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Getting insights into the multi-faceted role of Notch3 in different tumor contexts

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CONTENTS

SUMMARY

ABSTRACT

Notch receptor family comprises evolutionary conserved single-pass transmembrane proteins which are involved in several cellular processes during embryogenesis and in adult tissues. Given their pleiotropic effects, their de-regulation is associated to the development of several diseases including cancer. Since Notch receptors are differentially implicated in essentially all of the hallmarks of cancer, dissecting every facets of each receptor in different tumor contexts could help to foster effective and specific targeted therapies.

In keeping with this consideration, during my PhD project I specifically focused my attention on Notch3 receptor in three different tumor contexts: Triple-Negative Breast Cancer (Project n°1), T-cell Acute Lymphoblastic Leukemia (Project n°2), and Ovarian Cancer (Project n°3).

The main objective of this work, performed in the laboratory of Prof. Isabella Screpanti (at the Department of Molecular Medicine of Sapienza University) under the direct supervision of Dr. Saula Checquolo, was to broaden the knowledge of Notch3 receptor, trying to puzzle out its role in cancer, mainly focusing on how it is specifically regulated at the post-translational level, which still represents an unknown field of Notch3 regulation process.

Indeed, Project n°1 and Project n°2 report two different crosstalk between Notch3 and other signaling pathways, the EGFR signaling and the Unfolded Protein Response (UPR), respectively. Specifically, on the one hand, we demonstrate that Notch3 regulates EGFR localization in Triple-Negative Breast Cancer, making the receptor unavailable to be targeted by the anti-EGFR agents, such as tyrosine kinase inhibitors, thus highlighting how Notch3 could be crucial in promoting drug-resistance. On the other hand, we document that Notch3 is involved in the activation of pro-survival UPR by directly interacting with an UPR "effector", thus sustaining cancer cell growth under ER stress conditions.

Interestingly, in both tumor contexts Notch3 fulfils its function in a transcription-independent manner, paving the way for the study of a mostly untouched aspect of Notch3 function in cancer.

Moreover, in Project n°3 we cover a largely unstudied but crucial layer of fine-tuning and regulation of Notch3: its post-translational modifications (PTMs). To date, little is known about the potential different PTMs of Notch3, mainly regarding the glycosylation of its extracellular domain and the acetylation of its intracellular domain. Here we focus our attention on the study of the phosphorylation *status* of Notch3 intracellular domain showing how it influences its longevity and who are the actors of this regulation, thus finally fostering a novel therapeutic approach to target Notch3-dependent tumors through the modulations of these specific Notch3 protein regulators.

All in all, there are still lots of gaps in the puzzle of "Notch3 world" but some small pieces were falling neatly into place.

ORGANIZATION OF THE WORK

This thesis will start with a First Chapter providing an overall introduction to Notch receptors concerning their structure, their canonical signaling pathway and how their de-regulation is associated to several diseases.

The Second Chapter will be devoted to a brief explanation of the major objectives of each project in order to show the golden thread that runs through them.

The Third Chapter will cover the results obtained in each project. The results of the Project n°1 were reported in one publication (Diluvio G et al., Oncogenesis 2018) and the ones of Project n°2 are currently under revision (Giuli MV et al., manuscript submitted to Haematologica Journal). Therefore, in the Section 3.1, I will insert these two papers preceded by a brief introductory paragraph which will summarize the state-of-the-art of the studied tumor context, the role of the

involved pathways and the novelty of each project. The manuscript on the Project n°3 is in preparation so the Section 3.2 will be more detailed and it will be divided in several paragraphs.

Since each project has its own conclusion included in the Third Chapter, in the Conclusion Section of the present PhD Thesis (Forth and last Chapter), I will briefly discuss the obtained results focusing on their potential therapeutic application based on anti-Notch3 strategies.

Finally, in the Appendix I, I will insert a recent published review on the role of Notch receptors in Triple-Negative Breast Cancer (Giuli MV et al., Journal of Oncology 2019).

1. INTRODUCTION

1.1 NOTCH RECEPTORS

The Notch receptors are single-pass transmembrane proteins involved in several cellular processes during development and in adult tissues **(Louvi A et al., 2012)**.

For instance, Notch signaling is implicated in several organ and tissue development programs. On the one hand, Notch pathway impedes differentiation and secures a pool of stem or progenitors cells; on the other hand, Notch signaling promotes differentiation and it is involved in cell commitment. Moreover, increasing evidence suggests that Notch receptors orchestrate tissue homeostasis, both under normal conditions and during repair and regenerative processes **(Siebel C and Lendhal U, 2017)**.

Notch signaling is evolutionary conserved across the metazoan spectrum and occurs via short-range cell-cell communication between transmembrane ligands on one cell and transmembrane receptors on the neighboring one **(Artavanis-Tsakonas et al., 1999)**.

The first observations of Notch receptors derived from genetic studies of mutants of the fruit fly Drosophila melanogaster. The fruit fly genome encodes one Notch protein whereas two receptors (LIN-12 e GLP-1) with redundant roles were discovered in Caenorhabditis elegans **(Fitzgerald K et al., 1993)**. Moreover, in mammals four Notch paralogs are synthesized and they have only partly shared functions **(Wu J and Bresnick EH, 2007)**.

1.1.1 Notch structure

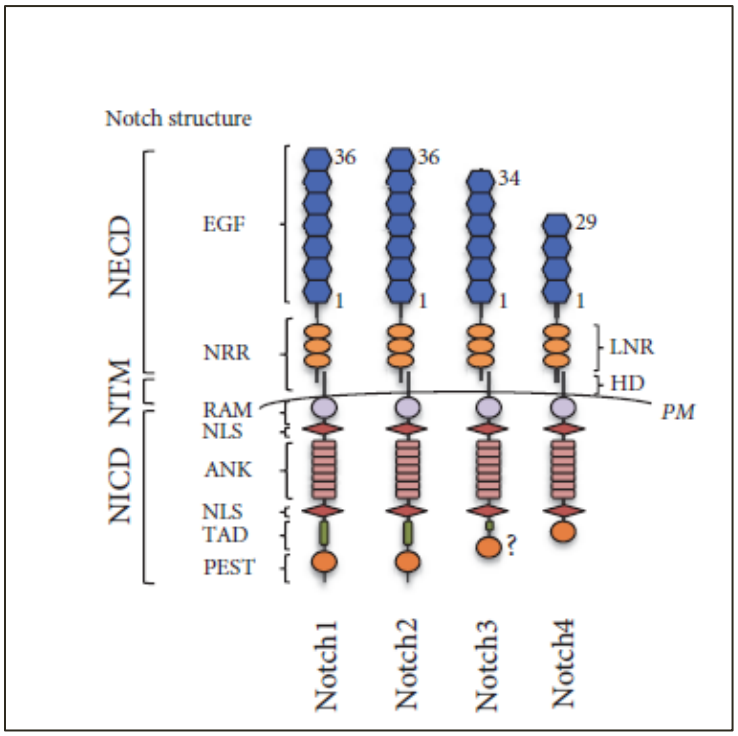

Figure 1. Notch family receptors in mammals. Schematic representation of the Notch receptors structure in mammals (Giuli MV et al., 2019).

The Notch locus encodes a large (~300 kDa) multidomain protein which is expressed on the plasma membrane as processed heterodimer. It is possible to distinguish a large extracellular domain (NECD), a transmembrane region (TM) and a large intracellular domain (NICD) **(Hori K et al., 2013)**.

The NECD comprises several tandem Epidermal Growth Factor (EGF)-like repeats followed C-terminally by a negative regulatory region (NRR) **(Gordon W et al., 2008)**.

The EGF-like domain is an evolutionarily conserved structure and it is formed by nearly 40 amino acid residues, among them 6 cysteines that are responsible for 3 characteristic disulphide bonds (**de Celis JF et al., 1993)**. Calcium-binding EGF repeats are functionally important to interact with the ligands **(de Celis JF et al.,**

1993) and, as a result, it has been proposed that extracellular Ca^{2+} concentration might influence Notch signaling **(Raya A et al., 2004)**. Non-calcium-binding EGF repeats are not able to coordinate Ca^{2+} ions and they are present in flexible regions of the ECD **(Hambleton S, 2004)**. The number of EGF-like repeats is not conserved across the metazoan: 11-14 in C. elegans and 29-36 in D. melanogaster and in mammals **(Rana NA and Haltiwanger RS, 2011)**. Despite the large number of EGF-like repeats, only two, 11 and 12, are essential for mediating interactions with the ligands **(Rebay I et al., 1991; Xu A et al., 2005).**

The NRR comprises 3 Lin-12-Notch repeats (LNRs) and an heterodimerization domain (HD). Structural studies revealed that in the resting state, the LNRs masks the heterodimerization domain, thus preventing ligand-independent proteolysis **(Gordon WR et al., 2009).** This region contains two proteases sites, known as S1 and S2, which are involved in the processing and signaling of Notch receptors **(Gordon WR et al., 2008).**

The ECD undergoes several processing events during their intracellular routing to the cell surface **(Kadesch T, 2000)**.

These processes comprise N- and O-glycosylation and occur in the Endoplasmic Reticulum (ER) and in the Golgi apparatus **(Jafar-Nejad H et al., 2010).** Early work on the Notch receptors documented that the EGF-like repeats are decorated with N-glycans **(Kornfeld K et al., 1981).** In addition to N-glycosylation, several O-linked glycosylation (O-fucosylation, O-glucosylation and O-GlcNAcylation) occurred in the ECD **(Moloney DJ et al., 2000; Matsuura A et al., 2008)**. A growing piece of evidence highlighted that glycosylation affects Notch signaling and it is crucial for their functions **(Stanley P, 2007; Luther KB and Haltiwanger RS, 2009)**.

Besides glycosylation, Notch receptors are cleaved at the S1 site in the Golgi apparatus by a furin-like convertase and reach the plasma membrane as heterodimers **(Logeat F et al., 1998).**

The NICD is formed by several regulatory motifs, including RAM (RBP-jk-Associated Molecule), TAD (Transcriptional Activation Domain) and PEST (P: Proline; E: Glutamic Acid; S: Serine; T: threonine) degron sequence. Between RAM and TAD there are 7 tandem Ankyrin repeats (ANK) flanked by 2 Nuclear Localization Signal (NLS) sequences **(Rana NA and Haltiwanger RS, 2011)**. The RAM and the ANK domains are essential to recruit transcriptional co-activators whereas PEST domain is involved in NICD degradation **(Hori K et al., 2013)**.

The 4 Notch receptors in mammals display subtle structural differences. For instance, Notch1 and Notch2 have 36 EGF-like repeats while Notch3 and Notch4 have 34 and 29 repeats, respectively **(Previs RA et al., 2015).** These differences correlate with diverse affinity for their ligands **(Rebay I et al., 1991)**. Moreover, another source of diversity rests within TAD resulting in differential transactivation activity: it is absent in Notch4 **(Kurooka H et al., 1998)** and Notch3 TAD is shorter with respect to Notch1 and Notch 2 **(Beatus P et al., 2001)**.

Furthermore, the NICD may differ between Notch paralogues **(Chillakuri CR et al., 2012)** since amino acid sequence identity is partial across the 4 Notch receptors **(Bellavia D et al., 2008)**. For instance, Beatus and colleagues identified a novel region located C-terminally to ANK domain known as RE/AC (REpression/ACtivation) which is necessary for N1ICD's ability to activate and for N3ICD's ability to repress a HES promoter **(Beatus P et al., 2001).**

Taken together, these structural differences amplify and diversify the signaling output.

1.1.2 Canonical Notch signaling pathway

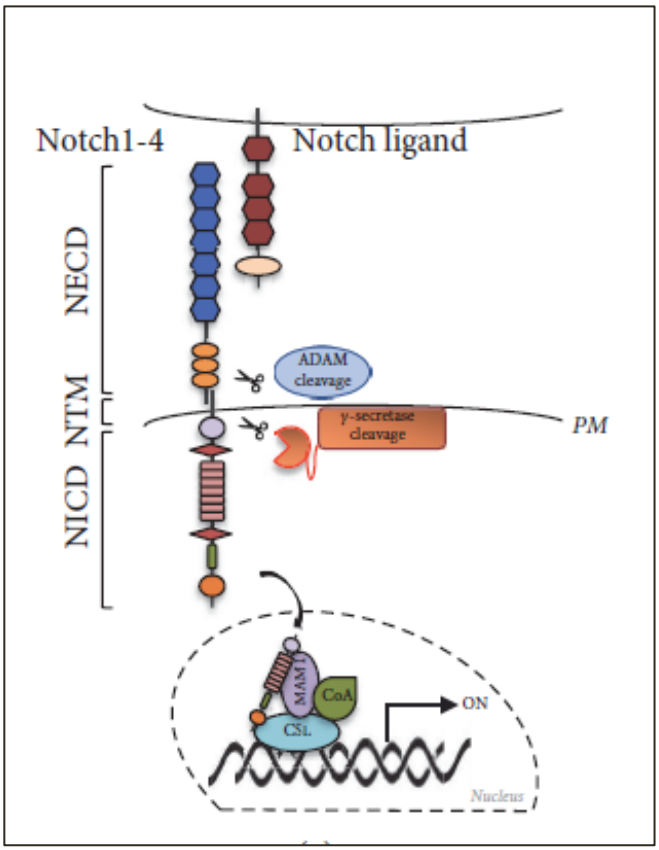

Figure 2. The canonical Notch signaling pathway. Ligand binding triggers two sequential proteolytic cleavages (by ADAM and γ-secretase complexes), resulting in the release of NICD which translocates to the nucleus where interacts with transcriptional regulators (CSL, MAM1 and CoA) (Giuli MV et al., 2019).

Notch signaling is made by 3 key steps: 1. ligand recognition; 2. conformational exposure of the S2 protease site and 3. assembly of transcriptional complexes.

Activation of the canonical pathway requires the *trans*-interaction of a Notch receptor in signal-receiving cell with a ligand on signal-sending neighboring cell **(D'Souza B et al., 2010).** *Cis*-interactions between Notch receptors and ligands expressed in the same cell also occur and they inhibit Notch1 signaling **(Sprinzak D et al., 2010)** while activating Notch3 signaling in a subset of T-cell acute lymphoblastic leukemia (**Pelullo M et al., 2014).**

Several genetic studies revealed that Notch ligands have been conserved across metazoan. Mammalian Notch ligands fall into two classes, depending on their homologies with the ones found in D. melanogaster (Delta and Serrate). Mammals have three Delta-like proteins, called Delta-like 1 (DLL1), Delta-like 3 (DLL3), and Delta-like 4 (DLL4), and two homologues of Serrate, called Jagged-1 (JAG1) and Jagged-2 (JAG2) **(D'Souza B et al., 2008).** Canonical Notch ligands are single-pass transmembrane proteins and exhibit a modular domain arrangement. The extracellular domain comprises an N-terminal MNNL (Module at the N-terminus of Notch ligands) domain and a cysteine-rich Delta–Serrate–LAG2 (DSL) domain **(Kovall RA and Blacklow SC, 2010).** The DSL domain is evolutionary conserved and it is crucial for the interaction with Notch receptors. Moreover, all ligands contain several EGF-like repeats followed by the transmembrane domain and a short C-terminal cytoplasmic tails **(D'Souza B et al., 2008).** Despite the similar overall modular organization, a number of structural differences exists among the DSL ligands. For instance, Serrate family (Serrate, JAG1 and JAG2) proteins and DLL1 have specific EGF-like repeats which form the DOS (Delta and OSM-11-like proteins) domain. Furthermore, Serrate family proteins are characterized by a large number of EGF-like repeats and a cysteine-rich region, partially homologous with the von Willebrand Factor C (VWFC) **(D'Souza B et al., 2010)**. DLL1, DLL4, and JAG1 contain PDZL motifs (PSD-95/Dlg/ZO-1 ligand) that recognize PDZ domain of several cytoskeletal proteins responsible for cell-cell junctions **(Popovic M et al., 2012)**.

Notch receptors have distinct ligand affinities, thus resulting in differential downstream transcriptional outcome **(Shimizu K et al., 2002)**.

Upon ligand binding, the key step in the activation of the pathway is a conformational change of the NRR domain, following ligand endocytosis within signal-sending cell, which unmasks the S2 protease site. Notch receptor is further

cleaved by two metalloproteases: ADAM10 (A Disintegrin And Metalloprotease 10) e ADAM17/TACE (A Disintegrin And Metalloprotease 17/TNFα Converting Enzyme) **(Gordon WR et al., 2008).** This cleavage creates a truncated membrane-bound form of Notch known as NEXT (Notch EXtracellular Truncation) which is progressively cleaved by γ-secretase complex (Presenelin/Nicastrin/APH1/PEN2) at S3 site and S4 to release NICD in the cytoplasm **(Gordon WR et al., 2008)**. Subsequently, NICD translocates to the nucleus where it forms a binary complex with DNA-binding factor CSL (CBF1/Suppressor of Hairless/Lag1), converting it from a repressor to an activator of transcription **(Wang H et al., 2015)**. Indeed, prior to pathway activation, CSL directly interacts with transcriptional co-repressor proteins and histone deacetylases **(Wang H et al., 2015),** which are displaced after NICD binding. The binary complex CSL-NICD is then recognized by co-activators belonging to the Mastermind (MAM) family. The formation of the CSL-NICD-MAM ternary complex leads to the recruitment of various transcriptional co-activators (CoA) and chromatin remodeler **(Wilson JJ and Kovall RA, 2006)**, finally resulting in the transcription of several Notch target genes, among them transcriptional repressors of the HES and HEY families, MYC, NF-κB, cyclinD1 **(Bray SJ, 2006).** The target genes activated by Notch are differential depending on ligand-receptor interaction and cell-type-specific transcriptional programs **(Bray SJ, 2016)**, thus partially explaining Notch pleiotropic effects.

1.2 THE OTHER SIDE OF THE COIN: NOTCH DYSREGULATION

Consistent with the crucial role of Notch pathway in many aspects of cellular processes, increasing evidence links perturbations in the pathway to various diseases, including several inherited syndromes and cancer.

1.2.1 Notch signaling and genetic disease

Some monogenic disease are related to mutations in the Notch signaling pathway, among them Alagille syndrome, Hajdu-Cheney syndrome and CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarts and Leukoencephalopathy) disease.

