dual-faced molecule acting in a cell-dependent fashion both as a transcriptional suppressor or co-activator, depending on its interaction with other PRC2 components. Apart from its histone methyltransferase (HMT) catalytic function carried out in the nucleus, EZH2 acts also in the cytosol as a methyltransferase of non-histone protein. EZH2 directly methylates the cardiac transcription factor GATA4 at Lys299 attenuating its p300-mediated acetylation and therefore repressing its transcriptional activity (He A, et al. 2012). Similarly, EZH2 methylates and counteracts the activity of RAR-relate

orphan receptor alpha (RORα) (Lee JM, et al. 2012) and Talin protein which are key factors in the regulation of cell migration (Gunawan M, et al. 2015). Besides the well studied nuclear activity of EZH2, it has been described also its cytoplasmic function on non-histone proteins and in a PRC2-independent manner. Recently, it has been shown that EZH2 is a substrate of the enzymatic activity of AKT, and that the AKT-dependent phosphorylation of EZH2 at Ser21 is critical to promote Signal Transducer And Activator Of Transcription 3 (STAT3) transcriptional activation (STAT3) in glioblastoma and glioblastoma stem-like cells (GSCs) (Kim E, et al. 2013). In addition EZH2 acts as co-activator for a series of other transcription factors such as the androgen receptor (AR)-associated protein complex (Xu K, et al. 2012), NF-κB, TCF/β-catenin, PCNA complex (Lee ST, et al. 2011), β-catenin and ERα complex (Shi B, et al. 2007; Jung HY, et al. 2013).

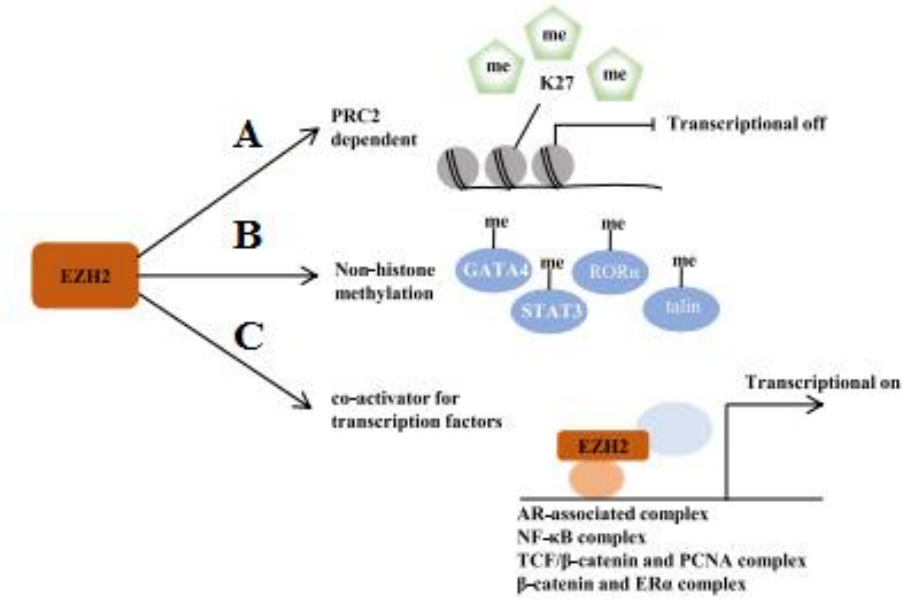


Figure 1.15: The action modes of EZH2. *(**A**) EZH2 catalyzes H3K27me3 dependent on PCR2, which contributes to transcriptional silencing. (**B**) EZH2 is also capable of methylating several non-histone protein substrates (e.g. STAT3, GATA4, talin, and RORα), which contributes to both transcriptional silencing and transcriptional activation. (**C**) EZH2 also has a PRC2-independent role in transcriptional activation, acting as co-activator for transcription factors, such as AR-associated complex, NF-κB signaling, TCF/β-catenin, and PCNA, and β-catenin and ERα. Adapted from: Gan L, et al. 2018.*

1.4.4 Demethylase Jumonji domain-containing 3 (JMJD3)

The proteins JMJD3 (Jumonji domain-containing 3 KDM6B) and UTX (Ubiquitously Transcribed X-Chromosome tetratricopeptide) are two epigenetic modulators that catalyze the demethylation reaction of the H3K27 residues (Agger K, et al., 2007). After their discovery in 2007, the role of these two demethylases has been deeply studied due to their involvement in various physiological processes such as embryonic development, cell plasticity and the regulation of the immune response as well as in some neurodegenerative diseases and specific types of cancer (Burchfield JS, et al. 2015). JMJD3 is a protein of 1697 amino acid, that owes its name to the presence of a Jumonji C domain (JmjC), that, catalyzes the demethylation of H3K27me2 / 3 (Klose RJ, et al. 2006), For this reason is defined as an activator of the gene expression (Barski A, et al. 2007). Different genetic sequencing studies based on chromatin immunoprecipitation (ChIP-Seq) carried out in murine macrophages, have solidly demonstrated that JMJD3 is associated to fragment of DNA characterized by low occupancy of the chromatin repressor mark H3K27me3 (De Santa F, et al. 2009). JMJD3 is expressed in multiple tissues, and its activity is crucial during the first phases of the morphogenic development of the three germinal layers. In adults, it contributes to a greater extent to the definition of terminal cell differentiation processes (Burchfield JS, et al. 2015). In the case of thymocytes differentiation, the demethylase JMJD3 does not seem to have a critical specific role, at least during the double-positive phase (see point 1.2.3), while it seems to play a role in the SP CD4 + specification. It has been shown that JMJD3 silencing prevents the expression of proteins necessary for the last phases of the thymocyte specification, such as S1pr1 (sphingosine-phosphate receptor), which is involved in extra-thymic migration of mature thymocytes (Manna S, et al. 2015). In addition, JMJD3 deletion induces the preferential differentiation of CD4 + cells towards T-helper2 (Th2) and Th17 lines, at the expense of Th1 thymocytes (Li Q, et al. 2014). In the pathological contexts, JMJD3 plays oncogenic or oncosupressive function depending on the tumor tissue. Indeed, it exerts anti-cancer activity in transformed diploid fibroblasts by demethylating the INK4A-ARF gene locus, thus inducing senescence and death of tumor cells (Agger K, et al. 2009). Supporting its oncosuppressive role, following DNA damage JMJD3, is recruited to the p53 responsive elements by interacting with p53 and allowing p53 transcriptional activity (Williams K, et al. 2014). Conversely, in T-ALL, JMJD3 is involved in the onset and progression of this disease, therefore, acting as an oncogene.

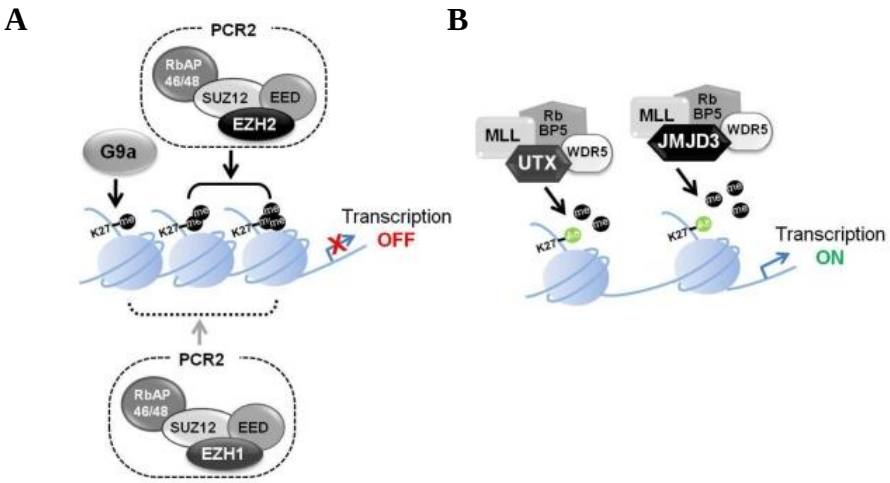


Figure 1.16: Current model of transcriptional regulation by PRC2 and JMJD3. *(**A**) PRC2 represses gene transcription through its catalytic subunits EZH1 and EZH2. The PRC2 complexes and G9a methyltransferase compact chromatin and silence genes through the methylation of K27 lysine residues of H3. (**B**) Conversely, UTX, and JMJD3, in complex with MLL, RbBP5, and WDR5, promote the relaxation of the chromatin-histone structure by demethylating H3K27me3 and therefore promote the gene transcription. Adapted from: Yoo KH, Hennighausen L. 2012.*

1.4.5 Epigenetic regulation of Notch signaling

The recruitment of epigenetic modulators, including acetyltransferases (HATs), deacetylases (HDACs), lysine-methyltransferases (KMTs) and demethylases over histones is a highly controlled process, that facilitates either gene expression or repression (Yun M, et al. 2011). Several pathways have been demonstrated to influence the chromatin landscape by activating transcription factors which DNA-binding activity is critical to the recruitment of chromatin modifiers. During the last decade, many studies have shown that the interplay between Notch and histone modification/modifiers on the regulatory regions of Notch target genes is critical to promote Notch transcriptional program. In the absence of Notch signaling activity, CSL is bound to the regulatory regions of its target genes, including promoters and distal enhancers together with a set of epigenetic repressors such as HDAC, SKIP (Ski-Interacting Protein) and SHARP (SMRT and HDAC Associated Repressor Protein) (Borggrefe T, et al. 2009; Borggrefe T, et al. 2012). Notch signaling activation leads to NICD nuclear translocation, which results in the displacement of the corepressors and the recruitment of coactivators such as GCN5 / PCAF, MAML and p300 / CBP (Creb Binding Protein) on the regulatory gene regions. In this scenario, CSL changes its function from a

transcriptional repressor to activator in a Notch-dependent fashion (Bray SJ, et al. 2006). The following figure 1.17 represents the protein interactions affecting the Notch target genes expression.

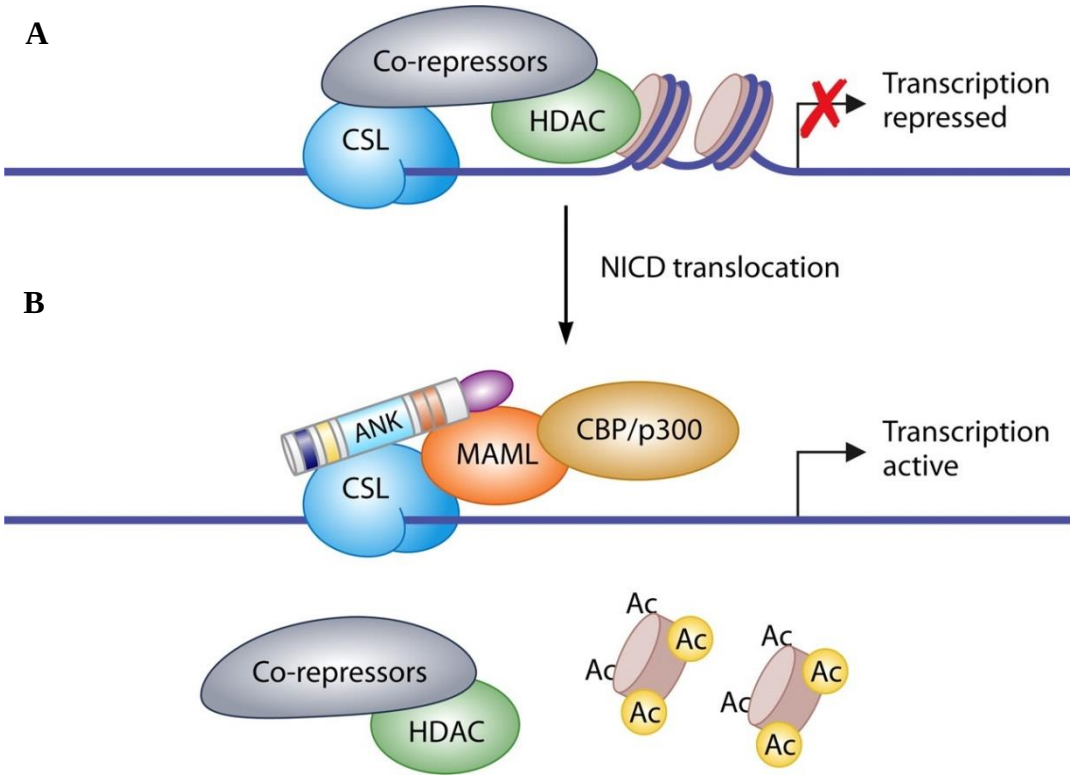
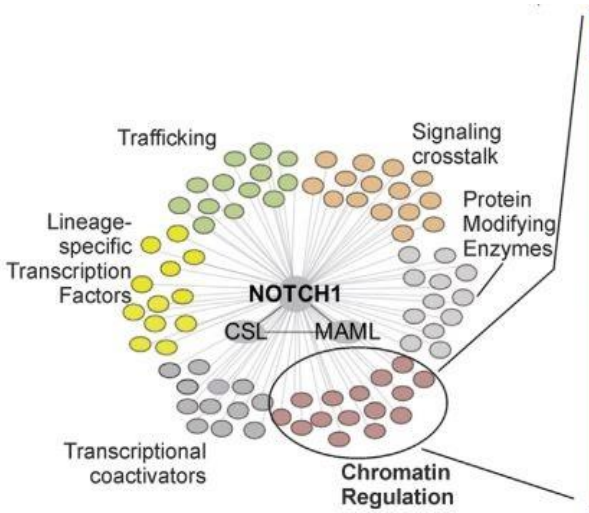


