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Notch1 is induced by p53 as a possible anti-apoptotic mechanism in Chronic Lymphocytic Leukemia

Master's Thesis

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1. ABBREVIATIONS

°C	Degree Celsius
7-AAD	7-Aminoactinomycin D
ABB	Annexin Binding Buffer
ADAM	A-disintegrin and Metalloproteinase
AGO	Argonaute
APAF-1	Apoptosis Protease-Activating Factor
BCA	Bicinchoninic Acid
BCL-2	B-cell Lymphoma Apoptosis Regulator-2
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CDK	Cyclin-dependent Kinase
cm^2	Square Centimeter
CLL	Chronic Lymphocytic Leukemia
CO_2	Carbon Dioxide
ddH_2O	Distilled Water
Del17p	Deletion of the Short Arm of Chromosome 17
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum

FITC	Fluorescein Isothiocyanate
g	Gram(s)
GFP	Green Fluorescent Protein
h	Hour(s)
H_2O	Water
HBS	HEPES-buffered saline
HEY	Hairy/Enhancer of Split related to YRPW motif
HES	Hairy/Enhancer of Split
HRP	Horseradish Peroxidase
hs	Homo Sapiens
kDa	Kilo Dalton
L	Liter
L-Glut	L-Glutamine
LARII	Luciferase Reagent II
Luc	Firefly Luciferase
M	Molar
MAML	Mastermind-like protein
MDM-2	Mouse double minute 2
miRNA, miR	MicroRNA
mL	Milliliters
mm	Millimeter
MOPS	3-(N-morpholino)propanesulfonic Acid
MOMP	Mitochondrial Outer Membrane Permeabilization
mRNA	Messenger RNA

mut	Mutated
n	Nano
NaCl	Sodium Chloride
NICD	Notch1 Intracellular Domain
Notch1-EC	Notch1 Extracellular Domain
Notch1-TMIC	Notch1 Transmembrane and Intracellular Domain
Notch1-TM	Transmembrane Portion Notch1
ns	Not Significant
Nut	Nutlin-3a
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline Tween
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene Fluoride
Ren	Renilla Luciferase
RBP-J	Recombination Signal Protein-J
RNA	Ribonucleic Acid
rpm	Rounds Per Minute
RPMI-1640	Roswell Park Memorial Institute 1640
RT	Room Temperature
sec	Second(s)
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
TF	Transcription Factor
UTR	Untranslated Region
WB	Western Blot
wt	Wild Type

2. ABSTRACT

Background: *TP53* is the most commonly mutated gene in cancer. In Chronic Lymphocytic Leukemia (CLL), loss of p53 function is associated with poor prognosis and lower levels of miRNA-34a (miR-34a), a well-known tumor suppressor. A major target of miR-34a is Notch1, which is frequently hyperactivated in CLL, promoting cell survival. Therefore, lower levels of miR-34a might be a mechanism that increases Notch1 activity and might partially explain why CLL patients with *TP53* alterations display a worse prognosis. Consequently, miR-34a levels can be a biomarker to assess prognosis and miR-34a restoration might represent a novel therapeutic approach for this subset of patients.

Aim: The main goal of this study was to better characterize the complex crosstalk between p53, Notch1 and miR-34a. A better understanding of the relationship between these entities is necessary to understand the role that miR-34a may play in the pathogenesis and progression of CLL.

Methods: Different CLL cell lines were either treated with the compound Nutlin-3a, which activates p53 by inhibiting MDM-2, or transfected with constructs overexpressing or silencing p53. We investigated how p53 activation/silencing affects Notch1 signaling activity by Luciferase Assay, protein levels by Western Blot (WB) and miR-34a expression by RT-qPCR in different CLL cell lines. Next, we analyzed the effects of miR-34a overexpression/inhibition on Notch1 signaling activity by Luciferase Assay and Notch1 and BCL-2 protein levels by WB. Finally, we characterized Notch1 and BCL-2 protein levels by WB and miR-34a levels by RT-qPCR in both *TP53* wt and *TP53* mut CLL patients.

Results: We confirmed that miR-34a is a transcriptional target of p53 and we found that p53 activation correlates with higher Notch1 protein levels and signaling activity. Furthermore, prolonged p53 activation by Nutlin-3a treatment and consequently higher Notch1 protein levels correlated with higher BCL-2 protein levels. However, we did not observe any significant effects of miR-34a on Notch1 signaling activity and Notch1 or BCL-2 protein levels.

Conclusions: Consistent with previous findings, Notch1 induction by p53 might represent an anti-apoptotic mechanism in CLL cells in which p53 is still functional. We hypothesize that this mechanism may be further supported by Notch1-dependent BCL-2 induction. Furthermore, we assume that the induction of miR-34a by p53 represents a system exploited by B cells to control Notch1 protein levels and signaling under physiological conditions.

2. ABSTRACT (ITALIAN TRANSLATION)

Background: *TP53* è il gene più comunemente mutato nel cancro. Nella leucemia linfatica cronica (LLC), la perdita della funzione di p53 è associata a una prognosi sfavorevole e a livelli più bassi di miRNA-34a (miR-34a), un noto soppressore tumorale. Uno dei principali bersagli di miR-34a è Notch1, via di segnalazione spesso iperattivata nella LLC che favorisce la sopravvivenza delle cellule. Pertanto, livelli più bassi di miR-34a potrebbero essere un meccanismo che aumenta l'attività di Notch1 e potrebbe spiegare in parte perché i pazienti affetti da LLC con alterazioni di *TP53* presentano una prognosi peggiore. Di conseguenza, i livelli di miR-34a possono essere un biomarker per valutare la prognosi e il ripristino di miR-34a potrebbe rappresentare un nuovo approccio terapeutico per questo sottogruppo di pazienti.

Aim: L'obiettivo principale di questo studio è stato quello di caratterizzare meglio il complesso crosstalk tra p53, Notch1 e miR-34a. Una migliore comprensione della relazione tra queste entità è necessaria per capire il ruolo che il miR-34a può svolgere nella patogenesi e nella progressione della LLC.

Methods: Diverse linee cellulari di LLC sono state trattate con il composto Nutlin-3a, che attiva p53 inibendo MDM-2, o trasfettate con costrutti che sovraesprimono o silenziano p53. Abbiamo studiato come l'attivazione/silenziamento di p53 influisca sull'attività di segnalazione di Notch1 mediante saggio della luciferasi, sui livelli proteici mediante Western Blot (WB) e sull'espressione di miR-34a mediante RT-qPCR in diverse linee cellulari di LLC. Successivamente, abbiamo analizzato gli effetti della sovraespressione/inibizione del miR-34a sull'attività di segnalazione di Notch1 mediante saggio della luciferasi e sui livelli proteici di Notch1 e BCL-2 mediante WB. Infine, abbiamo caratterizzato i livelli proteici di Notch1 e BCL-2 mediante WB e i livelli di miR-34a mediante RT-qPCR in pazienti affetti da LLC con *TP53* wt e *TP53* mut.

Results: Abbiamo confermato che il miR-34a è un bersaglio trascrizionale di p53 e abbiamo scoperto che l'attivazione di p53 è correlata a livelli più elevati di proteina Notch1 e all'attività di segnalazione. Inoltre, l'attivazione prolungata di p53 da parte del trattamento con Nutlin-3a e, di conseguenza, l'aumento dei livelli di proteina Notch1 sono stati correlati a livelli più elevati di proteina BCL-2. Tuttavia, non abbiamo osservato alcun effetto significativo del miR-34a sull'attività di segnalazione di Notch1 e sui livelli di proteina Notch1 o BCL-2.

Conclusions: Coerentemente con i risultati precedenti, l'induzione di Notch1 da parte di p53 potrebbe rappresentare un meccanismo anti-apoptotico nelle cellule LLC in cui p53 è ancora funzionale. Ipotizziamo che questo meccanismo possa essere ulteriormente supportato dall'induzione di BCL-2 dipendente da Notch1. Inoltre, ipotizziamo che l'induzione di miR-34a da parte di p53 rappresenti un sistema sfruttato dalle cellule B per controllare i livelli di proteina Notch1 e la segnalazione in condizioni fisiologiche.

3. INTRODUCTION

The genome has been frequently described as the "instruction manual" of cells, containing all the information needed to build complex organisms. For a long time, however, it remained a mystery how the same genome could lead to different phenotypes in different cell types within the same organism. This puzzle was solved by adding pieces of biological modulation between the different steps of the central dogma of biology, which is defined as the process by which DNA is transcribed into RNA and finally translated into proteins. All these steps are regulated by the cooperation of multiple molecules and biological processes. Importantly, these mechanisms have been evolutionarily selected to fine-tune how much a given gene is translated into the respective protein. This multi-layered regulation involves several mechanisms such as: chromatin modifications; cell signaling; mRNA splicing, polyadenylation and localization; protein localization, modification and degradation. Among the gene regulatory mechanisms, transcription factors and miRNAs are two of the best studied[1].

Studying the different levels of gene regulatory mechanisms is not only important for understanding how a single genome can give rise to a complex organism composed of many different cell types, but it is also fundamental for understanding how and why the phenotype of cells can be completely altered under pathological conditions such as cancer.

3.1 p53 is a master transcription factor involved in cancer suppression

P53 is the most common transcription factor (TF) found to be mutated in tumors[2,3]. Knockouts or mutations of *TP53* predispose organisms to develop cancer at a young age[4,5]. It has been hypothesized that the evolutionary role of p53 in higher organisms is to prevent tumor formation. In this context, the activation of p53 is triggered by several malignancy-associated stress signals, leading to the inhibition of cell growth and to the induction of apoptosis[6]. Besides tumor-associated activation, other cellular stresses such as hypoxia, DNA damage and nutrient deprivation can lead to the activation of p53 signaling. Activation of p53 mostly affects p53 stability, nuclear localization or interaction with other proteins, triggering a signaling cascade that leads to the induction of apoptosis and inhibition of cell growth[2,6].

Cell growth inhibition is a generalization of several more specific cellular responses that can be elicited by p53, such as cell cycle arrest, senescence, differentiation and apoptosis. Depending on many different intrinsic and extrinsic cellular factors, repair of genotoxic stress, angiogenesis

and feedback regulation of p53 function itself can also be induced by p53 signaling[2,6,7] and thereby it drives various cell fates.

3.1.1 MDM-2 keeps p53 inactive under resting conditions

Owing to the central role of p53 in the induction of cell cycle arrest and apoptosis, it is crucial that p53 activity is kept low under resting conditions[8,9]. Although several levels of regulation have been described in order to modulate p53 activity, the most important is the control of p53 protein stability[10,11]. In this context, the p53-specific E3 ubiquitin ligase MDM-2 plays a pivotal role in inhibiting p53 activity[12,13,14]. MDM-2 binds to the transactivation domain of p53[15,16] and thereby targets p53 for ubiquitination and subsequent proteasomal degradation[17,18]. In addition, MDM-2 interaction with p53 inhibits p53 acetylation and induces shuttling to the cytoplasm preventing the activation of p53 signaling[11,19,20]. Inhibition of MDM-2 via Nutlins, which are potent and selective inhibitors of MDM-2, has been shown to effectively cause non-genotoxic induction and activation of p53[21] (**Fig.1**).

3.1.2 p53 activation induces cell cycle arrest and apoptosis

Activation of p53 in response to cellular stress is initiated by phosphorylation of p53 at Serine (Ser)15 and Ser37[8]. This interferes with the ability of MDM-2 to interact and consequently inhibit p53 activity[22]. Upon activation, p53 protein accumulates in the nucleus and transactivates the expression of several genes that are involved in various cellular processes, two of the most important being cell cycle arrest and apoptosis[23]. Cell cycle arrest is mainly driven by p53-dependent expression of p21[24], which is a cyclin-dependent kinase inhibitor[25]. Instead, p53-dependent apoptosis is multifaceted because it is governed by multiple pro-apoptotic genes that are induced upon p53 transactivation[7]. The major pro-apoptotic genes that are influenced by p53 activation belong to the BCL-2 family and are: *BAX*[26], *PUMA*[27] and *NOXA*[28]. The proteins encoded by these three genes trigger apoptosis by promoting the loss of mitochondrial potential and the release of cytochrome c, which induces the activation of the apoptotic cascade led by the formation of the apoptosome[29] (**Fig. 1**).

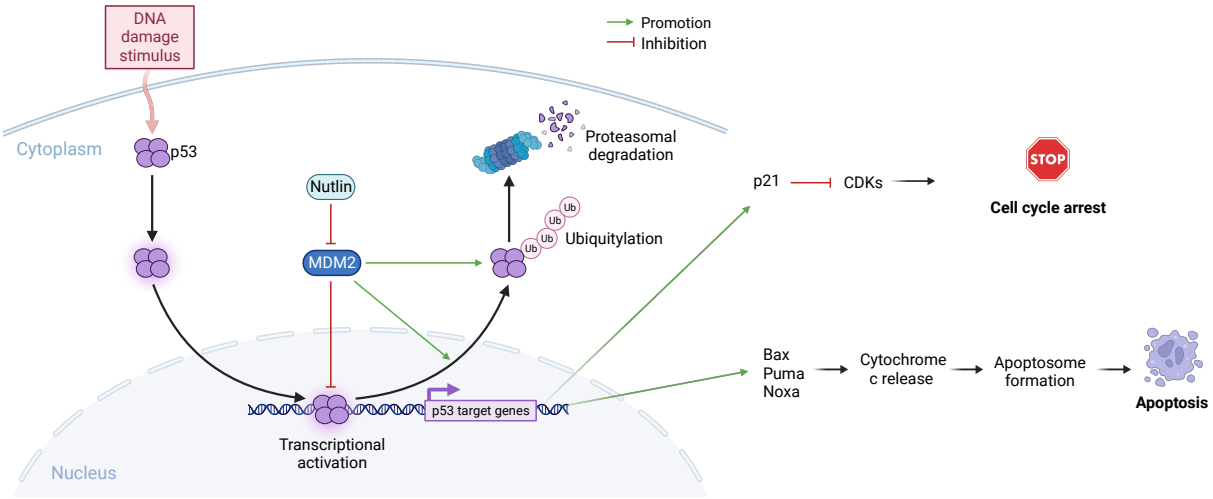


Figure 1 | p53 activation leads to cell cycle arrest or apoptosis.
p53 is activated either by genotoxic stress or via non-genotoxic MDM-2 inhibition (Nutlin treatment). Upon activation, p53 translocates in the nucleus and induces the transcription of target genes that are responsible for cell cycle arrest or apoptosis. The activity of p53 is modulated by the control of p53 protein stability. MDM-2 mediates the polyubiquitination of p53 that leads to p53 proteasomal degradation.
MDM-2, Mouse double minute 2 ; CDKs, Cyclin-dependent kinases. According to Chène et al. 2003 (13) ; Moll and Petrenko 2003 (14), created with BioRender.com.

Considering the importance of the p53 signaling pathway in cell cycle and apoptosis, *TP53* alterations and subsequent deregulation of the p53 signaling pathway lead to uncontrolled cell growth, which is a hallmark of cancer.

3.2 Chronic Lymphocytic Leukemia is aggravated by *TP53* alterations

Chronic Lymphocytic Leukemia (CLL) is a blood malignancy characterized by a progressive accumulation of B-lymphocytes that are morphologically identical to normal mature B-cells[30]. Typically, the onset of CLL is marked by asymptomatic peripheral blood lymphocytosis but, with increasing accumulation of non-functional lymphocytes in secondary lymphoid tissues, symptoms such as lymphadenopathy, hepatosplenomegaly, bone marrow failure and recurrent infections occur. Notably, the genetic causes of CLL are highly heterogeneous, varying from chromosomal aberrations to single gene mutations. As a result, the clinical course and response to treatment among CLL patients is highly individualized, ranging from patients with a slow-growing chronic tumor to patients with rapidly evolving disease that may even progress to a more aggressive state, such as Richter's Transformation[30].

Among CLL patients who present with a more aggressive form of the disease, there is an overrepresentation of *TP53* alterations that occur either as deletion of chromosome 17p13, affecting the genomic locus of *TP53*, or as mutations in the *TP53* gene itself[31,32]. *TP53* alterations in CLL are associated with lack of response to chemotherapeutic agents and short

event-free and overall survival of patients[33]. This explains why the frequency of p53 dysfunction increases up to 50% in CLL patients who experience disease progression upon initial treatment[34]. However, in previously untreated CLL patients approximately 10-15% show alterations in *TP53*[35–37].

Interestingly, CLL patients with *TP53* alterations show lower expression levels of miR-34a[33], which is considered to be a tumor suppressor. Thus, the impairment of this fundamental miRNA in CLL patients with *TP53* alterations, in addition to the loss of p53 function, may explain why these patients manifest such an aggressive form of the disease.

3.3 miRNAs play a fundamental role in CLL

It is now well established that microRNA (miRNA) dysregulation is associated with tumorigenesis[38–40]. The first evidence pointing this direction came from a study in CLL in 2002, making CLL the first model in which the role of miRNAs in oncogenesis was established[41]. After the first study, more and more studies have correlated miRNAs with pathogenesis and clinical outcome of CLL patients[42]. Consequently, the evaluation of the expression levels of miRNAs, found to play a role in CLL pathogenesis, has often been proposed as a useful tool in order to predict patient outcome and response to treatment[43].

3.3.1 miRNAs represent a finely tuned mechanism to post-transcriptionally regulate gene expression

As part of the gene expression regulatory system, miRNAs are involved in many biological processes[44]. Consequently, miRNAs transcriptional regulation has to be finely tuned depending on the cell type[45]. For this reason, different tissues in the body exhibit specific miRNA expression profiles[46].

Mature miRNAs are a class of small (approximately 20 nucleotides long), non-coding, endogenous RNAs[47] and are the result of a multi-step process. The first step is the transcription of the primary transcript (pri-miRNA), mainly performed by RNA Polymerases II and III. Subsequently, the pri-miRNA is processed into a stem-loop-structured miRNA precursor (pre-miRNA) by the nuclear RNAse III enzyme Drosha. Pre-miRNA is then transported from the

nucleus to the cytoplasm, where its harpin-structured part is cleaved by the cytosolic RNAse III Dicer, leading to the generation of a short-lived dsRNA of about 20-25 nucleotides[47] (**Fig. 2**).