Given the role of Notch pathway in organogenesis, Alagille syndrome affects multiple organs and clinical features may differ significantly. The initial diagnosis is based on the presence of intrahepatic bile duct paucity and at least of 3 other clinical features: chronic cholestasis, cardiac disease, ocular abnormalities, skeletal abnormalities, and peculiar facial features **(Reyes-de la Rosa ADP et al., 2018)**. Alagille-bearing patients are characterized by mutations in JAG1 and rarely in Notch2 **(Grochowski CM et al., 2016).**

In the Hajdu-Cheney syndrome, the excessive bone resorption is caused by losso ofo Notch2 PEST domain **(Simpson MA et al., 2011).**

Furthermore, CADASIL displays degenerated vascular smooth muscle cells in the brain and recurring small brain infarcts. This disease is associated with frequent missense mutations in Notch3 which alter the number of cysteine residues in the N3ECD **(Rutten JW et al., 2014).**

1.2.2 Notch signaling and cancer

Since this conserved pathway is very dosage-sensitive, too much or too little can lead to cancer. Indeed, functional studies highlighted that Notch signaling is

implicated in essentially all of the hallmarks of cancer, playing oncogenic or tumor suppressive role depending on cell type **(Aster JC et al., 2017)**.

Notch receptors as oncoproteins

One of the first evidence that dysregulation of Notch signaling is linked to carcinogenesis derived from studies on the genetic signature of T-cell acute lymphoblastic leukemia (T-ALL) cells when Ellisen and colleagues described a chromosomic translocation involving Notch1 locus **(Ellisen LW et al., 1991)**.

From that moment on, numerous reports have associated alterations in the Notch pathway with the pathogenesis of T-ALL, since activating Notch1 mutations are found in approximately 55–60% of T-ALL cases **(Weng AP et al., 2004)**. Indeed, Notch1 is a well-characterized oncoprotein in this type of cancer. Interestingly, functional studies suggested also a pro-tumorigenic effect of Notch3 in T-ALL. For more details on the relationship between Notch3 and T-ALL see **Section 3.1.2**.

Extensive research over the past decades highlighted that dysregulation of Notch signaling is implicated also in the pathogenesis of B-cell malignancies **(Mirandola R et al.,** 2011).

Furthermore, up-regulated Notch signaling has been found in multiple solid malignancies such as breast cancer, prostate cancer, lung cancer and ovarian cancer.

The possibility that Notch could function as proto-oncogene in human breast cancer development came from studies on mouse mammary tumor virus-induced cancer **(Gallahan D et al., 1987)**. Indeed, Notch signaling is frequently up-regulated, either for activating mutations of Notch receptors or for high levels of Notch ligands such as JAG1 **(Dickson BC et al., 2007; Reedijk M et al., 2005; Stylianou S et al., 2006)**. Moreover, Notch receptors are implicated in tumor initiation and progression of the most aggressive breast cancer subtype, the triple-

negative breast cancer (TNBC), as we described in a recently published review (see **Appendix I - Giuli MV et al., Journal of Oncology 2019**). In particular, for more details on the relationship between Notch3 and TNBC see **Section 3.1.1**.

Despite the fact that further investigations are needed, high levels of Notch1 and JAG1 correlate to prostate cancer progression and recurrence **(Carvalho FL et al., 2014)**. Similarly, the aberrant activation of the Notch pathway is a very common feature of non-small cell lung cancers (NSCLC) **(Westhoff B et al., 2009).** For instance, high expression of Notch1 increases lymph- and tumor-node metastasis **(Yuan X et al., 2015).**

Deregulated expression of all Notch receptors and their ligands has been associated with high grade ovarian carcinoma and carcinogenesis **(Rose SL, 2009)**. For instance, Notch1 is involved in ovarian cancer (OC) proliferation **(Hopfer O et al., 2005)** and chemoresistance **(Rose SL et al., 2010)** whereas Notch3 is implicated in OC growth, drug resistance and OC stem cell maintenance. For more details on the relationship between Notch3 and Ovarian cancer see **Section 3.2**.

Notch receptors as tumor suppressors

Notch receptors can act also as tumor suppressors since a number of mutations loss-of-function have been unexpectedly linked to cancer development.

Loss of Notch1 signaling causes skin cancer **(Stransky N et al., 2011)** and myeloid malignancies **(Lobry C et al., 2014)**. Moreover, Viatour and colleagues proposed a novel tumor suppressor role for Notch signaling in hepatocellular carcinoma (HCC) **(Viatour P et al., 2011).**

1.2.3 Highlights on anti-Notch therapies

Collectively, there is mounting evidence that the level of Notch signaling is critical within the cells and dramatic up- or down-regulation of this pathway can lead to

disease **(Guruharsha K et al., 2012).** Indeed, it is necessary to precisely fine-tune it with *ad-hoc* therapies.

A number of interesting avenues have been tested rendering the development of Notch-based therapies a very active area of research **(Braune EB and Lendhal U, 2016)**.

Briefly, the therapeutic strategy that has been explored more extensively is based on the use of γ-secretase inhibitors (GSIs). GSIs block the release of NICD in the cytoplasm by inhibiting the final proteolytic cleavage. Nevertheless, GSIs prevent the activation of all Notch receptors thus contributing to the onset of several adverse side effects *in vivo*, likely dependent on the fact that Notch receptors are involved in tissue homeostasis **(Shih IeM and Wang TL, 2007)**. For instance, Notch signaling plays a homeostatic role in the intestine where it not only secures the stem cell pool in the crypts but also it sustains enterocytes differentiation at the expense of secretory lineages **(Siebel C and Lendhal U, 2017)**. Therefore, GSIs-dependent blockade of Notch signaling results in secretory cells differentiation and excessive mucus production which explain the intestinal toxicity observed in many clinical trials **(van Es JH et al., 2005).** Moreover, the use of GSIs is further complicated by the fact that more than 40 other proteins are cleaved by the γ-secretase complex **(Selkoe DJ and Wolfe MS, 2007)**.

In spite of these potential shortcomings, a number of clinical trials of GSIs have been proceeded. However, it would be desirable to modulate Notch in more specific ways to avoid the issues related to pan-Notch inhibition.

Receptor- or ligand-specific antibodies have been recently developed. On one hand, anti-Notch antibodies block individual Notch receptors by binding NRR and preventing the proteolytic processing by ADAM10 and ADAM17/TACE, thus reducing the cleavage upon ligand binding **(Wu Y et al., 2010).** On the other hand,

anti-ligand antibodies impair the *trans*-interaction between receptors and ligands which is required for the proper activation of the signaling **(Hoey T et al., 2009).**
A third potential avenue is to target Notch pathway together with another signaling mechanism. Combination therapy might be particularly suitable since several studies documented the cross-talk between Notch signaling and other pathways **(Braune EB and Lendhal U, 2016)**. Moreover, it is noteworthy that in combination therapies it is possible to use lower doses of each inhibitor circumventing some of the high-dosage-dependent side effects.

Up to date, any anti-Notch therapies are in routine clinical use, so dissecting new facets of Notch signaling in different cancer contexts becomes a paramount issue in order to ameliorate the current treatments.

2. AIMS OF THE WORK

Decades of detailed studies revealed that Notch receptors are deeply involved in several processes, from cell commitment during embryogenesis to homeostasis maintenance in adult tissues.

Such pleiotropy is unexpectedly based on a simple core pathway where few proteins are involved **(Palermo R et al., 2014)** without any step of amplification such as phosphorylation cascades or second messengers **(Braune EB and Lendhal U, 2016)**. A noteworthy source of diversity relies on the fact that in mammals there are four Notch paralogs characterized by variable structural homology **(Bellavia D et al., 2008)** which contributes to partial shared functions **(Wu J and Bresnick EH, 2007)**. Nevertheless, it is only the tip of the iceberg since the structural variability cannot be an exhaustive answer. This consideration hints at a complex underlying network circuitry that diversifies the signaling output. Indeed, the downstream diversity may be achieved by multiple mechanisms derived from the crosstalk between Notch and other signaling pathways also involving different post-translational modifications (PTMs) of Notch receptors **(Braune EB and Lendhal U, 2016)**.

Despite a lot of effort has been put in dissecting every facets of each receptor, there is a long way to go before they are fully understood. Therefore, in the development of my PhD project, I focused my attention on the Notch3 receptor and how the afore-mentioned mechanisms influence its function in different tumor contexts.

Crosstalk between Notch3 and other signaling pathways

Triple-negative breast cancer (TNBC) is characterized by the poorest prognosis **(Dawson SJ et al, 2009)**. Since the tyrosine kinase receptor EGFR overexpression accounts for 45–70% of TNBC **(Hoadley KA et al., 2007)**, anti-EGFR therapies (such as tyrosin-kinase inhibitors, TKIs, or monoclonal antibodies) have been

developed in the recent years but the activation of compensatory pathways led to disappointing results **(Masuda H et al, 2012)**. Therefore, effective therapeutic strategies for overcoming drug-resistance are urgently required.

As a result, the main aim of the research **Project n°1** (where I participated to the last part of the project and during the revision process) was to evaluate the Notch3-EGFR interplay in TNBC and its involvement in the resistance to TKIs. All the data obtained were included in a recent published paper where I am a co-author ***(Diluvio G et al., 2018)*** and it is inserted in this PhD Thesis at **Section 3.1.1b**.

Cells adapt to stress and re-establish of ER homeostasis by activation of an integrated signal transduction pathway called Unfolded Protein Response (UPR) **(Ron D and Walter P, 2007)**. Since malignant cells could rely on UPR for their survival **(Papaioannou A and Chevet E, 2018)**, understanding how oncogenes regulate these pathways might help in searching for a novel therapy. To date, no relevant data are known about the possible involvement of Notch proteins and UPR system in cancer.

The main aim of the research **Project n°2** was to get new insights into the role of Notch3 in T-cell Acute Lymphoblastic Leukemia (T-ALL) regarding the adaptive response of Notch3-dependent cancer cells to further stimulate the development of Notch3-targeted therapies. All the data obtained were included in a new paper in submission, where I am the first author ***(Giuli MV et al., Submitted paper)*** and it is inserted in this PhD Thesis at **Section 3.1.2b**.

Post translational modifications (PTMs) of Notch3

Since PTMs are known to modulate protein activity, localization and stability inside a cell **(Walsh CT, 2005)**, the characterization of these modifications plays an important role in understanding their function, thus predicting cell behavior.

Among PTMs, phosphorylation (the addition of phosphate groups from ATP to specific Serine, Threonine, and Tyrosine residues) is one of the key mechanisms for tight dynamic regulation of protein activity in eukaryotic cells as one third of all eukaryotic proteins undergo reversible phosphorylation **(Antfolk D et al., 2019)**.

Several proteins, such as the peptidyl-prolyl *cis*/*trans* isomerase PIN1, recognize and bind specific phosphorylated residues affecting the protein function of their targets **(Zannini A et al., 2019).** Studies documented that Pin1 enhances the oncogenic potential of Notch receptors by phosphorylation-dependent prolyl isomerization in breast cancer context **(Rustighi A et al., 2009; Rustighi A et al., 2014)**. In this scenario we have recently demonstrated a specific functional Pin1-Notch3 crosstalk in T-cell leukemia **(Franciosa G et al., 2016)**. Based on these observations, the main aim of the research **Project n°3** (see **Section 3.2**) is to investigate in more detail the Notch3 – Pin1 relationship by focusing our attention on: 1. the underlying molecular mechanism; 2. the possibility of exploiting this relationship in order to develop a novel therapeutic strategy for the treatment of Notch3-overexpressing tumors which rely on Notch3 protein function to survive and spread to secondary organs ***(Giuli MV et al., Manuscript in preparation)***.

3. RESULTS

3.1 CROSSTALK BETWEEN NOTCH3 AND OTHER SIGNALING PATHWAYS

3.1.1 Notch3 and EGFR in Triple Negative Breast Cancer

3.1.1a Glance at Project n°1

- **General features of Triple Negative Breast Cancer (TNBC)**

  Breast cancer (BC) is one of the most commonly diagnosed cancer in women worldwide (**Siegel R et al., 2017; Torre LA et al., 2016**). BC is classified into different subtypes according to the presence or absence of estrogen receptors (ERs), progesterone receptors (PRs) and the human epidermal growth factor receptor 2 (Her2/neu) (**Carlson RW et al., 2009**). Triple Negative Breast Cancer (TNBC) is characterized by the lack of expression of ER, PR and, Her2 (**Abramson VG et al., 2015**), and it accounts for 15-20% of diagnosed breast cancer (**Lehmann BD et al., 2014**). TNBC is an heterogeneous tumor and displays aggressive phenotype and high relapse rates (**Dent R et al., 2007**), resulting in shorter overall survival when compared to other subtypes (**Prescott JD et al., 2007**). Since the inter-tumoral and intra-tumoral heterogeneity is one of the major issue to deal with, the development of targeted-therapies, less toxic and more effective than chemotherapy, is urgently needed.

- **Overview of the role of EGFR in TNBC**

  The Epithelial Growth Factor Receptor (EGFR) belongs to Tyrosine Kinase (TK) Receptors **(Schlessinger J et al., 2000)**. Several studies reported that EGFR is frequently overexpressed in TNBC and this is due principally to gene amplification (**Rakha EA et al., 2009**) and, to a lesser extent, to gene mutation (**Teng YH et al., 2011**).

Furthermore, TNBC cases with EGFR expression are associated with a poor prognosis **(Nicholson RI et al., 2001)**, thus rendering EGFR a hallmark of this BC subtype. Despite the reliance of TNBC on EGFR downstream signaling, anti-EGFR therapies are ineffective mainly because of the activation of compensatory pathways that induce drug resistance (**Bernsdorf M et al., 2011**). Indeed, this complicates matters further and cell re-sensitization to anti-EGFR therapies becomes a paramount issue.

- **Overview of the role of Notch3 in TNBC**

  Recently, we provided a detailed overview of the specific role of all four Notch receptors in TNBCs (see **Appendix I**, ***Giuli MV et al., Journal of Oncology 2019***). Regarding Notch3, several studies provide evidence of the correlation between Notch3 signaling and TNBC: in particular, Notch3 activating mutations (**Wang K et al., 2015**) and gene amplification (**Turner N et al., 2010**) are quite recurrent in TNBC context. Moreover, Notch3 displays an oncogenic role by sustaining TNBC cells proliferation (**Hirose H et al., 2010; Choy L et al., 2017**), the acquirement of the metastatic phenotype (**Zhang Z et al., 2010; Leontovich AA et al., 2018**) and chemo-resistance (**Boelens MC et al., 2014**).

  To sum up, Notch3 is known to be associated with TNBC tumor initiation and progression.

- **Novelty of the Project n°1**

  We reported a non-canonical role of Notch3 in TNBC: it is involved in the resistance to TKI-gefitinib (Tyrosine Kinase Inhibitor - gefitinib) by regulating EGFR intracellular localization. Therefore, Notch3 depletion

makes the cells sensitive to TKI-gefitinib, thus suggesting a potential novel combined therapeutic approach in the treatment of TNBC-bearing patients.

3.1.1b Paper

"NOTCH3 inactivation increases triple negative breast cancer sensitivity to gefitinib by promoting EGFR tyrosine dephosphorylation and its intracellular arrest"

G. Diluvio, F. Del Gaudio, M. V. Giuli, G. Franciosa, et al. Oncogenesis. 2018;7:42

Diluvio et al. *Oncogenesis* (2018)7:42
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NOTCH3 inactivation increases triple negative breast cancer sensitivity to gefitinib by promoting EGFR tyrosine dephosphorylation and its intracellular arrest

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Abstract

Notch dysregulation has been implicated in numerous tumors, including triple-negative breast cancer (TNBC), which is the breast cancer subtype with the worst clinical outcome. However, the importance of individual receptors in TNBC and their specific mechanism of action remain to be elucidated, even if recent findings suggested a specific role of activated-Notch3 in a subset of TNBCs. Epidermal growth factor receptor (EGFR) is overexpressed in TNBCs but the use of anti-EGFR agents (including tyrosine kinase inhibitors, TKIs) has not been approved for the treatment of these patients, as clinical trials have shown disappointing results. Resistance to EGFR blockers is commonly reported. Here we show that Notch3-specific inhibition increases TNBC sensitivity to the TKI-gefitinib in TNBC-resistant cells. Mechanistically, we demonstrate that Notch3 is able to regulate the activated EGFR membrane localization into lipid rafts microdomains, as Notch3 inhibition, such as rafts depletion, induces the EGFR internalization and its intracellular arrest, without involving receptor degradation. Interestingly, these events are associated with the EGFR tyrosine dephosphorylation at Y1173 residue (but not at Y1068) by the protein tyrosine phosphatase H1 (PTPH1), thus suggesting its possible involvement in the observed Notch3-dependent TNBC sensitivity response to gefitinib. Consistent with this notion, a nuclear localization defect of phospho-EGFR is observed after combined blockade of EGFR and Notch3, which results in a decreased TNBC cell survival. Notably, we observed a significant correlation between *EGFR* and *NOTCH3* expression levels by in silico gene expression and immunohistochemical analysis of human TNBC primary samples. Our findings strongly suggest that combined therapies of TKI-gefitinib with Notch3-specific suppression may be exploited as a drug combination advantage in TNBC treatment.

Introduction

Triple-negative breast cancer (TNBC), which lacks estrogen receptor (ER), progesterone receptor, and human epidermal growth factor 2 receptor (HER2), accounts for about 15–20% of breast cancers and represents the most aggressive breast cancer (BC) subtype[1]. To date, no molecularly targeted agents have been approved for TNBC, leaving to the conventional chemotherapy the

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Fig. 1 Notch3 and EGFR levels correlate in TNBC primary samples. a Upper panel: summary of the *NOTCH3-EGFR* and *NOTCH1-EGFR* gene expression levels correlation obtained by an in silico analysis from two TNBC tissue arrays (GSE76124 and GSE31519). Lower panels: representative graphs showing correlation between *NOTCH3* (left) or *NOTCH1* (right) and *EGFR* gene expression levels from GSE31519 dataset in a cohort of 579 TNBC patients. In both graphs, each dot corresponds to one patient and the expression value of *NOTCH3*, *NOTCH1*, and *EGFR* is given in log2 scale after normalizing data with justRMA algorithm normalization. The *X*–*Y* axis represent *NOTCH3* (left) or *NOTCH1* (right) and *EGFR* (both) expression levels, respectively. The index Pearson's R indicated expresses the linear relation between paired samples and *P*-values were calculated using Student's *T*-test, as described in Material and Methods section. **b** Upper panel: heatmap representing the protein levels of EGFR, Notch3, and Notch1 obtained by immunohistochemical analysis (IHC) in a cohort of 18 TNBC patients. The colors represent positive (red) or negative (blue) protein levels according to protein expression cutoff (see Materials and Methods section). Lower panel: summary of the Notch3-EGFR and Notch1-EGFR protein expression levels correlation showing percentage of each category calculated on the precedent category of patients. **c** Pattern of immunostaining in two different cases of TNBC. In case 1 (upper panels), there is a strong and diffuse staining of neoplastic cells both for EGFR (A) and Notch3 (B), whereas Notch1 is completely negative (C). In case 2 (lower panels), the neoplastic cells are negative for both EGFR (D) and Notch3 (E), whereas Notch1 (F) shows a weak positivity in about 20% of the cells

role of primary option for systemic treatment. Although TNBC-bearing patients better respond to current chemotherapy than do non-TNBC ones, patients with TNBC experience a more rapid relapse evolving as metastatic disease. For this reason, this BC subtype suffers from the poorest prognosis[1]. Therefore, targeted therapeutic strategies for TNBC are urgently needed.