Figure 1.17: Activation of transcription by the Notch intracellular domain (NICD). (A) *Under basal, unstimulated conditions, the absence of NICD permits CSL to maintain its inhibitory effect. The corepressors of transcription are bound to the CSL and recruit histone deacetylase (HDAC) to suppress transcription.* ***(B)*** *Following nuclear translocation of NICD (see* ***Fig. 2*** *for domain organization) into the nucleus, a ternary complex composed of NICD, CSL, and Mastermind-Like (MAML) is formed. This complex displaces the corepressors and the HDAC, occasioning in the recruitment of CBP/p300, acetylation (Ac) of histones, and gene expression. Adapted from: Zanotti S, Canalis E, 2009.*

The basal transcriptional machinery driven by Notch is sustained by the coordinate recruitment of the NICD / MAML1 /RBP-J ternary complex at the regulatory regions of its target genes in combination with the acetyltransferases p300 / CBP and PCAF that results in the transcriptional activation of the chromatin (Wallberg AE, et al. 2002; Jin Q, et al. 2010). However, it is not clear whether acetylation is the direct cause of the increased transcription of a gene, or is instead the result of its over-expression. In Drosophila, it has been demonstrated that Bre1 protein, a direct partner of N1ICD, is critical for the monoubiquitination of histone H2B at the K123 and, indirectly, for the H3K4 trimethylation is necessary for the transcription of Notch target genes (Bray SJ, et al. 2005; Vitaliano-Prunier, et al. 2008).



	Gene symbol (Protein*)	Description
Chromatin regulation	*SMARCA4* (BRG1)	ATP-dependent helicase BRG1 (component of the PBAF complex)
	PBRM1 (PB1)	Polybromo-1/BAF180 (component of the PBAF complex)
	SMARCC2 (BAF170)	SWI/SNF complex subunit BAF170 (component of the PBAF complex)
	SMARCC1 (BAF155)	SWI/SNF complex subunit BAF155 (component of the PBAF complex)
	HLTF	Helicase-like transcription factor (SWI/SNF-related)
	SMARCA5 (SNF2H)	Sucrose nonfermenting protein 2 homolog (SWI/SNF-related)
	SMC1A	Structural maintenance of chromosomes protein 1A (component of cohesin complex)
	SMC3	Structural maintenance of chromosomes protein 3 (component of cohesin complex)
	PDS5A	Sister chromatid cohesion protein PDS5A (component of cohesin complex)
	KIAA0892 (MAU2)	Cohesin loading complex subunit MAU2 (also known as SCC4)
	SMCHD1	Structural maintenance of chromosomes flexible hinge domain-containing protein 1
	TRIM28	Tripartite motif-containing protein 28 (also known as KAP1)
	WIZ	Widely-interspaced zinc finger-containing protein
	RB1	Retinoblastoma-associated protein
	PHF8	Histone lysine demethylase PHF8
	RNF40	E3 ubiquitin-protein ligase BRE1B
	CHD4	Mi2-beta. Component of the histone deacetylase NuRD complex
	RBBP4	RB-binding protein 4. Component of the histone deacetylase NuRD complex
	GATAD2B	GATA domain-containing 2B. Component of the histone deacetylase NuRD complex
	HDAC1	Histone deacetylase 1. Component of the histone deacetylase NuRD complex
	KDM1 (LSD1)	Lysine-specific histone demethylase 1A
	RING1	histone H2A ubiquitin ligase RING1. Component of the PRC1 complex
	RNF2	histone H2A ubiquitin ligase RNF2. Component of the PRC1 complex

Figure 1.18: Interaction network of ICN1-associated proteins identified by Mass Spectrometry (MS). *The study conducted by Yatim et al. revealed that Notch partners in the context of T-ALL were related to lineage-specific transcription factors, crosstalk signaling, protein-modifying enzymes, transcriptional regulators, and epigenetic modulators* (*Yatim A, et al. 2012). Adapted from: Schwanbeck R, 2015.*

Several independent studies conducted on murine experimental models demonstrated the importance of the H3K4me3 signal in the specific genetic response of the Notch signal. During the Notch induction, H3K4 tri-methylation undergoes a high fluctuation in the binding sites of RBP-J due in part to the action of the demethylase KMD5A (Liefke R, et al. 2010). Schwanbeck and colleagues found a 95% correlation between the expression Notch direct target genes and the H3K4me3 modifications at their promoter regions in ESC (Meier-Stiegen F, et al. 2010; Schwanbeck R, et al. 2011). In addition, they found that several Notch-regulated genes poised for activation show high levels of occupancy of bivalent domains H3K4me3 / H3K27me3 the regions of these promoters in combination with the binding of the repressor complex PRC2 (Schwanbeck R, et al. 2011). These data suggest the NICD / MAML / RBP-J complex could play a crucial role in the resolution of the bivalent state of these domains.

The NAD+-dependent deacetylase and gene repressor SIRT1 erases histone H4K16 acetylation marks and permits heterochromatin formation. Mulligan and colleges described a SIRT1 corepressor complex containing the histone H3K4 demethylase LSD1/KDM1A and several other LSD1-associated proteins. SIRT1 and LSD1 interact directly and play conserved and concerted roles in H4K16 deacetylation and H3K4 demethylation to repress genes regulated by the Notch signaling pathway (Mulligan P, et al. 2011). The intracellular domain of Notch, when it is translocated into the nucleus, is able to displace the repressor complex formed by RBP-J / PRC / HDACs / SIRT1 / LSD1 / KDM5 (Liefke R, et al. 2010). LSD1 recognizes and demethylates mono- and dimethylated H3K4 variants at the promoters of Notch targets while the demethylase KDM5A / Lid specifically recognizes H3K4me2 / 3 (Shi Y, et al. 2004). This suggests that these two enzymes may act in concert in order to convert H3K4me3 to the unmethylated state in a step-wise manner (Mulligan P, et al. 2011) (figure 1.19). The SIRT1 complex also has a deacetylase function of H4K16 and it can interact with the methyltransferase of H3K9 SUV39H1, histone H1 and with PRC2, leading to the formation of facultative heterochromatin (Vaquero A, et al. 2007).

In T-ALL, the induction of Notch1 antagonizes the activity of PRC2 resulting in the reduction of the inhibitory mark H3K27me3 in the promoters of its target genes. EZH2 and SUZ12 have been branded as gene suppressors, which turn out to be mutated in about 25% of the T-ALL samples analyzed. Added to this, the coupling of Notch1 to the Hes1 promoter region leads to the displacement of EZH2 and SUZ12 (Ntziachristos P,

et al. 2012). Another potential mechanism by which Notch is able to interact with chromatin to reshape it is through the NICD itself. We know that the intracellular domain contains a region of ankyrin repeats that turns out to be part of the binding center of RBP-J, MAML and NICD (Del Bianco et al., 2008). It has been demonstrated how Notch's ankyrin repeats interact with mono- or dimethylated lysines of H3K9 histone residues (Kutateladze TG, 2001; Schwanbeck R, 2015).

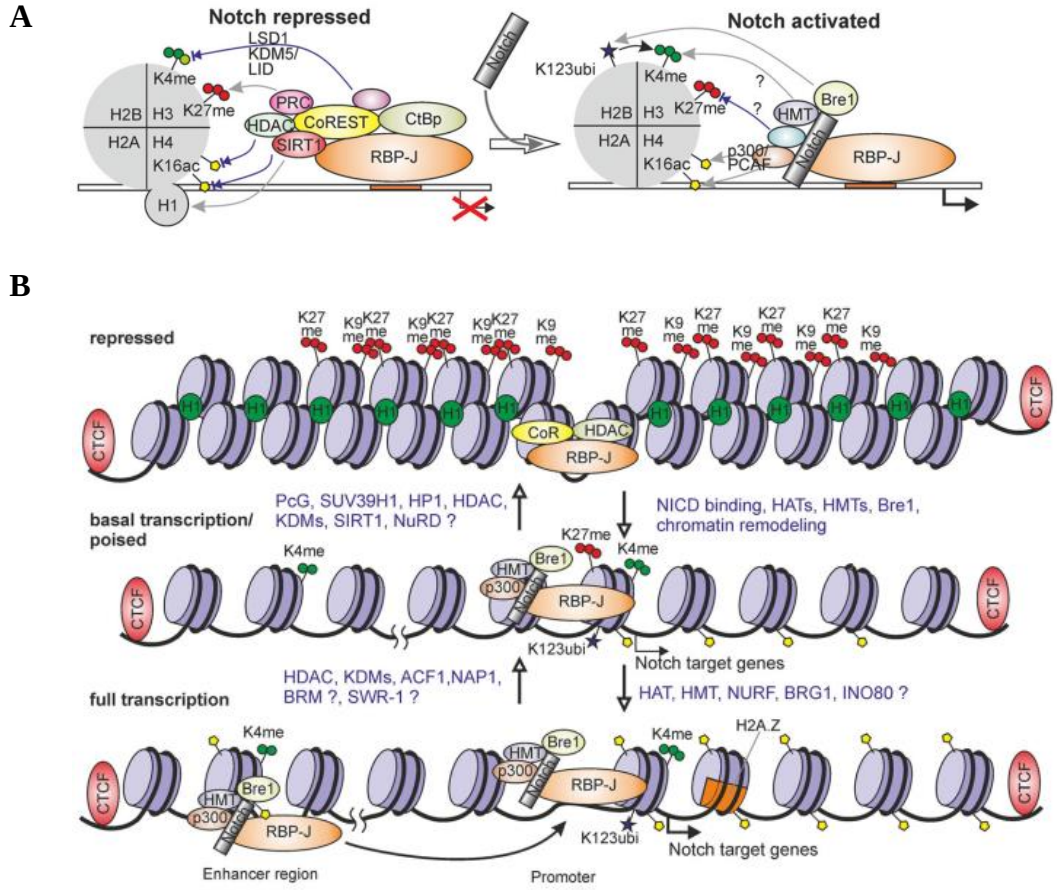


Figure 1.19: Notch signaling in the chromatin context. (A) *In the absence of NICD, the RBP-J / PRC / HDACs / SIRT1 / LSD1 / KDM5 complex is blocking the Notch target genes by adhesion of repressive marks. When NICD translocates in the nucleus, it displaces all repressors and recruits coactivators such as Bre1, HMTs, and p300 / PCAF, resulting in positive regulation of Notch target genes by monoubiquitination, acetylations and lysine-activating methylations.* ***(B)*** *The basal transcription of the Notch targets can be modulated by the histonical repression marks associated with H1 and the methylation of the DNA produced by a set of chromatin-modifying enzymes or by a looser state of the chromatin caused by histonical marks, activators and the variable H2A.Z near the promoter or the enhancer region. The activity of this enhancer region is determined and limited by the CTCF binding sites. Adapted from: Schwanbeck R, 2015.*

1.4.6 Epigenetic control of Notch

In addition to its role as an "effector" of epigenetic modifications on the regulatory region of its target genes, several epigenetic mechanisms have been identified to regulate the transcription of Notch genes. H3K4me3 and H3K9/14ac accumulate in combination with the transcriptional activation complex at the regulatory region of the Notch1 gene in cell contexts in which it is actively transcribed (Guenther MG, et al. 2007). In double-positive pre-leukemic cells derived from the murine model knock out for Ikaros ($Ikzf1^{-/-}$). N1ICD accumulation is associated with the progressive development of a tumorigenic phenotype (figure 1.20 A) (Gómez-del Arco P, et al. 2010). The absence of Ikaros results in the accumulation of the epigenetic marks H3K4me3 and H3K9 / 14ac at the intragenic region of the exon 25 and at a region upstream the promoter of the canonical transcript of Notch1. Both these Notch1 gene regions act as promoters for the expression of two alternative transcripts that encode ligand-independent constitutive active Notch1 receptors that lack the N-terminal peptide. (Gómez del Arco P, et al. 2010). Consistently, it has been shown that the loss of the first exon/promoter of Notch1 accelerates leukemogenesis in mice. In addition, more than two-thirds of the tumors induced by loss of function of Ikaros are characterized by the accumulation of this Notch1 truncated isoform that is susceptible to the proteolytic cleavages S2 and S3 but the activation is ligand-independent. The alternative transcription of the Notch1 receptor seems to be promoted by the acetylation of histones H3 at the 3 'end of the gene. The strong acetylation of the gene allows the expression of this truncated form only in the thymus, so these data define a specific origin of the process of leukemogenesis in terms of transcriptional and tissue regulation (Jeannet R, et al. 2010). The following figure 1.20 B describes the alternative isoforms of the Notch1 receptor transcribed from the different described promoters.