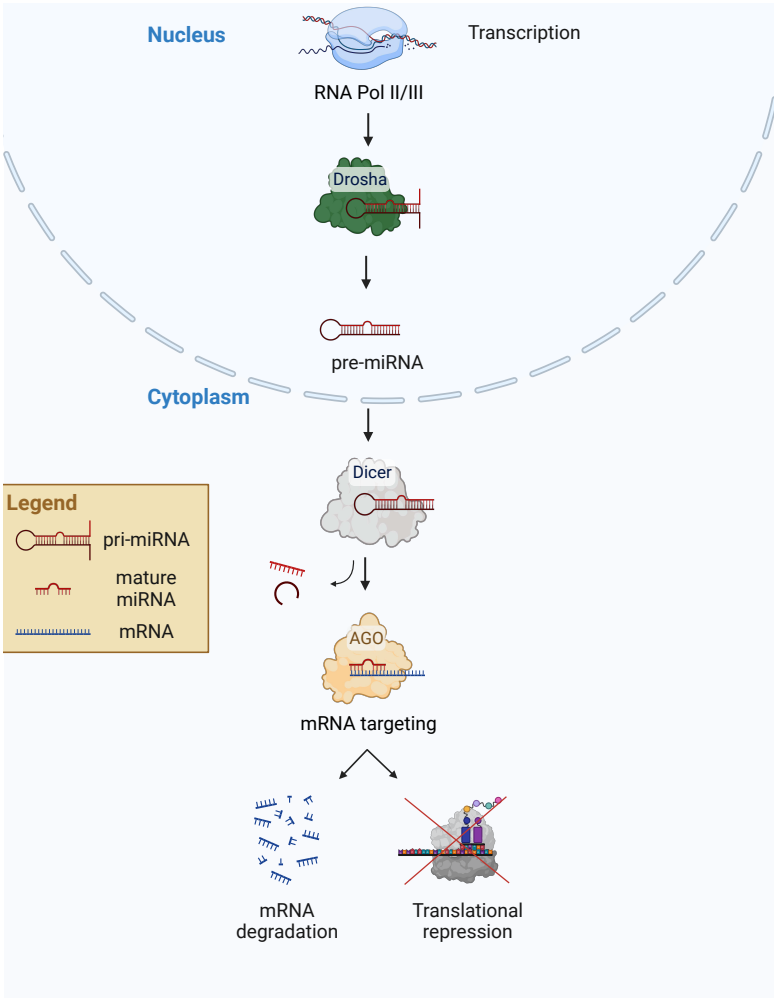


Figure 2 | From miRNA maturation to gene regulation: a multi-step process.
Pri-miRNAs are transcribed by RNA Polymerase II or III and processed into pre-miRNAs by Drosha. Pre-miRNAs are then transported form the nucleus to the cytoplasm and processed into small dsRNAs by Dicer. Only one RNA strand represent the mature miRNA that is loaded onto the Argonaute protein and guides mRNA targeting. Once targeted, mRNA is either degraded or its translation is repressed.
RNA Pol II/III, RNA Polymerase II/III; miRNA, microRNA; mRNA, messenger RNA, ds, double stranded; AGO, Argonaute protein. Modified from template created by Andris Finkbeiner (creator) and Wendy Jiang. Created with BioRender.com.

After processing, only one strand of the short-lived dsRNA is loaded on the Argonaute protein (AGO) as a mature miRNA to target the specific transcript. In order to recognize and target a specific mRNA, the seed region in the 5'-end of the miRNA has to be complementary to a binding site in the 3'-untranslated region (3'-UTR) of the targeted mRNA[48]. Depending on the complementarity between these two sequences, the miRNA can have different effects on the

targeted mRNA[47]. If there is sufficient complementarity between the miRNA and the target mRNA, the mRNA is cleaved and degraded. If there is insufficient complementarity, silencing occurs by translational repression and the specific mRNA is not cleaved[49,50]. Since in most of the cases human miRNAs imperfectly pair with their targets[51], translational repression is a common mechanism by which miRNAs regulate gene expression.

3.3.2 Different models may explain miRNAs effects in cells

In the last years, several models have been proposed to explain the purpose of miRNAs in the regulation of gene expression:

1. miRNAs can act as "binary off-switches" that reinforce the repression of certain genes that should not be expressed in specific cell types[52].
2. miRNAs can help cells to distinguish between real and stochastic fluctuations in gene expression. In this scenario, the presence of a basal level of a miRNA sets a stricter threshold for target expression and subsequent activity[53].
3. In the case of proteins that are not normally expressed in certain cells, the expression of miRNAs against these protein targets can further prevent their expression. This mechanism may represent an additional safety measure by which cells avoid the expression of aberrant proteins[54].
4. miRNAs can act as "rheostats" to dampen and fine-tune the levels of a specific protein[55].

It is important to consider the complementarity of these models, as none of them excludes the others. In fact, for the same miRNA, different models may apply depending on the different targets, cell types and developmental stages[44].

These models explain how miRNAs are involved in the modulation of gene and protein expression and the resulting regulation of signaling pathways. In pathological conditions such as cancer, this ideal balance between gene regulation and signaling networks can be disrupted. This overall imbalance in tumors is reflected by dysregulated expression levels of miRNAs which can be either "oncomirs" that promote oncogenesis or be tumor suppressors that suppress oncogenesis[45].

3.3.3 miR-34a is a tumor suppressor

MiR-34a belongs to the group of miRNAs that act as tumor suppressors. As a direct transcriptional target of p53, miR-34a mediates some key processes triggered by p53 transactivation[56]. In fact, miR-34a induction by p53 has been shown to extensively influence gene expression[57]. Genes that are targeted by miR-34a regulate: proliferation (*Notch1*, cyclins, cyclin-dependent kinases, *MYC*...), apoptosis (*BCL-2*, *SIRT1* and *BIRC5*), senescence (*E2F3*), stemness (*CD44*, *NANOG* and *SOX2*), motility (*SNAI1*, *MET* and *AXIN2*) or immune evasion (such as *PD-L1*)[56]. Further evidence highlighting the role of miR-34a as a tumor suppressor comes from clinical data where miR-34a levels are found to be reduced in several cancer entities[56].

In CLL, miR-34a expression is reduced in those patients with impaired p53 activity. The fact that miR-34a targets Notch1 and BCL-2, two important proteins involved in the pathogenesis of CLL[58,59], might explain, in addition to p53 dysfunction, why CLL patients with *TP53* alterations present with a more aggressive form of the disease.

3.4 Notch1 hyperactivation promotes leukemic development

Notch1 is a major target of miR-34a. Therefore, impairment of miR-34a expression levels may result in dysregulation of the Notch1 signaling pathway. Dysregulation of Notch1 signaling promotes proliferation and survival in several cell types under pathological conditions[60]. Notably, Notch1 is overexpressed in B-CLL cells compared to circulating normal B lymphocytes and it plays a critical role in leukemic development by promoting survival of malignant cells through inhibition of apoptosis[61].

3.4.1 Notch1 signaling is activated by several cleavage steps and regulated via proteasomal degradation

The Notch1 signaling pathway is involved in a variety of biological processes in cells[62] and the response that is elicited by Notch1 activation depends on several aspects, including interactions with other signaling pathways and the developmental stage[63,64]. In order to initiate the juxtacrine Notch1 signaling pathway, cell-cell contact is required. Specifically, signal-sending cells expose Notch1-ligands on the membrane, which belong to either the JAGGED or DELTA family. These are directly presented to the signal-receiving cells, which instead presents the

heterodimeric Notch1-receptor on the membrane[65] (**Fig.4**). The mature form of Notch1 consists of two subunits held together by non-covalent interactions. In particular, the N-terminal extracellular domain of Notch1 (Notch1-EC), contains a series of epidermal growth factor (EGF)-like repeats which are responsible for ligand recognition. The C-terminal domain of Notch1 consists of a small transmembrane and the intracellular domain of Notch1 (Notch1-TMIC). The intracellular domain of Notch1 (NICD) is composed of several parts that are important to mediate signaling. Among these, the very C-terminal PEST domain, a region rich in proline (P), glutamic acid (E), serine (S) and threonine (T), is critical for regulating Notch1 stability and proteasomal degradation[58] (**Fig.3**).

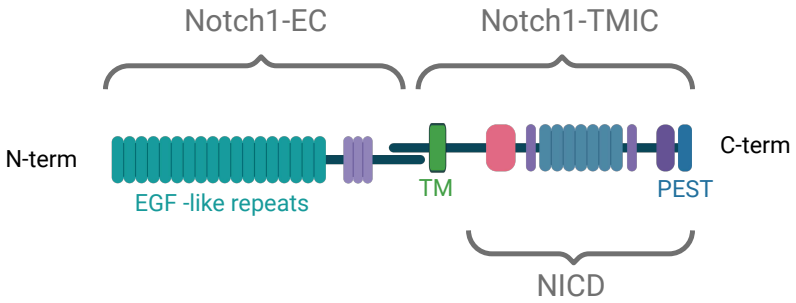


Figure 3 | Notch1 is composed of different domains.
Notch1 is a heterodimeric receptor consisting of an extracellular domain (Notch1-EC) and a transmembrane and intracellular domains (Notch1-TMIC). Notch1-EC is formed by several EGF-like repeats that are important for ligand recognition. Notch1-TMIC is composed of a small transmembrane portion (TM) and the intracellular domain of Notch1 (NICD). The most C-terminal part of the NICD contains the PEST domain, a region rich in proline (P), glutamic acid (E), serine (S) and threonine (T), that regulates Notch1 stability. According to Rosati et al. 2018 (58), created with Biorender.com

Upon interaction with the ligand, the Notch1 receptor undergoes a conformational change, which exposes the normally hidden Notch1 S2 cleavage site to ADAM metalloproteases. The S2 cleavage results in the separation of the Notch1-EC from the Notch1-TMIC. Next, Notch1-TMIC undergoes a further cleavage step (S3 cleavage) mediated by the γ-secretase. Following S3 cleavage, the NICD is released from the cell membrane and translocates into the nucleus where it accumulates.

In the nucleus, the DNA-bound RBP-J (Recombination Signal Binding Protein-J) recruits corepressor proteins and histone deacetylases to prevent Notch1 signaling in the absence of NICD. Once NICD accumulates in the nucleus, it binds to RBP-J and causes its conformational

change. Following this conformational change, RBP-J stop to interact with repressor proteins and recruits transcriptional coactivators, including Mastermind-like protein (MAM-L). The newly formed complex is responsible for the transcriptional induction of Notch1 target genes belonging to the HES (Hairy/Enhancer of Split) or HEY (Hairy/Enhancer of Split related to YRPW motif) gene families and others[66] (**Fig. 4**).

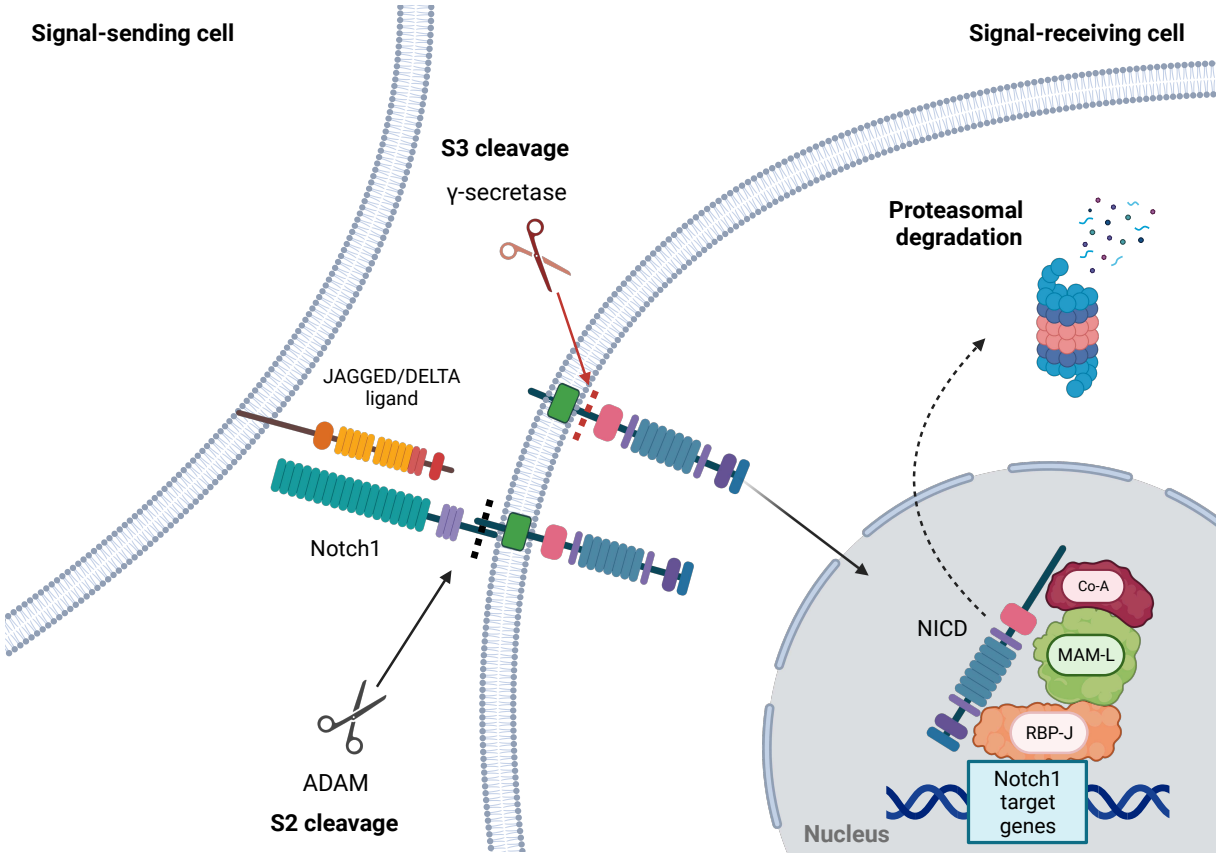


Figure 4 | Notch1 signaling is activated by several cleavage steps and regulated via proteasomal degradation.
Upon interaction with the ligand (either JAGGED or DELTA family), the Notch1 receptor undergoes S2 cleavage and S3 cleavage. Upon S3 cleavage, the intracellular domain of Notch1 is released into the cytoplasm and translocates to the nucleus. In the nucleus, NICD interacts with RBP-J, MAM-L and other co-activators, leading to the formation of a transcriptional complex that induces the expression of the Notch1 target genes. NICD signaling activity is finely tuned by controlling NICD stability through proteasomal degradation. ADAM, A-disintegrin and Metalloproteinase; NICD, Notch1 intracellular domain, RBP-J, Recombinant signal protein-J, MAM-L, Mastermind-like protein; Co-A, Co-activators. According to Rosati et al. 2018 (58), created with BioRender.com.

Under physiological conditions, cells tightly control Notch1 signaling by regulating Notch1 protein synthesis and degradation[67]. Notch1 is a short-lived protein that undergoes rapid ubiquitin-mediated degradation by the proteosome[68]. The PEST domain is responsible for controlling E3-ubiquitin ligase mediated turnover of Notch1[69] and its disruption results in increased NICD half-life[70].

3.4.2 NICD PEST domain mutations cause increased NICD signaling in CLL

In CLL, *Notch1* mutations account for 6-12% of the genetic aberrations found in patients at initial diagnosis[58,71]. Most *Notch1* mutational events, approximately 80%[58], cause truncation of the PEST domain of NICD[71,72]. The loss of the PEST domain results in impaired NICD degradation, which increases NICD stability and consequently hyperactivates NICD signaling. Hyperactivated NICD signaling is reflected by increased survival and apoptosis resistance of the malignant clone in CLL[73]. Therefore, *Notch1* mutations represent a poor prognostic factor in CLL[74]. Indeed, CLL patients with *Notch1* mutations at the time of CLL diagnosis show a more aggressive disease with a higher risk of progression to Richter's Transformation[75].

To date, no Notch1-targeted therapies have reached the clinic for the treatment of CLL patients with *Notch1* mutations. However, compounds targeting the B cell receptor (BCR) signaling pathway such as Ibrutininb or BCL-2 such as Venetoclax have been shown to be highly effective in the treatment of CLL patients. *Notch1* alterations have been shown to have no negative impact on the therapeutic efficacy of Ibrutinib[76]. Therfore, Ibrutininb is used to treat the subset of CLL patients with *Notch1* mutations. Interestingly, *Notch1* mutations associated with a low BAX/BCL-2 ratio correlated with poorer prognosis in CLL patients treated with Ibrutininb[77]. These findings may suggest the possibility of using BCL-2 inhibitors, such as Venetoclax, to improve the efficacy of Ibrutinib in *Notch1*-mutated CLL patients[58].

3.5 BCL-2 is highly expressed in CLL and inhibits apoptosis

In CLL, the BCL-2 protein has been found to be highly expressed[78–80] and therapies with selective BCL-2 inhibitors such as Venetoclax have been shown to be highly effective in treating the disease[81]. Since the discovery of BCL-2 in non-Hodgkin's B-cell lymphoma[82], the involvement of BCL-2 in programmed cell death has been well established. In particular, BCL-2 plays a major role in the inhibition of the intrinsic apoptotic pathway[83].

3.5.1 BCL-2 inhibits the intrinsic apoptosis pathway

The intrinsic or mitochondrial pathway of apoptosis is a type of programmed cell death that occurs mainly within the mitochondria. In particular, various types of stress such as DNA

damage can be sensed by sensor proteins such as p53, which induce the expression of effector proteins. The effector proteins mediate cellular changes characteristic of apoptotic cells[83]. In this process, the BCL-2 family proteins are particularly important effectors.

BCL-2 family proteins can be divided into three distinct groups according to their function:

- Pro-apoptotic BH-3-only proteins such as BAD, BIM, BID and NOXA.
- Pro-apoptotic pore-forming proteins such as BAX and BAK.
- Anti-apoptotic proteins such as BCL-2, BCL-X_L and MCL-1[84].