The overexpression of the tyrosine kinase receptor epidermal growth factor receptor (EGFR) is a hallmark of TNBC (45–70%) and exhaustive gene expression profiling has identified several EGFR-associated poor prognostic signatures[2]. Anti-EGFR therapies, including tyrosine kinase inhibitors (TKIs) and monoclonal antibodies, have been developed and are already available for treatment of different cancers such as non-small cell lung cancer (NSCLC) and colorectal cancer, making EGFR inhibitors an attractive option for TNBC therapy[3]. Unfortunately, no EGFR inhibitory therapies are currently approved for BC treatment, including TNBC, as results from clinical trials are disappointing[4]. This limited clinical activity is often due to the existence of compensatory pathways that confer resistance to EGFR inhibition, thus allowing continued cancer cell growth and survival[5–7].

Notch signaling dysregulation is often associated with tumor transformation[8], including the TNBC pathogenesis and progression[9–11]. In particular, TNBCs show Notch3 amplification and overexpression[12,13], and Notch3 knockdown has been shown to reduce the proliferation of ErbB2-negative breast tumor cells[9,14]. More recently, these data have been strongly supported by Choy et al.[15] who demonstrated that constitutive Notch3 signaling can drive an oncogenic program in a subset of TNBCs, thus suggesting that Notch3 activity (and not others Notch paralogues) may be clinically relevant in this BC subtype. There is a growing body of evidence that Notch hyperactivation or mutation results in several events that enable BC cells to become resistant to targeted treatments through different mechanisms[16,17], thus suggesting that the inactivation of Notch signaling could be a potential therapeutic approach for overcoming resistance to drugs[7]. Interestingly, more recently, it has been demonstrated that Notch3 pathway is strongly involved in the stroma-mediated expansion of therapy-resistant TNBC cells[18].

Notch-EGFR interplay occurs in different cellular contexts[19,20], including BC[16], raising the possibility that Notch signaling could be involved in the above mentioned resistance to EGFR inhibition. Arasada et al.[21] first reported that the EGFR inhibition by erlotinib treatment is able to activate Notch signaling in human lung cancer, resulting in an enriched stem cell-like populations in a Notch3, but not Notch1-dependent manner. In TNBC, it has been demonstrated that combined Notch-EGFR pathway inhibition is a rational treatment strategy for this type of tumors[22]. Pan-Notch inhibition using γ-secretase inhibitor (GSI) treatment supports this conclusion. Unfortunately, the use of GSIs fails to distinguish the particular Notch receptor driving growth, besides eliciting severe side effects.

Here we analyze the effects of a selective Notch3 inhibition in the response to gefitinib (GEF) treatment of resistant TNBC cells. We show that Notch3 (but not Notch1) depletion enhances the therapeutic target activity of the EGFR, by inducing its dephosphorylation via protein tyrosine phosphatase H1 (PTPH1), finally leading to an increased TNBC sensitivity to TKI-GEF.

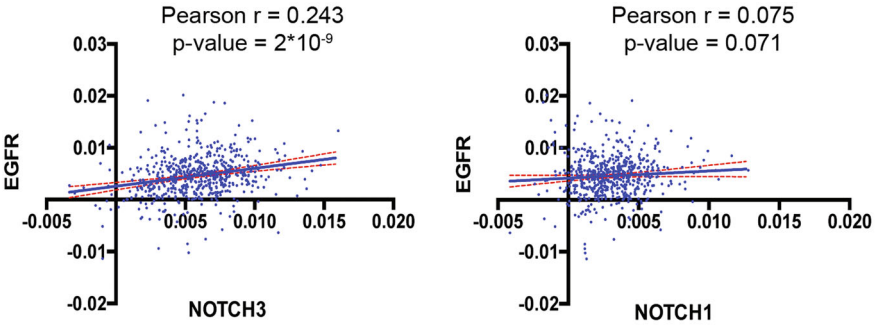
Results

Notch3-EGFR correlation in primary TNBC samples

To deepen the understanding of the possible Notch3-EGFR crosstalk in TNBC context, we first performed an in silico analysis of the *NOTCH3* and *EGFR* gene expression levels in two cohorts of TNBC patients, collectively consisting of 777 individuals[23–26] (Fig. 1a). The summary of the obtained results (Fig. 1a, upper panel) highlights a direct correlation between *EGFR* and *NOTCH3* gene expression levels in both datasets analyzed, while a weaker correlation between *EGFR* and *NOTCH1* is observed. This is also evident by the graphs included in the Fig. 1a (lower

a

Tumor Dataset	TNBC samples	Notch3-EGFR correlation	P-value	Notch1-EGFR correlation	P-value	Authors
GSE76124	198	21,1%	$2,9*10^{-3}$	12,7%	0,07 (ND)	Brown et al
GSE31519	579	24,3%	$2*10^{-9}$	7,5%	0,071 (ND)	Karn et al



b

TNBC patients

# sample	EGFR (IHC)	NOTCH3 (IHC)	NOTCH1 (IHC)
TNBC 17763/15	90%	12%	40%
TNBC 13709/14	15%	30%	
TNBC 22286/15	60%	15%	15%
TNBC 3500/15	50%	10%	
TNBC 7578/16	50%	8%	
TNBC 15481/14	40%	30%	15%
TNBC 2454/15	60%	2%	
TNBC 25353/15	90%	5%	5%
TNBC 9583/14			20%
TNBC 13502/14		15%	
TNBC 14884/14	70%	10%	50%
TNBC 1245/15	80%	5%	
TNBC 19168/15	90%	25%	10%
TNBC 18725/15	60%	15%	
TNBC 17493/14	90%		
TNBC 19910/15	70%	5%	20%
TNBC 27240/15	95%	10%	40%
TNBC 3528/16			1%

negative

positive

TNBC samples	EGFR⁺ samples	Notch3-EGFR⁺ samples	Notch1-EGFR⁺ samples
18	15	14 (93%)	8 (53%)

c

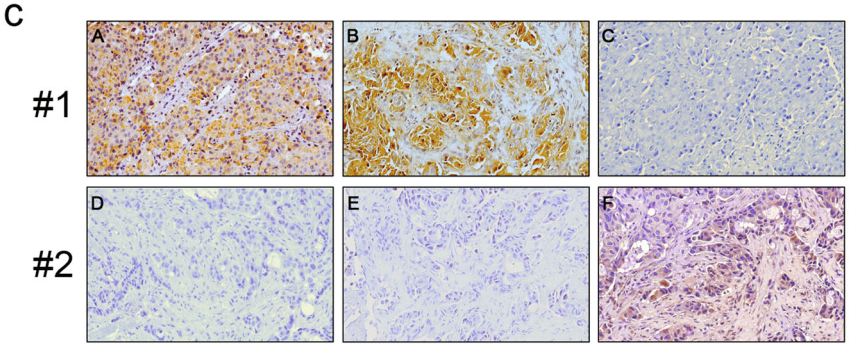


Fig. 1 (See legend on next page.)

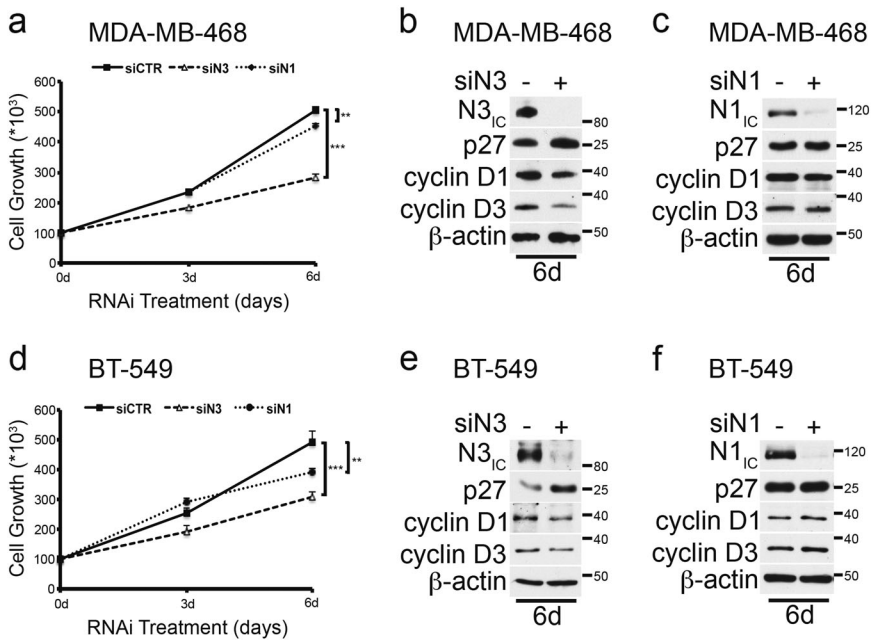


Fig. 2 Notch3 downregulation by siRNA affects TNBC cells survival. a, **d** Analysis of cell growth after 0–3–6 days of Notch3 and Notch1 silencing in **a** MDA-MB-468 and **d** BT-549 cells. **b**, **c** Whole cell extracts from **a** MDA-MB-468 or **d** BT-549 cells at 6 day of silencing were used for western blot against Notch3 ($N3_{IC}$) and Notch1 ($N1_{IC}$), to control the efficiency of the **b**, **e** Notch3 and **c**, **f** Notch1 silencing, respectively. Extracts were then immunoblotted with anti-p27, anti-cyclin D1, and anti-cyclin D3 antibodies. Anti-β-actin was used as a loading control. **b**, **c**, **e**, **f** are representative of three separate experiments. The statistical analysis associated is available in the Supplementary Figure S2

panels), representative of the larger dataset. These data indicate that in a consistent proportion of TNBC-bearing patients (about 23%) the presence of *EGFR* coexists with *NOTCH3* gene expression, allowing us to hypothesize a possible direct relationship between EGFR and Notch3 at the protein level in TNBC. To test this hyphotesis, we then analyzed the pattern of immunohistochemical expression of Notch3, Notch1, and EGFR in tissue samples of 18 human TNBCs. In the majority of cases (15/18), we found EGFR positivity in neoplastic cells. Notch3 is expressed in a higher percentage of EGFR-positive tumors as compared with Notch1 (93% vs. 53%) (Fig. 1b, lower panel). Figure 1c (case 1) shows an example of TNBC tumor expressing both EGFR (panel a) and Notch3 (panel b) but not Notch1 (panel c), representative of 6 out of 15 TNBC $EGFR^+$ tissue samples analyzed. Interestingly, two out of three TNBC samples not expressing EGFR are also Notch3 negative but express Notch1 (Fig. 1c, case 2), thus reinforcing the relevant Notch3-EGFR direct correlation in this cancer subtype.

Notch3 inhibition by siRNA sensitizes TNBC cells to EGFR-TKI-GEF treatment

To examine whether Notch3 could be involved in the mechanism of resistance to EGFR TKI, we first selected a group of TNBC cells expressing EGFR at various levels and known to be EGFR-TKI-resistant cells[27,28], and then we analyzed the expression of both Notch3 and Notch1 proteins (Supplementary Figure S1a). Almost all TNBC cells expressed activated Notch1 and/or Notch3 protein ($N3_{IC}$), thus confirming the hyperactivation of Notch signaling observed in this BC subtype[14], mainly involving the upregulation of $N3_{IC}$ expression, as it appears at undetectable levels in MCF10A, a normal immortalized mammary epithelial cell line. This also occurs in MDA-MB-453 cells, which express lower EGFR expression (data not shown) (Supplementary Figure S1a).

As drug resistance commonly involves several mechanisms that are often closely interconnected with their genetic profile, for our next analysis we chose the MDA-MB-468 and BT-549 cells, as they show a "similar" genetic background (i.e., phosphatase and tensin homolog (*PTEN*), *RB1*, and *P53* mutations)[29], which could help us to predict a "similar" sensitivity to TKIs[27]. We first evaluated whether the knockdown of Notch3 (siN3) or Notch1 (siN1) by small interfering RNA (siRNA) could affect cell growth or viability in such cells (Fig. 2a, d). Notably, both MDA-MB-468 (Fig. 2a) and BT-549 (Fig. 2d) cells display a more significant cell growth reduction after the selective depletion of Notch3 with respect to Notch1, measured by counting cell number until 6 days from the starting point, day 0 thus confirming previous data[14]. This effect could be due to the growth arrest of the cells, as the absence of Notch3 in both cell lines correlates

with a significant upregulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and downregulation of both the cyclins D1 and D3, known to be important protein regulators that exhibit dynamic changes during the cell cycle (Fig. 2b, e and Supplementary Figure S2a and c). Notably, the Notch1 silencing does not correlate with any significant changes of the same cell cycle regulators analyzed (Fig. 2c, f and Supplementary Figure S2b and d). These results demonstrate a specific role of Notch3 in the regulation of TNBC cell growth, as confirmed by the absence of viability of MDA-MB-468 clones stably deleted for *NOTCH3* (but not for *NOTCH1*), generated by using genome-editing CRISPR/Cas9 technique (data not shown).

Previous studies suggested that selective Notch3 inhibition (rather than pan-Notch inhibition) combined with EGFR TKI therapy should be explored as a novel strategy in the treatment of lung cancer patients[21]. In keeping with these data, we observed that Notch3 silencing significantly enhances the gefinitib (GEF)-induced growth inhibition in both MDA-MB-468 and BT-549 cells (Fig. 3a, c, left panels: compare siCTR + GEF vs. siN3 + GEF), in a similar or even more extensive way observed after combined treatment with GSI plus TKI-GEF (Supplementary Figure S3a and b, left panels: compare siCTR + GEF vs. GSI + GEF). These data thus strongly suggests that Notch3 depletion rather than pan-Notch inhibitor is sufficient to sensitize TNBC to TKI-GEF (Fig. 3a vs. Supplementary Figure S3a; Fig. 3c vs. Supplementary Figure S3b, left panels: compare siN3 + GEF vs. GSI + GEF). The quality of Notch(s) silencing were monitored until 6 days by evaluating the expression of both Notch3 and Notch1 proteins (Fig. 3 and Supplementary Figure S3, all the right panels).

In addition, although Notch1 silencing does not induce any significant changes in BT-549 GEF-treated cells with respect to control cells (Fig. 3d, compare siCTR + GEF vs. siN1 + GEF), it seems to paradoxically increase the MDA-MB-468 cell growth in response to GEF (Fig. 3b, left panel: compare siCTR + GEF vs. siN1 + GEF). These data suggest a potential different role of the different Notch receptors expressed in the same TNBC context relative to the TKI-response, which remains to be fully elucidated.

Dual targeting of EGFR and Notch3 increases both EGFR internalization and dephosphorylation, and decreases the EGFR nuclear localization

To understand how the Notch3-dependent TKI resensitization observed above could occur in TNBC cells, we initially examined whether EGFR turnover could be influenced by the absence of Notch3 rather than Notch1. To this purpose we focused our next studies on MDA-MB-468 cells, by evaluating both the EGFR subcellular localization and its tyrosine phosphorylation status, which is essential for EGFR to activate downstream mitogenic pathways and represents the basis for targeted therapy with TKIs[30].

The MDA-MB-468 cells were treated with GEF, alone or in combination with Notch3 or Notch1 silencing (siN3 + GEF or siN1 + GEF, respectively) for 6 days, followed by the analysis of the following: (1) the EGFR surface expression ($EGFR_{EC}$) by fluorescence-activated cell sorting (FACS) analysis (Figs. 4a) and (2) the tyrosine-phosphorylated EGFR expression at 1173 residue ($pEGFR_{Y1173}$) in both whole cell (Fig. 4b) and nuclear extracts (Fig. 4c). Notably, the absence of Notch3 amplifyies the GEF-dependent decrease of $EGFR_{EC}$ surface-expressing cells (siN3 + GEF: 54,4% vs. GEF: 66,9%) (Fig. 4a, left panels) whereas Notch1 silencing does not (siN1 + GEF: 69% vs. GEF: 70%) (Fig. 4a, right panels). Similarly, Notch3 depletion leds to a significant decrease of $pEGFR_{Y1173}$ expression, which appears rarely detectable in both total and nuclear extracts of GEF-treated Notch3-silenced cells (Fig. 4b, c, left panels, respectively). On the contrary, Notch1 silencing does not induce important alterations of $pEGFR_{Y1173}$ expression neither in whole cell nor in nuclear extracts from GEF-treated cells (Figs. 4b, c, right panels, respectively). It has been reported that the full-length form of nuclear EGFR is involved in several mechanisms including cell proliferation[31]. Consistent with this, by measuring bromodeoxyuridine (BrdU)-positive cells during the combined experiments (after 4 days), we observed a significant decrease in the percentage of cells entering the S phase in GEF-treated Notch3-silenced cells with respect to GEF-treated cells (Fig. 4d, compare siCTR + GEF vs. siN3 + GEF). Notably, in keeping to what is shown above (Fig. 3b), we observed an increase in the percentage of proliferative GEF-treated Notch1-silenced cells with respect to their counterpart treated with GEF alone (Fig. 4e, compare siCTR + GEF vs. siN1 + GEF).

All these data suggest an important correlation between EGFR behavior and Notch3 receptor in TKI-response of TNBC cells, thus providing a rationale for a combined therapy approach with TKI-GEF and Notch3 inhibition.

Rafts depletion correlates with EGFR dephosphorylation by PTPH1 phosphatase in TKI-resistant TNBC cells

In order to further understand the molecular mechanism underlying the EGFR-Notch3 crosstalk in TNBC, we investigated in more detail whether and how Notch3 may be involved in the regulation of the above described processes of EGFR subcellular localization and its phosphorylation/activation status.