In addition to the described mechanism of regulation, the demethylase JMJD3 has been found highly expressed in T-ALL when compared with physiological T-cell subsets or other leukemic subtypes and it has been demonstrated to sustain the expression of the oncogenic transcriptional program of Notch (Ntziachristos P, et al. 2014; Tottone L, et al. 2019). Notably, former studies have unveiled that the epigenetic modifications occurring at *Notch3* gene locus drive its transcription in leukemia. It has been shown that it is hypermethylated in not expressing *Notch3* B-ALL samples, while unmethylated in T-ALL context in which is highly expressed (Kuang SQ, et al. 2013).

Moreover, it has been demonstrated that the binding of BORIS/CTCFL at its *gene locus* is necessary to sustain high levels of H3K4me3 and gene expression in cancer cells (Zampieri M, et al. 2014) and that *NOTCH3* gene expression has been found associated with high occupancy of H3K27ac at its enhancer region in T-ALL cells (Yatim A, et al. 2012; Wang H, et al. 2014).

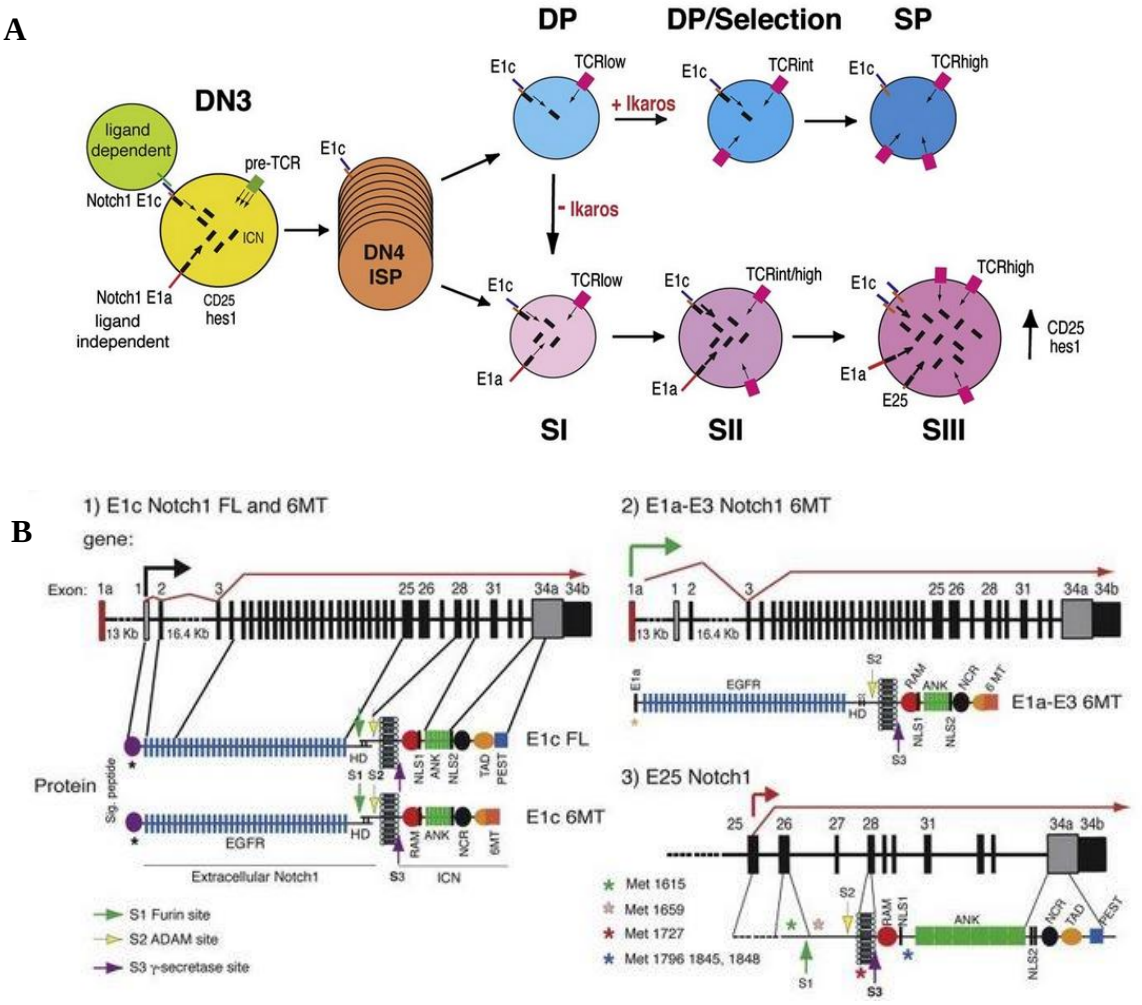


Figure 1.20: Expression of Notch1 transcripts during T cell development and leukemogenesis and the ligand-independent signaling provided by alternative Notch1 isoforms. *(**A**) The relevance of Notch during the development of T cells and the stages in which Ikaros is crucial. The expression and signaling of the canonical and alternative forms of Notch1 are present during the transition of the DN3-SP phases and during the process of leukemogenesis. (**B**) Composition of the exons and protein domains of the canonical (E1c FL) and alternative (E1a-E3 and E25) forms of the Notch1 transcripts. Panel 1 presents an isoform lacking the PEST domain (E1c 6MT). In panel 2, this mutated isoform E1a-E3 Notch1 6MT also lacks the signal peptide that localizes the receptor in the membrane. Panel 3 represents the alternative transcript of the Notch1 receptor from exon 25, absolutely devoid of the membrane location signal peptide, of the ligand-dependent domain and of the PEST domain. The truncated isoforms of panels 2 and 3 are considered constitutively active since their activation is ligand-independent. Adapted from: Gómez-del Arco P, et al. 2010.*

1.3.7 Misregulation of EZH2 in cancer

Varambally and colleagues firstly elucidated the association between EZH2 and prostate cancer prognosis, describing that the upregulation of EZH2 is associated with advanced stage and poor prognosis of the disease (Varambally S, et al. 2002). Subsequently, other studies have unveiled EZH2 upregulation in additional solid tumors including lung, hepatocellular, colorectal (CRC), breast, cervical (CC) or pancreatic cancers, and indicated the prognostic significance of its deregulation in the disease progression (Pasini D, 2016; Puppe J, et al. 2019; Yamagishi M, et al. 2017). The biological functions of EZH2 in diverse types of tumor contexts are under deep investigation. However, it has been suggested that EZH2 can be considered as a tumor suppressor gene or as an oncogene depending on the tumor context, indicating that the critical consequences of its dysregulation are strictly dependent on the tissue context (Veneti Z, et al. 2017). In the following figure 1.21, the mutations corresponding to subunits of the PRC2 complex associated with different types of cancer are compiled.

Among the many roles of EZH2 in cancer, it is also involved in the regulation of cell cycle progression, and consistently, dysregulation due to mutations accelerates cell proliferation and promotes survival, thus resulting in tumor development. EZH2 inhibitor 3-Deazaneplanocin A (*DZNep*) or gene knock-down, induces autophagy, cell cycle arrest in G1 phase and apoptosis in many different cell contexts such as CRC, melanoma, breast cancer or cholangiocarcinoma (Nakagawa S, et al. 2013; Zingg D, et al. 2015; Mahara S, et al. 2016; Yao Y, et al. 2016).

Gene	Cancer	Notes	References
Hyperactivating mutations			
EZH2	DLBCL	Y646N/F/S/H/C A682G A692V	Morin et al. 2010, 2011; Lohr et al. 2012; McCabe et al. 2012a; Okosun et al. 2014
	FL	Y646N/F/S/H/C A682G A692V	Morin et al. 2010, 2011; Bodor et al. 2011, 2013; McCabe et al. 2012a; Okosun et al. 2014
	NHL ("other")	Y646F/S A692V	Morin et al. 2011
	B-ALL (patient-derived cell lines)	Y646N A692V	Ott et al. 2014
	Sporadic parathyroid adenomas	Y646N	Cromer et al. 2012
	Melanoma	Y646N/F/S	Hodis et al. 2012; Krauthammer et al. 2012; Alexandrov et al. 2013; Zingg et al. 2015
Loss-of-function mutations			
EZH2	Myeloid malignancies, including MDS, MPN, MF, CMML, and AML	Correlated with poor survival	Ernst et al. 2010; Nikoloski et al. 2010; Abdel-Wahab et al. 2011; Bejar et al. 2011; Guglielmelli et al. 2011; Jankowska et al. 2011; Score et al. 2012; Muto et al. 2013; Lindsley et al. 2015;
	T-ALL		Ntziachristos et al. 2012; Neumann et al. 2015
EED	MDS/MPN		Score et al. 2012
	MPNST		De Raedt et al. 2014; Lee et al. 2014
	GBM		De Raedt et al. 2014
	Melanoma		De Raedt et al. 2014
SUZ12	MDS/MPN		Score et al. 2012
	T-ALL		Ntziachristos et al. 2012; Simon et al. 2012; Neumann et al. 2015
	MPNST		De Raedt et al. 2014; Lee et al. 2014; Zhang et al. 2014
	GBM		De Raedt et al. 2014
	Melanoma		De Raedt et al. 2014
JARID2	MDS/MPN		Puda et al. 2012; Score et al. 2012
	T-ALL		Simon et al. 2012
AEBP2	MDS/MPN		Puda et al. 2012

Figure 1.21: PRC2 subunits mutations involving different types of cancer. *(A) Abbreviations: AML, Acute myeloid leukemia; B-ALL, B-cell acute lymphoblastic leukemia; CMML, chronic myelomonocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; GBM, glioblastoma multiforme; MDS, myelodysplastic syndrome; MF, myelofibrosis; MPN, myeloproliferative neoplasm; MPNST, malignant peripheral nerve sheath tumor; NHL, non-Hodgkin lymphoma; T-ALL, T-cell acute lymphoblastic leukemia. Adapted from: Laugesen A, et al. 2016.*

EZH2 overexpression was found closely associated with the carcinogenesis and poor prognosis of cervical cancer and its overexpression is strictly related to clinical stage, poor differentiation, infiltration depth, lymph node metastasis, and lower patient survival (figure 1.22) (Yueyang L, et al. 2014). While benign epithelial cells are characterized by low EZH2 expression, increased expression of this lysine methyltransferase is associated with poor prognosis in solid tumors of the uterine cervix, prostate, breast and bladder (Liu Y, et al. 2014; Varambally S, et al. 2002; Dong M, et al. 2014; Martínez-Fernández M, et al. 2015). Despite several studies indicate that EZH2 induces the aggressiveness and invasiveness of tumor cells in different cancer types, key molecular aspects of its function remain to be clarified. It has been demonstrated that EZH2 mediates the transcriptional silencing of the tumor suppressor gene E-cadherin (Cao Q, et al. 2008) suggesting that the interplay between EZH2 and E-cadherin may be a potentially effective target for developing novel anti.-cancer strategies. Similarly, since the chemical inhibition of the histone deacetylases prevents the transcriptional repression induced by EZH2, the interplay between epigenetic modulators such as EZH2, JMJD3, and HDACs could be investigated to design novel therapeutic approaches in this malignancies (Cao Q, et al. 2008). Confirming the critical role of EZH2 in tumor progression, its silencing by a small interfering RNAs (siRNAs) counteracts the proliferation of cervical, pancreatic, breast and prostate cancer cells as well as lung adenocarcinoma cells (Liu Y, et al. 2014; Mallen-St, et al. 2012; Ren G, et al. 2012; Lv Y, et al. 2012), while conversely, its overexpression promoted EMT in tumoral epithelial cells. In this regard, Qi Zhang and colleges demonstrated that the EZH2 silencing, as well as its chemical blockade with EZH2-inhibitors GSK126 or DZNep in endometriotic epithelial cells, decreased levels of EMT-related transcription factors such as Snail and Slug and mesenchymal markers including Vimentin, N-cadherin, Fibronectin and PAI-1, while conversely resulted in increased level of expression of E-cadherin (Zhang Q, et al. 2017). A recent study described that EZH2 expression gradually increases during the progression of cervical cancer and that it acts as an oncogene accelerating the cell cycle transition from the G0/G1 to S phase through activating the Wnt/β-catenin pathway (Chen Q, et al. 2016). Similarly, it has been indicated as a poor prognostic factor in different other malignancies including gallbladder adenocarcinoma, gastric and bladder cancers (Liu D-C, et al. 2011; Lee H, et al. 2012; Wang H. et al 2011; Liu Y, et al. 2014).