BH-3-only pro-apoptotic proteins are responsible for the activation of pro-apoptotic pore formers, which upon induction oligomerize and form pores in the mitochondrial outer membrane. This process, called Mitochondrial Outer Membrane Permeabilization (MOMP), results in the release of cytochrome c and other proapoptotic factors into the cytoplasm[85]. In this context, anti-apoptotic proteins, such as BCL-2, prevent apoptosis by sequestering both pro-apoptotic BH-3-only proteins and pro-apoptotic pore-forming proteins[83–85] (**Fig. 5**).

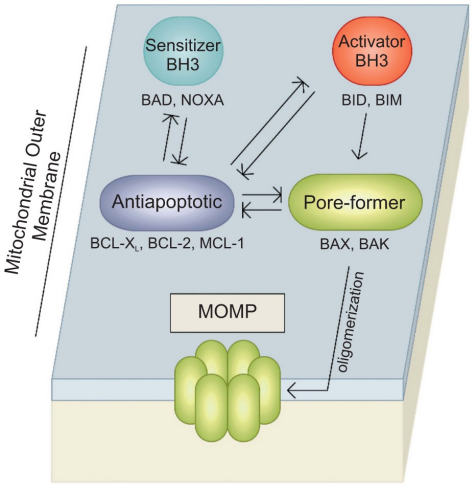


Figure 5 | Mitochondrial Outer Membrane Permeabilization is regulated by BCL-2 family proteins.
Mitochondrial Outer Membrane Permeabilization (MOMP) consists of pore formation by oligomerization of pro-apoptotic pore-forming proteins such as BAX and BAK in the mitochondrial outer membrane. This process is finely regulated by pro-apoptotic BH3-only proteins (sensitizers and activators), which promote pore formation, and by antiapoptotic BH3 proteins such as BCL-2, which inhibit pore formation. Taken from Fig. 1a, Kale et al. 2018 (85).

3.6 Aim of the thesis

The incessant discovery of new branches of the p53 signaling network continues to add further complexity to this crucial cellular pathway. In this study, we aimed to better characterize the crosstalk between p53 and Notch1.

We started from the following previous findings:

1. *Notch1* mutations and *TP53* alterations are two independent markers of poor prognosis in CLL.
2. CLL patients with *TP53* alterations show lower miR-34a expression levels.
3. Notch1 is a major target of miR-34a.

We hypothesized that CLL patients with *TP53* alterations and consequently low levels of miR-34a would express higher levels of Notch1 protein and show higher NICD signaling.

In CLL, hyperactivated NICD signaling is involved in the survival of malignant cells. Therefore, higher levels of Notch1 protein and NICD signaling in patients with low levels of miR-34a due to *TP53* alterations would be another reason for a more aggressive form of the disease in this subset of patients. Consequently, restoring miR-34a levels might represent a novel therapeutic approach to treat CLL patients with *TP53* alterations.

To better characterize the role of miR-34a in the crosstalk between p53 and Notch1, we investigated how p53 activation/silencing affects Notch1 signaling, protein levels and miR-34a expression in different CLL cell lines. Next, we analyzed the effects of miR-34a overexpression/inhibition on Notch1 signaling and Notch1 and BCL-2 protein levels. Finally, we characterized Notch1 and BCL-2 protein levels and miR-34a levels in both *TP53* wt and *TP53* mut CLL patients.

4. MATERIALS

4.1 Chemical reagents

Amersham ECL™ Western Blotting Detection Reagents	*GE Healthcare* *Little Chalfont, UK*
Ampicillin Natriumsalz	*Carl Roth GmbH* *Karlsruhe, Germany*
Chloroform	*VWR chemicals* *Fontenay-sous-Bois, France*
Complete™ Mini EDTA-free protease inhibitor cocktail	*Roche* *Mannheim, Germany*
Dimethyl sulfoxide (DMSO)	*Sigma-Aldrich* *Steinheim, Germany*
Distilled water (ddH2O)	*B. Braun* *Melsungen, Germany*
Dithiotreitol (DTT)	*Thermo Scientific* *Carlsbad, CA, USA*
Ethanol	*Sigma-Aldrich* *Steinheim, Germany*
FITC-Annexin V	*BD Biosciences* *East Rutherford, NJ, USA*
Glacial acetic acid	*VWR chemicals* *Fontenay-sous-Bois, France*
LB-Agar	*Carl Roth GmbH* *Karlsruhe, Germany*
Methanol	*Sigma-Aldrich* *Steinheim, Germany*
NaCl	*Sigma-Aldrich* *Steinheim, Germany*

Nutlin-3a	*Sigma-Aldrich* *Steinheim, Germany*
Ponceau S	*Fluka* *Buchs, Switzerland*
Super Signal® West Femto ECL	*Thermo Scientific* *Rockford, IL, USA*
Skim milk powder	*Sigma-Aldrich* *Steinheim, Germany*
Tween20	*Sigma-Aldrich* *Steinheim, Germany*
7-Aminoactinomycin D (7-AAD)	*BD Biosciences* *East Rutherford, NJ, USA*

4.2 Buffers and solutions

1x PBS Dulbecco, w/o Ca^{2+} w/o Mg^{2+}	*Biochrom GmbH* *Berlin, Germany*
5x Passive Lysis Buffer	*Promega; Madison, USA*
10x PBS	*Gibco; Grand Island, USA*
HEPES Buffered Saline Solution	*Sigma-Aldrich* *Steinheim, Germany*
NuPAGE™ LDS Sample Buffer (4X)	*Novex by life technologies* *Carlsbad, USA*
NuPAGE™ LDS MOPS SDS Running Buffer (20X)	*Novex by life technologies* *Carlsbad, USA*
NuPAGE™ Transfer Buffer (20X)	*Novex by life technologies* *Carlsbad, USA*

PageRuler™ Prestained Protein Ladder	*Thermo Scientific* *Rockford, IL, USA*
QIAzol® Lysis Reagent	*Qiagen; Hilden, Germany*
RIPA Buffer	*Sigma-Aldrich* *Steinheim, Germany*
RNAse Away®	*Molecular BioProducts* *San Diego, CA, USA*

Table 1 | Composition of PBS-Tween.

PBS-Tween (PBS-T)	1000 mL
ddH_2O	900 mL
10x PBS	100 mL
Tween20	0.01%

Table 2 | Composition of Transfer Buffer.

Transfer Buffer	1000 mL
ddH_2O	850 mL
Methanol	100 mL
NuPAGE™ Transfer Buffer (20X)	50 mL

Table 3 | Composition of Annexin V Binding Buffer.

Annexin V Binding Buffer	1000 mL
ddH_2O	959.5 mL
5M NaCl	28 mL
1M HEPES	10 mL
1M $CaCl_2$	2.5 mL

Table 4 | Recipe for 5% Skim Milk in PBS-T.

5% Skim Milk in PBS-T	200 mL
PBS-T	200 mL
Skim Milk	10 g

Table 5 | Recipe for Ponceau S staining solution.

Ponceau S staining solution	1000 mL
ddH_2O	950 mL
Glacial acetic acid	50 mL
Ponceau S	1 g

4.3 Kits

Amaxa™ Cell Line Nucleofector™ Kit T	*Lonza; Cologne, Germany*
Dual Luciferase Assay Kit	*Promega; Madison, USA*
miRCURY LNA™ RT Kit	*Qiagen; Hilden, Germany*
miRCURY LNA™ SYBR Green Kit	*Qiagen; Hilden, Germany*
miRNeasy® Mini Kit	*Qiagen; Hilden, Germany*
Pierce™ BCA Protein Assay Kit	*Thermo Scientific Rockford, IL, USA*
ProFection® mammalian transfection system	*Promega; Madison, USA*
Qubit™ RNA BR Assay Kit	*Invitrogen Carlsbad, CA, USA*
Qubit™ dsDNA BR Assay Kit	*Invitrogen Carlsbad, CA, USA*
ZymoPURE™ II Plasmid Maxiprep Kit	*Zymo Research Freiburg in B., Germany*

4.4 Antibodies

Table 6 | Used primary and secondary antibodies.

Epitope	**Clone Number**	**Type**	**Species**	**Dilution**	**Manufacturer**
BCL-2	N-19	Primary polyclonal	Rabbit	1:1000	Santa Cruz Biotechnology

p53	7F5	Primary polyclonal	Rabbit	1:1000	Cell Signaling
Notch1-TM	D1E11	Primary monoclonal	Rabbit	1:1000	Cell Signaling
p21 Waf/Cip1	12D1	Primary monoclonal	Rabbit	1:1000	Cell Signaling
β-actin HRP-coupled	A3854		Mouse	1:20000	Sigma
Anti-Rabbit HRP-coupled	P0448	Secondary polyclonal	Goat	1:4000	Dako

4.5 Plasmids

Table 7 | Used plasmids.

Plasmid	**Name**	**Additional Information**
pcDNA3 Flag-p53	Flag-p53 (#441)	AddGene: #10838
pcDNA3 p53	p53 (#444)	AddGene: # 69003
pcDNA3 empty vector	Empty Vector (#478)	A kind gift from Franz Oswald, Internal Medicine I, University hospital Ulm
pcDNA3.1(-)hsNICD-EHEB	NICD	A kind gift from Sabrina Kugler, Internal Medicine III, University hospital Ulm. Derived from the EHEB cell line. The plasmid codes for wild type NICD starting at amino acid (aa) 1761 of human NOTCH1 (reference NP_060087) till the last amino acid (2555).
pcDNA3-dnMam-GFP	dnMAM	A kind gift from Franz Oswald, Internal Medicine I, University hospital Ulm
pLKO.1-TP53shRNA	p53 shRNA (#519)	Sequence targeting p53: CGGCGCACAGAGGAAGAGAAT
pLKO.1-TP53shRNA	p53 shRNA (#520)	Sequence targeting p53: TCAGACCTATGGAAACTACTT
pLKO.1-TP53shRNA	p53 shRNA (#521)	Sequence targeting p53: GTCCAGATGAAGCTCCCAGAA
pLKO.1-TP53shRNA	p53 shRNA (#522)	Sequence targeting p53: CACCATCCACTACAACTACAT
pLKO.1-scrambled shRNA	scr shRNA	Gift from Franz Oswald.
pGL3 Sensor 1.1	pGL3 Sensor 1.1	Firefly Luciferase reporter construct containing several miR-34a target sites. Gift from Franz Oswald.
hsa-miR-34a-Precursor	Pre-miR-34a (#1)	Thermo Fisher AM17100 PM11030

miR Precursor Negative Control	Pre-miR NC (#2)	Thermo Fisher AM17111
anti-hsa-miR-34a	Anti-miR-34a (#3)	Thermo Fisher AM17000 AM11030
anti-miR Negative Control	Anti-miR NC (#4)	Thermo Fisher AM17010
pcDNA3-USP28-GFP	USP28-GFP	Gift from Franz Oswald.
pGL3-Hes1-Luc	pGL3-Hes1-Luc	Firefly Luciferase reporter construct under the control of Hes1 promoter. Gift from Franz Oswald.
Renilla control	Renilla control	Renilla Luciferase reporter under the control of a constitutive promoter. Promega

4.6 Primers

Table 8 | Used primers.

Primer	**Target**	**Company**	**Catalog number**
miRCURY LNA miRNA Assay hsa-U6 snRNA	U6 snRNA	Qiagen Hilden, Germany	YP00203907
miRCURY LNA miRNA Assay hsa-SNORD 38b	SNORD 38b	Qiagen Hilden, Germany	YP00203901
miRCURY LNA miRNA Assay hsa-SNORD 44	SNORD 44	Qiagen Hilden, Germany	YP00203902
miRCURY LNA miRNA Assay hsa-miR-34a-5p	miR-34a	Qiagen Hilden, Germany	YP00204486
miRCURY LNA miRNA Assay hsa-miR-21-5p	miR-21	Qiagen Hilden, Germany	YP00204230
miRCURY LNA miRNA Assay hsa-miR-20a-5p	miR-20a	Qiagen Hilden, Germany	YP00204292
miRCURY LNA miRNA Assay hsa-miR-26b-5p	miR-26b	Qiagen Hilden, Germany	YP00204172
miRCURY LNA miRNA Assay hsa-miR-140-5p	miR-140	Qiagen Hilden, Germany	YP00204540

4.7 Cell culture solutions and media

DMEM (1X)	*Gibco by Life Technologies* *Grand Island, USA*
L-Glutamine	*Biochrom GmbH* *Berlin, Germany*
FBS Superior	*Biochrom GmbH* *Berlin, Germany*
RPMI 1640	*Gibco by Life Technologies* *Grand Island, USA*
Trypsin-EDTA (10X)	*Gibco by Life Technologies* *Grand Island, USA*

4.8 Cell lines

Table 9 | Cell lines used.

Name	***Type***	***TP53***	***Medium***
HEK293 *ATCC: #CRL-157* ™	Epithelial Cell Line	wt	DMEM supplemented with 10% FBS
HG-3 *DMSZ: #ACC 765*	B-CLL Cell Line	wt	RPMI 1640 supplemented with 10% FBS and 5% L-glutamine
EHEB *DMSZ: #ACC 67*	B-CLL Cell line	wt	RPMI 1640 supplemented with 10% FBS and 5% L-glutamine

WA-OSEL *DMSZ: #ACC 767*	B-CLL Cell Line	wt	RPMI 1640 supplemented with 10% FBS and 5% L-glutamine
MEC-1 *DMSZ: #ACC 497*	B-CLL Cell Line	mut*	RPMI 1640 supplemented with 10% FBS and 5% L-glutamine
MEC-2 *DMSZ: #ACC 500*	B-CLL Cell Line	mut*	RPMI 1640 supplemented with 10% FBS and 5% L-glutamine

*MEC-1 and MEC-2 represent two different subclones in different stages of progression. In both cell lines TP53 present the following mutations: 7,576,896 T>G amino acid Q317P homozygous[86].

4.9 Primary CLL patients cells

Primary CLL patient cells were obtained from patients at the outpatient clinic of the university hospital Ulm after written informed consent (Ulm Ethics Committee, Votum 96/08) in accordance with the Declaration of Helsinki. CD19+ lymphocytes were isolated from heparinized blood of CLL patients and viably preserved in the liquid nitrogen sample bank.

Table 10 | Patient cohort characteristics.

Patients Groups	p53 wt status	p53 altered status
Number of patients	10	10
Sex	Female: 4/10 Male: 6/10	Female: 2/10 Male: 8/10
Median age (y)	60,5 (51-75)	70 (66-86)
Binet stage	A: 6/10 B:4/10 C: 0/10 Undetermined: 0/10	A: 5/10 B: 1/10 C: 1/10 Undetermined: 3/10
Treatment	Yes: 6/10 No: 4/10	Yes: 7/10 No: 3/10
IGHV status	Mutated: 7/10 Unmutated: 3/10 n/a: 0/10	Mutated: 6/10 Unmutated: 1/10 n/a: 3/10
Chromosomal aberrations	Het 17p13 del: 0/10 Het 13q14.3 del: 8/10 (1/8 homozygote) Trisomy 12: 0/10 Het 11q22 del: 2/10 Normal karyotype: 2/10	Het 17p13 del: 8/10 (p53 remaining allele mutated in all cases, 2/10 patients only with p53 mut) Het 13q14.3 del: 5/10 Trisomy 12: 1/10 Het 11q22 del: 1/10 Normal karyotype: 0/10

4.10 Consumables

6-well plates	*Greiner Bio-One* *Kremsmünster, Austria*
48-well plates	*Greiner Bio-One* *Kremsmünster, Austria*
96-well plates	*Thermo Scientific* *Rockford, IL, USA*
ClipTip 384 with filter (12.5 µL)	*Thermo Scientific* *Rockford, IL, USA*

Combitips advanced® (0.2, 0.5, 10 mL)	*Eppendorf; Hamburg, Germany*
Falcon® tubes (15, 50 mL)	*Corning Science; Mexico*
Flow Cytometry tubes	*VWR; Radnor, USA*
Luciferase 96-well plates (white)	*Greiner Bio-One* *Kremsmünster, Austria*
MicroAmp® Optical 384-Well Reaction Plates	*Applied Biosystems* *Foster City, CA, USA*
Nitril gloves	*B. Braun* *Melsungen, Germany*
Nunc™ EasYFlask™ (25, 75, 175 cm^2) Nuclon Delta Surface	*Thermo Scientific* *Rockford, IL, USA*
NuPAGE™ 4-12% Bis-Tris Protein Gels 1.5 mm , 10-well	*Novex by Life Technologies* *Carlsbad, USA*
NuPAGE™ 4-12% Bis-Tris Protein Gels 1.0 mm , 12-well	*Novex by Life Technologies* *Carlsbad, USA*
NuPAGE™ 4-12% Bis-Tris Protein Gels 1.0 mm , 15-well	*Novex by Life Technologies* *Carlsbad, USA*
NuPAGE™ 4-12% Bis-Tris Protein Gels 1.0 mm , 26-well	*Novex by Life Technologies* *Carlsbad, USA*
PCR tubes	*Nerbe Plus GmbH* *Wissen/Luhe, Germany*
Pipette tips with filter (10, 20, 100, 200, 1000 µL)	*Sarsedt AG;* *Nürnbrecht, Germany*
Plates for bacterial culture (100 x 15 mm)	*Falcon® Corning Incorporated* *Durham, NC, USA*
Polystyrene tubes	*Sarsedt AG;* *Nürnbrecht, Germany*

PVDF-membrane	*Merck Millipore; Burlington, USA*
Reaction tubes (0.5, 1.0, 1.5 mL)	*Eppendorf; Hamburg, Germany*
Serological pipettes (2, 5, 10, 25 mL)	*Falcon® Corning Incorporated Durham, NC, USA*
T-Shaped Cell Spreaders	*VWR; Radnor, USA*
Whatman® gel blotting paper	*Sigma-Aldrich Steinheim, Germany*