It has been shown that EGFR localizes within lipid rafts in different cell lines[32] and this specific localization could induce different functional effects[33,34]. More recently, Irwin et al.[35] have shown that EGFR localization to lipid

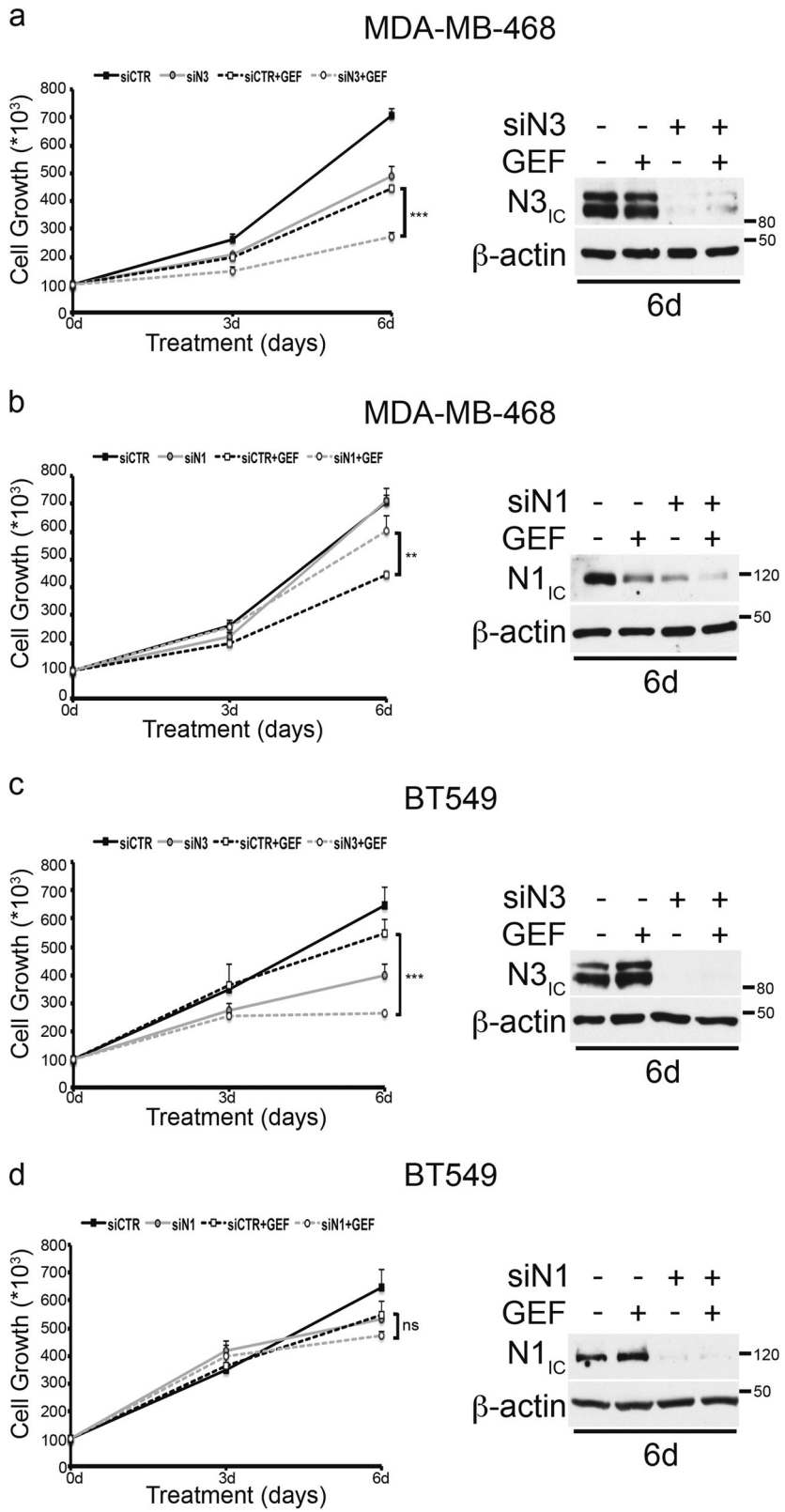


Fig. 3 (See legend on next page.)

Fig. 3 Notch3 downregulation (but not Notch1) sensitizes TNBC cells to TKI-gefitinib. a–**d** Left panels: inhibition of **a**, **b** MDA-MB-468 and **c**, **d** BT-549 cell growth was observed after gefitinib (GEF) treatment combined with Notch3 silencing in **a**, **c** but not with **b**, **d** Notch1 silencing. All the right panels showed in the figure represent western blotting of total extracts from cells described above against Notch3 ($N3_{IC}$) and Notch1 ($N1_{IC}$), to control the efficiency of the **a**, **c** Notch3 and **b**, **d** Notch1 silencing, respectively. Anti-β-actin was used as a loading control. All data are representative of at least three independent experiments, each in triplicate. Results shown in **a**, **b**, **c**, and **d** are expressed as the means average deviations and *P*-values were calculated using Student's *T*-test (i.e., ns not significant; $P > 0.05$, $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$)

rafts of TNBC cells may correlate with their resistance to EGFR TKI-induced growth inhibition. First, we confirmed the presence of EGFR within lipid rafts by using biochemical and confocal microscopy analyses: Fig. 5a shows that EGFR (green) strongly colocalizes with GM1 (red), a lipid raft glycosphingolipid specifically recognized by the Cholera toxin subunit B. Biochemical rafts isolation shown in the Fig. 5b confirms these data. Notably, the tyrosine-pEGFR expression, essential for its functional activity[36] and predictive for target therapy efficiency with TKIs[30] appears to be exclusive of raft compartment, as it moved to the non-rafts fractions in the presence of Methyl-β-cyclodextrin (MβCD), a drug which removes cholesterol from the plasma membrane, thus disrupting the integrity of membrane rafts microdomains (Fig. 5b). Interestingly, after MβCD treatment, we observe a clear defect in the increase of EGF-induced tyrosine phosphorylation of EGFR at 1173 residue ($pEGFR_{Y1173}$) but not at 1068 residue ($pEGFR_{Y1068}$) (Fig. 5c), thus suggesting the presence of potential different roles between EGFR phosphorylation pattern and function of different tyrosine phosphorylation sites[30]. These data indicate a

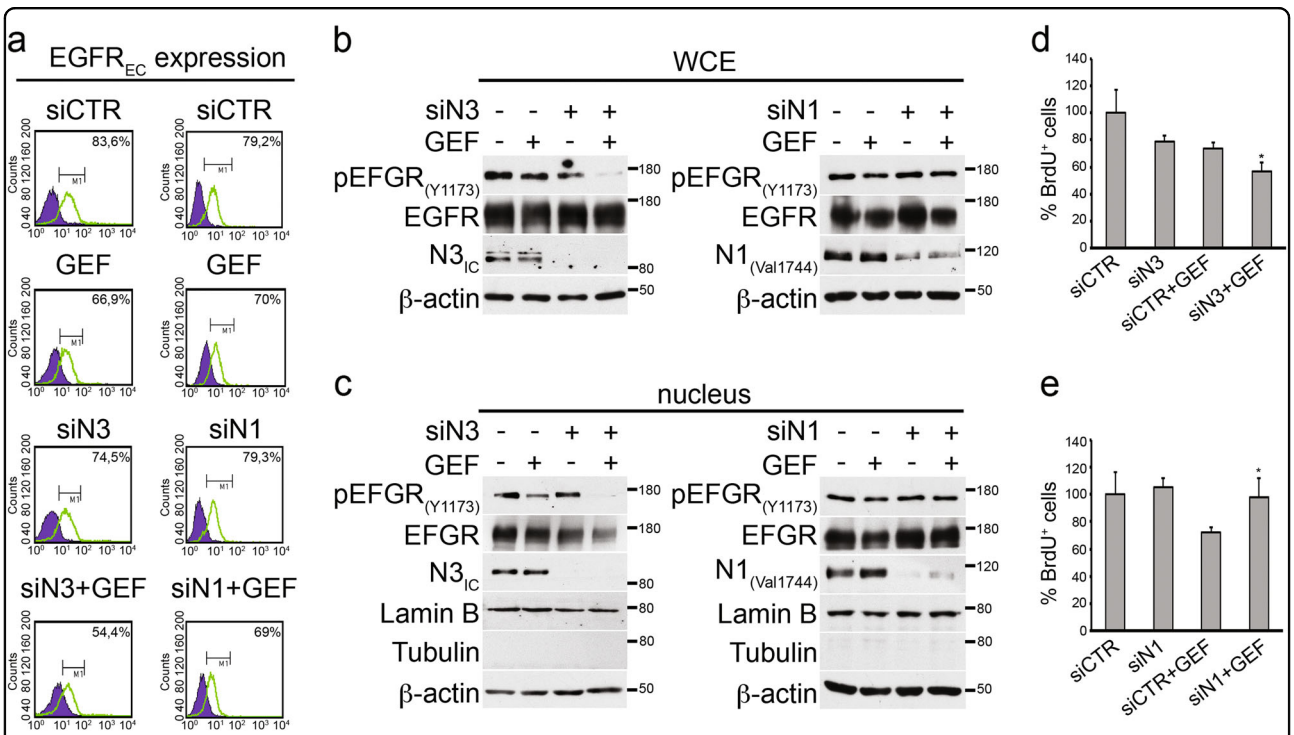


Fig. 4 Combined Notch3 and EGFR targeting induces EGFR internalization but defect the nuclear-activated EGFR localization. a FACS analysis of the EGFR surface expression ($EGFR_{EC}$) in MDA-MB-468 cells treated with gefitinib (GEF) alone or in combination with Notch3-silencing (siN3 + GEF) or Notch1 silencing (siN1 + GEF) for 6 days. **b** Whole cell extracts (WCE) and **c** nuclear extracts from the same cells used in **a** were immunoblotted with anti-EGFR and anti-$pEGFR_{(Y1173)}$ antibodies, to evaluate the EGFR expression and phosphorylation, and with anti-$N3_{IC}$ or anti-$N1_{Val1744}$ antibody to control the efficiency of Notch3 (left panels) or Notch1 (right panels) silencing, respectively. Anti-lamin B and anti-tubulin were used as fraction markers; anti-β-actin was used as a loading control. **d**, **e** Proliferation analysis by BrdU assay (see Matherials and Methods section): compared with control cells (siCTR + GEF), the percentage of $BrdU^{+}$ cells is lower after Notch3 silencing plus **d** GEF (siN3 + GEF) and not after Notch1 silencing plus **e** GEF (siN1 + GEF). All data are representative of at least three independent experiments, each in triplicate. Results are expressed as the means average deviations and *P*-values were calculated using Student's *T*-test (i.e., $^{*}P \le 0.05$)

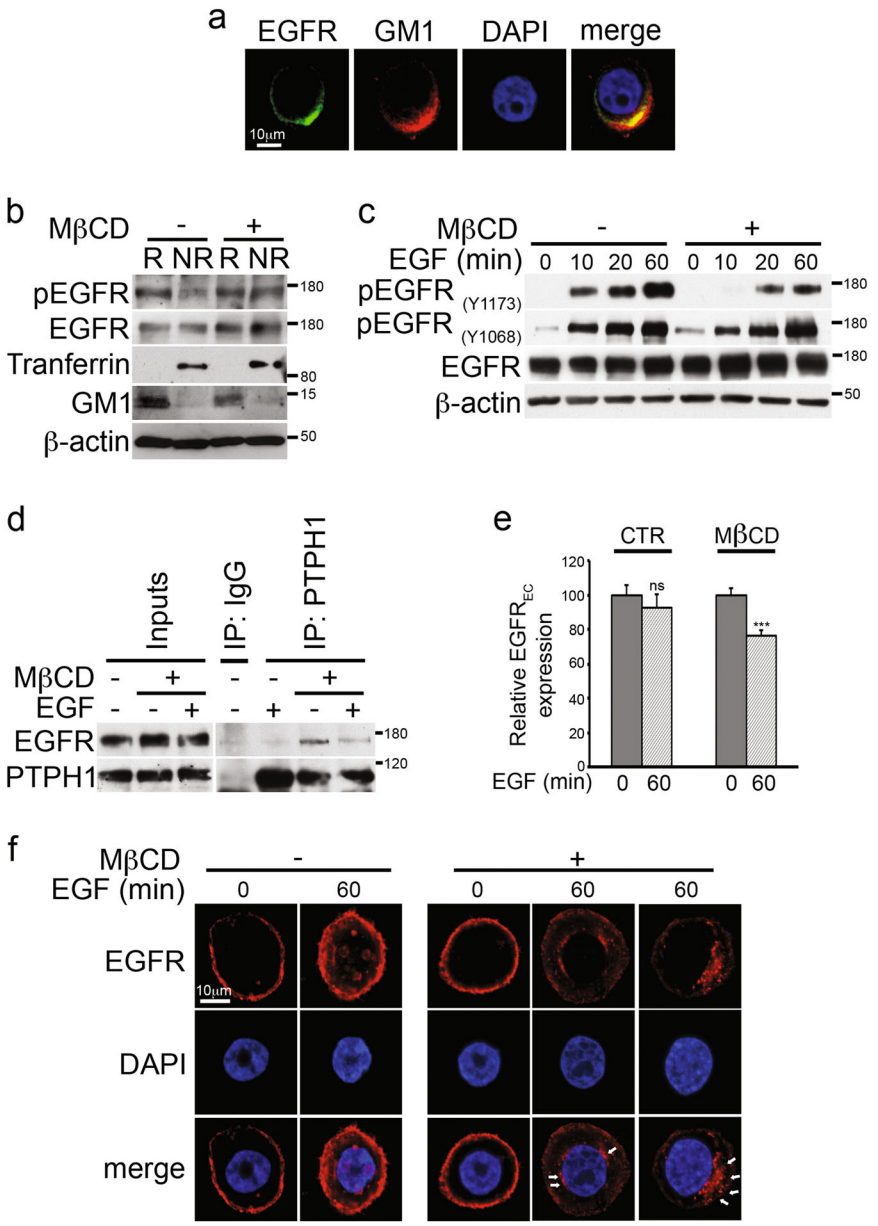


Fig. 5 Rafts depletion induces endogenous EGFR-PTPH1 interaction, EGFR dephopshorylation, and its intracellular arrest in MDA-MB-468 TNBC cells. a Immunofluorescence assay (IF) was performed by using anti-EGFR (green) and anti-GM1 (red) antibodies to reveal the endogenous EGFR-rafts colocalization, shown in yellow (merge). Nuclei were DAPI labeled (blue). **b** Raft (R) and non-raft (NR) fractions derived from Methyl-β-cyclodextrin (MβCD)-treated and untreated cells were used for immunoblot assay with anti-pEGFR$_{(Y1173)}$ (indicated as pEGFR) and anti-EGFR antibodies, to test activated and total EGFR expression in rafts compartment, respectively. Anti-transferrin and anti-GM1 antibodies were used as a fraction markers. **c** Cells have been activated with EGF ligand for the times indicated, in the presence or absence of MβCD: the expression of phospho-EGFR at tyrosine 1173 and 1068 residues and total EGFR was determined in whole cell extracts by immunoblot analysis using the specific indicated antibodies. **d**–**f** MDA-MB-468 cells were treated with MβCD and stimulated with EGF for 60 min: control or anti-PTPH1 antibody immunoprecipitates were probed with anti-EGFR, to detect the EGFR-PTPH1 binding, and with the anti-PTPH1 antibody, to show PTPH1 immunoprecipitated protein levels. The inputs indicated in the panel shows 5% of each total lysate **d**. Relative EGFR extracellular expression (EGFR$_{EC}$) was evaluated by FACS **e**. IF assay was performed by using anti-EGFR (red) antibody to reveal the endogenous EGFR intracellular localization. Nuclei were DAPI labeled (blue). White arrows indicated peri-nuclear EGFR localization in EGF stimulated MβCD-treated cells (**f**). **a**, **f** Representative single plane confocal IF images captured using a × 60 oil objective. Scale bar: 10 μm. In both **b** and **c**, western blotting against the anti-β-actin was used as a loading control. All data are representative of at least three independent experiments, each in triplicate. Results shown in **e** are expressed as the means average deviations and *P*-values were calculated using Student's *T*-test (i.e., ns, not significant $P > 0.05$, $^{**}P \leq 0.01$)

possible relationship between rafts compartment integrity and EGFR/Y1173 dephosphorylation, which is known to have an important role in the therapeutic activity of EGFR TKI inhibition through the involvement of the tyrosine phosphatase H1 (PTPH1)[37].

Several protein tyrosine phosphatases (PTPs) dephosphorylate EGFR at Y1173 (alone or together with other residues)[38,39]. Among them, the PTPH1 specifically catalyzes EGFR/Y1173 dephosphorylation (and not EGFR/Y1068 dephosphorylation), thus finally increasing non-TNBC BC sensitivity to TKIs, including GEF[37]. The EGFR/PTPH1 direct interaction is closely required to favor the therapeutic targeting of EGFR itself[37]. In agreement with this, we observed that MβCD-treated TNBC cells showed high levels of endogenous EGFR-PTPH1 interaction (Fig. 5d), thus suggesting the possible PTPH1 involvement in the observed decreased levels of EGFR/Y1173 phosphorylation after rafts depletion (Fig. 5c). Interestingly, the EGFR-PTPH1 interaction disappears after MβCD plus EGF ligand (Fig. 5d), probably due to the EGF-dependent endocytic events of ligand-activated EGFRs which may influence the kinetics of EGFR availability to PTPs-mediated dephosphorylation[40]. In keeping with this, we observed a decreased extracellular EGFR expression ($EGFR_{EC}$) in MβCD-treated cells with respect to untreated cells, upon stimulation with EGF (Fig. 5e), despite the natural slowdown of EGFR endocytic trafficking in MDA-MB-468 cells due to their known saturated endocytic machinery[41]. In agreement with previous data[42], our results suggest that rafts depletion may allow the internalization of ligand-occupied EGFR. Following ligand binding and receptor phosphorylation/activation, pEGFR is endocytosed and commonly transported to lysosome where it is degraded[43]. In our experiments, we do not observe decreased levels of total EGFR expression after rafts depletion (Fig. 5c, d), thus suggesting that removal of EGFR from the cell surface observed in MβCD-treated cells may be correlated to a different mechanism of EGFR downregulation, not involving receptor degradation. Since it has been demonstrated that many tumor cells which overexpress EGFR, including the MDA-MB-468 cells, have limited ligand-stimulated EGFR degradation[44] and that tyrosine dephosphorylation of EGFR is correlated with an increased EGFR stability[37], we wanted to know where the EGFR accumulated after MβCD treatment, in order to completely understand how signaling by the EGFR is terminated. To this purpose, cells were treated with or without MβCD and stimulated with EGF ligand for 60 min, followed by the immunostaining with anti-EGFR antibody (Fig. 5f): confocal analysis shows that rafts depletion correlates with the accumulation of EGFR at a peri-nuclear level (white arrows) whereas the majority of MβCD-untreated cells (EGF stimulated) show spots of nuclear EGFR, which represents a specific localization known to be associated with resistance to EGFR-targeted therapies[31]. In addition, the same control cells stimulated with EGF ligand show persistent high levels of EGFR cell surface expression (Fig. 5f), thus confirming the saturation of the endocytic machinery previously mentioned[41].

Together, these data indicate that EGFR trafficking is retained outside the nucleus in MDA-MB-468 TNBC cells in response to the rafts-disrupting agent, MβCD.