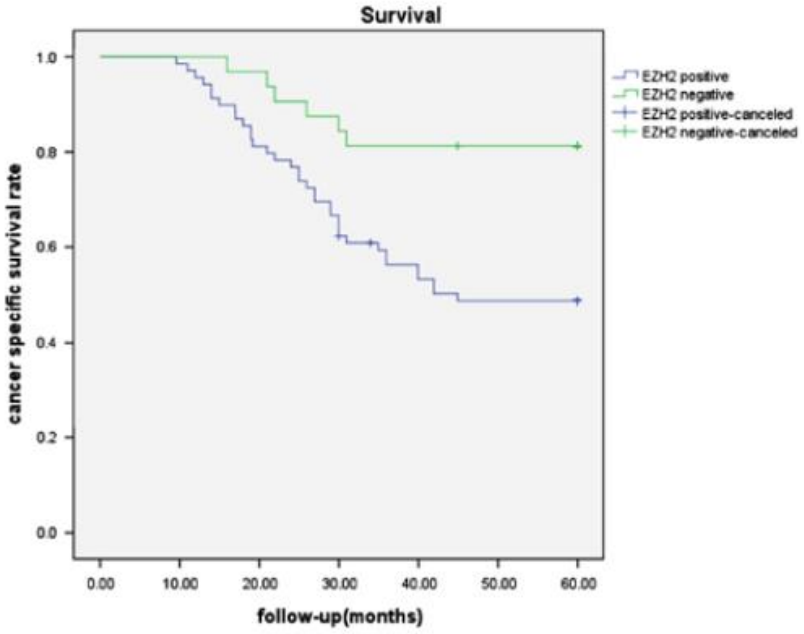


***Figure 1.22**: Kaplan-Meier survival curve analysis indicating how cervical cancer patients with EZH2 increased expression present a worse overall survival rate than those with a negative expression of EZH2. Adapted from: Liu Y, et al. 2014.*

Persistent human papillomavirus (HPV) infection leading to dysplasia and tumorigenic cervical lesions has been associated with high levels of EZH2 expression (Holland et al. 2008). HPV has shown to play a role in the aberrant expression of EZH2, as HPV viral oncoprotein E7 directly induces EZH2's upregulation by interacting with pathways responsible for EZH2 transcription (Holland D, et al. 2008; Hyland PL, et al. 2011). Sartor and colleagues reported strongly methylated promoter regions of PRC2 targets in HPV-positive cell lines relative to the HPV-negative variants, indicating enhanced activity of this methyltransferase (Sartor MA, et al 2011). Assumed the evidence of EZH2 potential in the control of CpG methylation through direct physical contact with DNA methyltransferases (DNMT), EZH2 is considered as an attractive chemotherapeutic target for both HPV-positive and HPV-negative squamous cell carcinoma (SCC) (Vire E, et al. 2006; Lindsay CD, et al. 2017).

In addition to this, recent studies also revealed that aberrant EZH2 expression might have critical roles in tumor immunity modulation and immunotherapy response. Peng et al. demonstrated how abnormal expression and activity of EZH2 in immune cells within

the tumor microenvironment affect tumor progression and therapy (Peng D, et al. 2015). Former evidence has also shown that EZH2 has direct roles in T cell response. EZH2 expression in naïve T cells promotes survival, proliferation, and function of effector CD4+ and CD8+ T cells but inhibits Th1 and Th2 differentiation (Karantanos T, et al. 2016). Moreover, EZH2 is believed to be critical for the recruitment and immunosuppression function of activated regulatory T cells (Tregs) at the sites of inflammation, as soon as EZH2-deficient Tregs fail to protect mice from the development of autoimmunity in a model of naïve T cell-mediated colitis (Long H, et al. 2016; DuPage M. et al. 2015). Yin et al. have recently provided insight into an epigenetic-based mechanism regulating natural killer (NK) cell development and NK-based cancer immunotherapies. Inhibition of EZH2 expression or activity in hematopoietic stem and progenitor cells (HSPCs) provoked increased NK precursors and mature progeny, which exhibit enhanced cytotoxicity against tumor cells with up-regulation of IL-15R (CD122) and the NKG2D-activating receptor (Yin J, et al. 2015). Gunawan et al. studies revealed that EZH2 regulated the integrin signaling and adhesion dynamics of dendritic cells (DCs) which promote the development of experimental autoimmune encephalomyelitis (Gunawan M, et al. 2015), while Doñas et al. demonstrated that the inhibition of H3K27 demethylation induced tolerogenic DCs to inhibit inflammation and the development of experimental autoimmune encephalomyelitis (Doñas C, et al. 2016).

2. Materials and Methods

Cell Lines and Treatments

MOLT3, DND41, KOPTK1, P12/Ichikawa, and TALL-1 cells were maintained in RPMI-1640 (31870025; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (10270106; Gibco). Hela and Siha cell lines were grown in DMEM (Dulbecco's Modified Eagle Medium) (Gibco) culture medium, with the addition of 10% fetal bovine serum (FBS), L-glutamine 1 nmol / L (EuroClone) and 10 nmol / Penicillin-streptomycin L (EuroClone), and remove with Trypsin-EDTA 1X in PBS w/o Calcium, w/o Magnesium, w/o Phenol Red (EuroClone). All lines were grown at 37 ˚C and 5% CO_2.

To inhibit Notch S3 cleavage, MOLT3 and TALL-1, as well as HeLa and SiHa cells were treated with 10 μM gamma-secretase-inhibitor IX (DAPT) (565770; Calbiochem, Darmstadt, Germany) or with vehicle (DMSO) (D8418; Sigma-Aldrich, St Louis, MO, USA) for 48 h.To inhibit the enzymatic activity of JMJD3 or of EZH2, cells were exposed to 2 μM GSK J4 HCL (GSKJ4) (S7070; Selleckchem, Houston, TX, USA) or to 5 μM GSK 126 (S7061; Selleckchem, Houston, TX, USA), respectively, for times indicated in figures.

Protein and Histones Extracts Preparation, Western Blot, and Antibodies

To obtain total protein extracts, cells were lysed in Laemmli buffer (2X Laemmli Sample Buffer (1610737; Biorad, Hercules, CA) by sonication and clarified at 10.000 × g for 10 min. Before immunoblotting, samples were added with 0.04% β-mercaptoethanol (M6250; Sigma-Aldrich) and boiled for 10 min. To analyze histone mark levels, histones were extracted by using the acid extraction protocol by Abcam (Cambridge, UK). For immunoblotting, protein and histone extracts were run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (1620115; Biorad). Blots were incubated with antibodies against: Jagged1 (HPA021555, Sigma-Aldrich), Notch1Val1744 (4147; Cell Signaling), Notch1 (3608; Cell Signaling Technology), Notch3 (2889; Cell Signaling Technology), H3K27me3 (9733; Cell Signaling Technology), H3K27ac (4353; Cell Signaling Technology), H3 (9714, Cell Signaling Technology), PARP (9542; Cell Signaling Technology), $p27^{Kip1}$ (3688; Cell Signaling

Technology) $p21^{Waf1/Cip1}$ (2947; Cell Signaling Technology), E-cadherin (3195; Cell Signaling Technology), Vimentin (5741; Cell Signaling Technology), p53 (sc-6243; Santa Cruz Biotechnology), β-actin (A5441; Sigma-Aldrich), Myc Tag (06-549; Sigma-Aldrich), HA (16B12; Covance Inc, Princeton, NJ, USA) followed by hybridization with Antibodies HRP conjugated: anti-rabbit (A120-108P; Bethyl Laboratories, TX, USA) or anti-mouse (A90-116P; Bethyl Laboratories).

RT-PCR Analysis

Total RNA was isolated by using TRIZOL reagent (15596018; Invitrogen, Carlsbad, CA, USA) as described previously (Vargas Romero P, et al. 2015). cDNA was synthesized by using High Capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Taqman Gene Expression Master Mix (4304437) and Taqman Gene Expression Assays for NOTCH1 (Hs01062014_m1), NOTCH3 (Hs00166432_m1), DELTEX1 (Hs01092201_m1), HES1 (Hs00172878_m1), JAGGED1 (Hs01070032_m1), GAPDH (Hs02758991_g1), and 18S (4352930E) were purchased from Applied Biosystems. Relative quantification was carried out using the comparative ΔΔCT method, as previously described (Coni S, et al. 2017). GAPDH or 18S were used to normalize mRNA levels. Measurements were performed in technical triplicates and figures show the averages ± SEM of at least 3 biological replicates.

Plasmids and Transfections

To generate a construct expressing GFP-fused constitutively active human Notch3 intracellular domain (hICN3), the human N3ICD fragment was amplified by using primers FW 5′-GTCATGGTGGCCCGG-3′ and REV 5′- CAACACTTGCCTCTTGGG-3′, and cloned into the mammalian expression vector pcDNATM3.1/CT-GFP TOPO according to the manufacturer's instructions (K4820-01, Invitrogen). The constitutively active human Notch1 intracellular domain (hICN1) was previously cloned into the mammalian expression vector pcDNATM3.1/CT-GFP TOPO according to the manufacturer's instructions (K4820-01, Invitrogen). The expression vector PIRVNeoSV containing the human c-Myc cDNA coding sequence (c-Myc) was kindly provided by Dr. Giuseppe Giannini (Sapienza University, Rome, Italy). pCMV3-HA vector containing the human EZH2 coding sequence (HA-EZH2) was purchased from Sino Biological (HG11337-CY; Sino Biological, Beijing, China). Gene silencing was

performed by using EZH2 Silencer® Select Pre-design (Ambion, Cat#AM16708) or with its negative control (Ambion, Cat#AM4611).

Transient transfections of T-ALL cell lines with expression vectors or with the relative control plasmids were carried out by using Neon Transfection System (MPK5000; Invitrogen) as described by the manufacturer. Following 24 h of transfection of hICN3 plasmid and the relative control plasmid in DND41, about 40–50% of transfection efficiency was detected by fluorescence microscopy. The strength of HA-EZH2, hICN3, and c-Myc transfection was confirmed by western blotting shown in figures 1C and 3A, 3B and 3C, respectively.

Transient transfection of HeLa and SiHa cells was performed using either with Lipofectamine® 2000 Transfection Reagent (11668027, Invitrogen, Carlsbad, CA, USA) for hICN1 ectopic expression, or with Lipofectamine® RNAiMAX Transfection Reagent (1952335, Invitrogen, Carlsbad, CA, USA) for EZH2 gene silencing, according to the manufacturers' instructions for use.

Cell Viability Assay

Human T-ALL cell lines were seeded in 96-well plate at 5 × 105 cells/ml and treated with 2 μM GSKJ4 for times indicated in figures. As control samples, cells were treated with equal volumes of the vehicle (DMSO). Cell viabilities were assayed by using the MTS-based assay CellTiter 96® AQueous One Solution Cell Proliferation Assay (G3580; Promega, Madison, WI, USA), as previously described (Colicchia V, et al. 2017). Absorbances were measured at 490nm by using GloMax Multidetection System (Promega). Data were collected as units of absorbance (ABS) and normalized to cell proliferation percentage by following equation: % Cell Viability = (ABScells+compound – ABSmedium+compound) / (ABScells+DMSO – ABSmedium+DMSO) X 100. Measurements were performed in technical duplicates or triplicates and figures show the averages ± SEM of at least 3 biological replicates. Trypan blue (T8154, Sigma-Aldrich) was used to count viable and dead cells.