4.11 Instruments

Amaxa Nucleofector II	*Lonza; Cologne, Germany*
Balance	*Sartorius; Göttingen, Germany*
Biometra Fastblot blotting chamber	*Analytic Jena; Jena, Germany*
Centrifuge, Allegra® X-12	*Beckman Coulter; Brea, USA*
Centrifuge, Galaxy Mini	*VWR; Radnor, USA*
Centrifuge, multifuge 3 X3R	*Heraeus; Hanau, Germany*
Centrifuge, Pico 17	*Heraeus; Hanau, Germany*
Centrifuge, Z 233 MK-2	*Hermle; Wehingen, Germany*
Cold room, 4°C	*Weiss Technik Reiskirchen-Lindenstruth, Germany*
Cytoflex S Flow Cytometer	*Beckman Coulter; Brea, USA*
E1-ClipTip Pipette	*Thermo Scientific Rockford, IL, USA*
Electrophoresis power supply, EV231	*Consort; Belgium*
Freezer -20°C	*Bosch; Gerlingen, Germany*
Freezer -80°C, U725 Innova	*New Brunswick Scientific Co. Edison, New Jersey, USA*
Fridge 4°C	*Liebherr; Germany*

Fusion FX6 Edge	*Vilber; France*
WB gel running chamber XCell SureLock™ Mini-Cell	*Invitrogen* *Carlsbad, CA, USA*
Glomax Discover	*Promega; Wisconsin, USA*
Heating block	*Grant; Cambridge, UK*
Incubator for Bacteria and BCA Assay, 37°C	*Heraeus; Hanau, Germany*
Incubator for Cell Line, 37°C	*Thermo Scientific; USA*
Ice machine AF124	*Scotsman Ice Systems; Milan, Italy*
Labcycler Gradient	*SensoQuest GmbH* *Göttingen, Germany*
Laminar flow hood for bacteria LaminAir®, HA 2448 GS	*Heraeus; Hanau, Germany*
Laminar flow hood for cell lines sterilGARD III Advance	*The Baker Company* Stanford, USA
Magnetic Stirrer	*IKAmag; Staufen, Germany*
Microscope, Olympus CK2	*Zeiss; Oberkochen, Germany*
Multipipette® E3	*Eppendorf; Hamburg, Germany*
Needles, Sterican® (0.70 x 30 mm)	*B. Braun* *Melsungen, Germany*
Pipetboy	*Integra Biosciences; Germany*
Pipettes (2, 10, 20, 100, 200, 1000 µL)	*Gilson; Middleton, WI, USA*
QuantStudio™ 5 Real-Time PCR	*Applied Biosystems* *Foster City, CA, USA*
Qubit™ 2.0 Fluorometer	*Invitrogen* *Carlsbad, CA, USA*
Shaker	*NeoLab®; Heidelberg, Germany*

Sunrise absorbance plate reader	*Tecan Trading AG; Switzerland*
Plastipak™ Syringes	*BD Biosciences* *East Rutherford, NJ, USA*
Tube rotator	*Thermo Fisher; USA*
Vi-Cell XR Cell Counter	*Beckman Coulter; Brea, USA*
Vortex Genie 2	*Scientific Industries* *Bohemia, NY, USA*
Water bath	*Julabo; Seelbach, Germany*

4.12 Software

CytExpert	*Beckman Coulter; Brea, USA*
Glomax	*Promega; Wisconsin, USA*
GraphPad Prism	*GraphPad Software Inc.* *San Diego, CA, USA*
Image J	*NIH*
Magellan 3.11	*Tecan Trading AG; Switzerland*
Microsoft Excell	*Microsoft Corp.* *Washington, USA*
QuantStudio™ Design & Analysis Software	*Applied Biosystems* *Foster City, CA, USA*
RepDilPCR*	*German Cancer Research Center (DKFZ)* *Heidelberg, Germany*

**Link for RepDilPCR: https://repdilpcr.eu/app/repDilPCR*

5. METHODS

5.1 Cell Culture Methods

5.1.1 Cell thawing

Cryo-vials containing cells frozen in medium supplemented with 10 % DMSO were removed from the liquid nitrogen storage and placed in the water-bath at 37°C until the cell suspension was completely thawed. The cell suspension was then mixed with 1 mL of the dedicated cell medium and centrifuged for 5 min at 1200 rpm. The supernatant was discarded and the cell pellet was resuspended in 7 mL of medium and seeded in T25 flasks. The next day, the cells were transferred to T75 flasks.

CLL primary cells were thawed using the same procedure as for the cell lines, except that the cell pellet was resuspended in 5 mL of medium. After overnight (ON) culture, primary cells were directly harvested, counted and pelleted for the subsequent analysis.

5.1.2 Cell culturing

All the cell lines were maintained in the incubator under the following conditions: 37°C, 5% CO_2 and saturated humidity.

Cells were split twice a week according to the following steps:

- Collection of cells in Falcon tube.
- Centrifugation at 1200 rpm for 5 min.
- Removal of the supernatant.
- Resuspension of cells in 1 mL of medium.
- Counting of the cells (see **5.1.3**).
- Resuspension of cells in 15 mL of medium and transfer to a new T75 flask.

The splitting ratio varied according to the required cell density and number for the planned experiments.

5.1.3 Cell counting

Cells were collected in Falcon tubes and centrifuged at 1200 rpm for 5 minutes. After removing the supernatant, cells were resuspended in 0.5 mL of fresh medium. A 1:10 dilution was prepared by mixing 50 µL of the resuspended cell solution with 450 µL of sterile 1x PBS. This solution was used to obtain the cell count using the Vi-Cell XR Cell Counter (Beckman Coulter).

5.1.4 Cell harvesting

Cells were collected in 1.5 mL tubes and centrifuged at 2800 rpm for 5 minutes. After removal of the supernatant the cells were washed by resuspending the pellet in 200 µL of sterile 1x PBS followed by centrifugation. The PBS was discarded and the pellet was either used directly for the experiments or stored at -80°C until sample processing.

5.1.5 Nutlin-3a treatment

3 or 4 x 10^6 cells were seeded in 3 mL of medium per well of 6-well plates the day before the treatment. The next day, the medium was replaced with either Nutlin-3a (Nut) or DMSO containing medium. The volume of DMSO used as control was equal to the volume used of Nutlin-3a.

The concentration of Nutlin-3a varied in different experimental settings and is specified in the text and figure legends.

In addition, the time cells were exposed to Nutlin-3a varied between experiments. When cells were treated for more than 24h, the medium was substituted with fresh medium containing either Nut or DMSO every 24h.

5.1.6 Calcium phosphate transfection

HEK293 cells were seeded at a density of 0.08 x 10^6 cells in 0.5 mL of medium per well of 48-well plates the day before the transfection.

Table 11 | Composition of the solution for calcium phosphate transfection of HEK293 cells in 48-well plates.

Solution	**Concentration (ng/µL)**	**Volume (µL)**
Nuclease free H_2O		7.65
Construct to transfect	100	2.5
Luc-Reporter	100	2.5
Renilla	2.5	2.5
TOTAL VOLUME	17.25	

The transfection procedure was performed using the ProFection® mammalian transfection system (Promega) according to the following steps:

1. The transfection solution was prepared in a polystyrene tube according to the volume specifications (**Table 11**) and vortexed.
2. Another polystyrene tube was filled with a volume equal to the total volume of the transfection solution of HEPES-buffered Saline (HBS).
3. The transfection solution was pipetted dropwise into the tube containing HBS while vortexing to create the final transfection mix.
4. The final mix was incubated for 30 min at RT to let DNA-calcium phosphate complexes form.
5. After incubation, the transfection mix was added dropwise to the cells seeded in the well the day before.
6. The cells were then incubated in the incubator for 24h.

5.1.7 Nucleofection

CLL cell lines were seeded one day prior to transfection at a density of 3 or 4 x 10^6 cells in 3 mL of medium per well of 6-well plates.

The transfection procedure was carried out after the following steps using the Amaxa™ Cell Line Nucleofector™ Kit T (Lonza):

1. Cells were harvested out of the wells into falcon tubes and centrifuged for 5 min at 1200 rpm.

2. The supernatant was discarded and the cell pellet was resuspended in 100 µL of transfection buffer.
3. The constructs to be transfected were added to the cell suspension. Two µg each of the overexpression/shRNA constructs and the Luc-Reporter and 50 ng of the Renilla luciferase were used.
4. The solution containing the cells and DNA was then transferred to electroporation cuvettes included in the transfection kit.
5. Cells were transfected using the Amaxa Nucleofector II (Lonza), program X-001.
6. After transfection, the cells were transferred to 6-well plates in fresh medium and incubated in the incubator for 24h.

5.2 Protein Methods

5.2.1 Cell lysis for protein extraction

After the cells were harvested (see **5.1.4**), the cell pellet was resuspended in RIPA Lysis Buffer (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitors. The volume of RIPA buffer varied from 50 to 80 µL, depending on pellet size. Subsequently, the resuspended cells were incubated on ice for 10 min and then centrifuged at 13000 rpm for 15 min at 4 °C. The supernatant containing the cell lysate was transferred to a new 1.5 mL tube and stored at -80°C until further use.

5.2.2 Determination of protein concentration

The protein concentration of the cell lysates was measured by using the BCA Assay Kit (Thermo Fisher Scientific). A protein standard of eight different dilutions of bovine serum albumin (BSA) was used (2000µg/mL, 1500µg/mL, 1000µg/mL, 750µg/mL, 500µg/mL, 250µg/mL, 152µg/mL, 62.5mg/mL).

One replicate of 10 µL from each dilution of protein standard was pipetted to a 96-well plate. While for the cell lysates, triplicates of 1 µL were added.

Next, the BCA Reagent Solutions A and B (part of the BCA assay kit) were mixed 50:1 and 200 µL were pipetted to each well containing either protein standard or cell lysate. The plate was then incubated at 37°C for 15 min. The Sunrise absorbance plate reader (Tecan, Switzerland) and Magellan 3.11 software were used to measure the absorbance at 495nm of

each well. Linear regression of the absorbance values of the standard curve was performed to calculate the protein concentrations of the samples.

5.2.3 WB sample preparation

In order to prepare the protein samples for WB analysis, 10-35 µg of protein were mixed with NuPAGE™ LDS Sample Buffer (4X) supplemented with 50 mM DTT. Water was used to adjust all samples to the same volume. The mixed samples were heated to 95°C for 5 min and subsequently placed on ice. Samples were stored at -20°C or directly used in WB analysis.

5.2.4 Western Blot

For protein separation, samples were loaded onto NuPAGE Bis-Tris 4-12% gels (Thermo Fisher Scientific) and separated by applying constant voltage. Afterwards, the proteins were transferred from the gel to a PVDF membrane using a semi-dry blotting method.

After blotting, the membranes were washed once with distilled water and immersed in Ponceau S staining solution to check the effectivity of the blotting. After three washes in distilled water to remove the stain, the membranes were blocked by incubation in 5% skim milk in PBS-T for 45 min at RT. Subsequently, the membranes were incubated ON at 4°C with primary antibodies (Abs) (**Table 6**) diluted in 5% skim milk-PBS-T. The next day, the membranes were washed three times for 5 min in PBS-T and incubated for 45 min with the secondary mouse anti-rabbit HRP-conjugated polyclonal antibody diluted 1:4000 in 5% skim milk PBS-T. After three washes in PBS-T, the membranes were incubated with ECL substrate and the chemiluminescence signal was detected using the Fusion FX6 Edge imaging system (Vilber).

After detection, the membranes were air dried and reactivated in MeOH over the following days for further analyses. After three washes in PBS-T , the membranes were incubated in BSA-diluted HRP-coupled anti-β-actin Abs for 15 min. After three washes in PBS-T, the β-actin signal was detected with ECL and used as a loading control.

5.2.5 Quantification of WB

The protein signal detected by WB analysis was quantified measuring band densitometry using the "Analyze Gels" function in ImageJ (NIH). The densitometry values of the analyzed proteins were then normalized to the corresponding β-actin values. Finally, graphs were generated using GraphPad Prism (GraphPad Software Inc.).

5.3 miRNA Methods

5.3.1 miRNA extraction

After the cells were harvested (see **5.1.4**), the pellet was lysed in 700 µL of QIAzol® Lysis Reagent (Qiagen) using a syringe and needle. Next, miRNA extraction was performed according to the protocol of the miRNeasy® Mini Kit (Qiagen).

5.3.2 Determination of miRNA concentration

After extraction, the miRNA concentration was determined according to the manufacturer's instructions using the Qubit™ RNA BR Assay Kit (Invitrogen) together with the Qubit™ 2.0 Fluorometer (Invitrogen).

5.3.3 Reverse transcription and RT-qPCR

The reverse transcription of miRNA samples was performed using the miRCURY LNA™ RT Kit (Qiagen) according to the protocol provided with the kit. The different temperature steps were carried out using the Labcycler Gradient (SensoQuest GmbH) PCR machine.

After reverse transcription, the samples were diluted to have three different concentrations (in respect to the original solution: 1:60, 1:120 and 1:600). The RT-qPCR was performed following the protocol of the miRCURY LNA™ SYBR Green Kit (Qiagen) using a 1:4 primer dilution. The QuantStudio™ 5 Real-Time PCR (Applied Biosystems) thermocycler and the QuantStudio™ Design & Analysis Software were used to set up and carry out the measurement. Finaly, the obtained data were analysed by RepDilPCR (DKFZ), which is a tool for automated analysis of qPCR assays by the dilution-replicate method (https://repdilpcr.eu/app/repDilPCR).

5.4 Luciferase Assay

5.4.1 Cell lysis with Passive Lysis Buffer

After the cells were harvested (see **5.1.4**), the pellet was resuspended in 50-80 µL of 1x Passive Lysis Buffer (Promega). Next, the samples were incubated on a shaker for 15 min at RT and afterwards centrifuged at 2800 rpm for 5 min. The supernatant was then transferred to a new Eppendorf tube and used for the luciferase assay.

5.4.2 Luciferase measurement

For measurement, 10 µL of the samples lysed with Passive lysis buffer were transferred in duplicate to a white 96-well plate. Four wells were kept free and used as blanks to calibrate the measurement according to the background signal of the plate. The Dual Luciferase Assay Kit containing the LAR II and Stop&Glo® luciferase substrate buffers (Promega), in combination with the Glomax Discover Luminometer (Promega), was used to assess the luciferase activity according to the manufacturer's instructions. To prime the instrument, 600 µL of both substrates were used. The parameters shown in **Table 12** were used to measure the activity of the Firefly and Renilla Luciferases.

Table 12 | Parameters used to set Glomax Discover.

Parameter	**Value**
Injection Volume	50 µL
Time between injection and measurement	5 sec
Integration time	5 sec

For instrument maintenance 4 wash steps were performed after the analysis using different agents in the following order: ddH_2O, EtOH 70%, ddH_2O and air.

Data analysis was performed using a Microsoft Excel spreadsheet in which the Firefly Luciferase signal was normalized to the corresponding Renilla signal to control for transfection efficiency. The firefly-renilla ratios were then represented as individual points on graphs generated with GraphPad Prism.

5.5 Flowcytometry Methods

5.5.1 Viability Assay

The Annexin V/7-Amminoactinomycin D staining is a cytofluorimetric assay for the detection, quantification and discrimination of apoptosis *in vitro*[87].

One day prior to the assay, CLL cell lines were seeded at a density of 3 or 4 x 10^6 cells in 3 mL of medium per well of 6-well plates.

To assess cell viability, 1x10^6 cells were harvested for each of the following staining conditions:

1. Unstained
2. FITC-Annexin V
3. 7-Aminoactinomycin D (7-AAD)
4. FITC-Annexin V + 7-AAD

After washing the cells once with 1 mL of ice-cold 1x PBS the staining solution was prepared as following. To achieve a total volume of 50 µl staining solution Annexin Binding Buffer (ABB) was mixed with either 1 µl FITC-Annexin V, 1.5 µl 7-AAD or both. The staining solution was added to the cells which were then incubated for 15 min at RT in the dark. Next, 150 µl of ABB were added to each tube and the measurement was performed with the Cytoflex S flow cytometer (Beckman Coulter).

The data were analyzed using the CytExpert Software (Beckman Coulter) and GraphPad Prism.

5.5.2 Assessment of transfection efficiency

Cells were transfected using nucleofection (see **5.1.7**) with a USP28-GFP expressing plasmid (**Table 7**), which was selected as it resembles the size and other properties of the constructs used during the following experiments.

The day after transfection, the harvested cells were washed once with 1 mL of 1x PBS and resuspended in 200 µL of 1x PBS that had been pre-warmed in the water-bath at 37°C. Subsequently, the GFP fluorescence was measured using the Cytoflex S flow cytometer (Beckman Coulter).

5.6 DNA Methods

5.6.1 Transformation of bacteria

To amplify the plasmids used for the transfection experiments chemically competent *E. Coli* bacteria were transformed according to the "Chemical transformation procedure" of the Zero Blunt® PCR Cloning protocol (Thermo Scientific).

5.6.2 DNA isolation from bacteria

The DNA from transformed bacteria was isolated with the ZymoPURE™ II Plasmid Maxiprep Kit (*Zymo Research*) according to the manufacturer's instructions.

5.6.3 Determination of DNA concentration

After DNA isolation, the DNA concentration was determined using the Qubit™ dsDNA BR Assay Kit (Invitrogen) Together with the Qubit™ 2.0 Fluorometer (Invitrogen) following the instructions provided with the kit.

5.7 Statistical Analysis

Different statistical tests were used to evaluate the significance of data. Generally, no asterisks or ns (not significant) were used when the data were not significant with $p > 0.05$, * when $p \leq 0.05$, ** when $p \leq 0.01$, *** when $p \leq 0.001$.

5.7.1 Luciferase data

The statistical significance of the luciferase data was evaluated by applying the Kruskal-Wallis Test using GraphPad Prism. The choice of the Kruskal-Wallis test is due to the nature of the Luciferase data, which is derived from a ratio involving two variables: Firefly Luciferase and Renilla Luciferase. Due to the non-normal distribution of the data in question, the use of a non-parametric test such as the Kruskal-Wallis Test was essential for accurate assessment of its statistical significance.

5.7.2 WB quantification

The significance of the WB quantification data was evaluated using the Student's t-test within GraphPad Prism. This choice of the Student's t-test was driven by the comparison of two groups along a single variable, involving a comparison of their means.

5.7.3 RT-qPCR data

The significance of the statistical differences in the RT-qPCR data was determined using a one-way ANOVA calculated directly by RepDilPCR. The use of one-way ANOVA was appropriate when comparing the means of an independent variable across three or more different groups.