Notch3 inhibition by siRNA mimics rafts depletion effects on EGFR in TKI-resistant TNBC cells

We have previously shown that Notch3 receptor constitutively localizes to lipid rafts of Notch3 overexpressing lymphocytes, thus contributing to sustain the signaling pathways responsible of the T-cell leukemia development[45]. Here we first hypothesized that both Notch3 and EGFR receptors could share the same localization to directly interact, leading to the observed EGFR-TKI resistance process in TNBC cells. Surprisingly, both confocal analysis (Supplementary Figure S4a, upper panels) and biochemical rafts isolation with or without MβCD treatment (Supplementary Figure S4b) show that in MDA-MB-468 cells Notch3 receptor ($N3_{EC}$) is widely expressed in all the cell surface, whereas Notch1 receptor appears to be restricted to lipid rafts microdomains (Supplementary Figure S4a, lower panels, and S4b). Thus, Notch3 and EGFR do not completely colocalize (Supplementary Figure S4c, upper panels), whereas Notch1 shows a strong rafts colocalization with EGFR (Supplementary Figure S4c, lower panels). Notably, by in situ proximity ligation assay (PLA), we still observed the endogenous Notch3/EGFR complex all around the cell membrane (Supplementary Figure S4d) while Notch1/EGFR complex seems to be mainly restricted to a limited portion of the membrane (Supplementary Figure S4e), reflecting their strictly shared localization (Supplementary Figure S4c, lower panels).

These results suggest the existence of a different relationship between Notch3 or Notch1 and EGFR in TNBC. However, in order to deepen inside the molecular mechanism related to the TKI-GEF resensitization of TNBC cells observed only when Notch3 (and not Notch1) is depleted (Fig. 3), here we further investigated how the EGFR-rafts localization could be influenced when Notch3 is depleted. Similar to what happens in MβCD-treated cells (Fig. 5b-d), in the absence of Notch3 the tyrosine phosphorylation of EGFR at 1173 residue ($pEGFR_{Y1173}$) disappears and this event does not involve receptor degradation, as EGFR total levels remain unchanged (Fig. 6a). Notably, after Notch3 depletion, we observed a clear defect in the increasing levels of EGF-induced tyrosine phosphorylation of EGFR at 1173 residue ($pEGFR_{Y1173}$)

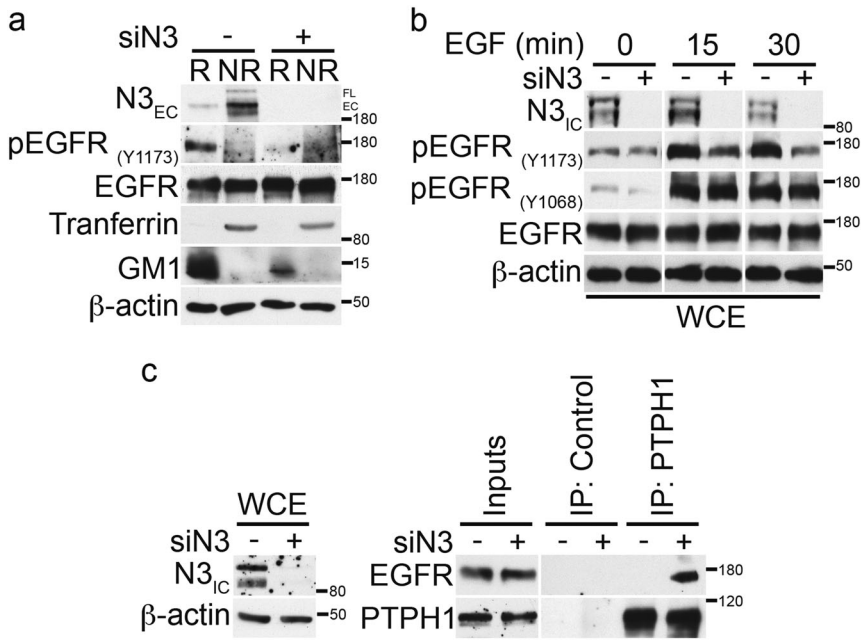


Fig. 6 Notch3 downregulation induces EGFR dephosphorylation by promoting the endogenous EGFR/PTPH1 interaction. a Raft (R) and non-raft (NR) fractions derived from 6 days of Notch3-silenced cells were used for immunoblot assay with anti-$N3_{EC}$, anti-$pEGFR_{(Y1173)}$, and anti-EGFR antibodies, to test the effect of Notch3 downmodulation on EGFR-rafts localization. Anti-transferrin and anti-GM1 were used as a fraction markers. **b** Cells have been activated with EGF ligand for the times indicated, combined or not with Notch3 silencing for 3 days: the expression of phospho-EGFR at tyrosine 1173 and 1068 residues and total EGFR was determined by immunoblot analysis using the specific indicated antibodies. **c** Control or anti-PTPH1 antibody immunoprecipitates from control and Notch3-silenced cells were probes with anti-EGFR, to detect the EGFR-PTPH1 binding, and with the anti-PTPH1 antibody, to show PTPH1 immunoprecipitated protein levels. The inputs indicated in the panel shows 5% of each total lysate (right panels). Whole cell extracts (WCE) were incubated with anti-$N3_{IC}$ antibody to control the efficiency of Notch3 silencing (left panels). In all panels **a**, **b** and **c**, western blotting against the anti-β-actin was used as a loading control. The results are representative of three independent experiments

but not at 1068 residue ($pEGFR_{Y1068}$) (Fig. 6b), as already shown after rafts depletion (Fig. 5c). For this reason, we further investigated whether Notch3 could influence the EGFR/Y1173 dephosphorylation by the phosphatase PTPH1, by using co-immunoprecipitation assay. In agreement with the above results (Fig. 5d), we observed that the absence of Notch3 is able to induce the endogenous EGFR/PTPH1 interaction (Fig. 6c), thus suggesting a possible link between Notch3, EGFR-rafts localization and EGFR dephosphorylation event by PTPH1.

In addition, we also observed that the absence of Notch3 correlates with a rapid and persistent EGFR downregulation from the cell surface, as revealed by the decrease of $EGFR_{EC}$ mean fluorescence intensity (MFI) in Notch3-depleted cells (siN3) with respect to control cells (siCTR) after EGF stimulation (Fig. 7a, upper panel). As expected, treatment of MDA-MB-468 cells with EGF until 270 min results in an increased EGFR surface expression (Fig. 7a, upper panel), also supported by the unchanged levels of total EGFR protein (Fig. 7a, lower panels), as previously reported (Fig. 5f and[41]). Interestingly, despite the increased EGFR internalization observed in the absence of Notch3, although the $pEGFR_{Y1173}$ expression decreases, the EGFR total levels does not change, thus suggesting that Notch3 depletion (such as rafts depletion) could correlate with an increased dephopshorylated EGFR endocytosis followed by its intracellular shuttling blockade rather than sorting for intracellular degradation. Using immunofluorescence staining, we obtained additional evidence in support of the Notch3-depletion dependence of EGFR intracellular fate. As shown in the Fig. 7b, after 2 h of EGF stimulation combined with Notch3 silencing, we observed that EGFR localizes preferentially at a peri-nuclear level, similarly to what observed after MβCD treatment (Fig. 5f). Interestingly, a few cells show a similar EGFR staining also without EGF stimulation (Fig. 7b, see white arrows), thus suggesting that Notch3, alone, may influence the EGFR internalization also through ligand-independent mechanisms (data not shown).

Together these results demonstrate that Notch3 depletion mimics the effects of rafts depletion on EGFR, as the Notch3 silencing correlates with EGFR dephosphorylation (by PTPH1) and its persistent internalization, followed by intracellular arrest.

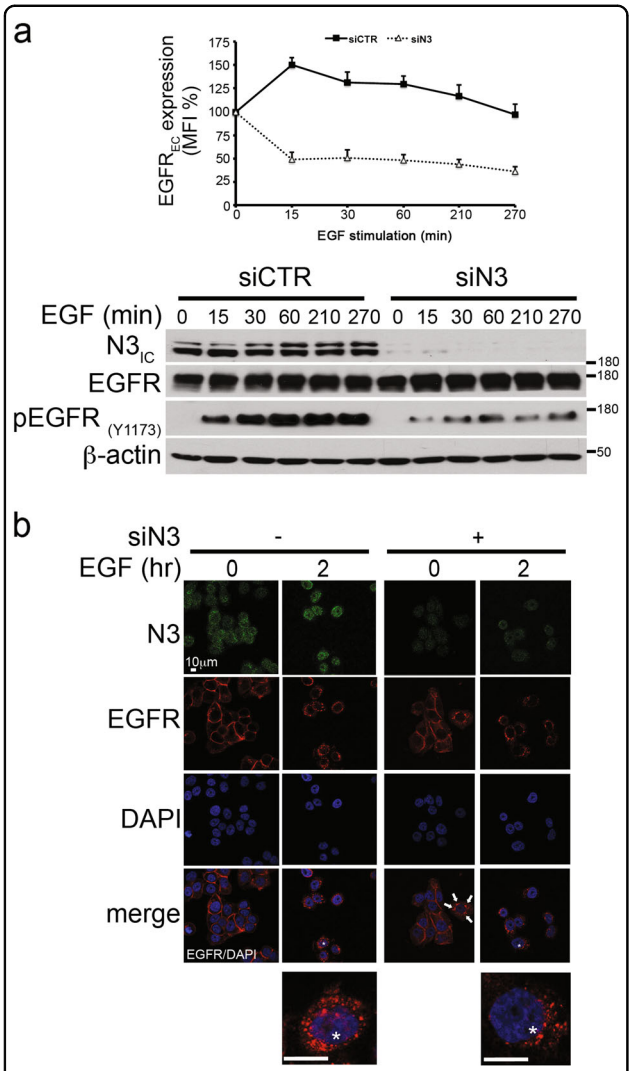


Fig. 7 Notch3 downregulation induces EGFR internalization and intracellular arrest. a Upper panel: FACS analysis of the EGFR surface expression ($EGFR_{EC}$) in control (siCTR) and Notch3-silenced (siN3) cells after EGF stimulation for the time indicated, shown as percentage of the mean fluorescence intensity (MFI) respect to the EGF-untreated cells ($t = 0$). lower panel: Western blot analysis of the total extracts from the same cells probed with anti-Notch3 ($N3_{IC}$) antibody, to test the efficiency of Notch3 silencing, and with anti-EGFR and anti-$pEGFR_{(Y1173)}$ antibodies, to evaluate the EGFR expression. The β-actin expression was used as loading control. **b** MDA-MB-468 cells were Notch3-silenced for 48 h and EGF-treated for 2 h: Immunofluorescence assay (IF) was performed by using anti-Notch3 green) or anti-EGFR (red) antibodies to test the efficacy of Notch3 silencing and to reveal the endogenous EGFR intracellular localization, respectively. Nuclei were DAPI labeled (blue). EGFR/DAPI merge is shown. White arrows indicate peri-nuclear EGFR localization in Notch3-silenced cells. The * indicate the higher magnification of a single EGF-stimulated control cell (left) and Notch3-silenced (right) cell. All the panels are representative single plane confocal IF images captured using a ×60 oil objective. Scale bar: 10 µm. The results are representative of three independent experiments

Discussion

Among the EGFR TKIs, GEF and erlotinib were the first to be approved by Food and Drug Administration for treatment of NSCLC[46]. These drugs inhibit the EGFR kinase activity, finally resulting in proliferation inhibition, cell cycle progression delay, and apoptosis[47]. Although EGFR TKIs show good response rates and progression free survival in NSCLC patients with *EGFR* gene mutations, acquired resistance to TKIs therapy is commonly reported, often due to multiple mechanisms including *EGFR* additional mutations[48,49], activation of redundant kinase signaling pathway, or EGFR downstream molecules[2]. As activating mutations of *EGFR* in BC are rare, it is uncertain whether some of the above mentioned mechanisms observed in NSCLC are involved in the failure of clinical trials with TKIs in TNBC. One possible explanation for the lack of response to current targeted therapies is that most TNBCs are not exclusively dependent on EGFR signaling for their survival but involve the activation of alternative receptors and pathways. As some NSCLC patients with wild-type *EGFR* gene amplification and wild-type *KRAS* also respond to EGFR TKIs[50], it may be that these alternative resistant pathways need to be blocked in wild-type EGFR overexpressing TNBC patients to increase TKIs therapy efficacy.

Here we demonstrate that Notch3 (but not Notch1) is strongly involved in the TNBC resistance to TKIs, as Notch3 depletion induces the resensitization of TNBC cells to GEF treatment. These results indicate that Notch3 specifically functions in these cells without invoking contributions from other Notch receptors, thus supporting the importance of a selective Notch3 therapeutic targeting in order to avoid the known toxicity associated with pan-Notch inhibition[51]. The significant correlation observed between Notch3 and EGFR in a large group of human TNBC patients supports these data.

Mechanistically, we show that Notch3 depletion induces the downregulation of EGFR cell surface expresssion and function by promoting its dephosphorylation via PTPH1 and its intracellular arrest, similar to what observed after rafts-disrupting treatments. Interestingly, we have shown that MDA-MB-468 TNBC-resistant cells show a strong lipid rafts localization of the activated EGFR. In addition, it has been demonstrated that EGFR overexpression correlates with the natural saturation of its endocytic trafficking[41]. Consequently, in these cells EGFR seems not to be available to be downregulated, thus finally retaining a constitutive higher surface expression, known to be associated with cell growth[44]. In this scenario, the Notch3 silencing is able to favor a hypothetical shift of the EGFR from rafts to non-rafts compartment, thus moving it to a membrane localization, which may be available to subsequent downregulating events. Possible

mechanisms able to attenuate EGFR signaling include dephosphorylation of the EGFR, removing it from the cell surface and allowing degradation of the receptor or sequestration into intraluminal vesicles[52]. PTPH1 is a phosphatase able to specifically dephosphorylate EGFR at tyrosine Y1173 residue[53], thereby regulating EGFR interaction with ER and the subsequent ER^+ BC sensitivity to TKIs treatment[37]. In both Notch3-silenced and MβCD-treated cells, we observed a strong EGFR-PTPH1 endogenous interaction which is correlated with a significant decrease of $pEGFR_{Y1173}$ expression, thus suggesting that EGFR dephosphorylation by PTPH1 may represent an important event of the observed Notch3-dependent increased response to TKI-GEF. Further studies are required to understand if PTPH1 could be recruited into lipid rafts before EGFR moving, as PTPH1 is involved in the non-clathrin endocytosis of EGFR in lung cancer[54] and/or if the EGFR internalization is required for the PTPH1-EGFR interaction, as occurs for other tyrosine phosphatases function on EGFR itself[55].

EGFR dowregulation commonly involves a clathrin-mediated endocytosis event dependent on physiological concentrations of growth factors[42]. Moreover, it has been demonstrated that EGFR internalization occurs also under various stress conditions (such as treatment with drugs) through the involvement of p38 mitogen-activated protein kinase (MAPK), finally leading to its arrest in endosomes, without recycling[56]. Here we demonstrate that Notch3 depletion induces an increased EGFR internalization, more evident in combination with GEF, thus leading to the EGFR intracellular accumulation and not degradation. Interestingly, Notch3 is able to positively control the levels of MAPK phosphatase 1 (MKP-1), thus decreasing the levels of phosphorylated p38, a canonical MKP-1 target[57]. These data suggest that the absence of Notch3 may mimic a stress condition able to activate p38 and favor the EGFR non-canonical internalization. Moreover, it has been also shown a direct crosstalk between PTPH1 and p38 MAPK in promoting Ras oncogenesis and regulating stress response: in particular, PTPH1 represents a p38γ-specific phosphatase[58] and PTPH1 phosphorylation by p38 is required to favor the PTPH1 dephosphorylation activity on its targets, such as EGFR/Y1173[53]. These observations further support our hypothesis of an important involvement of Notch3-PTPH1 axis in the regulation of EGFR internalization machinery in TNBC. Moreover, the EGFR intracellular accumulation observed in Notch3-depleted cells correlated with a defect in its nuclear localization, further suggesting that removal of EGFR from the cell surface may help to evade survival signaling and enhances drug-induced cell death, in accordance with a previous report[42].

Collectively, our data suggest an important role of Notch3 in regulating the EGFR subcellular localization and function in TNBC cells, thus contributing as an intrinsic resistant factor to anti-EGFR therapies, whose failure is often dependent on different EGFR subcellular localization that elicit distinctly different and also overlapping signals[35,59] and can make the receptor unavailable to be targeted.

Due to the heterogeneity of the TNBC and its poor outcome, subtyping through robust predictive and prognostic biomarkers that may contribute to therapy resistance is crucial for understanding the molecular mechanism underlying EGFR inhibitors sensitivity and further discuss the possible perspective on anti-EGFR therapies in TNBCs. In this view, here we observed an overlapped TKI-response in MDA-MB-468 and BT-549 cell lines, which both express a constitutive EGFR activation in a *PTEN*-null background, that is a known common combination of aggressive and drug-resistant subset of TNBC[60]. Based on our results, we can suggest Notch3 as one driver of an oncogenic signaling network, which may influence this intrinsic EGFR-TKI drug resistance in TNBC cells with such a similar molecular signature, finally allowing the design of specific target therapy protocols, which may include Notch3 inhibition as a potential approach for overcoming TKIs resistance.

Materials and methods

Cell culture and treatments

Human BC MDA-MB-468, MDA-MB-231, HCC1143, and BT-549 TNBC cell lines were obtained from ATCC; HCC38, BT-20, HS578T, and MDA-MB-453 TNBC cell lines were kindly provided by Professor JV Olsen (Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Denmark). All TNBC cells were maintained in accordance with the ATCC's instructions and all are mycoplasma free.

Cell viability was measured by the Trypan blue dye exclusion assay (Sigma-Aldrich, St Louis, MO, USA, Catalog number T8154). Cells were treated with the following compounds: 5 mM MβCD (Sigma-Aldrich, Catalog number C4555); 3 μM GEF (Iressa, Selleckem, Houston, TX, USA; Catalog number ZD1839), 100 nM EGF Ligand (EGF; Gibco, Life Technologies, Carlsbad, CA, USA; Catalog number PHG0315); 10 μM of GSI IX (DAPT) (Calbiochem, Darmstadt, Germany; Catalog number 565770).

siRNA silencing

Cell were transfected as previously described[61] with siRNAs anti-Notch3 (Catalog number sc-37135), Notch1 (Catalog number sc-36095), and corresponding control scrambled siRNAs (Catalog number sc-37007), all from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA).

Protein extract preparation, immunoprecipitation, and immunoblot analysis

Protein extract preparation[62], immunoprecipitation assay[63], immunoblotting assays[64], and sucrose gradient for rafts isolation[45] were performed as described elsewhere. Primary antibodies were as follows: anti-Notch3 (Catalog number 2889), anti-Notch1 (Catalog number 2421), anti-activated Notch1 ($N1_{Val1744}$), anti-EGFR D38B1 (Catalog number 4267S), anti-phospho-EGFR Y1173 (53A5-Catalog number 4407S), and anti-phospho-EGFR Y1068 (Catalog number 2234S), all from Cell Signaling (Danvers, MA, USA); anti-α-tubulin (Catalog number sc-8035), anti-Lamin B M20 (Catalog number sc-6217), anti-p27 C19 (Catalog number sc-528), anti-cyclin D1 M20 (Catalog number sc-718), anti-cyclin D3 C16 (Catalog number sc-182), anti-Notch1 L18 ($N1_{EC}$) (Catalog number sc-23299), anti-transferrin H65 (Catalog number sc-21011), and anti-PTPH1 (Catalog number sc-515181), all from Santa Cruz Biotechnology; anti-β-actin (Catalog number A5441) and anti-cholera toxin B subunit peroxidase conjugate (GM1, Catalog number C3741) from Sigma-Aldrich; and anti-Notch3 5E1 ($N3_{EC}$) antibody was kindly provided by Professor A Joutel[65].