Rescue Assays

Growth sensitivity to treatment with 2 μM GSKJ4 was compared between DND41 cells transduced with retroviral construct encoding the entire murine Notch1 intracellular fragment CMMP-ICN1-IRES-EGFP (mICN1) and the empty relative control vector

CMMP-IRES-EGFP (empty) previously described (Mori M, et al. 2017), and between DND41 cells transiently transfected with hICN3 expression plasmid and the relative control plasmid, and between TALL-1 cells transiently transfected with the expression vector c-Myc and the cell counterparts transfected with the empty control vector. Trypan blue (T8154, Sigma-Aldrich) was used to count viable cells.

Wound healing assay

A total of 5×10^4 cells were plated in each well of a 24-well plate. The cells were treated the next day with DMEM with either GSK126 (5 μmol/L) alone or in combination with GSI (10 μmol/L) or vehicle control (DMSO) for 48 h. The cells were scraped with a 200-μL pipette tip in each well to create an artificial wound. The migration of the cells was quantified by taking pictures of the cells at 0 and 48 h under microscopy. All of the experiments were performed in triplicate. Later, collected data were analyzed with ImageJ Software package freely downloadable from the US National Institute of Health website (http://imagej.nih.gov/ij/) with the MRI wound healing tool.

Statistical Analysis

All statistical tests were carried out by using GraphPad Prism version 6.0 (GraphPad Software, San Diego California, USA). Statistical analysis of data between the two groups was carried out by two-tailed Student's unpaired t-test. Multiple comparisons analysis was carried out by one-way ANOVA followed by Tukey's posthoc test. Differences were considered significant when P-values < 0.05. Values significance: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3. Results

3.1 JMJD3 down-regulation abrogates Notch signaling in T-ALL

To evaluate whether lysine demethylase JMJD3 is necessary for NOTCH genes transcriptional activation and to study their functional role in Notch-dependent T-ALL cell context, we assessed the effects of the inhibition of its enzymatic activity on the expression of NOTCH1, NOTCH3 and the Notch target gene DELTEX1 and on the proliferation rate in TALL-1 and MOLT3 T-ALL cell lines (figure 3.1 A, B). Additionally, we evaluated whether the overexpression of histone-lysine N-methyltransferase EZH2 (the JMJD3 counterpart) modulated the expression of NOTCH1, NOTCH3, and Notch target gene DELTEX1 in TALL-1 cells, harboring *EZH2* missense mutations (Van der Meulen et al. 2015). The inhibition of histone modifiers activity was achieved by treating for 48 h cells with the commercially available specific inhibitor of JMJD3 GSKJ4 (Ntziachristos P, et al. 2014), whereas transient overexpression of ectopic EZH2 was performed by Neon transfection system.

GSKJ4 treatment abrogated the expression of NOTCH1 and NOTCH3 at both gene and protein levels and reduced the transcription of Notch target gene DELTEX1 in both the T-ALL cell lines (figures 3.1 A, B). Confirming the bonafide of the treatment, we found that GSKJ4 administration correlated with increased H3K27me3 and decreased H3K27ac global accumulation (figures 3.1 A, B, lower panels). The ectopic expression of the wild type form of EZH2 reduced the levels of NOTCH3 and DELTEX1 in TALL-1 cells. Unexpectedly, it did not influence significantly NOTCH1 expression (figure 3.1 C).

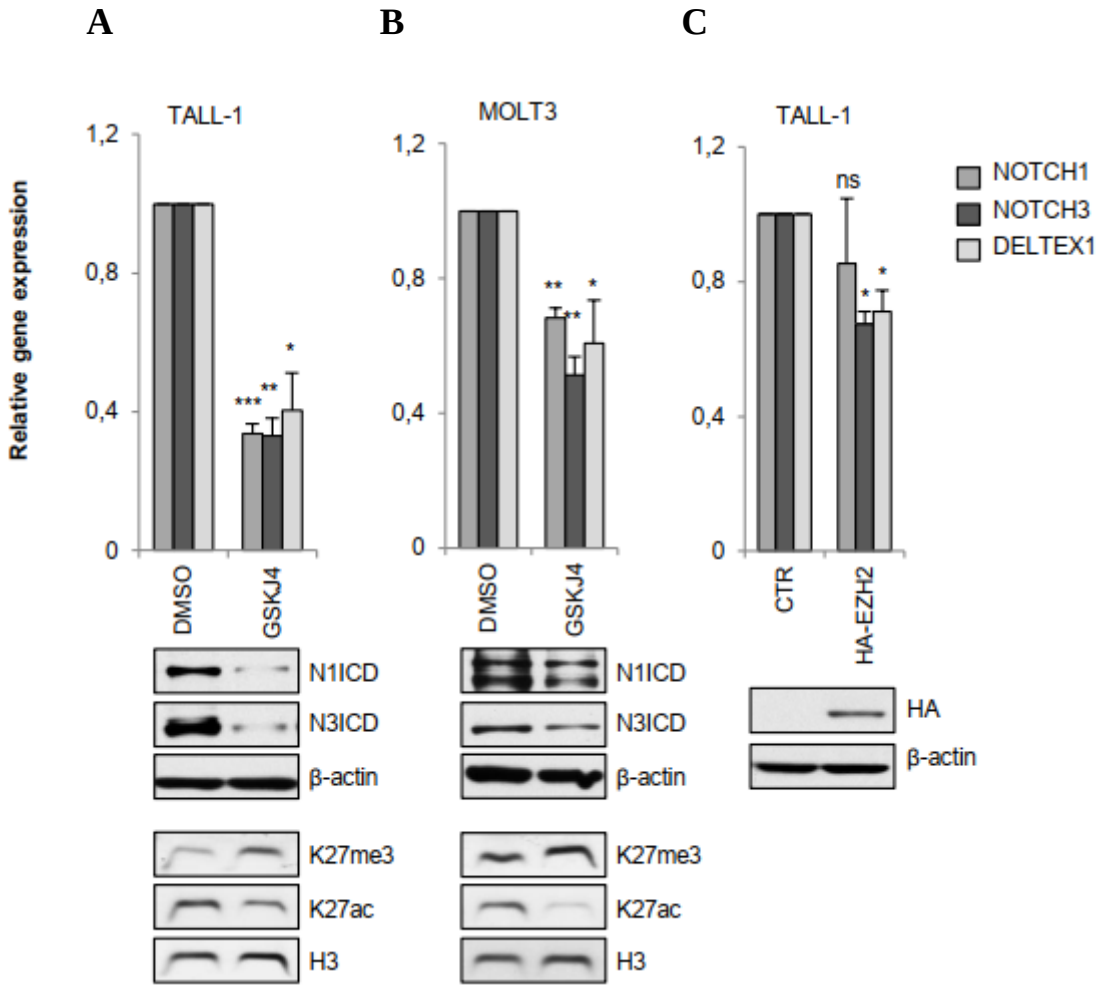


Figure 3.1. GSKJ4 modulates Notch receptors expression and activity. *Relative NOTCH1, NOTCH3, and DELTEX1 gene expression and N1ICD, N3ICD, β-actin, H3K27ac (K27ac), H3K27me3 (K27me3) and H3 total protein expression levels in* ***(A)*** *TALL-1 or* ***(B)*** *MOLT3 cells treated for 48h with 2μM GSKJ4 or with DMSO.* ***(C)*** *Relative NOTCH1, NOTCH3, and DELTEX1 gene expression and HA and β-actin protein levels in TALL-1 cells transfected with HA-tagged EZH2 expression vector (HA-EZH2) or with the empty control vector. β-actin and histone H3 were used as loading control. Data represents mean values of three biological replicates ± standard error of the mean (S.E.M.); (n = 3) *P < 0.05, **P < 0.01, ***P < 0.001.*

3.2 Pharmacological inhibition of JMJD3 decreases viability of T-ALL cell lines

Aiming to evaluate whether JMJD3 is implicated in sustaining cell proliferation and studying its functional part in Notch-dependent T-ALL cell contexts, we assessed the effects of the inhibition of its enzymatic activity on cell viability by MTS assay (figure 3.2 A), and on proportion of viable and dead cells by trypan blue exclusion assay after 48h of GSKJ4 treatment (figure 3.2 C) in different T-ALL cell lines after 48 and 72h of exposure to 2μM of GSKJ4. In addition, we evaluated the expression of the pro-apoptotic cleaved form of poly ADP-ribose polymerase (PARP), the negative regulator of cell cycle progression $p27^{KIP1}$ (p27), and the oncogenic protein c-Myc (figure 3.2 B).

In line with the anti-Notch signaling activity mentioned in point 3.1, the treatment with low doses of GSKJ4 decreased cell viability by promoting cell death and decreasing the number of viable cells in different T-ALL cell contexts (figure 3.2 A, C). Consistently, the treatment reduced the expression of oncogenic c-Myc protein and augmented the levels of the anti-proliferative cyclin-dependent kinase inhibitor p27 and the pro-apoptotic cleaved form of PARP (figure 3.2 B).

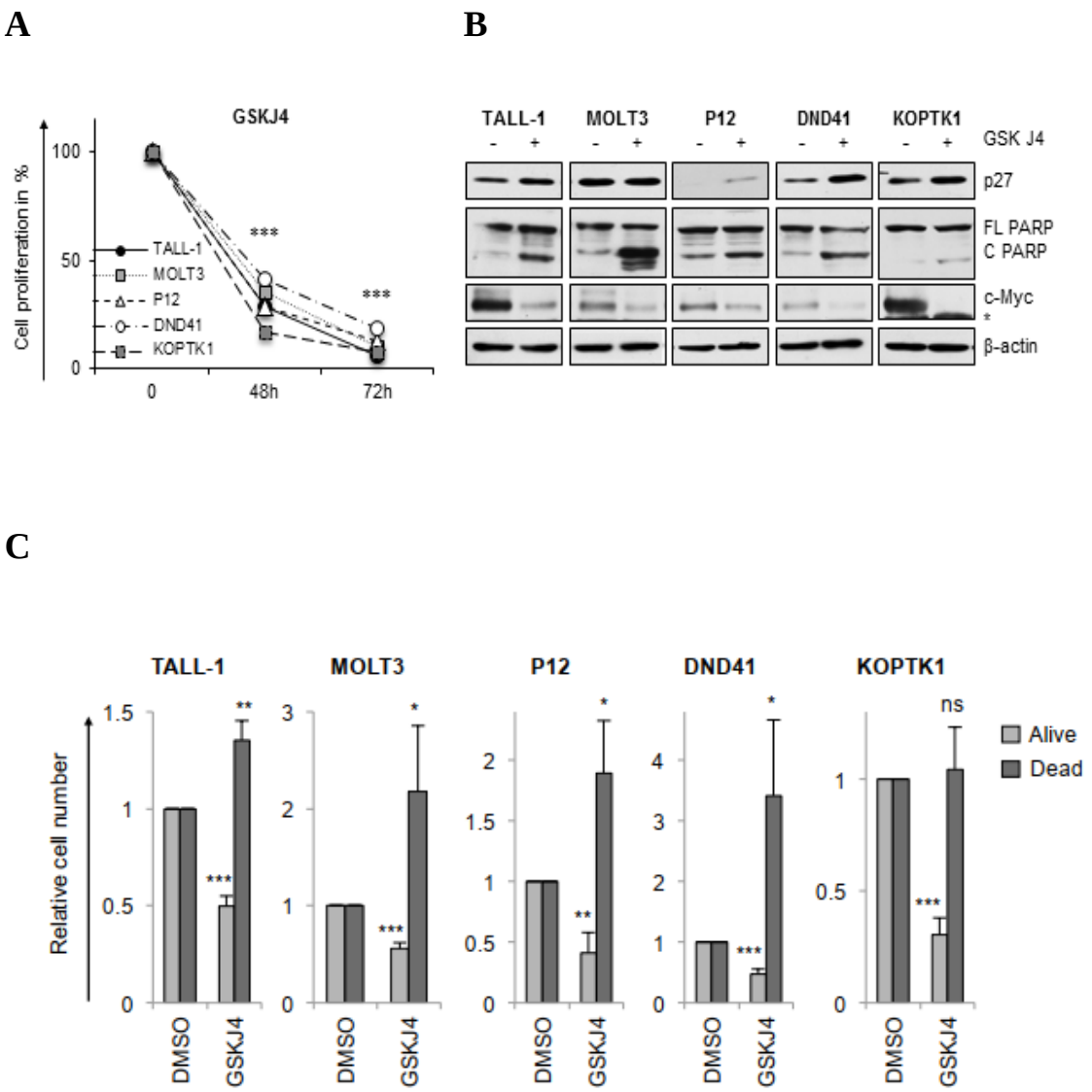


Figure 3.2. GSKJ4 treatment inhibits viability in T-ALL cell lines. (A) *Cell viability measurement by MTS assays in TALL-1, MOLT3, P12, DND41, and KOPTK1 cell lines treated with 2µM of GSKJ4 for times indicated in the figure.* ***(B)*** *Protein expression levels of p27, cleaved and full-length form of PARP (C PARP and FL PARP, respectively), and c-Myc. β-actin expression was used as loading control, and* ***(C)*** *Number of viable and dead cells detected via trypan blue exclusion assay in TALL-1, MOLT3, P12, DND41, and KOPTK1 cell lines treated with 2µM of GSKJ4 or DMSO for 48h. Relative cell viability reported in panel A represents the mean of 3 replicates normalized to cell number in DMSO-treated cells at corresponding time points ± standard deviation of the mean (S.D.) Relative cell number reported in charts in panel C represents the mean of 4 replicates normalized to cell number in DMSO-treated cells ± standard deviation of the mean (S.D.); *P < 0.05, **P < 0.01, ***P< 0.001. ***P < 0.001. Non-specific bands are indicated with an asterisk (*).*