6. RESULTS

Notch1 and p53 represent distinct, independent prognostic markers in the context of CLL. Both *Notch1* mutations and *TP53* aberrations are associated with an unfavorable prognosis due to resistance to chemotherapeutic agents, increased aggressiveness and enhanced persistence of the leukemic clone. In particular, *TP53* aberrations are associated with decreased levels of miRNA-34a (miR-34a), a well-established tumor suppressor in CLL. Notably, aberrant activation of Notch1 signaling promotes leukemic development. Notch1 has been identified as a major target of miR-34a. This raises the possibility that reduced levels of miR-34a in CLL patients with *TP53* aberrations may contribute to the increased activity of Notch1, potentially providing insight into the poorer prognosis observed in this subset of CLL patients.

To study how p53 activation affects NICD signaling and Notch1 protein levels, we first treated CLL cell lines with Nutlin-3a, a potent and selective small-molecule inhibitor of MDM-2.

6.1 Assessment of the optimal Nutlin-3a concentration to treat CLL cell lines

In the literature several studies commonly used 10 μM as the concentration of Nutlin-3a to treat primary CLL cells or cell lines[9,88–90].

In order to define the optimal concentration of Nutlin-3a to be used in our experimental setting, two CLL cell lines with wild type or mutated p53 (HG-3, p53 wt; MEC-1, p53 mut) were exposed to different Nutlin-3a concentrations for 24 hours. Subsequently, viability of the treated cells was analyzed by Annexin V/7-Amminoactinomycin D staining. As expected, MEC-1 viability was not markedly affected by increasing Nutlin-3a concentrations since p53 is not functional in this cell line. However, HG-3 viability was affected in a dose-dependent manner (**Figure 6a**). Specifically, we observed that:

- 90.55 ± 0.28 % (mean ± SD) of cells were alive after 24h exposure to 1%DMSO in RPMI.
- 82.60 ± 0.45 % of cells were alive after 24h exposure to 1 μM Nutlin-3a.
- 73.48 ± 0.07 % of cells were alive after 24h exposure to 5 μM Nutlin-3a.
- 69.83 ± 0.60 % of cells were alive after 24h exposure to 10 μM Nutlin-3a.

- 66.19 ± 0.56 % of cells were alive after 24h exposure to 15 µM Nutlin-3a.

Furthermore, protein levels of p53 and one of its main transcriptional targets p21[24] were assessed by WB in the cell lines HG-3, WA-OSEL, EHEB, MEC-1 and MEC-2 exposed to the different Nutlin-3a concentrations for 24h. Higher Nutlin-3a concentrations correlated with higher p53 and p21 levels in p53 wt cell lines (HG-3, WA-OSEL and EHEB) but, as expected, not in p53 mut cell lines (MEC-1 and MEC-2). Particularly, from the concentration of 5 µM on high levels of p53 and p21 were observed (**Fig. 6b**).

Next, the effects of each concentration of Nutlin-3a on the expression levels of different miRNAs were evaluated by RT-qPCR. In addition to the major p53 transcriptional target miR-34a, the following miRNAs were analyzed: miR-21, miR-20a, miR-26b and miR-140. miR-21 was selected because it has been shown to be overexpressed in several cancer entities, including CLL[42,91], and has been presented as a possible prognostic marker in del17p CLL patients[43]. While miR-20a and miR-26b were included as possible positive controls of p53 induction, miR-140 was included as a negative control[47]. Nutlin-3a treatment induced a slight increase of miR-34a expression levels in all the cell lines, except MEC-2. Surprisingly, this increase was more pronounced in MEC-1 cells in which p53 is mutated. In contrast, miR-21 did not show any specific change in expression after treatment. However, the positive controls miR-20a, miR-26b and the negative control miR-140 did not respond to Nutlin-3a treatment as we would have expected. This was probably due to the specificity of miRNA expression profiles among different cell types and they were therefore successively excluded in the final experiments (**Fig. 6c**).

In conclusion, Nutlin-3a at a concentration of 5 µM strongly induced p53 expression and activity, measured by the induction of p21. Furthermore, 5 µM Nutlin-3a induced a slight increase of miR-34a expression in all p53 wt cell lines and did not notably affect the viability of HG-3 (still around 75% of cells alive), which was used as a model for p53 wt cell lines. Therefore, Nutlin-3a was used at a concentration of 5 µM in all following experiments.

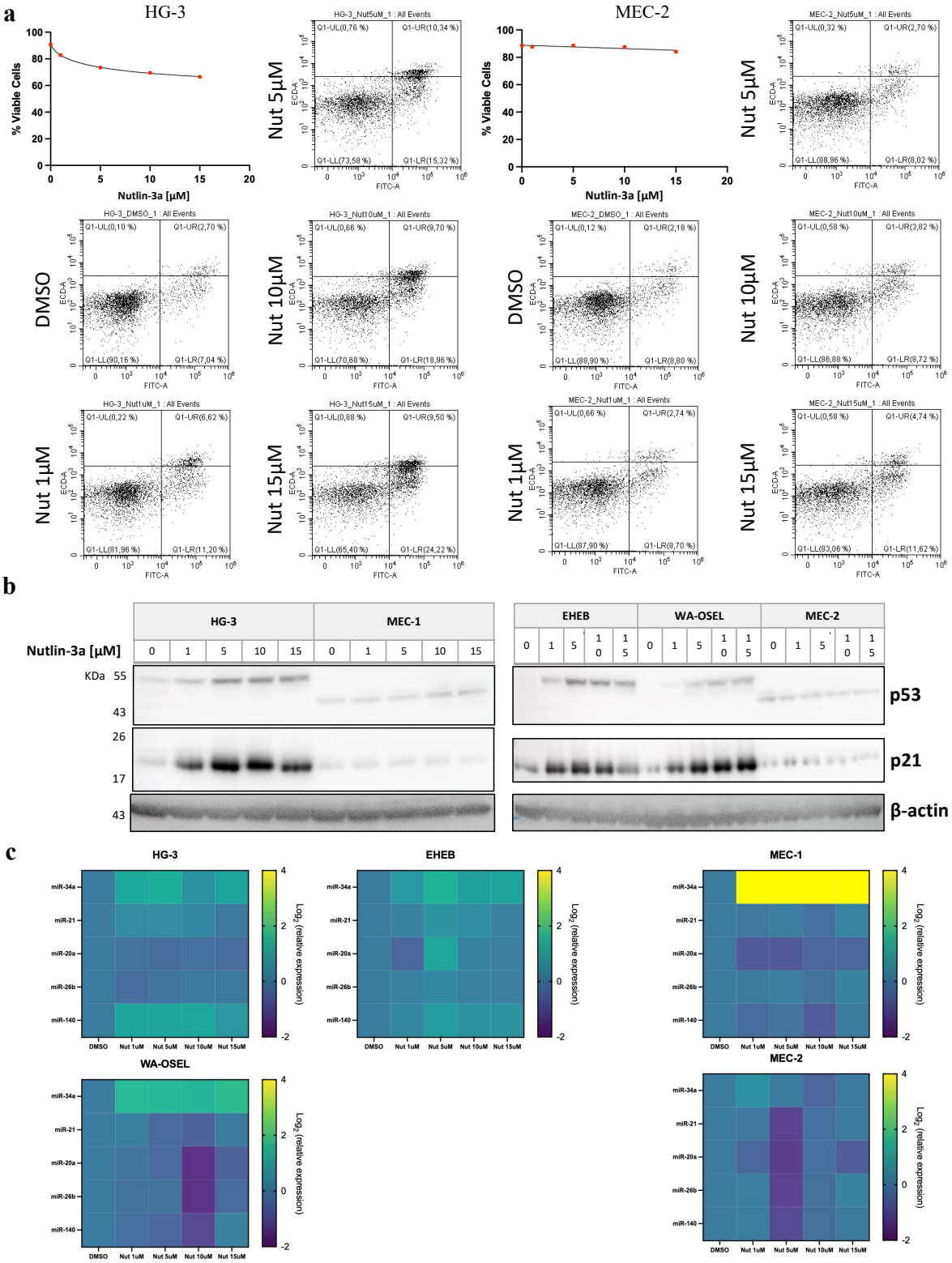


Figure 6 | 5μM showed to be the optimal Nutlin-3a concentration to treat CLL cell lines with.
(**a**) Determination of MEC-2 (p53 mut) and HG-3 (p53 wt) viability upon treatment with the indicated Nutlin-3a concentrations via 7-AminoActinomycin D/Annexin V Assay (n=2). (**b**) Western Blot analysis of p53 and p21 protein levels in HG-3, WA-OSEL, EHEB (p53 wt) and MEC-1, MEC-2 (p53 mut) upon treatment with the indicated Nutlin-3a concentrations (n=1). (**c**) RT-qPCR analysis of miR-34a, miR-21, miR-20a, miR-26b and miR-140 expression in HG-3, WA-OSEL, EHEB (p53 wt) and MEC-1, MEC-2 (p53 mut) upon treatment with the indicated Nutlin-3a concentrations (n=1).

6.2 Assessment of the optimal time point to evaluate Nutlin-3a dependent effects on CLL cell lines

Once the optimal concentration of Nutlin-3a had been determined, the next step was to define the correct time point for evaluating Nutlin-3a-dependent effects. Therefore, all cell lines were exposed to Nutlin-3a for different time windows. The effects of various exposure times to Nutlin-3a on NICD signaling, p53 and p21 protein levels and miRNAs expression were evaluated.

First, a dual Luciferase Assay system was used to assess Nutlin-3a effects on NICD signaling. In addition to Nutlin-3a or DMSO treatment, cells were transfected with a Notch1 specific Firefly-luciferase reporter and a constitutively active Renilla luciferase construct. An alteration in NICD signaling was observed in MEC-1, WA-OSEL and EHEB after 20 or 24h Nutlin-3a exposure when compared to the respective DMSO control condition (**Fig. 7a**).

Second, the analysis of p53 and p21 protein levels by WB showed a clear induction of both in p53 wt cell lines already after 6h of Nutlin-3a treatment, but the strongest induction was seen after 24h (**Fig. 7b**).

Third, the expression levels of the selected miRNAs (more details in **Section 6.1**) were analyzed by RT-qPCR and miR-34a was found to be induced mainly after 24h of Nutlin-3a treatment (**Fig. 7c**).

Thus, 24h was identified as the ideal exposure time of CLL cell lines to Nutlin-3a treatment in order to assess the effect on NICD signaling activity, p53 expression and activity and miR-34a induction.

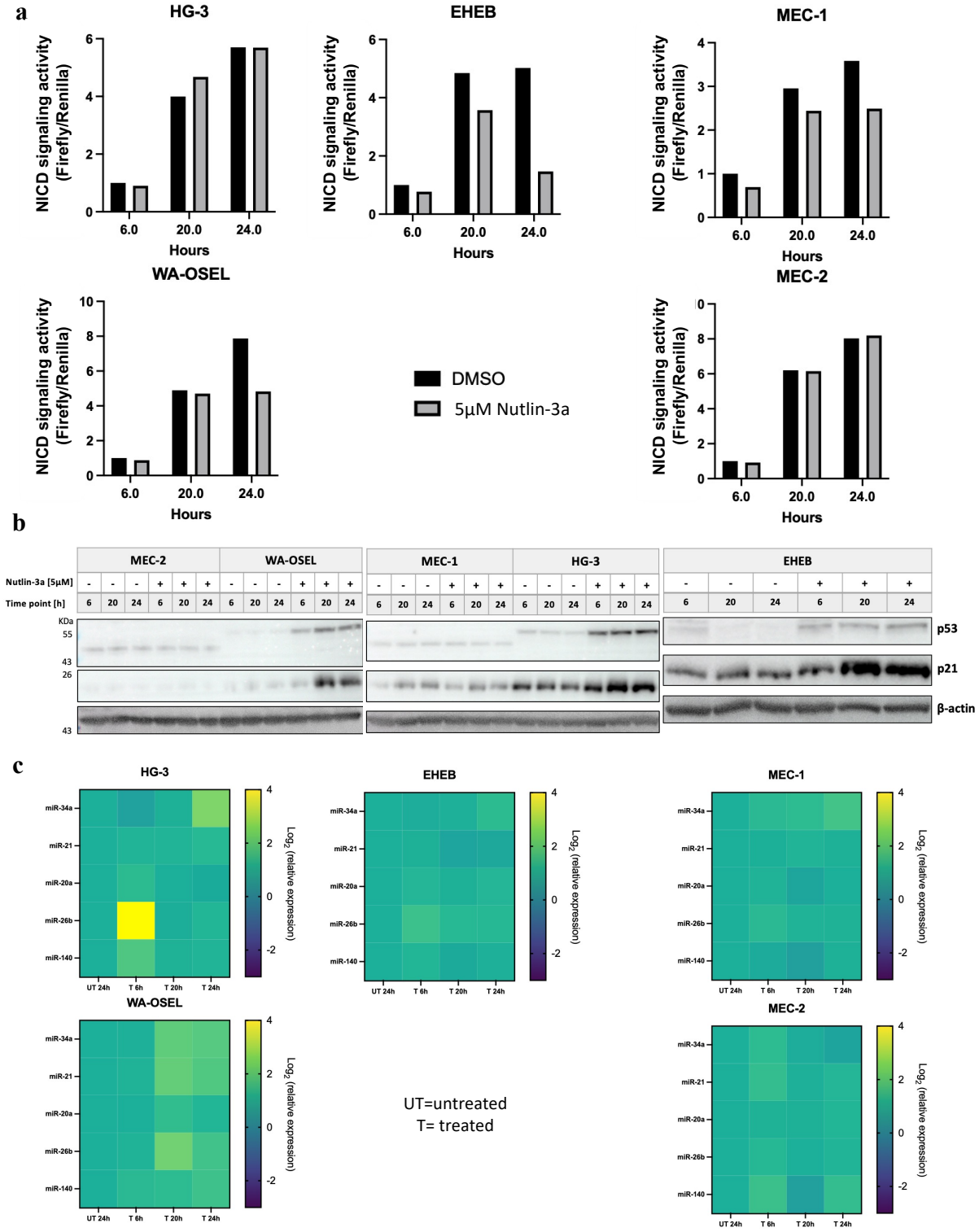


Figure 7 | 24h was selected as ideal time point to evaluate Nutlin-3a dependent effects on CLL cell lines.
(**a**) Luciferase assay of Nutlin-3a effects on NICD-signaling in HG-3, WA-OSEL, EHEB (p53 wt) and MEC-1, MEC-2 (p53 mut) treated at the indicated time points. The values showed are the result of the signal coming from the Hes1-Firefly Luciferase reporter normalized to the constitutive Renilla Luciferase control (n=1). (**b**) Western Blot analysis of p53 and p21 protein levels in HG-3, WA-OSEL, EHEB (p53 wt) and MEC-1, MEC-2 (p53 mut) upon treatment with Nutlin-3a at the indicated time points (n=1). (**c**) RT-qPCR analysis of miR-34a, miR-21, miR-20a, miR-26b and miR-140 expression in HG-3, WA-OSEL, EHEB (p53 wt) and MEC-1, MEC-2 (p53 mut) upon treatment with Nutlin-3a at the indicated time points. Only 24h time point showed for untreated condition (n=1).

6.3 Nutlin-3a dependent effects are cell line specific

Based on the exploratory experiments (**Sections 6.1** and **6.2**), the different cell lines were finally treated with either 5 μM Nutlin-3a or DMSO as control for 24h to evaluate the impact of p53 induction on NICD signaling, Notch1 protein levels and expression of miR-34a and miR-21.

Compared to the DMSO control, Nutlin-3a treatment did not induce significant changes in NICD signaling detected by Luciferase Assay in all the cell lines (**Fig. 8a**). In contrast, the overexpression of NICD, used as positive control, and the overexpression of dnMAM (dominant negative Mastermind-like), used as negative control, respectively up- or downregulated NICD signaling compared to cells transfected with an empty vector construct.

The protein levels of the transmembrane portion of Notch1 were affected by Nutlin-3a treatment differently depending on the cell line. In WA-OSEL and EHEB an increase in Notch1-TM was observed, a decrease in HG-3 and no difference in the p53 mut cell lines MEC-1 and MEC-2. Protein levels of p53 and consequently its target p21 were induced in all p53 wt cell lines, as expected. In addition, we assessed the protein levels of BCL-2, since it is reported to be a target of miR-34a[92] and it is involved in CLL pathogenesis[79]. However, BCL-2 protein levels were not affected by Nutlin-3a treatment in any particular pattern of variation (**Fig. 8b**).

Furthermore, RT-qPCR data showed no significant differences in miR-34a and miR-21 expression levels after Nutlin-3a treatment in the different cell lines. Though as expected, p53 mut cell lines showed significantly lower levels of miR-34a compared to p53 wt cell lines (**Figure 8c**).

In summary, Nutlin-3a did not significantly affect NICD signaling in CLL cell lines but affected Notch1 protein levels differently depending on the cell line. Thus, the effects of Nutlin-3a-dependent p53 induction on Notch1 protein levels showed cell line specificity. In addition, we were unable to identify a specific pattern of variation in BCL-2 protein levels upon Nutlin-3a treatment. Finally, although Nutlin-3a treatment did not significantly influence miR-21 and miR-34a expression, we found significantly lower miR-34a expression levels in p53 mut compared to p53 wt cell lines.