Immunofluoresce assay and confocal imaging

Immunofluorescence staining and in situ PLA were performed as described elsewhere[66]. Primary antibodies were as follows: rabbit anti-Notch3 M-134 (Catalog number sc-5593), mouse anti-EGFR 528 (Catalog number sc-120), and rabbit anti-Notch1 L18 (Catalog number sc-23299) from Santa Cruz Biotechnology; anti-GM1 (Life Technologies; 595-Cy3 conjugated, Catalog number C34777); mouse anti-Notch3EC, Clone 1E4 (Millipore, Billerica, MA, USA; Catalog number MABC594); and rabbit anti-EGFR (Proteintech, Rosemont, IL, USA; N-Terminal, Catalog number 22542–1-AP). Secondary antibodies were as follows: Alexa Fluor 594- and 488-conjugated, respectively, both anti-mouse and anti-rabbit (Molecular Probes, Life Technologies). Nuclei were counterstained with Hoechst reagent. Single, plane confocal images in the center of the cell were acquired using an inverted Olympus iX73 microscope equipped with an X-light Nipkow spinning-disk head (Crest Optics, Rome, Italy) and Lumencor Spectra × Led illumination. Images were collected using a CoolSNAP MYO CCD camera (Photometrics, Tucson, AZ, USA) and MetaMorph Software (Molecular Device, Sunnyvale, CA, USA) with a × 60 oil objective.

Immunohistochemistry

Studies on human samples (already obtained for diagnostic purposes) were performed according to the standards of the local ethical committee. Immunoistochemistry was performed as previously described[11], by using the following antibodies: anti-Notch1 C-20R (Santa Cruz Biotechnology; Catalog number sc-6014, 1:50 dilution), anti-Notch3 M-134 (Santa Cruz Biotechnology; Catalog number sc-5593, 1:50 dilution), and anti-EGFR D38B1 (Cell Signaling; Catalog number 4267S, 1:50 dilution). The sample was defined as negative when the number of stained cells was < 1% of the tumor cell population. The percentage of positive cells for each marker analyzed is reported in the Figure.

In vitro BrdU assay and FACS analysis

In vitro BrdU assay was performed as described elsewhere[67]. For $EGFR_{EC}$ expression, cells were stained with anti-EGFR antibody (Santa Cruz Biotecnology; Catalog number sc-528) or normal mouse IgG (Santa Cruz Biotecnology; Catalog number sc-2025) used as a negative control. Data were analyzed on a FACS-Calibur with CellQuest software (BD Bioscience, San Jose, CA, USA), as previosuly described[68].

In silico analysis of TNBC patients' deposited data

Tumor samples from a cohort of 198 TNBC patients (GEO ID: GSE76124) and 579 TNBC patients (GEO ID: GSE31519) were selected and analyzed for the correlation between *NOTCH1* or *NOTCH3* and *EGFR* genes. The expression values were filtered in each analysis utilizing the expression probe set 218902_at representing *NOTCH1*, 203238_s_at representing *NOTCH3*, and 201983_s_at representing *EGFR*. The expression value of *NOTCH1*, *NOTCH3*, and *EGFR* for both datasets was in log2 scale after normalization of the data with the RMA algorithm. The index Pearson's r coefficient correlation and reported P-values were calculated using GraphPad Prism software Version 6.0 (La Jolla, CA, USA) for both datasets. Plots were generated and data were converted to standard score for the plots using GraphPad Prism Version 6.0.

Statistical analysis

All results were reported as the mean ± SD of at least three independent experiments, each performed in triplicate. Student's t-test for unpaired samples was used to assess differences among groups, with similar variance. A P-value of 0.05 was considered statistically significant (NS $P > 0.05$; $^{*}P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$). We estimated the sample size considering the variation and mean of the samples. No statistical method was used to predetermine sample size. No samples were excluded from any analysis.

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Authors' contributions:
Study concept and design: S.C. and C.C. Experiments and procedures: G.D., F.D. G., M.V.G., G.F., E.G., Z.M.B., and M.G.P. Data analysis: C.T., D.B., R.P., G.D., A.V., and M.M. Writing of the manuscript: S.C., G.D., F.D.G., D.B., and C.C.

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Conflict of interest
The authors declare that they have no conflict of interest.

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In the Supplementary Information there are included 5 files, as follow:

- Supplementary Figure S1 (.jpg)

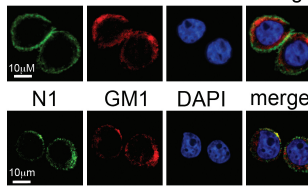
- Supplementary Figure S2 (.jpg)

- Supplementary Figure S3 (.jpg)

- Supplementary Figure S4 (.jpg)

- Supplementary Figures Legends (.docx)

a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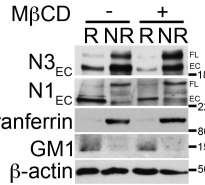
b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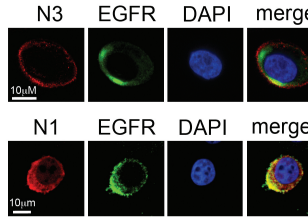
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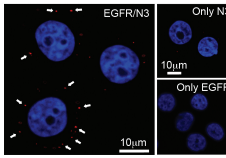
d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Only EGFR
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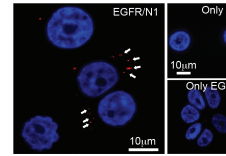
e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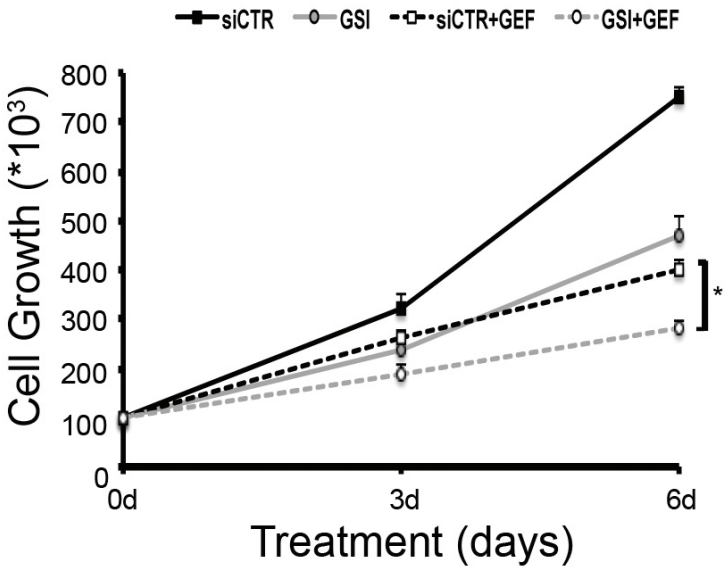
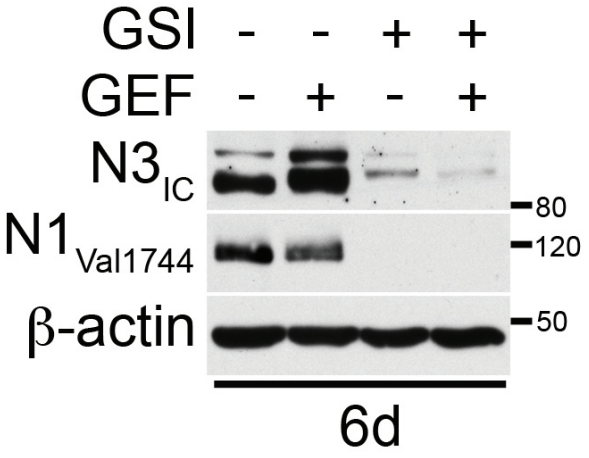
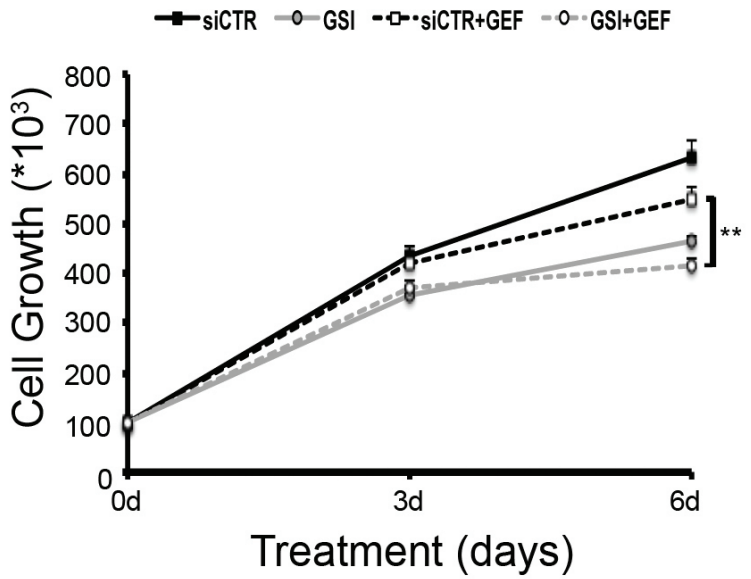
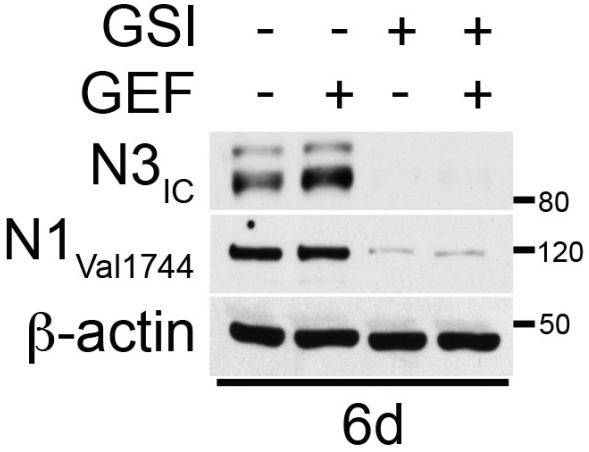
a MDA-MB-468



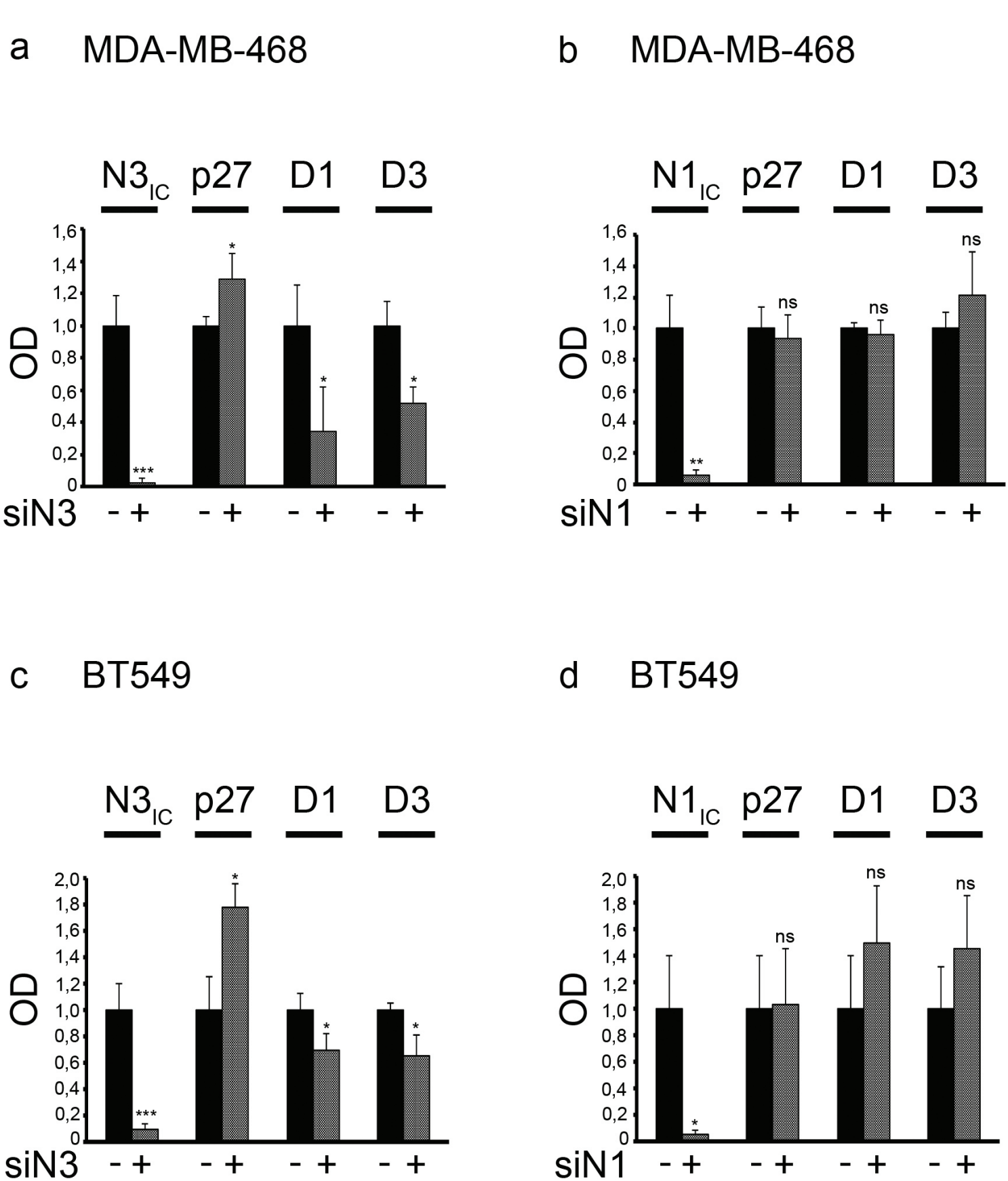


b BT549





Diluvio et al. Supplementary Figure S3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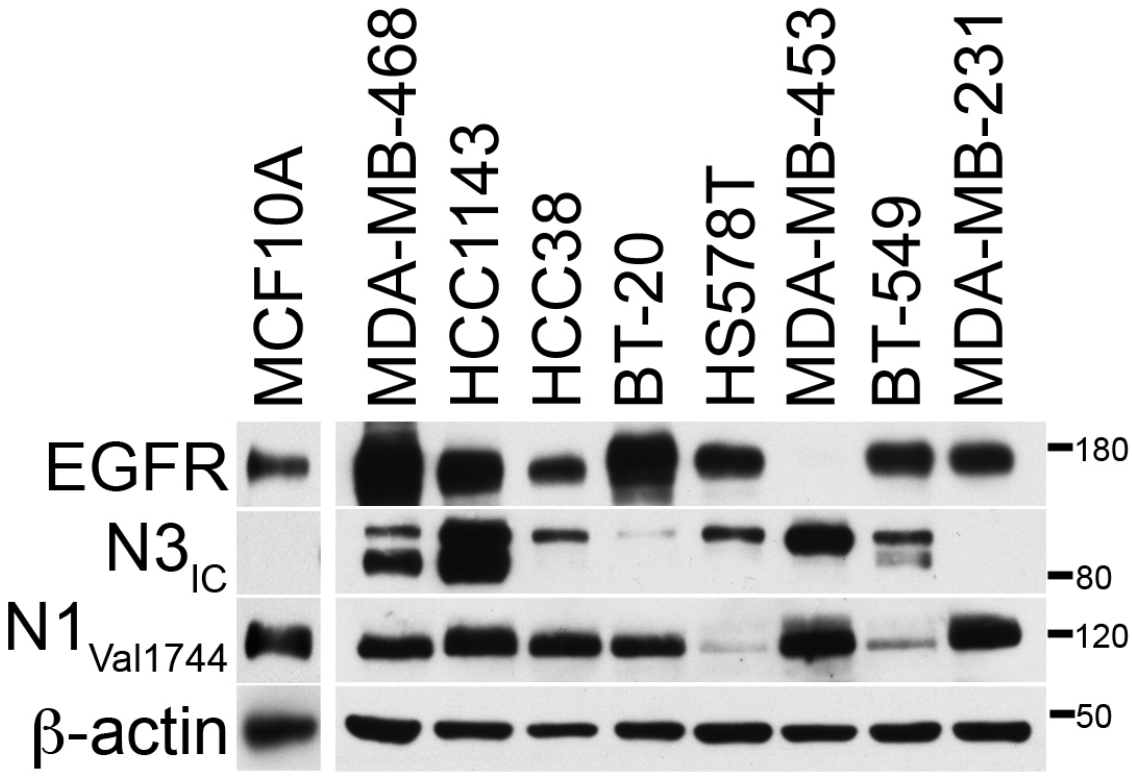
BT549

a



Diluvio et al. Supplementary Figure S1

Supplementary Figure S1. TNBC screening for EGFR, Notch3 and Notch1 protein expression. (a) Western blot analysis of EGFR, Notch3 and Notch1 proteins expression on a subset of TNBC selected cells (MDA-MB-468; HCC1143; HCC38; BT-20; HS578T; MDA-MB-453; BT-549; MDA-MB-231) compared to MCF10A cell line used as normal breast sample.

Supplementary Figure S2. Notch3 silencing (rather than Notch1) influences the TNBC cell growth. In all panels (a), (b), (c), (d) is shown the relative Optical densitometry (OD) of $N3_{IC}$ (a,c) or $N1_{IC}$ (b,d), p27, cyclin D1 (D1) and cyclin D3 (D3) protein expression levels represented in the Figure 2b, 2c (for MDA-MB-468 cells) and 2e, 2f (for BT-549 cells). Results are shown as means average deviations of three separate Notch silencing experiments and P-values were calculated using Student's T-test (i.e., ns, not significant P>0,05; * P≤0.05; **P≤0.01; ***P≤0.001).

Supplementary Figure S3. Effects of combined GSI IX (DAPT) plus GEF treatment on TNBC cells. (a,b) Left panels: analysis of MDA-MB-468 (a) and BT-549 (b) cell growth after 0-3-6 days of gefitinib (GEF) treatment combined with γ-secretase inhibition (GSI+GEF) in MDA-MB-468 (a) and BT-549 (b) cells. Right panels represent western blot of total extracts from the same cells in (a) and (b) at 6 days, against Notch3 ($N3_{IC}$) and activated-Notch1 ($N1_{Val1744}$), to control the efficiency of the GSI treatment. Anti-β-actin was used as a loading control. All data are representative of at least three independent experiments, each in triplicate. Results shown in panels (a) and (b) are expressed as the means average deviations and P-values were calculated using Student's T-test (i.e., *P≤0.05; **P≤0.01).