3.3 Enforced Notch-cMyc axis partially rescues T-ALL cells from anti-growth effects of JMJD3 inhibition

To further evaluate whether the above-mentioned biological effects occurred via Notch inhibition, we compared the effects of JMJD3 inhibition on cell viability in DND41 cells transduced with retroviruses encoding murine N1ICD (mICN1) and with the counterparts transduced with the empty retroviral vector (empty) after 48h of exposure to GSKJ4. As we expected, mICN1 expression moderately rescued DND41 cells from the antiproliferative effects of GSKJ4 (figure 3.3 A, upper panel) and, interestingly, it preserved N1ICD endogenous expression from the effects of the drug (figure 3.3 A, lower panel). Similarly, the decrease of viable cells induced by 48 h of treatment with GSKJ4 in DND41 cells was in part prevented by the reintroduction of exogenous human N3ICD (hICN3) (figure 3.3 B, upper panel) and the transient introduction of plasmid vector coding hICN3 protected Notch3 endogenous expression (Figure 3.3 B, lower panel).

Since GSKJ4 treatment decreased c-Myc levels in T-ALL cells (figure 3.2 B), we suggested that the axis between Notch signaling and c-Myc could be a critical target of this inhibitor and that c-Myc reduced expression might be essential for its biological effects. We evaluated this hypothesis by comparing the number of viable TALL-1 cells transiently transfected with an expression vector encoding human c-Myc with their counterpart transfected with the empty vector after 48 h of treatment with GSKJ4. Remarkably, the expression of exogenous c-Myc partially protected the viability of Notch3-dependent TALL-1 cells exposed to GSKJ4 (figure 3.3 C).

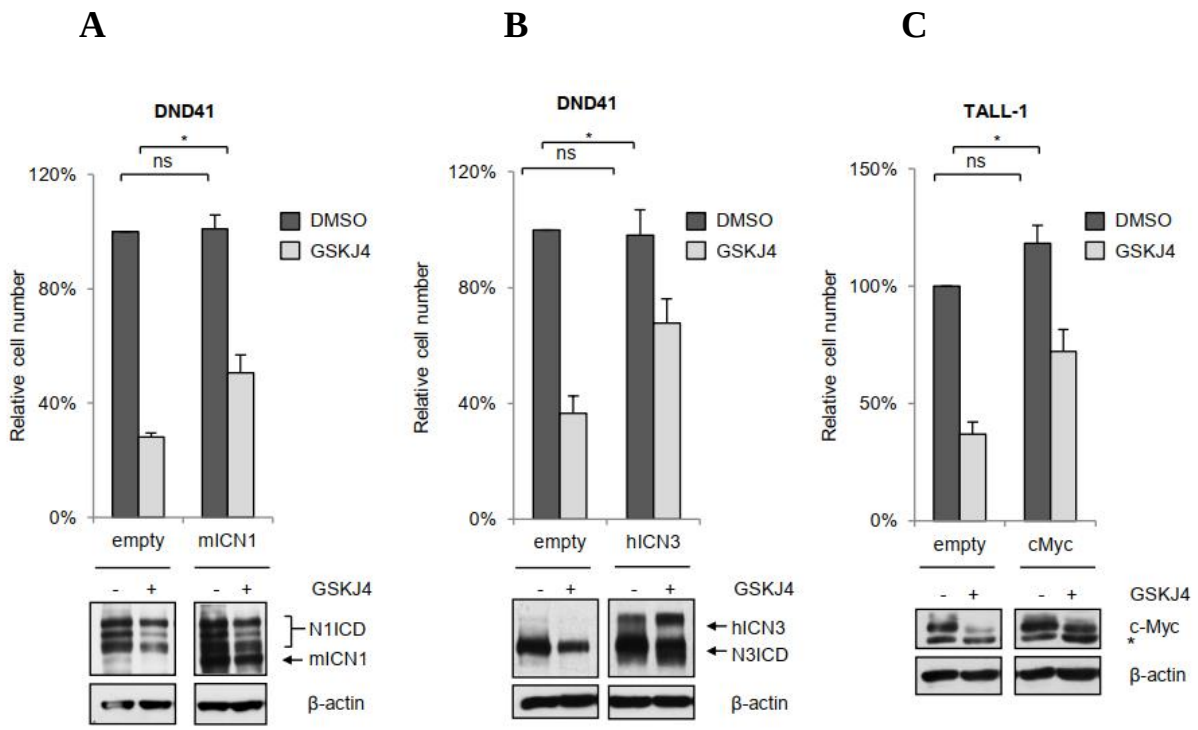


Figure 3.3. Enforced Notch/c-Myc axis partially shields from inhibitory effects of GSKJ4 on T-ALL cell viability. *Change in number of viable cells after 48 h of exposure to 2 μM GSKJ4 in DND41 cells transduced with the mICN1 or with the empty control retroviruses (**A, upper panel**), in DND41 cells transiently transfected with hICN3 or with the empty vector (**B, upper panel**), and in TALL-1 cells transfected with c-Myc or with the control plasmid (**C, upper panel**). The number of viable cells was calculated via trypan blue exclusion assay. Relative cell number reported in charts represents the mean of at least four biological replicates normalized to cell number in DMSO-treated cells transduced or transfected with empty control ± standard error of the mean (S.E.M.); *P < 0.05, **P < 0.01. Western blot analysis by using antibodies against Notch1 in mICN1 transduced DND41 cells (**A, lower panel**), Notch3 in hICN3 transfected DND41 cells (**B, lower panel**) and c-Myc in TALL-1 cells transfected with the c-Myc expression vector (**C, lower panel**). β-actin expression was used as loading control. Non-specific bands are indicated with an asterisk (*).*

3.4 EZH2 blockade stimulates Notch pathway activation in cervical cancer cells

Considering the presence of chromatin-modification based circuit coordinating JMJD3 recruitment to loci of NOTCH genes and Notch transcriptional targets in T-ALL where Notch is a recognized oncogene, we questioned if a specular mechanism involving Polycomb Repressor Complex could act in the context of HPV-driven cervical cancer where Notch might be an oncosuppressor. To evaluate this hypothesis, we performed both transient knockdown of EZH2 with siRNA and chemical inhibition of its enzymatic activity with commercially available compound GSK126 in HeLa (adenocarcinoma) and SiHa (squamous cell carcinoma) cell lines. 72h of EZH2 silencing in these cell lines resulted in upregulated expression of NOTCH1, NOTCH3, and Notch target gene HES1, as well as in increased protein levels of Jagged1, the activated domain of Notch1 (N1VAL), and the intracellular domain of Notch3 (N3ICD) (figure 3.4 A).

On the other hand, after 48h of GSK126-mediated EZH2 inhibition, we spotted increased expression of JAG1, NOTCH3, and HES1 genes and enhanced protein levels of Jagged1, N1VAL, and N3ICD in both HeLa and SiHa cell lines (figure 3.4 B). Unexpectedly, GSK126 exposure influenced NOTCH1 expression in HeLa but not in SiHa cells (figure 3.4 B, upper panel). Confirming the specific effect of the treatments, EZH2-silenced cells showed decreased expression of EZH2 (figure 3.4 A, lower panel) and GSK126 exposure decreased H3K27me3 and increased H3K27ac global accumulation relative to total H3 (figure 3.4 B, right panel).

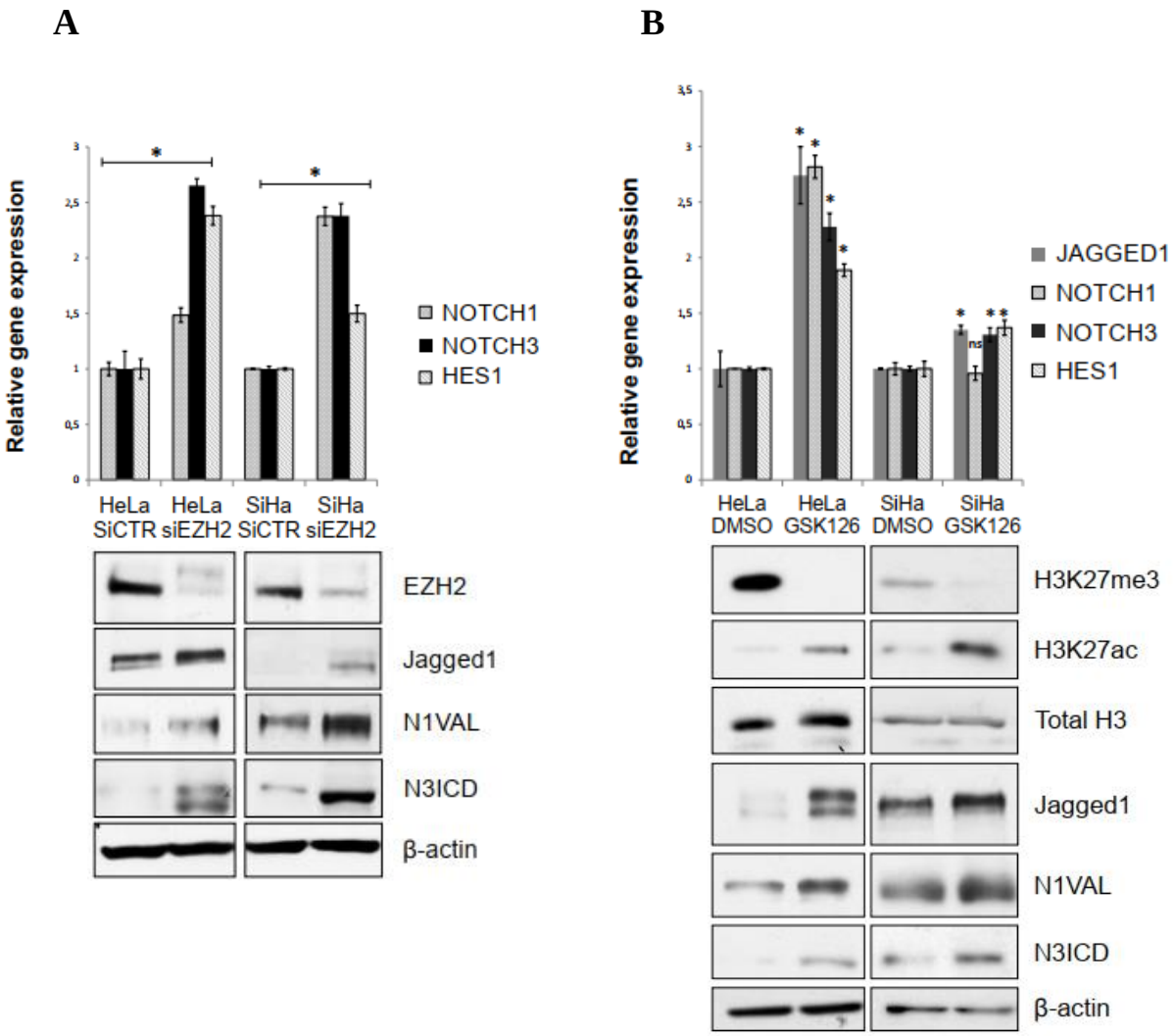


Figure 3.4: EZH2 inhibition activates Notch signaling in cervical cancer cell lines. *Relative gene expression levels of Jagged1, Notch1, Notch3 and Hes1 (left panels) and protein levels of H3K27me3 (K27me3), H3K27ac (K27ac), H3 total, Jagged1, the activated domain of Notch1 (N1VAL), the intracellular domain of Notch3 (N3ICD) (right panels) in HeLa and SiHa cervical cancer cell lines following EZH2 inhibition carried out by:* ***(A)*** *72h of transient knockdown with siRNA against EZH2 or* ***(B)*** *48h of treatment with 5μM of the EZH2 inhibitor GSK126. β-actin expression was used as loading control. Confirming the bonafide of the treatments, (A, right panel) EZH2 silenced cells showed decreased expression of EZH2 and (B, right panel) GSK126 exposure resulted in decreased H3K27me3 and increased H3K27ac global accumulation. Data represent mean values of three biological replicates ± standard deviation of the mean (S.D.); (n = 3) *P < 0.05.*

3.5 Notch signaling blockade partially abrogates anti-viability effects of EZH2 inhibition in cervical cancer cell lines

To appraise if PRC2 activity sustains the proliferation program in cervical cancer, we performed a 72h transient knockdown of EZH2 with siRNA in HeLa and SiHa cells and evaluated changes in some markers of cell cycle progression. As shown in panel A of figure 3.5, EZH2 depletion resulted in increased expression of the cyclin-dependent kinase inhibitors $p21^{Cip1}$ (alternatively $p21^{Waf1}$) (p21) and $p27^{KIP1}$ (p27) and the oncosuppressive protein p53 together with the downregulation of oncogenic protein c-Myc. Confirming the bonafide of the treatment, siRNA transfected cells expressed lower levels of EZH2 when compared to the control cells (figure 3.5 A).