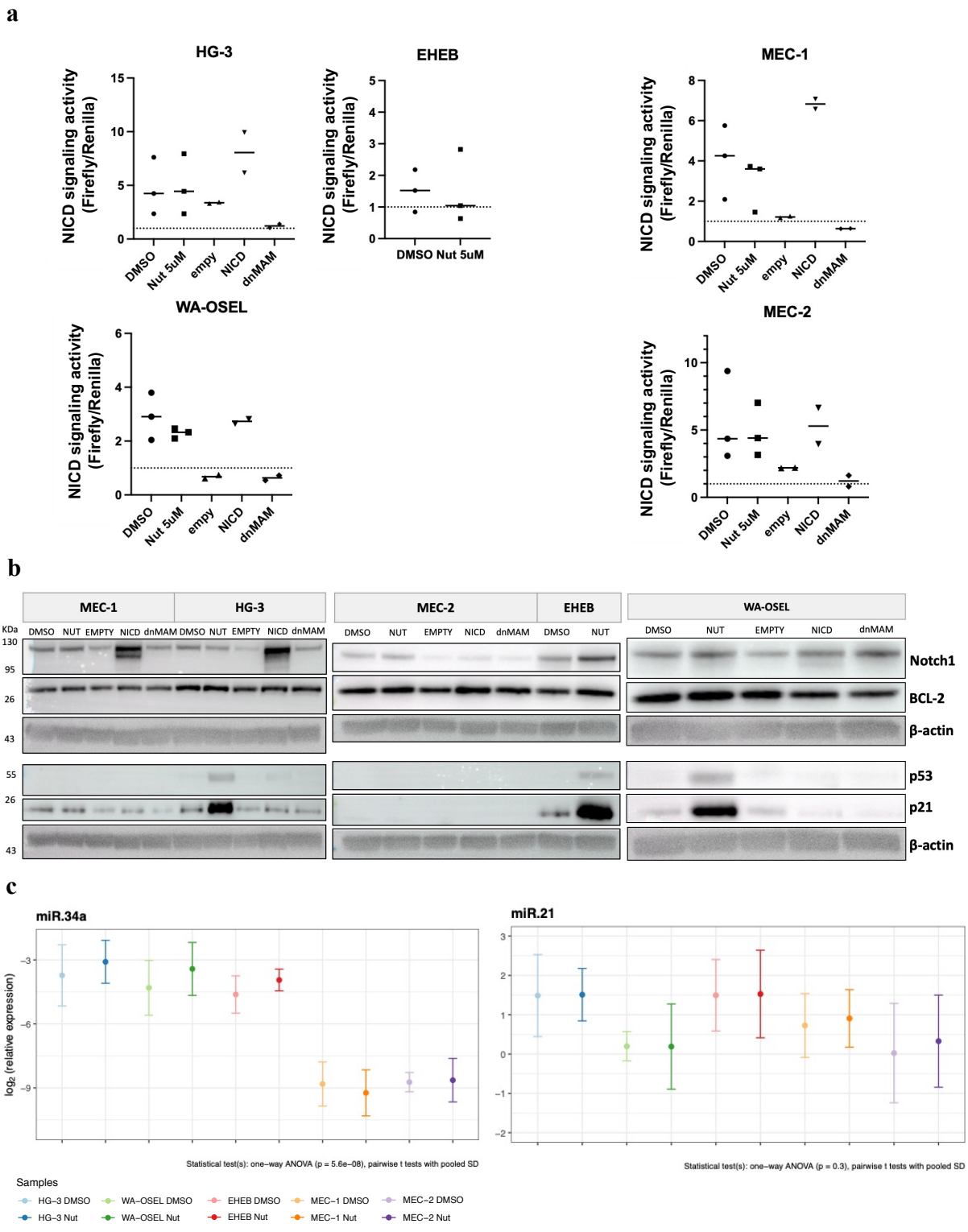


Figure 8 | Nutlin-3a dependent effects are cell line specific.
(**a**) Luciferase assay of Nutlin-3a effects on NICD-signaling in HG-3, WA-OSEL, EHEB (p53 wt) and MEC-1, MEC-2 (p53 mut). The values showed are the result of the signal coming from the Hes1-Firefly Luciferase reporter normalized to the constitutive Renilla Luciferase control (n=3). (**b**) Western Blot analysis of Notch1, BCL-2, p53 and p21 protein levels in HG-3, WA-OSEL, EHEB (p53 wt) and MEC-1, MEC-2 (p53 mut) upon treatment with Nutlin-3a (n=3). (**c**) RT-qPCR analysis of miR-34a and miR-21 expression in HG-3, WA-OSEL, EHEB (p53 wt) and MEC-1, MEC-2 (p53 mut) upon treatment with Nutlin-3a. (n=3)

6.4 Prolonged Nutlin-3a treatment increased NICD signaling and Notch1 protein levels in EHEB and induced miR-34a expression in EHEB and WA-OSEL

The previous experiments showed that 24h Nutlin-3a treatment did not affect and induce significantly NICD signaling and miR-34a, respectively. However, although not significant, we observed the same trend in the induction of miR-34a expression in all p53 wt CLL cell lines upon Nutlin-3a treatment. Therefore, we attempted to expose two of the three p53 wt CLL cell lines to Nutlin-3a for a longer period of time to see if this could enhance the previously observed effects.

First, we evaluated the effects of prolonged Nutlin-3a treatment on NICD signaling by Luciferase Assay. Although not significant, we found a trend toward increased NICD signaling in EHEB upon Nutlin-3a treatment for 48 or 72h compared to DMSO control. This was not the case in WA-OSEL, where treatment at 72h appeared to decrease NICD signaling (**Fig. 9a**).

These results were supported by Notch1 protein levels detected by WB. Indeed, higher Notch1 protein levels were observed in EHEB after Nutlin-3a treatment for 48 and 72h compared to the control, whereas lower levels were observed in WA-OSEL (**Fig. 9b**). Furthermore, considering β-actin levels, BCL-2 protein levels were found to be higher in EHEB after prolonged treatment, probably due to increased Notch1 signaling. Indeed, Notch1 signaling has been shown to induce BCL-2 expression via NF-κB signaling[93,94].

Additionally, exposure to Nutlin-3a for 48 and 72h induced miR-34a expression in both EHEB and WA-OSEL. Similarly, miR-21 expression was induced in EHEB cells after Nutlin-3a exposure for 48 and 72h (**Fig. 9c**).

In summary, while prolonged Nutlin-3a treatment led to increased NICD signaling and Notch1 protein levels in EHEB, it appeared to have the opposite effect in the WA-OSEL cell line. However, in both EHEB and WA-OSEL, prolonged Nutlin-3a treatment led to an increase in miR-34a levels, which in the case of EHEB was also accompanied by an increase in miR-21 expression.

Taken together, we found that the p53 pathway can be manipulated in leukemic cell lines by treatment with the non-genotoxic agent Nutlin-3a. Additionally, some of our results indicated a link between the p53 and Notch1 signaling pathways, which we further characterized in the following experiments.

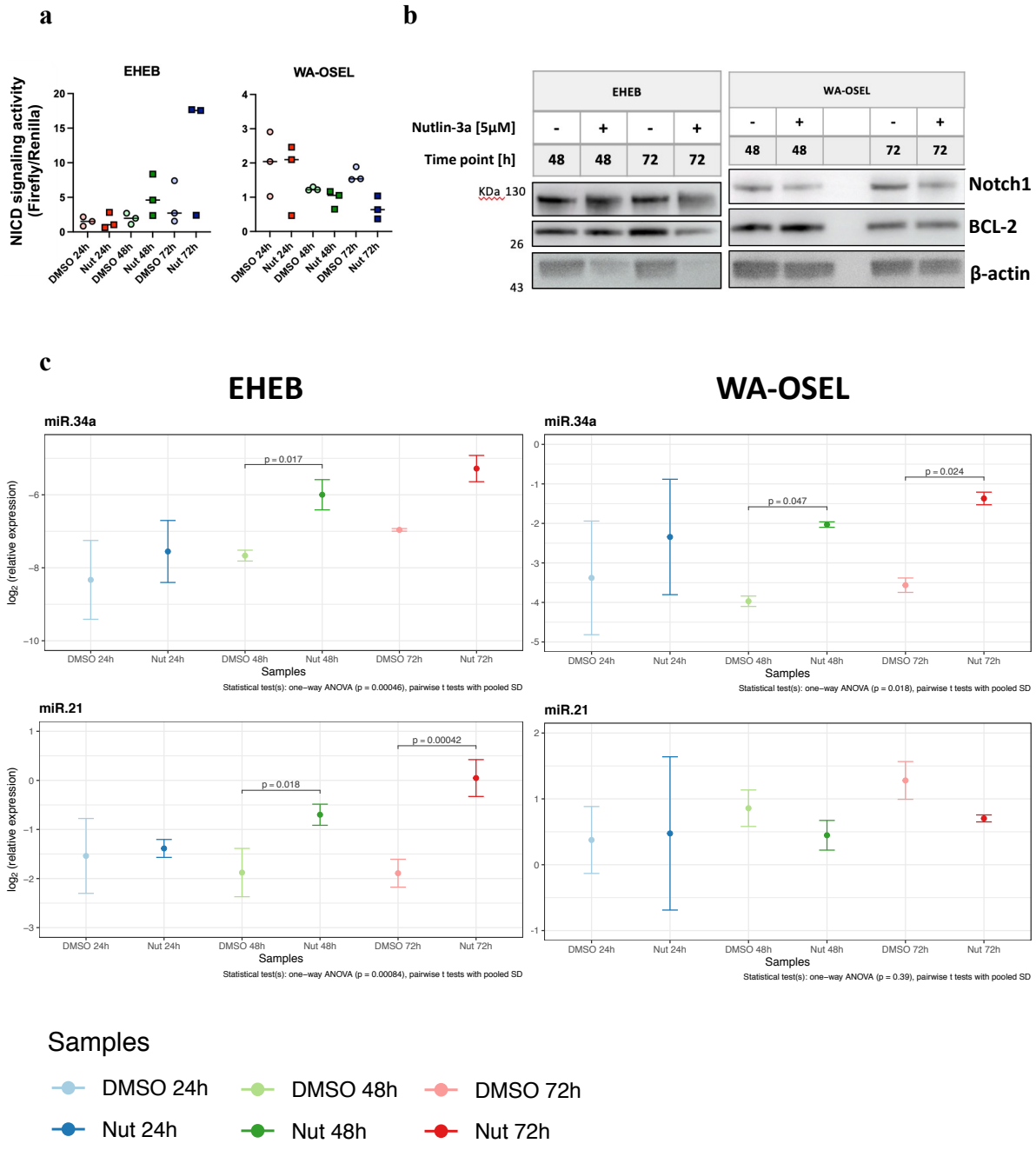


Figure 9 | Prolonged Nutlin-3a treatment increased NICD signaling and Notch1 protein levels in EHEB and induced miR-34a expression in EHEB and WA-OSEL.
(**a**) Luciferase assay of prolonged Nutlin-3a treatment effects on NICD-signaling in EHEB and WA-OSEL. The values showed are the result of the signal coming from the Hes1-Firefly Luciferase reporter normalized to the constitutive Renilla Luciferase control (n=3). (**b**) Western Blot analysis of Notch1 and BCL-2 protein levels in in EHEB and WA-OSEL upon prolonged treatment with Nutlin-3a (n=3). (**c**) RT-qPCR analysis of miR-34a and miR-21 expression in in EHEB and WA-OSEL upon prolonged treatment with Nutlin-3a (n=3).

6.5 Assessment of the optimal transfection conditions for p53 gain/loss and miR-34a overexpression/inhibition experiments

To further define the link between the p53 and Notch1 pathways and the role of miR-34a in this context, we decided to perform p53 gain/loss and miR-34a overexpression/inhibition experiments in CLL cell lines. To do so, we needed to establish some experimental conditions beforehand.

For the p53 gain/loss experiment, all cell lines were transfected with different p53 expressing constructs and p53 targeting shRNAs. The best working construct for both p53 overexpression and silencing was selected based on the WB results (**Fig. 10a**). Number #441 for p53 overexpression and #519 for p53 silencing were selected and used in the subsequent experiments.

Next, we determined the transfection efficiency of the different cell lines by quantifying the percentage of GFP-positive cells by flow cytometry after transfection with a GFP-carrying construct. Among the p53 wt cell lines, HG-3 and WA-OSEL displayed the best transfection efficiencies with 9.55 ± 2.20 % (mean ± SD) and 9.35 ± 1.83 % of cells transfected, respectively. Among the p53 mut cell lines, MEC-1 showed the best transfection efficiency with 7.00 ± 0.76 % of cells transfected (**Fig. 10b**). Therefore, WA-OSEL and MEC-1 were selected for the following experiments.

For the miR-34a overexpression/inhibition experiment, the optimal concentration of the different constructs had to be determined. The constructs data sheets suggested a concentration range between 3 and 30 nM for the miR-34a precursor and between 10 and 100 nM for the miR-34a inhibitor in the transfection volume of a lipid-based transfection approach. However, effective transfection of CLL cell lines requires an electroporation approach that uses a lower transfection volume. Therefore, we started transfecting WA-OSEL with a concentration of 15 nM pre-miR-34a and 50 nM anti-miR-34a calculated on the electroporation transfection volume of 100 µL. In addition, the cells were transfected with pGL3-sensor 1.1, a luciferase reporter construct carrying several miR-34a target sites. Thus, we expected a lower and higher luciferase activity in cells transfected with pre-miR-34a and anti-miR-34a, respectively. As shown in **Figure 10c**, we did not observe the expected patterns of variation in luciferase activity in cells transfected with pre-miR-34a or anti-miR-34a when compared to the respective negative controls (NC). Most likely, the observed effects were due to unspecific background noise, suggesting that the amount of each construct used was too low. This assumption was also

supported by the fact that RT-qPCR analysis did not show an increase in miR-34a expression levels in cells transfected with pre-miR-34a (**Fig. 10d**). Consequently, we repeated the experiment in HG-3 and WA-OSEL but calculated the concentrations for transfection of miR-34a precursor and inhibitor considering the culture volume of 3 mL. This corresponds to a 30-fold higher amount used for each construct than in the first approach. Nevertheless, also the higher transfection amount did not induce the expected decrease or increase in luciferase activity (**Fig. 10e**). However, the transfection with 30 times more pre-miR-34a than in the first approach consistently increased miR-34a expression levels in HG-3 and WA-OSEL (**Fig. 10f**). Therefore, we decided to use a concentration of 15 nM pre-miR-34a and 50 nM anti-miR-34a calculated on the culture volume of 3 mL for the following experiments.

In summary, the WA-OSEL and MEC-1 cell lines were selected for the following experiments due to their higher transfection efficiency among p53 wt and p53 mut cell lines, respectively. For the p53 gain/loss experiment, #441 and #519 were selected as the best performing constructs in p53 overexpression and silencing, respectively. For the miR-34a overexpression/inhibition experiment, we decided to continue with a concentration of 15 nM pre-miR-34a and 50 nM anti-miR-34a calculated on the culture volume of 3 mL for the following experiments.

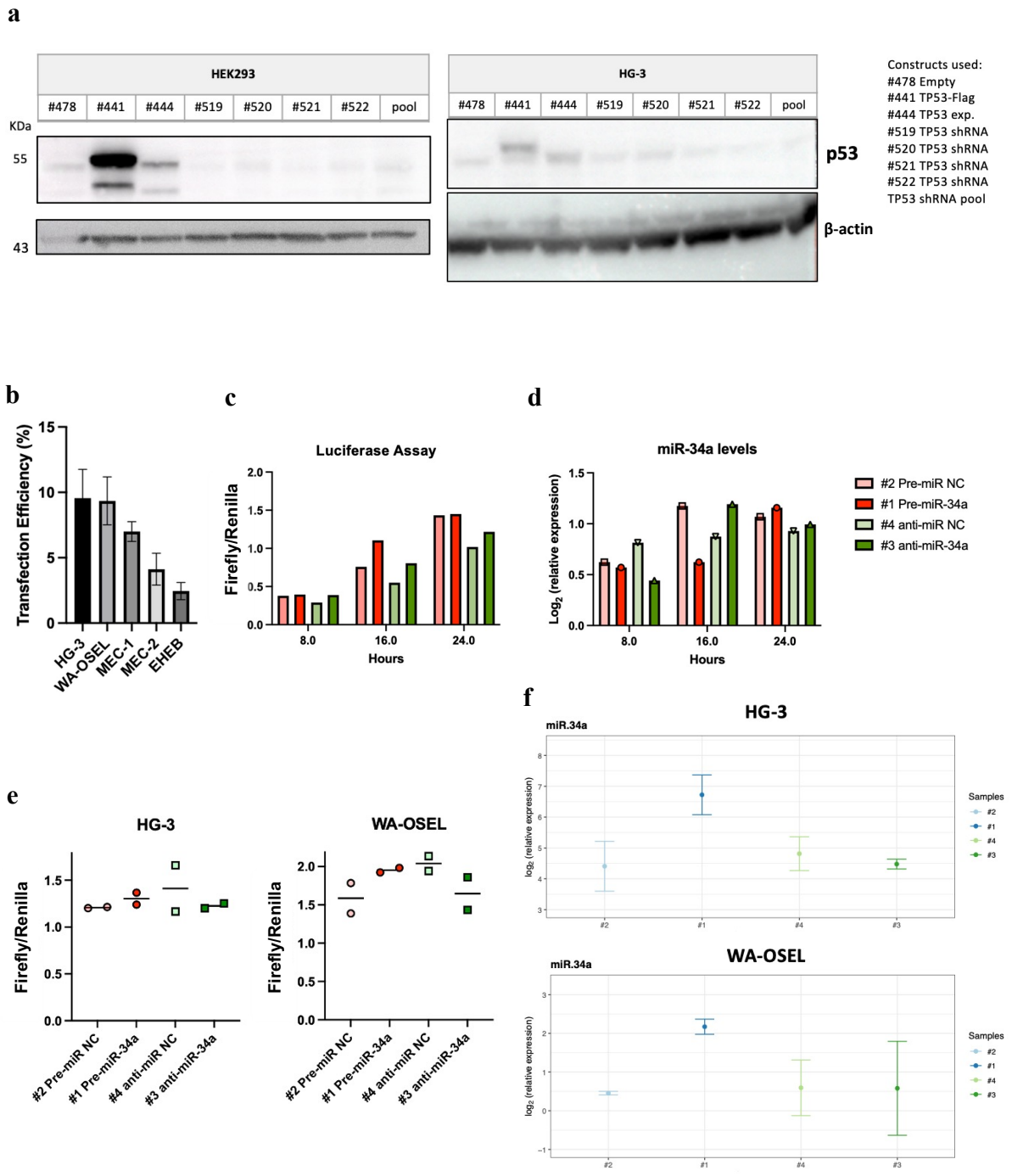


Figure 10 | Assessment of the optimal transfection conditions for p53 loss/gain and miR-34a overexpression/inhibition experiments.
(**a**) Western Blot analysis of p53 protein levels in HEK293 and HG-3 after transfection with the indicated constructs (n=1). (**b**) Cytofluorimetric quantification of GFP-positive cells after transfection with the USP28-GFP construct (n=2). (**c, e**) Luciferase Assay of miR-34a overexpression and inhibition effects on pGL3-Sensor 1.1 (specific Luc reporter construct having several miR-34a target sites). The values showed are the result of the signal coming from the Firefly Luciferase reporter normalized to the Renilla Luciferase control (n=1). In (**e**) used a 30X higher amount of each construct compared to (**c**). (**d,f**) RT-qPCR analysis of miR-34a expression in WA-OSEL (**d, f**) and HG-3 (**f**) upon miR-34a overexpression/inhibition (n=1, **d** ; n=2, **f**). In (**f**) used a 30X higher amount of each construct compared to (**d**).