Supplementary Figure S4. Notch3 and Notch1 membrane localization in MDA-MB-468 TNBC TKI-resistant cells. (a) Immunofluorescence assay (IF) was performed by using anti-Notch3 (grenn, upper panel) or anti-Notch1 (green, lower panel) and anti-GM1 (red) antibodies to reveal the endogenous Notch(s) receptor-rafts colocalization, shown in yellow (merge). Nuclei were

DAPI labeled (blue). (b) Raft (R) and Non-Raft (NR) fractions derived from Methyl-β□cyclodextrin (MβCD)-treated and untreated cells were used for immunoblot assay with anti-$N3_{EC}$ and anti-$N1_{EC}$ antibodies, to test Notch(s) expression in rafts compartment. Anti-transferrin and anti-GM1 antibodies were used as a fraction markers; anti-β-actin was used as a loading control. (c) IF assay was performed by using anti-Notch3 (green, upper panel) or anti-Notch1 (green, lower panel) and anti-EGFR (red) antibodies to reveal the endogenous Notch(s) receptor-EGFR colocalization, shown in yellow (merge). Nuclei were DAPI labeled (blue). (d,e) Endogenous Notch3/EGFR (d) and Notch1/EGFR (e) interaction analyzed by *in situ* proximity ligation assay (PLA), detecting single interaction pairs of native proteins displayed as red signal by confocal microscopy. Negative controls lacking one of the primary antobody (only Notch3 (d); only Notch1 (e); only EGFR (d,e)). Protein complexes were visualized in red and indicated with white arrows; nuclei were DAPI labeled (blue).

All the panels (a), (c) and (d) are representative single plane confocal IF images captured using a 60X oil objective. Scale bar: 10 μm. The results are representative of three independent experiments.

3.1.2 Notch3 and the Unfolded Protein Response in T-cell Acute Lymphoblastic Leukemia

3.1.2a Glance at Project n°2

- **General features of T-cell Acute Lymphoblastic Leukemia (T-ALL)**

  T-cell acute lymphoblastic leukemia (T-ALL) is a genetically heterogeneous and aggressive malignancy which is caused by the accumulation of lesions acting in a multistep pathogenic process involving cell growth, proliferation, survival, and differentiation during thymic development **(Chiaretti S et al., 2013).** It accounts for about 15% and 25% of ALL among pediatric and adult cohorts, respectively, and is slightly more frequent in males than females, particularly in older children and adolescents **(Van Vlierberghe P et al., 2012).**

  Current treatment of T-ALL consists of high intensity combination chemotherapy and results in a very high overall survival for pediatric patients. Unfortunately, this treatment comes with significant short term and long term side effects. In addition to the side effects, the occurrence of relapse is another important challenge as it is observed in up to 20% of pediatric and 40% of adult T-ALL **(Pui C-H et al., 2008).**

  To further improve the treatment of T-ALL and to reduce the toxicity of current treatment, the introduction of newer targeted agents is highly awaited **(Girardi T et al., 2017).**

- **Overview of the role of the Unfolded Protein Response (UPR) in T-ALL**

  Malignant cells activate various pathways to survive and proliferate in a hostile biological environment such as the adaptive response termed the unfolded protein response (UPR) **(Riha R et al., 2017)**. UPR is activated in a wide variety of tumor types and it is gaining increasing recognition as a key

targetable pathway **(Tameire F. et al., 2015).** Several studies have highlighted the importance of drugging UPR in acute leukemia **(Masouleh BK et al., 2015)**. Interestingly, the downregulation of UPR signaling causes apoptotic cell death in T-ALL **(DeSalvo J et al., 2012).**

Since malignant T-ALL cells may rely on UPR, understanding how oncogenes regulate this pathway might help in searching a novel therapy.

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- **Overview of the role of Notch3 in T-ALL**

  Notch1 and Notch3 are key oncogenes in the pathogenesis of T-ALL **(Oliveira ML et al., 2017)**. Indeed, constitutive activation of both receptors correlates with development of T-ALL in mouse models **(Bellavia D et al., 2000; Bellavia D et al., 2002)**. Moreover, T-ALL displays increased Notch1 activity in about 60% of cases, due to activating Notch1 mutations or alterations in the FBXW7 gene **(Weng AP et al., 2004; Mansour MR et al., 2006)**, and Notch3 overexpression **(Pinazza M et al., 2018)**. Interestingly, Bernasconi-Elias and colleagues identified activating mutations of Notch3 also in the absence of functional Notch1 **(Bernasconi-Elias P et al., Oncogene 2016)**. This study concurs with another one where several Notch3 mutations, not coincident with Notch1 ones, were described in infant cases of T-ALL **(Doerrenberg M et al., 2017)**.

  As a result, increasing evidence acknowledges the role of Notch3 in sustaining T-ALL but further investigations are needed to deepen the understandings of its function.

- **Novelty of the Project n°2**

  We documented an unknown role of Notch3 protein, but not of Notch1, in sustaining the activation of pro-survival UPR pathway under ER stress conditions in T-ALL. Indeed, Notch3 inhibition in combination of high ER

stress prevents leukemic cells to restore ER homeostasis, tipping the balance in favor of pro-death UPR pathway. As a result, this strategy could be adopted for inducing T-ALL cell death and ameliorate the treatment of Notch3-dependent TALL-bearing patients.

3.1.2b Paper

"Notch3 contributes to T-cell leukemia growth via regulation of the unfolded protein response"

M. V. Giuli, G. Diluvio, E. Giuliani, G. Franciosa, et al. Paper submitted to Haematologica Journal.

Notch3 contributes to T-cell leukemia growth via regulation of the unfolded protein response

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[*]MVG, GD and EG contributed equally to this work.

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Running title: Notch3 sustains UPR signaling in T-ALL

Conflict of Interest: The authors declare no competing financial interests.

Abstract

Unfolded protein response (UPR) is a conserved adaptive response which tries to restore protein homeostasis after Endoplasmic Reticulum (ER) stress. Recent studies highlighted the role of UPR in acute leukemias and UPR targeting has been suggested as a therapeutic approach. Aberrant Notch signaling is a common feature of T-cell acute lymphoblastic leukemia (T-ALL), as downregulation of Notch activity negatively affects T-ALL cell survival, leading to the employment of Notch inhibitors in T-ALL therapy. Here we demonstrate that Notch3 is able to sustain UPR in T-ALL cells, as Notch3 silencing favoured a Bip-dependent IRE1α inactivation under ER stress conditions, leading to increased apoptosis via upregulation of the ER stress cell death mediator CHOP. By using *Juglone*, a naturally-occurring naphtoquinone acting as anticancer agent, to pharmacologically decrease Notch3 expression and induce ER stress, we observed an increased ER stress-associated apoptosis, in both *in vitro* and *in vivo* systems. This suggests that Notch3 inhibition may prevent leukemia cells from engaging a functional UPR needed to compensate the *Juglone*-mediated ER proteotoxic stress. Notably, *in vivo* administration of *Juglone* to human T-ALL xenotransplant models significantly reduced tumor growth, finally fostering the exploitation of *Juglone*-dependent Notch3 inhibition to perturb the ER stress/UPR signaling in Notch3-dependent T-ALL subsets.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic tumor resulting from the malignant transformation of T-cell progenitors. T-ALL accounts for approximately 15% and 25% of ALLs seen in children and adults, respectively (1). Notch receptors have been implicated as oncogenic drivers in a number of different human cancers, including T-ALL, which shows increased Notch1 activity in about 60% of cases, due to activating Notch1 mutations or alterations in the FBXW7 gene (2, 3). By screening primary T-ALL tumors and orthotopic patient-derived xenograft models, activating mutations of Notch3 have been recently identified, also detectable in the absence of an activated Notch1 (4). Since constitutive activation of Notch signaling pathway confers to the leukemic cells a strong growth advantage, the Notch therapeutic targeting has assumed a considerable clinical relevance, especially for patients refractory to chemotherapy. However, the gamma-secretase inhibitors (GSIs) treatment, which blocks cleavage of Notch receptors, exhibits significant gastrointestinal toxicity, mainly due to the simultaneous inhibition of Notch1 and Notch2 signaling in gut epithelial stem cells (5).

The therapy of T-ALL patients has gradually improved in recent decades. In this scenario, the endoplasmic reticulum (ER) stress/unfolded protein response (UPR) pathway is gaining increasing recognition as a key targetable pathway in acute leukemias (6). Tumor cells are often exposed to different stimuli that cause ER stress: adaptation to stress and re-establishment of ER homeostasis is achieved by activation of an integrated signal transduction pathway called UPR (7). Three major

branches of the UPR have been identified: IRE1α (inositol-requiring enzyme 1 alpha), PERK (double stranded RNA-activated protein kinase (PRK)-like ER kinase) and ATF6 (activating transcription factor 6). Under unstressed conditions, the stress sensors are maintained inactive through binding to the ER chaperone GRP78/Bip. After ER stress induction, GRP78/Bip dissociates from UPR sensors, thereby leading to their activation. Importantly, by integrating transcriptional and translational responses, UPR makes life/death decisions for the cell and the final outcome of ER stress is either recovery and survival or apoptosis, depending on the severity and duration of ER stress (7). Targeting the UPR for cancer treatment is considered a promising approach, as the UPR appears to be activated in a variety of human tumors. Interestingly, the downregulation of UPR signaling was shown to drive apoptotic cell death in T-ALL (8, 9). However, it remains to be fully elucidated how different oncogenes are able to influence the UPR autonomously or through interaction with the ER sensors, raising the possibility to identify a new therapeutic opportunity for T-ALL bearing patients.

Here, we revealed an unknown role of Notch3 protein in sustaining the activation of UPR pathway through its involvement in the ER stress/UPR signaling network regulation. By using a canonical ER stress inducer, Tunicamicin, we observed that the combined downregulation of Notch3 protein expression (but not of Notch1) was able to favour the ER stress-associated IRE1α ubiquitination and inactivation, in a GRP78/Bip-dependent manner. This event prevented leukemic cells from engaging a functional UPR required to counteract the ER-mediated proteotoxic stress, finally leading to ER-associated pro-apoptotic events, represented by increased levels of the ER stress cell death mediator CHOP.

In order to evaluate *in vivo* the potential anti-leukemic effects derived from the previously reported combination of Notch3 downregulation under ER stress conditions, in this study we used the *Juglone* (5-hydroxy-1,4-naphthoquinone), a naturally occurring naphtoquinone derived from the *Juglans mandshruica Maxim*, that has shown a strong activity against cancer cells *in vitro*, including human leukemia cells (10, 11). Interestingly, we first demonstrated that *Juglone* treatment

resulted in the Notch3 downregulation, IRE1α ubiquitination/inactivation and amplification of ER-associated pro-apoptotic events, confirming the *in vitro* experiments. Furthermore, we also observed that *Juglone* was able to induce Notch3 dowmodulation and CHOP induction *in vivo*, finally exerting anti-leukemia growth in a human T-ALL xenograft mouse model. Taken together, our findings provided a rationale for the use of Notch3 inhibition and *Juglone*-based combined therapy protocols in the treatment of a Notch3-dependent subset(s) of T-ALLs.

Materials and Methods

Cell culture and treatments. Human leukemic cells (TALL-1, Jurkat, Ke37, KOPKT1, DND41, Molt3, P12-lchikawa and SIL-ALL) (12, 13) were maintained as described elsewhere and all are mycoplasma-free. Cells were treated with different doses (as indicated in some Figures) or fixed 2,5μM of *Juglone* (Calbiochem, San Diego, CA, USA, Cat#420120), 2,5μM Thapsigargin (Sigma, St Louis, MO, USA, Cat#T9033) or 5μM Tunicamycin (Sigma, Cat#T7765) for the times indicated, according to their datasheets' instructions. In some cases, cells were treated with 30μM MG132 (Z-Leu-Leu-Leu-al; Sigma, Cat#C2211) for 6 hours before harvesting. For survival analysis, cells were harvested at different time points and counted by using Trypan blue assay.

Flow cytometric analysis. To determine the extent of apoptosis induction after drug treatment, flow cytometric analysis of Annexin V (BD Pharmigen, San Diego, CA, USA, Cat#550474) /propidium iodide (PI) (BD Pharmigen, Cat#556463) stained samples was performed, as described elsewhere (14). In same cases, cells were incubated with human Notch3 antibody (R&D Systems, Minneapolis, MN, USA, Cat#MAB1559, clone #603532) or monoclonal mouse IgG1 isotype control (R&D Systems, Cat#MAB002, clone #11711) used as negative control, as previously described (15). Then, samples were analysed on a FACS-Calibur with CellQuest software (BD-Biosciences, San Jose, CA, USA).

RNA extraction, RT-PCR and qRT-PCR, Notch3 knockdown. Total RNA extraction and reverse transcription (RT-PCR) were previously described (16, 17). The expression levels of GRP78/Bip, CHOP and GAPDH mRNAs were determined by SYBR Green quantitative real-time RT-PCR (qRT-PCR) performed on cDNA according to the manufacturer's instructions (Applied Biosystems, Life Technologies Brand, Carlsbad, CA, USA) and using the ABI Prism 7900HT (Applied Biosystems). Data were analyzed by the □□Ct method and GAPDH was used to normalize the expression levels of mRNA (18). RT-PCR for XBP1 mRNA splicing analysis and β-actin was performed using Taq Gold polymerase. The amplicons were resolved using 2% agarose gel. The details of the primers for each gene are given in the Supplementary Table S1. Cells were silenced for Notch3 as previously described (19).

Western blot, Immunoprecipitation assay and Antibodies. Protein extracts preparation, immunoprecipitation and immunoblotting assays were performed as previously described (20, 21). Antibodies: from Cell Signalling (Danvers, MA, USA), anti-Notch3 (Cat#2889); anti-Notch1 (Cat#3608); anti-GRP78/Bip (Cat#3177); anti-IRE1α (Cat#3294); anti-CHOP (Cat#2895); from Santa Cruz Biotechnology (Dallas, TX, USA), anti-LaminB M20 (Cat#sc-6217), anti-ubiquitin (P4D1; Cat#sc-8017); anti-β-actin (Sigma-Aldrich, Saint Louis, MO, USA, Cat#A5316). The anti-$N3_{EC}$ (5E1) antibody was kindly provided by Professor A Joutel, as previosly described (22).

Immunohistochemistry. Tissue samples were fixed and paraffinized as described (23). The 4 μm thick sections were prepared from paraffin-embedded tissues and immunostained with anti-CD45 (X16/99, Novocastra, Leica biosystems, Newcastle, UK, Cat# NCL-L-LCA), anti-Notch3 (Santa Cruz Biotechnology, Dallas, TX, USA, Cat#5539) or anti-CHOP (Cell Signalling, Danvers, MA, USA, Cat#2895) antibodies. After washes, secondary biotinylated antibodies were applied. Binding

of antibodies was detected with the Mouse to Mouse HRP (DAB) Staining System (Scytek Laboratories, Inc., Logan, UT, USA) according to the manufacturer's protocol.

Animal studies. All animal experiments were approved by local ethic authorities and conducted in accordance with Italian Governing Law (D.lgs 26/2014; Prot. no. 03/2013). Animals were housed in the Institute's Animal Care Facilities, which meet international standards and were checked regularly by a certified veterinarian responsible for health monitoring, animal welfare supervision and revision of experimental protocols and procedures.

For TALL-1 xenograft models, all the studies were conducted by using TALL-1_luc cells, generated by lentivirus infection with pLENTI-CMV-Puro-LUC (Addgene, Watertown, MA, USA, Cat#17477) in order to track leukemia progression in vivo by optical imaging, according to previous data (4).

To assess *in vivo* activity of Juglone (Sigma-Aldrich, Saint Louis, MO, USA, Cat#H47003) on Notch3 protein expression, $10*10^6$ TALL-1_luc cells were resuspended in an equal volume of MEM medium and Matrigel (BD Biosciences, Heidelberg, Germany) and injected subcutaneously at the both posterior flanks of 6-week-old female NOD/SCID/gamma (NSG) mice (Charles River Laboratories, Lecco, Italy). Tumor growth was monitored weekly by caliper measurements and mice were weighed frequently to determine treatment-induced toxicity. Once tumors reached between 300 and 500mm^3 (after 21 days), mice were randomly assigned to receive a double intravenous 1mg/kg dose of *Juglone* or vehicle (Ethanol and physiological solution) for 48 hours plus 48 hours (n = 6 CTR, n = 6 *Juglone*-treated). At the end of the treatment, all the masses were excised and a portion of tumor was fixed in formalin and analyzed by IHC. To assess *in vivo* cytotoxic activity of *Juglone*, TALL-1_luc cells were intravenously injected (i.v.) in 6-week-old female NSG mice at $10*10^6$ cells/mouse (n=6 mice per group). On day 16, tumor-bearing animals were randomly assigned to receive intraperitoneal doses of either vehicle (Ethanol and physiological solution) or *Juglone* (1 mg/kg), 3 days per week until a maximum of 3 weeks. For

bioluminescence imaging performed at different times before killing, mice were anaesthetized, intraperitoneally injected with RediJect D-Luciferin Bioluminescence Substrate (PerkinElmer, Whaltam, MA, USA, Cat#770504), 150mg per Kg body weight, and scanned with IVIS Lumina III In Vivo Imaging System (Caliper Life Science, Waltham, MA, USA) after 10 minutes. Animals were imaged with an exposure time of 30 seconds. Both luminescence and image data were analyzed using Living Image software (Caliper Life Science). Total flux was calculated and expressed as photons per second.

Statistical Analysis. For all other experiments, P-values were determined using Student's t-test and statistical significance was set at $P \leq 0.05$. Results are expressed as mean ± SD from an appropriate number of experiments (at least three biological replicates).

For *in vivo* studies, statistical differences were determine by unpaired Student's t test. Differences were considered significant for $P \leq 0.05$.

Results

Notch3 modulation influences T-ALL cells survival in response to ER stress induction

To assess the possible involvement of Notch proteins in the ER stress/UPR signaling network in T-ALL, ER stress induction by Tunicamycin treatment was performed in different human T-ALL cell lines, all constitutively expressing Notch1-IC ($N1_{IC}$) and Notch3-IC ($N3_{IC}$) at various levels (Supplementary Figure S1A), and their survival was evaluated (Figure 1A). Interestingly, all the selected leukemic cells expressing higher levels of Notch3-IC (TALL-1, DND41, Molt3) appeared more resistant to increasing doses of Tunicamycin while $Notch3^{low}$ (Jurkat and KOPKT1) and $Notch3^{-}$ (Ke37) cells did not (Figure 1A), indipendently of Notch1 expression levels and its mutational *status* (Supplementary Figure S1A). In agreement with this, downregulation of Notch3 expression (but not of Notch1) seems to correlate with Tunicamycin response: indeed, Notch3-

silenced cells (siN3) displayed a significant decreased percentage of survival with respect to control cells (siCTR) (Figures 1B, 1D, 1F and Supplementary Figures S1B, S1D, S1F) while the Notch1-silenced cells (siN1) did not (Figures 1C, 1E, 1G and Supplementary Figures S1C, S1E, S1G). All together our observations suggested a potential novel role of Notch3 in sustaining the response to ER stress induction in T-ALLs.