In line with these results, we aimed to evaluate how the upregulation of Notch signaling could modulate the cell cycle on its own. To assess this, HeLa and SiHa cells were transiently transfected with an expression vector encoding human N1ICD (hICN1) for 48h and compared to the counterparts transfected with the empty vector (control). hICN1-transfected cells showed higher expression of cell cycle arrester p53, whereas p21 was modulated just in HeLa cells and not in SiHa. Surprisingly, p27 levels remained unchanged in both cell lines. Conversely, c-Myc expression was abrogated in SiHa but not in HeLa cells (figure 3.5 B).

To further estimate whether GSK126 biological effects, at least in part, fulfilled through Notch signaling activation, HeLa and SiHa cells were treated with DMSO or GSK126 alone or in combination with γ-Secretase inhibitor (GSI) LY-374973, alternatively named DAPT, for 48h (figure 3.5 C). Low doses of GSK126 decreased the total number of viable cells in both cervical cancer cell lines, and this effect was partially abrogated when the cells were treated with GSK126 in combination with GSI (COMBINED) (figure 3.5 C, upper panels). Coherently with this, N1VAL, p21, p27, p53 protein levels were upregulated in HeLa and SiHa after 48 h of EZH2 chemical blockade and returned to normal levels when the cells were treated with GSK126 in combination with GSI (figure 3.5 C, lower panels).

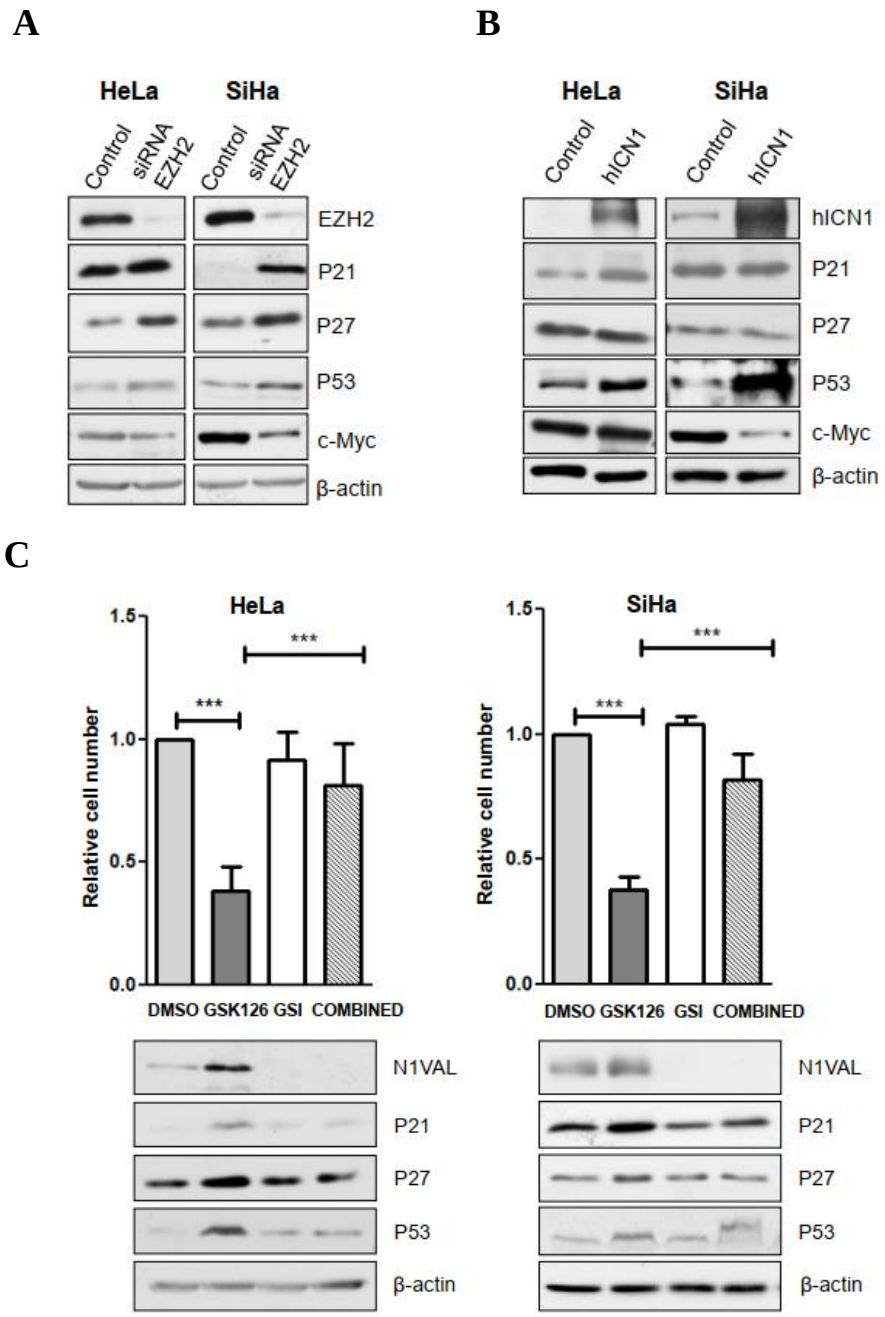


Figure 3.5. Notch signaling blockade partially abrogates anti-viability effects of EZH2 inhibition in cervical cancer cells. (A) *Protein expression levels of cell cycle regulators p21, p27, and p53 and oncogene c-Myc in HeLa and SiHa cells transfected with siRNA against EZH2 for 72h or* ***(B)*** *transfected with an expression vector encoding human N1ICD (hICN1) for 48h. β-actin expression was used as a loading control. Confirming the bonafide of the treatment, siRNA transfected cells express lower levels of EZH2 when compared to the control cells.* ***(C)*** *Change in the number of viable cells and N1VAL, p21, p27, p53 protein expression levels in HeLa (C, left panels) and SiHa cells (C, right panel) treated with DMSO or 5 μM of GSK126 and 10 μM of GSI alone or in combination (COMBINED) for 48h. The number of viable cells was detected via trypan blue exclusion assay. Relative cell number reported in charts represents the mean of 3 replicates normalized to cell number in DMSO-treated cells ± standard deviation of the mean (S.D.) ***P < 0.001.*

3.6 EZH2 sustains EMT program in cervical cancer cells through Notch signaling repression

In order to evaluate whether EZH2 function sustains the EMT program in cervical cancer cells, we carried out its gene silencing with siRNA for 72h and estimated the expression of some epithelial and mesenchymal markers. EZH2 depletion in both HeLa and SiHa cells increased the levels of E-cadherin (E-cadh) and decreased vimentin expression (figure 3.6.1 A). To further evaluate the grade of Notch-dependence of the EMT program, we transiently transfected HeLa and SiHa cells with an expression vector encoding human N1ICD (hICN1). Ectopic overexpression of Notch1 resulted in the upregulation of E-cadherin and downmodulation of Vimentin in both cell lines (figure 3.6.1 B) when compared to the counterparts transfected with the empty vector (control).

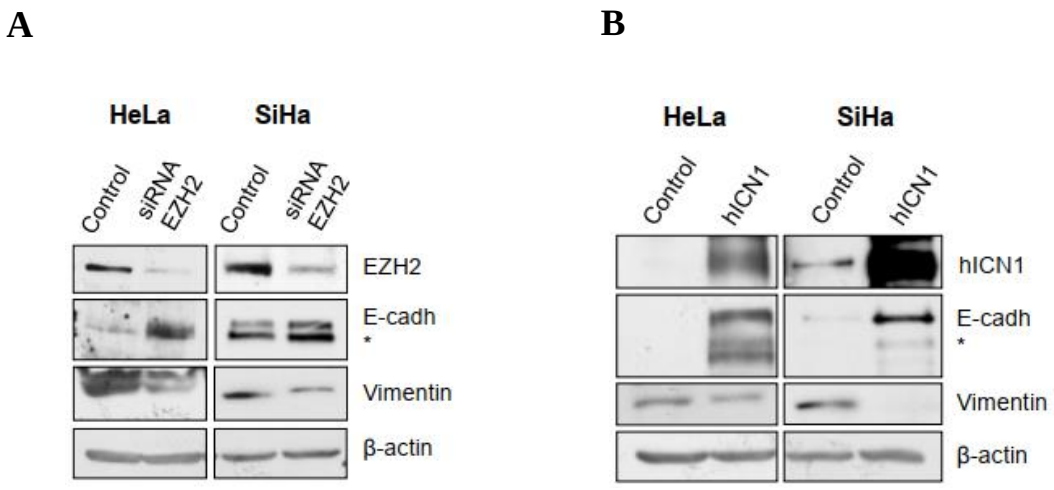
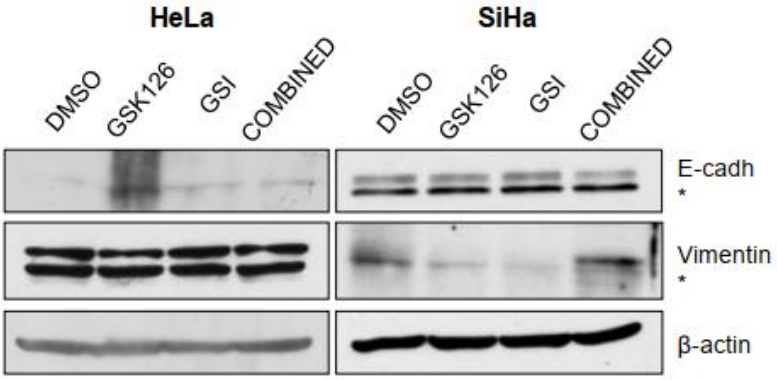


Figure 3.6.1. EZH2 sustains EMT program in cervical cancer cells through Notch signaling repression. ***(A)*** *Protein expression levels of the epithelial phenotype marker E-cadherin, the mesenchymal phenotype marker Vimentin in HeLa and SiHa cells transfected with siRNA against EZH2 for 72h or* ***(B)*** *transfected with hICN1. β-actin expression was used as loading control. Confirming the bonafide of the transfection, protein extracts were revealed for EZH2* ***(A)*** *and for N1IC antibodies* ***(B).*** *Non-specific bands are indicated with an asterisk (*).*

To confirm the hypothesis that EZH2 stimulates the EMT program, at least in part, through the inhibition of Notch1 expression in cervical cancer, we carried out a 48h GSK126 treatment, alone or in combination with GSI, in HeLa and SiHa cells. EZH2 chemical blockade induced the upregulation of epithelial protein marker E-cadherin accompanied by decreased expression of Vimentin in HeLa cells after 48h of treatment. The combination of GSK126 and GSI resulted in abrogated levels of E-cadh and restored Vimentin protein expression, (figure 3.6.2 C, left panel). On the other hand, GSK126 effect did not modulate the protein levels of E-cadh in SiHa cells, but it clearly decreased the global expression of vimentin which restored after 48h exposure to the combined treatment with GSI (figure 3.6.2 C, right panel). In line with these results, we focused our interest in the role of Notch signaling modulation in the migratory capacity of these cervical cancer cells. HeLa and SiHa cells treated with GSK126 presented sustained free area of the scratch surface which was significantly reduced under GSI-combined treatment conditions during 48h wound healing assays (figure 3.6.2 D, left panels). Both cell lines exhibited lesser wound healing capacity when compared to controls and the combined treatment with GSI dramatically annulled the inhibitory effects of GSK126 alone in HeLa cells and, in a moderate fashion, in SiHa cells (figure 3.6.2 D, right panels).