6.6 p53 activation correlated with increased Notch1 protein levels in WA-OSEL and MEC-1

To further characterize the link between the Notch1 and p53 pathways we performed a p53 overexpression/knockdown experiment in WA-OSEL and MEC-1 and analyzed NICD signaling activity via Luciferase Assay and Notch1 protein levels via WB. As negative controls we used (1) an empty vector construct to control the effects of p53 overexpression and (2) a construct expressing a scrambled shRNA (scr shRNA) to control the p53 knockdown.

Even though p53 overexpression/knockdown did not show significant effects on NICD-signaling by Luciferase Assay (**Fig. 11a**), it did affect Notch1 protein levels in both WA-OSEL and MEC-1. While in MEC-1 it was clear that p53 overexpression and knockdown led to increased and decreased Notch1 protein levels respectively, in the WA-OSEL cell line it was more difficult to appreciate the same effects (**Fig. 11b**). We were able to provide a plausible explanation by looking at two different replicates:

1. p53 knockdown correlated with reduced Notch1 protein levels (first replicate on left, **Fig. 11b**);
2. p53 overexpression correlated with increased Notch1 protein levels (second replicate on the right, **Fig. 11b**);
3. p53 overexpression did not correlate with increased Notch1 protein levels (first replicate on the left, **Fig. 11b**);
4. p53 knockdown correlated with increased Notch1 protein levels (second replicate on the right, **Fig. 11b**).

If points 1 and 2 are consistent with what we observed in MEC-1, points 3 and 4 are not. However, p21 levels, major p53 transcriptional target[24], must also be considered. When compared to the respective controls, there is a correlation between p53 and p21 levels for the points 1 and 2, but not for the points 3 and 4. This is probably related to the activity of p53. If so, higher p53 activity and consequently higher levels of p21 correlated with higher levels of Notch1. To strengthen this point, we tried to develop the membranes with primary antibodies against the form of p53 phosphorylated at Ser15, which is an activated form of p53, but we were unable to detect any bands (data not shown).

Lastly, p53 overexpression significantly induced miR-34a expression in MEC-1 but not in WA-OSEL (**Fig. 11c**), probably because MEC-1 have significantly lower levels of miR-34a under standard conditions (see **Fig. 8c**).

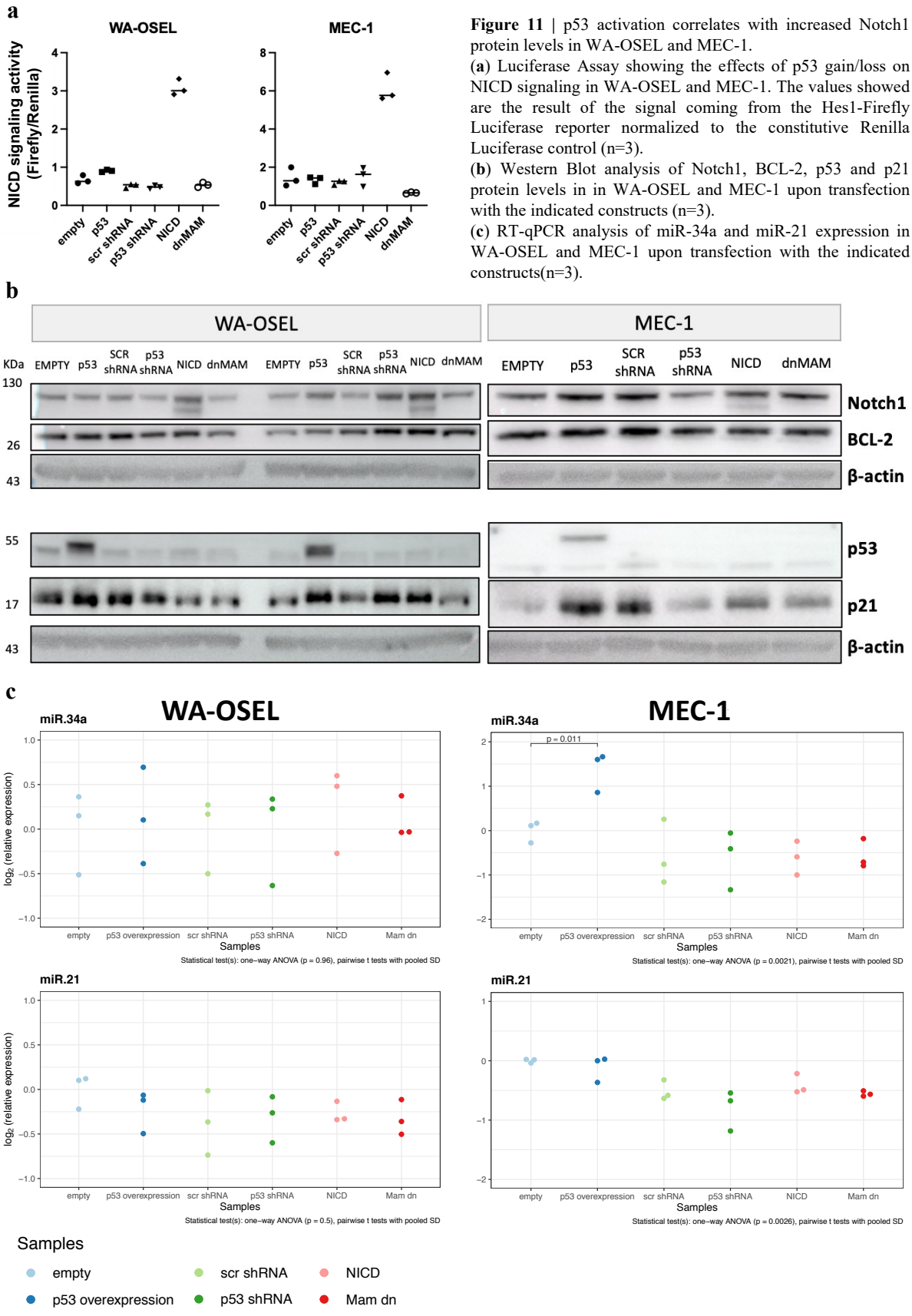


Figure 11 | p53 activation correlates with increased Notch1 protein levels in WA-OSEL and MEC-1.
(**a**) Luciferase Assay showing the effects of p53 gain/loss on NICD signaling in WA-OSEL and MEC-1. The values showed are the result of the signal coming from the Hes1-Firefly Luciferase reporter normalized to the constitutive Renilla Luciferase control (n=3).
(**b**) Western Blot analysis of Notch1, BCL-2, p53 and p21 protein levels in in WA-OSEL and MEC-1 upon transfection with the indicated constructs (n=3).
(**c**) RT-qPCR analysis of miR-34a and miR-21 expression in WA-OSEL and MEC-1 upon transfection with the indicated constructs(n=3).

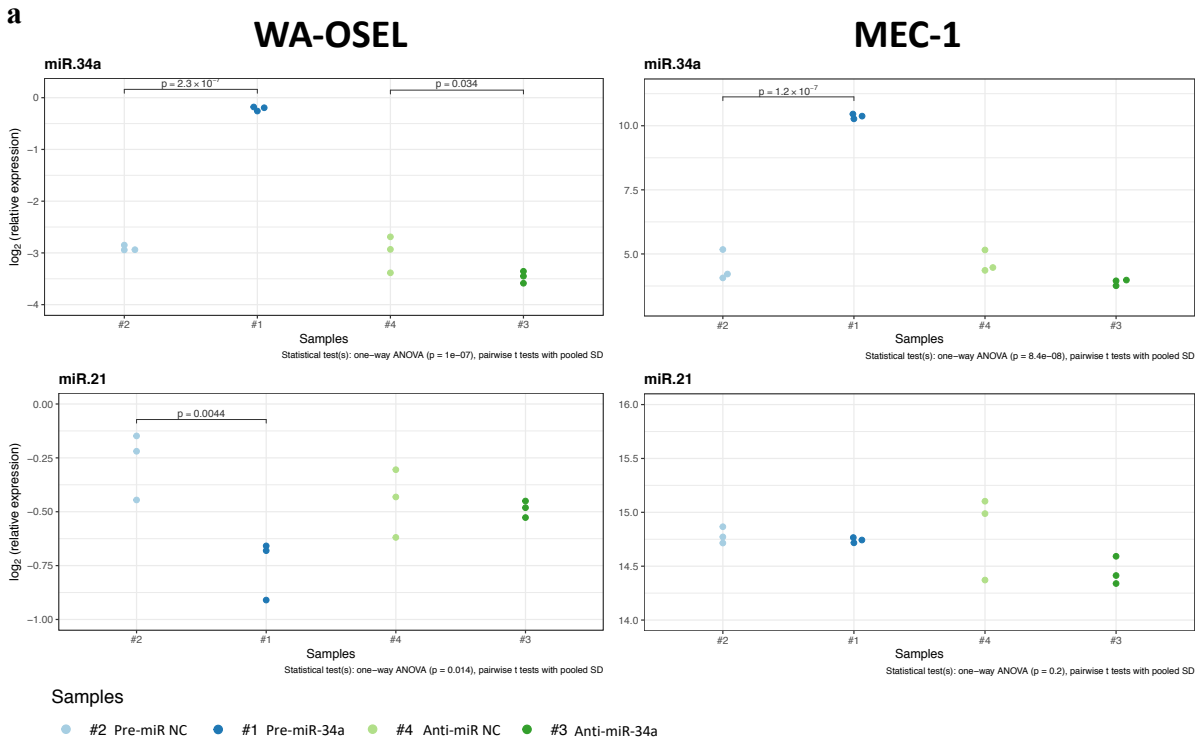
6.7 miR-34a overexpression/inhibition did not show significant effects on NICD signaling and Notch1 and BCL-2 protein levels in WA-OSEL and MEC-1

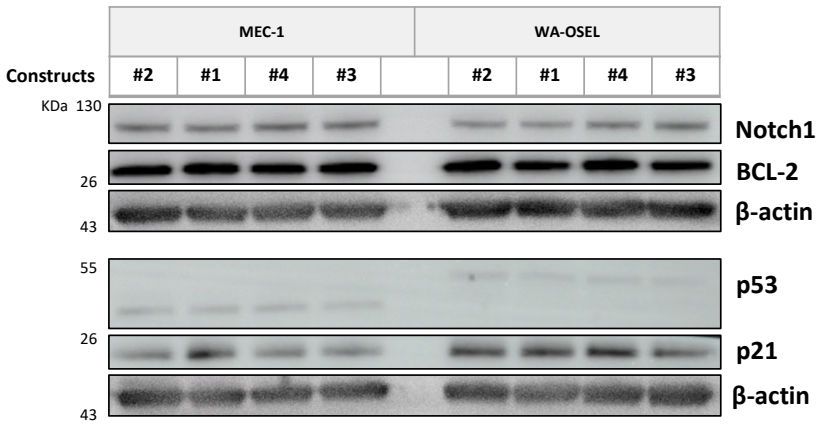
To determine whether miR-34a plays a role in the context of p53-Notch1 crosstalk, NICD signaling activity and Notch1 protein levels were analyzed in WA-OSEL and MEC-1 cells in which miR-34a was either overexpressed or inhibited.

RT-qPCR was performed to confirm the successful transfection of the pre-miR-34a or miR-34a inhibitor constructs in both the cell lines. Compared to the respective negative controls, miR-34a levels were increased in cells transfected with pre-miR-34a and decreased when the miR-34a inhibitor was transfected (**Fig. 12a**). Interestingly, higher levels of miR-34a correlated with significantly lower levels of miR-21 in WA-OSEL.

Even if the transfection was successful, no clear effects of miR-34a overexpression or inhibition on NICD signaling activity measured by Luciferase Assay (**Fig. 12c**) and on Notch1 and BCL-2 protein levels assessed by WB (**Fig. 12b**) were observed. Regarding the WB results, any difference in protein levels might not have been detected due to only a small proportion of cells being efficiently transfected in the whole cell population used for the analysis.

In summary, we did not observe any significant effects of miR-34a overexpression or inhibition on NICD signaling and Notch1 and BCL-2 protein levels in WA-OSEL and MEC-1.





Constructs used:

- #2 Pre-miRNA Negative Control
- #1 Pre-miR-34a
- #4 anti-miRNA Negative Control
- #3 anti-miR-34a

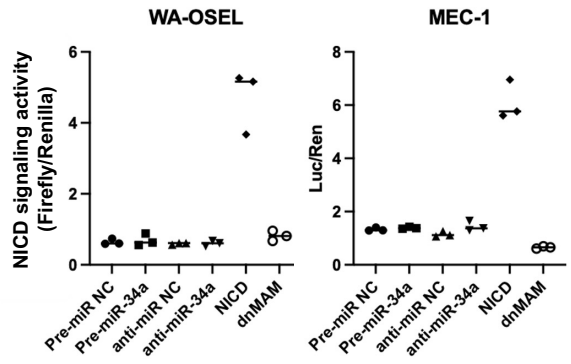


Figure 12 | miR-34a overexpression/inhibition did not show significant effects on NICD signaling and Notch1 and BCL-2 protein levels in MEC-1 and WA-OSEL.
(**a**) RT-qPCR analysis of miR-34a and miR-21 expression in WA-OSEL and MEC-1 upon transfection with the indicated constructs(n=3).
(**b**) Western Blot analysis of Notch1, BCL-2, p53 and p21 protein levels in in WA-OSEL and MEC-1 upon transfection with the indicated constructs (n=3).
(**c**) Luciferase Assay showing the effects of miR-34a overexpression/inhibition on NICD signaling in WA-OSEL and MEC-1 . The values showed are the result of the signal coming from the Hes1-Firefly Luciferase reporter normalized to the constitutive Renilla Luciferase control (n=3).

6.8 CLL patients with altered p53 status showed decreased Notch1 protein levels and miR-34a expression levels

Given the relevance of p53 and Notch1 as prognostic markers in the clinic, we aimed to characterize the effects of *TP53* mutation or deletion via del17p, which frequently disrupt the normal functionality of p53 in CLL patients, on Notch1 and BCL-2 protein levels and miR-34a expression (more details on patient cohort, **Table 10**). In this way, we also wanted to investigate whether the results obtained in the cell lines could be translated to what normally happens in CLL patients.

WB analysis of primary CLL cells from patients with p53 alterations showed dysregulated Notch1 and BCL-2 protein levels by WB compared to p53 wt patients' cells (**Fig. 13a**). Furthermore, quantification of the transmembrane portion of Notch1 (upper band), revealed it to be significantly decreased in primary CLL cells from patients with altered p53 status. However, no significant differences were detected when the lower bands of anti-Notch1 probed WB were quantified. Similarly, quantification of BCL-2 protein levels did not differ between p53 wt and mut patient cells, although there were some patients with altered p53 status who expressed noticeably higher levels of BCL-2, which seemed to correlate with high levels of Notch1 in these patients (**Fig. 13b**).

In the end, RT-qPCR analysis of primary CLL patient cells resulted in significantly lower expression levels of miR-34a in CLL patients with altered p53 status, reproducing previous findings by others[95]. Instead, no significant differences in miR-21 levels were observed (**Fig. 13c**), although we detected an anti-correlation with miR-34a expression in **Fig. 12a** (**Section 6.7**).

In the cell lines, we observed increased NICD signaling activity and Notch1 protein levels upon p53 induction. In agreement with these previous findings, we found that nonfunctional p53 in CLL patients correlates with lower levels of Notch1-TM protein levels. This suggests that Notch1 is induced by p53 in CLL.

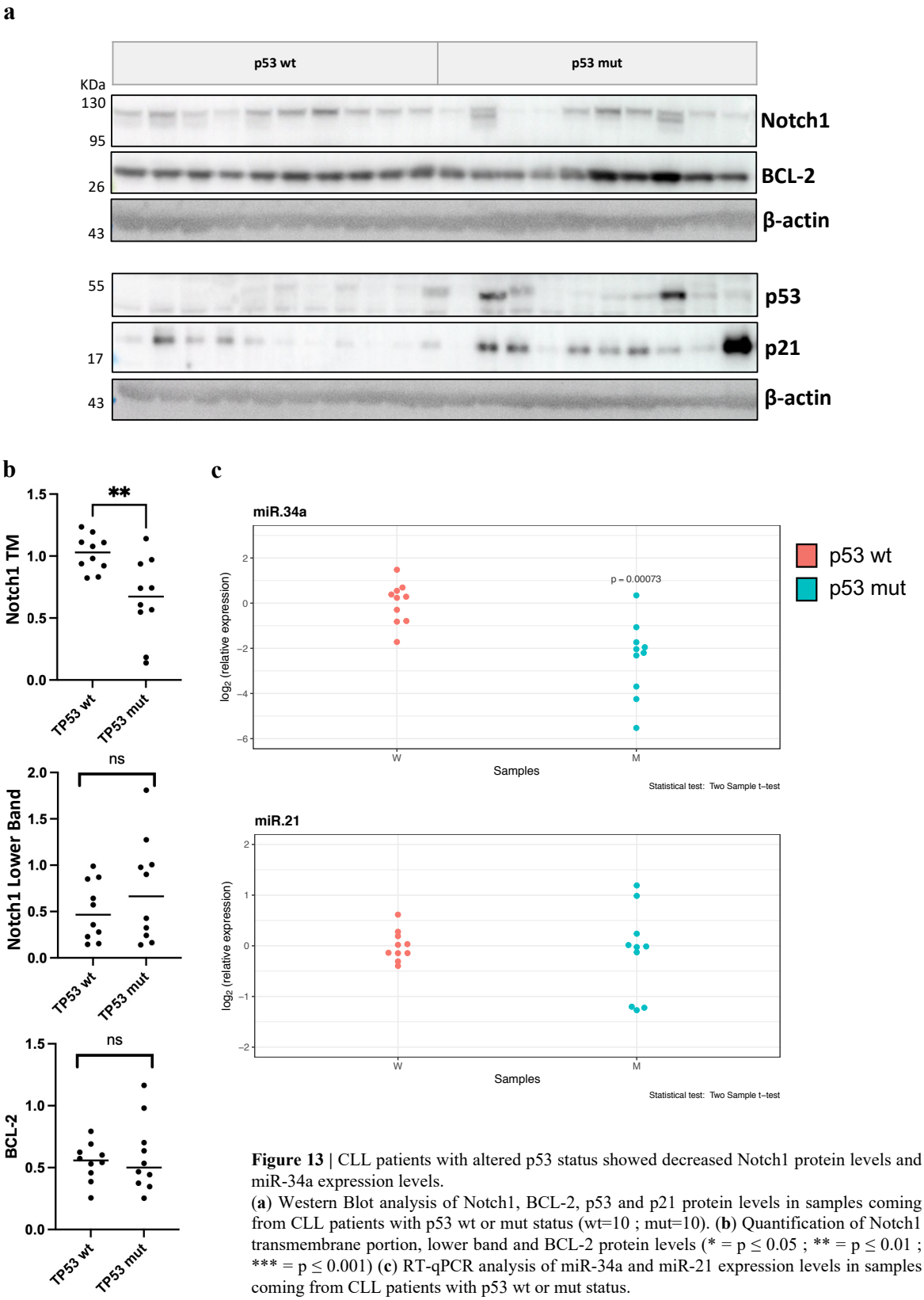


Figure 13 | CLL patients with altered p53 status showed decreased Notch1 protein levels and miR-34a expression levels.
(**a**) Western Blot analysis of Notch1, BCL-2, p53 and p21 protein levels in samples coming from CLL patients with p53 wt or mut status (wt=10 ; mut=10). (**b**) Quantification of Notch1 transmembrane portion, lower band and BCL-2 protein levels (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$) (**c**) RT-qPCR analysis of miR-34a and miR-21 expression levels in samples coming from CLL patients with p53 wt or mut status.

7. DISCUSSION

Notch1 and p53 are two independent prognostic markers in CLL[75]. Both Notch1 mutations and p53 disruption have been associated with poor prognosis due to chemotherapeutic resistance and increased aggressiveness and survival of the leukemic clone [89,95]. In this context, we wanted to understand whether lower levels of miR-34a in CLL patients with p53 alterations could cause an increase in Notch1 protein levels and NICD signaling activity, and whether this could be one cause explaining the poor prognosis of this subgroup of patients. Thus, we expected that Notch1 protein levels would be regulated by miR-34a upon p53 activation. Instead, our results showed the opposite. Specifically, we found that Notch1 is induced by p53 in CLL and we were unable to detect any significant effects of miR-34a on NICD signaling and Notch1 protein levels.

7.1 miR-34a is induced by p53

Several studies have shown that miR-34a is a direct transcriptional target of p53[33,57,96]. In order to verify this previous finding, we analyzed the effects of p53 activation via Nutlin-3a treatment and p53 overexpression or knockdown on the expression levels of miR-34a by RT-qPCR.

Unfortunately, 24h Nutlin-3a treatment failed to significantly induce miR-34a expression in all tested cell lines and p53 overexpression failed to significantly induce miR-34a expression in WA-OSEL cells. Possible explanations for these negative results might be both technical and molecular reasons. On the one hand, transfection efficiency might have been too low to sufficiently overexpress p53. Therefore, it would be interesting to repeat the experiment using a more efficient transfection method such as viral transduction[97]. On the other hand, p53 stabilization and activation is governed by a complex network of stimuli and regulators[98]. Thus, p53 overexpression might not always correlate with increased activity and vice versa. This might be particularly true when transfection efficiency is low.

However, in other experiments we found that:

- miR-34a levels were significantly lower in p53 mut CLL cell lines compared to p53 wt cell lines;
- p53 overexpression significantly induced miR-34a expression in MEC-1 cells;
- 48/72h Nutlin-3a exposure significantly induced miR-34a expression in both EHEB and WA-OSEL;

- CLL patients with p53 alterations showed significantly lower miR-34a expression levels compared to p53 wt CLL patients.

These findings may suggest that Nutlin-3a treatment alone and the resulting p53 activation are not sufficient to induce miR-34a levels and that the induction of miR-34a is controlled by multiple factors in cells. Another example of a factor that regulates the activity of miR-34a is given by an interesting study that showed the presence of mature, inactive, single-stranded miR-34a pools in the nuclei of four different cancer cell lines which are rapidly released upon DNA damage. In addition, they showed that the release of these miR-34a pools is not p53-dependent, but mediated by kinases activated upon DNA damage[99]. Therefore, it would be interesting to compare whether DNA damage induced by genotoxic agents such as fludarabine, which is used as a chemotherapeutic agent to treat CLL, is able to induce a more rapid and pronounced increase in miR-34a expression levels, in contrast to non-genotoxic Nutlin-3a treatment. Moreover, the difference in miR-34a induction between fludarabine alone and in combination with Nutlin-3a could be another experimental point worth investigating.

7.2 miR-34a did not affect Notch1 and BCL-2 protein levels

MiR-34a is a tumor suppressor involved in a wide variety of cellular processes such as proliferation, senescence and stemness[56]. Despite the fact that Notch1 and BCL-2 have been shown to be two of the major targets of miR-34a[92,100], in the present study we were unable to detect significant differences in Notch1, BCL-2 protein levels and NICD signaling upon transfection with miR-34a precursors or inhibitors in WA-OSEL and MEC-1. Several technical and molecular reasons might account for our inability to verify the findings of others.

First, the analysis of the cells 24h after transfection might have been too early to observe the effects elicited by miR-34a overexpression or inhibition. Therefore, the experiment should be repeated including longer time points after transfection to detect possible effects of miR-34a on Notch1 and BCL-2 protein levels that occur later than 24h.

Second, the effects of miRNAs on protein output have been reported to be in the range of a twofold change[101,102] and might therefore be difficult to detect in a population of cells in which only a small fraction was transfected due to a low transfection efficiency.

Last, we used a miR-34a inhibitor that acts as a "sponge" by binding to the mature form of miR-34a and depriving cells of this miRNA. It is important to consider that each miRNA can have many targets and consequently can bind to different transcripts. Therefore, another approach to disrupt the specific interaction between miR-34a and Notch1 would be to use antisense oligos that hybridize to the 3' UTR of the target instead of hybridizing to the miRNA itself[44].

Nevertheless, we do not exclude the possibility that our results of miR-34a not affecting Notch1 and BCL-2 protein levels were caused by molecular mechanisms. However, considering the potential technical issues and the fact that different publications show Notch1 and BCL-2 being targeted by miR-34a, it is more likely that the lack of effects we observed is due to technical issues rather than molecular reasons. Only further experiments using approaches that reduce technical issues would clarify this point.

7.3 p53 induces Notch1 expression in CLL cells

In this study, we confirmed that Nutlin-3a induces p53 expression and activation, as documented by increased levels of p21 in p53 wt cell lines, but not in p53 mut cell lines. We then sought to examine the effects of p53 expression and activation on NICD signaling activity and Notch1 protein levels.

Although 24h Nutlin-3a treatment did not affect NICD signaling in our experimental settings, prolonged Nutlin-3a treatment for 48 and 72h hours notably increased NICD signaling activity in EHEB cells. This was not the case in WA-OSEL cells, where 48 and 72h exposure to Nutlin-3a decreased NICD signaling activity. These different results in the two cell lines could be explained by differences in cell viability. Indeed, although the same number of cells was used, lower protein concentrations were obtained in EHEB samples after 48 or 72h Nutlin-3a treatment if compared to WA-OSEL samples. This is also clearly visible when β-actin bands between EHEB and WA-OSEL cells are compared in the WB results. Therefore, it is likely that 48 and 72h Nutlin-3a treatment substantially affected the viability of EHEB cells but not of WA-OSEL cells. It is possible that Nutlin-3a treatment selected a specific subclone of the EHEB cells in which enhanced NICD signaling sustained resistance to Nutlin-3a and cell survival. This hypothesis could be tested with the following experiments. First, cell viability should be tested with a dedicated assay. Second, the clonogenic potential of EHEB under Nutlin-3a treatment and whether this is connected to enhanced NICD signaling could be tested with a clonogenic assay. These assumptions may be further supported by the fact that, when

compared to the DMSO control, the prolonged Nutlin-3a treated surviving clone of EHEB cells showed higher Notch1 and BCL-2 protein levels and significantly higher expression levels of miR-21, which is considered to be an onco-miR[42]. All these molecular features, related to aggressiveness, resistance and survival of cancer cells, support the hypothesis that prolonged Nutlin-3a treatment selected a clone of EHEB cells that is more resistant to p53-dependent apoptosis. Hence, it could be interesting to investigate whether Nutlin-3a treatment can lead to cell resistance to treatment.

Consistent with increased NICD signaling in EHEB treated with Nutlin-3a, we found higher Notch1 protein levels in WA-OSEL and EHEB cells upon Nutlin-3a treatment, but not in the p53-mutated cell lines MEC-1 and MEC-2. In an antecedent study, Secchiero et al. observed increased Notch1 RNA and protein levels upon Nutlin-3a treatment in several leukemic cell lines and primary B-CLL cells. In addition, they showed that the induction of Notch1 upon Nutlin-3a treatment is prevented when p53 is silenced or when p53 transcriptional activity is chemically inhibited[89]. Our results from the p53 overexpression/knockdown experiment add further robustness to these findings. Indeed, we found that while p53 overexpression led to increased Notch1 protein levels in both p53 wt (WA-OSEL) and p53 mut (MEC-1) cells, p53 silencing led to decreased Notch1 protein levels. Taken together, p53 seems to induce Notch1 expression in CLL cells. This is supported by other studies in the literature showing Notch1 induction by p53[103] and the presence of p53 binding sites in the Notch1 promoter in keratinocytes[104].

Notch plays a critical role in many aspects of cell biology under physiological and non-physiological conditions. Deregulation of the Notch pathway can lead to different outcomes depending on the context and the cell type[105]. Although Notch signaling exerts an antiproliferative role in a limited number of malignancies such as hepatocellular carcinoma and small cell lung cancer, hyperactivation of Notch signaling sustains oncogenesis and promotes survival in many other malignancies[106]. This is the case in Chronic Lymphocytic Leukemia where *Notch1* is frequently found to be mutated. The oncogenic driving role of Notch1 in CLL is still poorly understood[74], but its influence on disease progression and poor patient outcome is evidenced by an enrichment of *Notch1* mutations over the course of the disease. Especially since *Notch1* mutations have been identified, together with *TP53* alterations, as hallmark genomic alterations in Richter's Transformation[107]. Consistent with these observations,

Secchiero et al. claimed that the upregulation of Notch1 by p53 is a negative feedback loop capable of inhibiting p53-induced apoptosis. They showed that the combination of Notch1 inhibition by γ-secretase inhibitors and p53 activation by Nutlin-3a treatment sensitizes leukemic cells to apoptosis[89]. In support of this point, other studies have shown the ability of Notch1 to suppress p53-induced apoptosis, even through a direct interaction between NICD and p53[108,109]. This may explain why higher Notch1 protein levels were observed in CLL patients carrying functional p53. Moreover, p53 wt CLL patients showed consistent Notch1 protein levels, whereas some CLL patients with p53 alterations showed extremely low Notch1 protein levels, suggesting that Notch1 is necessary for the survival of leukemic cells that still express functional p53. Indeed, it has been hypothesized that cancer cells, due to their numerous physiological aberrancies, rely on anti-apoptotic mechanisms in order to survive[110,111].

In contrast to these findings, we observed reduced Notch1 protein levels in HG-3 cells upon Nutlin-3a treatment. This highlights the complexity of the interaction between p53 and Notch1 and the need to be cautious in generalizing cell line-specific results.

7.4 BCL-2 may sustain Notch1 driven anti-apoptotic mechanism

In T-cell leukemia, Notch1 has been shown to induce BCL-2 signaling via the Nf-κB pathway[93]. Moreover, it has been shown that Notch1 knockdown sensitizes prostate cancer cells to docetaxel treatment through the regulation of BCL-2[94] and that the combination of Notch1 inhibitors with BCL-2 inhibitors has a synergistic cytotoxic effect on Multiple Myeloma cells both *in vivo* and *in vitro*[112]. These findings suggest a potential crosstalk between Notch1 signaling and BCL-2 in cancer.

Since BCL-2 is involved in CLL and is a major target of miR-34a, we examined BCL-2 protein levels by WB in the different experiments. We found that BCL-2 protein levels were upregulated in EHEB upon prolonged exposure to Nutlin-3a. In addition, higher BCL-2 protein levels correlated with the expression of Notch1 in most of CLL patients with p53 alterations. These findings further highlight a plausible link between Notch1 and BCL-2 in CLL.

BCL-2 is highly expressed in CLL and inhibits apoptosis induced by BH3-only family members. Specifically, BCL-2 can partially suppress p53-mediated apoptosis by inhibiting PUMA, which is a p53-upregulated modulator of apoptosis[90,113,114].

Taken together, we hypothesize that Notch1-dependent induction of BCL-2 may serve as an additional mechanism exploited by CLL cells to promote cell survival and resistance.

8. CONCLUSIONS AND OUTLOOK

In this study, we demonstrated that Notch1 is induced by p53, which is hypothesized to be an anti-apoptotic mechanism in CLL. In addition, we found a Notch1-dependent BCL-2 induction, which might in parallel enhance the Notch1-mediated anti-apoptotic mechanism. To our current knowledge, there are no studies in literature investigating the link between Notch1 and BCL-2 in CLL. It would be interesting to investigate whether cells from CLL patients expressing higher levels of Notch1 and BCL-2 proteins represent a subclone that is more aggressive and resistant to therapy. Furthermore, NICD signaling activity and Notch1 protein levels should be analyzed in CLL patients who show resistance to BCL-2 inhibitors such as Venetoclax. Increased NICD signaling and Notch1 protein levels may represent a novel mechanism by which CLL patients become resistant to BCL-2 inhibitors. These studies may provide new treatment options for this subset of patients who are resistant to current therapeutic regimens.

Both Notch1 and BCL-2 are direct targets of miR-34a. However, we could not detect any significant effects of miR-34a on NICD signaling activity and Notch1 and BCL-2 protein levels. Therefore, further experiments are needed to make more reliable assumptions about the role of miR-34a in regulating the p53-Notch1 crosstalk and BCL-2 levels in CLL. Since Notch1 is a direct transcriptional target of p53, p53 variation would directly translate into Notch1 oscillations, which could have deleterious effects due to the pro-apoptotic role of p53 and the oncogenic role of Notch1. For this reason, a mechanism is required to keep the p53-Notch1 axis under control. MiRNAs have been shown to be important in maintaining the robustness of biological processes in constantly fluctuating environments[115–117]. Hence, we can hypothesize that miR-34a, which is a transcriptional target of p53 and targets Notch1, might be important to keep Notch1 protein levels under control in physiological conditions.

If we hypothesize that miR-34a acts as a "rheostat"[44] in the p53-miR-34a-Notch1 axis in B cells, keeping Notch1 protein levels under control in physiological conditions, miR-34a dysregulation in CLL may result to be deleterious because it leads to an uncontrolled p53-Notch1 axis. An interesting question that arises is: why should the anti-apoptotic protein p53 induce the Notch1 oncogenic pathway in B cells under physiological conditions? Although p53 has mostly been studied for its role in directing genome repair and cell apoptosis, recently the role of p53 in the immune system has been established. It has been shown that p53 contributes to the immune response by regulating key components of immune signaling pathways[118]. Furthermore, p53 transcriptional activation can be triggered by a wide variety of stresses,

including viral infection[3]. In addition, Notch1 signaling has been shown not only to regulate genes important for B cell physiology[74] but also to enhance the immune response by upregulating immune-related genes[66]. Thus, it may be interesting to investigate whether the crosstalk between p53 and Notch1 somehow regulates B cell immune responses to pathogens.

As CLL patients with *TP53* and *Notch1* alterations exhibit a poor prognosis, further studies characterizing the p53-miR-34a-Notch1 axis are needed. In particular, the latter may provide new insights that can have a profound impact on clinical routine for these and other subgroups of patients. For example, the utility of miRNA profiling as a possible indicator of patient prognosis should be considered. In this regard, miR-34a and miR-21 expression levels could be evaluated to define specific subgroups of patients with better or worse prognosis and to understand the stage of the disease. This approach is very powerful for many reasons:

- miRNA levels are usually very informative, especially in CLL where miRNAs have been shown to be highly involved in disease pathogenesis;
- miRNAs are easy to assess in both cells and body fluids, as it is simple to access the samples form which miRNAs are extracted;
- the amount of miRNAs needed to evaluate miRNA profiles by RT-qPCR is very low (10ng)[119].

On the other hand, Notch1 induction by p53 has been shown to be a plausible mechanism by which leukemic cells escape p53-induced apoptosis. Therefore, inhibition of Notch1 signaling combined with p53 induction should be evaluated as a potential strategy to therapeutically target CLL cells.

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OFFICIAL DECLARATION

I hereby declare that I composed the present thesis with the topic:

Notch1 is induced by p53 as a possible anti-apoptotic mechanism in Chronic Lymphocytic Leukemia

Independently and that I have used no other sources than those indicated. The text passages which are taken from other works in wording or meaning I have identified in each individual case by stating the source.
I further declare that I have completed my academic work in line with the principles of good scholarly and scientific practice and in accordance with the valid ‘Article of the University of Ulm for Ensuring Good Scientific Practice’.

Ulm, 11.09.2023