C



D

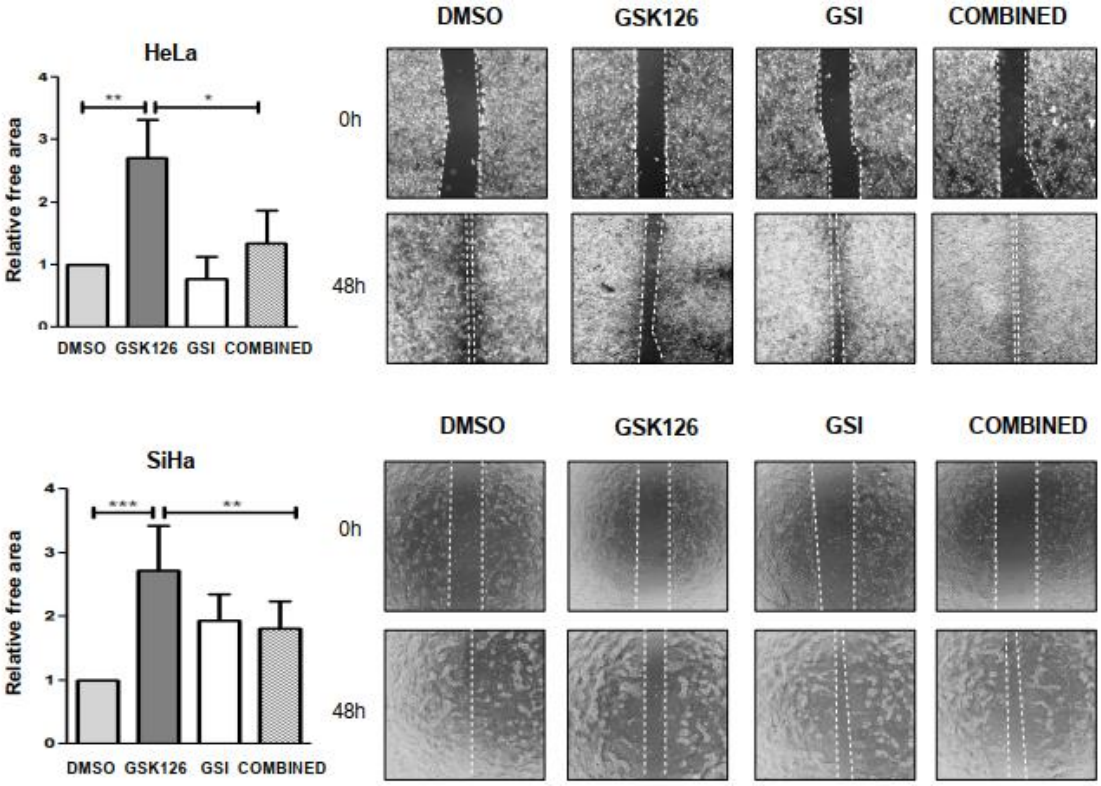


Figure 3.6.2. EZH2 sustains EMT program in cervical cancer cells through Notch signaling repression. (C) *Western blot analysis for epithelial to mesenchymal transition markers E-cadh and Vimentin on HeLa and SiHa cells treated with DMSO or GSK126 alone or in combination with GSI (COMBINED) for 48h.* ***(D)*** *Migratory capacity of HeLa and SiHa cells treated with DMSO or GSK126 alone or in combination with GSI (COMBINED) for 48h. Data reported in charts represents the mean of 3 replicates normalized to wound free area of DMSO-treated cells after 48h ± standard deviation of the mean (S.D.); *P < 0.05, **P < 0.01 ***P < 0.001. Non-specific bands are indicated with an asterisk (*).*

4. DISCUSSION

Previous evidence suggested an epigenetic machinery driving Notch expression in different cell contexts. However, not much is known yet about the specific histone modifiers writing or erasing the chromatin status of NOTCH1 and NOTCH3 genes. Here we highlighted additional mechanistic insights into Notch1-3 regulation, thus advancing the understanding of distinct interplays/relationships between Notch receptors and histone modifiers in T-ALL and cervical cancer.

Recent studies indicated that NOTCH3 gene locus is poorly methylated in the T-ALL context (Kuang SQ, et al. 2013). Methylation of H3K27 at gene loci results from the balance between the methyltransferase activity of the Polycomb-Repressive Complex 2 (PCR2) component EZH2 (Cao R, et al. 2004) and of the enzymatic activity of the demethylases JMJD3 and UTX (Hong S, et al. 2007). Considering that our previous data indicated that N3ICD, as well as N1ICD, allow the recruitment of JMJD3 to the NOTCH3 gene locus to preserve permissive local H3K27 trimethylation mark (Tottone et al. 2019) and that about 25% of T-ALL patients possess inactivating mutations of PRC2 components (Ntziachristos P, et al. 2012), we decided to focus on EZH2 antagonist JMJD3 favouring demethylation and chromatin opening at its target areas. In line with this aim, we performed pharmacological inhibition of JMJD3 with GSKJ4 and we found that GSKJ4 treatment suppressed Notch signaling activation and the viability of human T-ALL cell lines by promoting the accumulation of anti-proliferative factor p27 and apoptosis-associated cleaved form of PARP and suppressing the expression of the oncogenic target of Notch c-Myc. Notably, ectopic overexpression of the activated domain of both Notch1 and Notch3 partially rescued DND41 cell lines from anti-growth effects induced by JMJD3 blockade, thus indicating that Notch inhibition is responsible, at least in part, for the cell viability by reversing the effect of the drug. Moreover, the expression of exogenous c-Myc partly shielded the viability of Notch3-dependent TALL-1 cells exposed to GSKJ4, proposing that biological effects of this treatment were partially mediated through aiming c-Myc signal via Notch inhibition. Therefore these data suggest that growth arrest and apoptosis associated with JMJD3 inhibition may be possibly due to the impaired Notch/c-Myc regulatory axis (Tottone et al, 2019). These last results are in line with a well-established concept of c-Myc as a prominent mediator of Notch oncogenic program in Notch1-driven leukemia (Palomero

T, et al. 2006; Sharma VM, et al. 2006; Herranz D, et al. 2014) and with its capacity to rescue T-ALL cells from the anti-growth effects of Notch1 inhibition by GSI treatment (Weng AP, et al. 2006; Sharma VM, et al. 2006).

According to what we found in the T-ALL context, we aimed to unveil whether these mechanisms were somehow conserved, in a specular fashion, in tumor context in which Notch signaling might be deleterious. To note, in B-ALL where Notch1 and Notch3 are supposed to be oncosuppressors, Notch ligands, genes, and target genes are frequently hypermethylated (Kuang SQ, et al. 2013). EZH2 may work both as oncogene and oncosuppressor in different cancers (Gan L, et al. 2018). Whereas in T-ALL it has a tumor-suppressive effect (Leong KG, et al. 2006), in cervical cancer it is overexpressed and associated with tumor progression and development of more aggressive forms of cancer (Liu Y, et al. 2014). Several authors demonstrated how high EZH2 activity leads to cell invasion in cervical cancer (Adhikary G, et al. 2015; Liu Y, et al. 2014). However, the precise molecular mechanisms by which EZH2 contributes to the development of invasive cervical cancer remain still unclear.

In this framework, we investigated the relevance of EZH2 function in the epigenetic control of Notch signaling and its relevance in tumor progression and invasiveness capacity in human cervical cancer cell models. We examined the consequences of EZH2 inhibition with GSK126/siRNA-mediated gene silencing on Notch signaling activation, cell growth and viability, and migratory capacity in human cervical cancer cells. In our study, EZH2 expression was found to be a repressor of the Notch pathway, given that its inhibition or transient knockdown upregulated the levels of the Notch receptors, ligands, and target genes. As we expected, EZH2 inhibition potently suppressed the growth of cervical cancer cells in vitro, and it was supported with the upregulation of p53, negative regulators of cell cycle progression from G0/G1 to S phase $p21^{Cip1}$ and $p27^{Kip1}$ and reduced expression of c-Myc.

In order to determine whether Notch signaling activation affects cell cycle progression by itself, we carried out a transient transfection of HeLa and SiHa cells with hICN1. By this approach, we observed different cell line-dependent effects. Indeed, Notch1 ectopic introduction induced p53 expression in both cell lines, whereas its target $p21^{Cip}$ was upregulated in HeLa but not in SiHa cells, and the latter ones showed strong downmodulation of c-Myc, unlike HeLa. Probably, higher basal levels of Notch1 in

SiHa comparing with HeLa contribute to the expression of $p21^{Cip1}$ and it makes these cells less dependent on transient introduction of exogenous Notch1. Distinctly from EZH2 expression silencing, the ectopic introduction of hICN1 did not modulate the cell growth arrester $p27^{Kip1}$, possibly indicating that it is not completely dependent on Notch signaling in this context. Confirming the dependence of observed anti-growth effects of EZH2 inhibition on Notch activation, we combined the GSK126 treatment with γ-secretase inhibitor (GSI). The addition of GSI to GSK126 abrogated its antigrowth effects by restoring the number of viable cells and $p21^{cip}$, $p27^{kip}$, and p53 levels, thus indicating that the deleterious effect of EZH2 inhibition in cervical cancer is at least partially explained by the activation of Notch signaling.

Considering that E-cadherin gene locus is frequently hypermethylated in cervical cancer (Chen CL, et al. 2003), we decided to evaluate the effects of EZH2 blockade on the realization of the EMT program in model cell lines. Our data confirm that EZH2 inhibition shifts cervical cancer cells from a more aggressive mesenchymal phenotype to the epithelial one. Both transient EZH2 gene silencing and its pharmacological blockade with GSK126 resulted in upregulation of epithelial phenotype marker E-cadherin (E-cadh) and reduced expression of pro-mesenchymal and vimentin. Similar effects were observed under transient transfection with plasmid encoding hICN1.

In line with the above-mentioned association of EZH2 effects with Notch activation, the combination of GSK126 with GSI restored E-cadh and/or Vimentin proteins levels in both cell lines, confirming the relevance of Notch signaling in the epithelial phenotype maintenance and once more indicating the existence of cell line-dependent effects. Notably, modulation of markers of EMT under Notch activation correlated with reduced cell motility in scratch assay, even if basal migration velocity HeLa cells was higher than the one of SiHa probably due to different histological type of these cells. In particular, squamous carcinoma cell line SiHa had higher initial levels of E-cadh and lower ones of vimentin comparing with adenocarcinoma cells HeLa. Furthermore, although the EZH2 basal level of expression is generally similar in HeLa and SiHa cells, HeLa cells are more motile than SiHa cells. This may be attributable to other migration-related and phosphorylated proteins (integrin-linked kinase, p-p38, p38, p-Src, macrophage-capping protein, focal adhesion kinase, p-focal adhesion kinase, Cavelion-1) that provide HeLa cells with a series of metastatic potentials (Ding M, et al.

2015). The molecular nature of observed differences could be an interesting subject for our further studies.

The strong anti-growth potential of EZH2 inhibition/Notch activation associated with shifting from invasive mesenchymal phenotype to a less aggressive epithelial one could offer promising therapeutic strategies to detain tumor development and reverse the biological effects of EZH2 over-activation in cervical cancer.

5. CONCLUSIONS

In the past decades, we have begun to fully recognize the importance of the flexible nature of epigenetic changes in cancer cell development and maintenance and to understand that oncogenic or oncosuppressive function of certain transcription factors can be determined by the epigenetic landscape surrounding them and their target genes. In the present study, we confirmed the doubled-faced role of Notch in two different cancer contexts and highlighted how these oncogenic/oncosuppressive roles can be orchestrated by epigenetic regulators. The results of our work contribute to the increasing amount of evidence that pharmacological inhibition of histone methyltransferases and demethylases might be a promising strategy for controlling malignant cell growth and metastatic capacity thus opening new roads for creation of novel targeted cancer therapies.

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