Notch3 modulation affects ER stress/UPR signaling by regulating IRE1α protein expression

Digging deeper into the molecular mechanism underlying the possible Notch3-UPR cross-talk in T-ALL cells, we focused our *in vitro* studies on the Notch3-overexpressing TALL-1 leukemic cells, which display constitutive activation of Notch3 (24), while neither bearing Notch1-activating mutations nor displaying Notch1 activation (25). The specific Notch3 downregulation induced during ER stress conditions (siN3+Tun) was able to both enhance GRP78/Bip expression, a known marker of UPR activation (26), and to attenuate the Tunicamycin-induced increase of IRE1α expression (Figure 2A). In keeping with these data, by using the same experimental conditions, we also observed the increase of the endogenous IRE1α ubiquitination levels (Figure 2B), which may occur through a known mechanism of IRE1α protein regulation, GRP78/Bip-dependent, that was described only under ER stress conditions (27), when high levels of GRP78/Bip were induced (Figure 2A). These observations suggested a direct involvement of Notch3 in the regulation of the ER stress/UPR markers expression only within an ER stress-microenvironment, as the absence of Notch3 alone (siN3) did not correlate with any change in GRP78/Bip and IRE1α levels (Figure 2A and Supplementary Figure S2). In order to evaluate the relationship between Notch3 and the above-mentioned UPR sensors in TALL-1 cells, we further analysed its role with respect to the known GRP78/Bip-IRE1α cross-talk. As shown in the Figure 2C, we observed an unexpected endogenous Notch3-GRP78/Bip association in basal conditions and the absence of the known endogenous GRP78/Bip-IRE1α interaction, normally occurring in

unstressed cells, thus suggesting the hypothesis of a Notch3-dependent GRP78/Bip sequestering which may justify the presence of an active ER stress/UPR machinery in TALL-1 cells, finally responsible of sustaining their survival.

As a consequence, our data sustained that the Notch3 downmodulation under ER stress conditions may be correlated with an UPR defect by favoring the GRP78/Bip-overexpression effects upon IRE1α protein level, which resulted in IRE1α ubiquitination and inactivation (Figure 2). Notably, an *in silico* analysis performed in several T-ALL cell lines (28) (Figure 2D, left panel and Supplementary Table S2) and in a cohort of 53 T-ALL patients (1) (Figure 2D, right panel) highlighted a significant positive correlation between Notch3 and IRE1α gene expression levels, thus reinforcing the possible relationship between Notch3 and IRE1α at protein levels in T-ALL context.

Juglone acts simultaneously by inducing ER stress and downregulating Notch3 in TALL-1 cells

In order to evaluate the potential anti-leukemic effects of the Notch3 downregulation under ER stress conditions previously observed in TALL-1 cells (Figures 1-2, siN3+Tun) also in *in vivo* studies, we chose the natural compound *Juglone* for the subsequent experiments, since several literature's data (10, 29-31), including ours (15), showed its ability to induce different molecular mechanisms potentially resulting in the simultaneous ER stress induction and Notch3 blocking.

Several *in vitro* experiments were first performed to test the multiple effects of *Juglone* on TALL-1 cells. Since it has been shown that *Juglone* induces apoptosis in several cancer cells (32, 33), including leukemia (10), we first investigated its effects on TALL-1 leukemic cells survival: as shown in the Figure 3A, increasing doses of *Juglone* for 24 hours (h) correlated with progressive cell growth inhibition. Time-dependent anti-proliferative effects of fixed dose (2,5μM) of *Juglone* are shown in the Figure 3B. To evaluate whether the impact of *Juglone* on cell viability could be related to apoptosis, we performed time course experiments of *Juglone* treatments followed by flow

cytometric analysis after Annexin V/PI staining. We detected an increase in the percentage of early apoptotic (positive for Annexin V) and/or late apoptotic cells (positive for both Annexin V and PI) after 12 hours, progressively increasing up to 48 hours (Figure 3C). Notably, regarding ER stress/UPR balance, we also observed the increased expression of CHOP, a pro-apoptotic transcription factor induced by severe or prolonged ER stress conditions, both at mRNA (Figure 3D) and nuclear protein levels (Figure 3E), thus suggesting the involvement of ER stress in the *Juglone*-induced leukemia cell death.

Several cellular stimuli that perturb ER homeostasis may lead to ER stress, including proteasome function alteration (34), production of increased reactive oxygen species (ROS) (35) and $[Ca^{+2}]_{ER}$ depletion (36). In TALL-1 cells, we observed that *Juglone* treatment was able to induce at the same time: 1. the fast accumulation of poly-ubiquitinated proteins (Supplementary Figure S3A); 2. the increase in the Ca^{+2} release from the ER to the cytoplasm (Supplementary Figure S3B), probably due to the significant inhibition of the mRNA expression of sarco(endo)plasmic reticulum Ca^{+2} ATPase 3 (SERCA3), known to pump cytoplasmic Ca^{+2} to ER lumen and to be expressed at high levels in the hematopoietic cell lineage (37) (Supplementary Figure S3C). All these observations sustained the ability of the *Juglone* to induce ER stress through different mechanisms: the increased GRP78/Bip expression, both at mRNA (Supplementary Figure S3D) and protein (Supplementary Figure S3E) levels, also observed in combination with the widely used ER stress inducer Tapsigargin, TH (Juglone+TH) (Supplementary Figure S3E), validated these data.

Juglone is a natural inhibitor of Pin1 protein, a peptidyl-prolyl isomerase that we recently discovered as a novel regulator of Notch3-IC protein expression in T-ALL (15). Furthermore, others and we demonstrated its ability to inhibit SERCA activity (Supplementary Figure S3C and (31)). Since SERCA inhibition can modulate Notch function in T-ALL cell lines by affecting Notch1 maturation process (38), we can suppose the potential co-presence of Pin1-independent mechanisms mediating *Juglone* function upon Notch proteins inhibition in T-ALL, as previously described in different cellular contexts (39).

As expected, reduced levels of Notch3 receptor at the cell surface (N3EC) (Supplementary Figure S3F), resulting in the reduced Notch3-IC protein expression (Supplementary Figure S3G), were observed in *Juglone*-treated TALL-1 cells with respect to the control cells (Supplementary Figures S3F – S3G).

Together, all these findings supported the possibility of using the *Juglone* in order to induce the TALL-1 cells death through the simultaneous Notch3 downregulation under ER stress conditions.

Juglone affects ER stress/UPR signaling by regulating IRE1α protein expression

Since we have demonstrated that combined Notch3 downmodulation under ER stress conditions may be correlated with an UPR defect (Figure 2), we further evaluated the *Juglone* effects upon the ER stress/UPR signaling. As shown in the Figure 4A, the levels of ER stress/UPR markers were not significantly altered until 6 hours of *Juglone* treatment, while they were strongly modulated at 12-24 hours: in particular, *Juglone* significantly decreased the expression of Notch3 and IRE1α proteins while increasing GRP78/Bip levels (Figure 4A). In keeping with these results, similar time-dependent kinetics was observed for the ER stress-activated XBP1 splicing, which measures the IRE1α-dependent endoribonuclease activity during ER stress, as demonstrated by the modulation of the XBP1 spliced band (XBP1s) during *Juglone* treatment (Figure 4B): at very early time points (1-3 hours) XBP1s was produced while disappeared progressively at 12-24 hours (Figure 4B), possibly due to the observed defect in IRE1α expression (Figure 4A).

All these data raised the possibility that *Juglone* might also be able to suppress UPR at later time points through the regulation of the Notch3-GRP78/Bip-IRE1α axis previously described (Figure 2). As expected, *Juglone* treatment, by downregulating Notch3 and overexpressing GRP78/Bip at the same time, was able to restore the GRP78/Bip-IRE1α interaction (Figure 4C), which in turn correlated with an increased IRE1α ubiquitylation (Figure 4D) and inactivation, thus confirming previous mechanicistic data (Figure 2) and finally pointing out the potential involvement of Notch3 in the *Juglone*-dependent ER stress/UPR signaling regulation.

Notch3 silencing synergizes with *Juglone* and amplifies Juglone-induced TALL-1 cell apoptosis, ER-stress associated

We have demonstrated that the mechanism of Juglone-induced leukemia cell death could involve an induction/amplification of a pre-existing ER stress microenvironment (depicted by the high basal levels of UPR components), followed by a serious defect of the ER stress response, as evidenced by the strong down-regulation of IRE1α expression and function observed at late time points (Figure 4). This scenario renders leukemic cells unable to adequately respond to ER stress, thus finally leading to the cell-destroying pathway activation which prevails over the compensatory UPR (Figure 3). To define how Notch3 contributes to *Juglone*-induced T-ALL cell apoptosis, we examined the existence of a potential synergism between *Juglone* and the decreased Notch3 protein expression, by using Notch3-silenced TALL-1 cells incubated for the last 24 hours of silencing with *Juglone*. The absence of Notch3 in *Juglone*-treated (siN3+Jug) cells (thus under ER stress conditions) resulted in an increased percentage of the early apoptotic cells (Figure 5A), when compared to the cells treated with *Juglone* alone (Jug), which in turn promoted a significant decrease in cell count when Notch3 silencing was further prolonged for 96 hours (Figure 5B). More interestingly, the silencing of Notch3 strongly sinergized with *Juglone* treatment in both increasing GRP78/Bip expression and decreasing IRE1α expression (Figure 5C), thus reinforcing the potential role of Notch3 inhibition in the *Juglone*-dependent perturbation of ER stress/UPR signaling, which finally leads to a stronger ER-stress associated cell apoptosis induction, as demonstrated by the significant concomitant amplification of the mRNA levels of CHOP (Figure 5D).

Notably, despite the expected Notch1 downregulation observed after *Juglone* treatment, related to its previously described SERCA inhibition function (Supplementary Figure S3C and (38)), Notch1 silencing in the selected Notch3$^{low/-}$ cells (Ke37 and Jurkat) did not amplify the decreased cell count induced by *Juglone* itself (Supplementary Figures S4A and S4B, left panels). The possible reason of the different behaviour of Notch1 with respect to what observed for Notch3 could be related to

the inability of Notch1-IC to interact with GRP78/Bip protein (Supplementary Figures S4C), also in TALL-1 cells where we previously observed the endogenous Notch3-GRP78/Bip interaction in basal conditions (Figure 2C). Therefore, these findings dropped the hypothesis of a GRP78/Bip sequestration by every Notch proteins in T-ALL context, which seems to be specifically related to a novel and unknown role of Notch3. In keeping with these observations, no significant change of IRE1α expression was observed in Notch1-silenced T-ALLs analysed after *Juglone* treatment (Supplementary Figures S4A and S4B, right panels).

Juglone displays *in vivo* activity against TALL-1 tumor growth models by defecting Notch3 expression and inducing ER stress-associated apoptosis

The effects of the *Juglone* activity upon Notch3 protein expression were also confirmed in a human T-ALL xenograft mouse model. Mice were treated with intravenous (i.v.) injection of *Juglone* or vehicle alone (CTR) at days 21th and 23 th after subcutaneous leukemia cells implantation (Figure 6A) and, at day 25th, excised tumors were evaluated for the effects on Notch3 and CHOP expression (Figures 6B and 6C). As expected, comparable levels of CD45, used as marker of human TALL-1 injected cells, were observed between tumors after the short treatment performed (Figure 6B, upper panels and Figure 6C). Notably, staining of xenografts with anti-Notch3 antibody revealed a strong reduction of Notch3 expression following *Juglone* treatment with respect to the high levels observed in xenografts from animals treated with vehicle alone (Figure 6B, middle panels, and Figure 6C). Interestingly, a significant increased CHOP levels were observed in *Juglone*-treated tumors when compared to controls (Figure 6B, lower panels, and Figure 6C). These studies demonstrated that *Juglone* treatment is able to inhibit Notch3 expression *in vivo,* thus recapitulating the previously described perturbation of the ER stress/UPR signaling balance.

Based on the observed strong *in vitro* activity of *Juglone* on TALL-1 cell survival, we asked whether it also has antitumor effects in the TALL-1 xenograft mouse model, by using TALL-1 cells luciferized (TALL-1_luc) in order to evaluate xenograft growth *in vivo*.

Mice were injected intravenously (i.v.) with TALL-1_luc cells and were monitored by optical imaging at various time points after cells implantation (day 0): on day 15th after cell transfer, mice were randomly distributed in two groups to receive one intraperitoneal (i.p.) injection of *Juglone* or vehicle alone (CTR) every two days, starting from day 16th until day 38th, and were sacrificed at day 40th (Figure 6D). Results showed a strong and significant reduction in leukemia burden in *Juglone*-treated mice with respect to control mice at all time points analysed (Figures 6E and 6F).

Taken together, our data suggest that the *Juglone*-dependent inhibition of Notch3 might be a useful therapeutic strategy for Notch3-overexpressing T-ALLs.

Discussion

Increasing evidence supports an important role of the UPR in tumorigenesis through the identification and characterization of mechanisms by which tumor cells are able to promote their own survival in unfavorable conditions, leading to tumor progression and metastasis (40). In this view, targeting the UPR represents a new window of research focused on the identification of druggable targets against malignancies, including acute leukemia (6). The therapeutic potential of targeting UPR signaling in cancer could involve two main approaches: 1. inhibition of UPR to eradicate tumors that are strongly dependent on an activated UPR for their survival in unfavorable conditions (i.e. highly stressed ER) or 2. induction of a severe ER stress by the accumulation of misfolded protein in the ER in order to overload restoration ability of tumor cells with compromised UPR or to hyperactivate the UPR to kill cells through pro-apoptotic UPR signaling (40).

Small molecule inhibitors which target the UPR transducers (i.e. PERK/eIF2α and IRE-1α/XBP1 signaling axis) are currently available (41-43) and several compounds are found to block the functional activity of GRP78/Bip protein (44, 45), whose high levels are commonly related to tumor protection, survival and chemoresistance (46). In addition, it has been well documented that proteasome inhibitors may potentiate ER stress in cancer cells, thus promoting proteotoxic conditions (46). Combined therapies are also under investigation: the proteasome inhibitor

bortezomib combined with small molecules that inhibit IRE1α activity significantly decreased Multiple Myeloma growth *in vivo* (47) as well as combining bortezomib with the SERCA inhibitor Thapsigargin amplified ER stress and increased cancer cell death (48). In T-ALL context, it has been demonstrated that pharmacological inhibition of Casein Kinase 2 (CK2) through CX-4945 may be an efficient treatment for a subset of T-ALLs displaying upregulation of the CK2/PI3K/Akt/mTOR axis via downmodulation of the ER stress/UPR signaling (49). More recently, Huiting and colleagues clearly documented the cross-talk between MYC and UFD1, a component of the ER-associated degradation complex (ERAD) commonly involved in the pro-survival UPR signaling (50).

In this study we demonstrated that Notch3 may have a novel role in T-ALL, being important in sustaining the UPR through the regulation of IRE1α protein expression and function. The significant positive correlation observed between Notch3 and IRE1α expression levels in human T-ALL cell lines and primary tumor samples confirms the possible relevance of our observations for human T-ALL development. Previous data sustained a role of proteasome inhibitors (i.e. bortezomib) in disrupting the ER stress response in Myeloma cells by suppressing the endoribonuclease function of IRE1α, through an unknown mechanism possibly involving unknown protein(s) that may act by stabilizing IRE1α-GRP78/Bip association (51). In keeping with these findings, we observed that Notch3 (but not Notch1) is able to interact with GRP78/Bip in leukemia cells in basal conditions. As a consequence, the absence of Notch3 induced under ER stress conditions lets GRP78/Bip (overexpressed in the ER stress microenvironment) free to interact with IRE1α, thus leading to its ubiquitination and inactivation, as recently described (27).

All these data suggested the potential use of Notch3 targeting in combination with an ER stress inducer as a novel therapeutic approach of a subset of Notch3-overexpressing T-ALLs that rely on UPR for their survival. Such an approach would be based on the disruption of the pro-survival UPR signaling, partially represented by the Notch3-dependent IRE1α/XBP1 axis, thus forcing to switch

to UPR pro-apoptotic mode, mainly represented by the detectable increase of pro-apoptotic CHOP expression. To this purpose we exploited the observed abilities of the natural compound *Juglone* to act simultaneously 1. as an ER stress aggravator (ERSA), thus exacerbating pre-existing ER stress conditions in TALL-1 cells and 2. as a Notch3 inhibitor both *in vitro* and *in vivo* systems, thus defecting UPR by IRE1α downmodulation.

Finally, all these events contribute to the *Juglone*-dependent leukemia cell death via CHOP induction, also observed *in vivo* studies, thus confirming previous data obtained after treatment of tumor-bearing mice with ERSA compounds (48).

Juglone treatment abilities provided new insights unveiling a possible development of more effective therapies, exploiting the idea of aggravating ER stress and defecting UPR at the same time, thus preventing leukemic cells from engaging a functional UPR to restore ER homeostasis through Notch3 protein modulation. To our knowledge, this is the first study demonstrating a specific involvement of Notch3 in regulating the balance between UPR pro-survival and UPR pro-death under ER stress conditions. These findings suggest that, in addition to the currently established approaches (52), the modulation of the ER stress/UPR signaling through a selective Notch3 inhibition could be exploited for inducing T-ALL cell death (Figure 7), thus improving the outcomes of Notch3-dependent TALL-bearing patients. Our *in vivo* studies performed with chronic administration of *Juglone* showed a significant inhibition of Notch3-dependent leukemia growth through Notch3 downregulation, thus providing preclinical evidence of the efficacy of Notch3 targeting in T-ALL, which is becoming of great interest as a potential novel therapeutic approach (53, 54). Like other ERSAs compounds (i.e. thapsigargin, tunicamycin, brefeldin A, ...) which are under investigation for anticancer applications in clinics (46), *Juglone* may represent a new agent whose cancer therapeutic efficacy should be considered. Further studies will be required to fully understand its mechanism of action and to enable selective and target-specific drug delivery, in keeping with what it has been shown for similar compounds such as Thapsigargin (55), thus reducing drug-related toxicities. In this scenario, *Juglone* efficacy could be further increased via

combination with Notch3 inhibitors, as we showed that Notch3 silencing in TALL-1 cells amplifies the ER stress associated pro-apoptotic effects of the *Juglone*, thus resulting in antitumor synergy effects with potential lower toxicity. Moreover, since we demonstrated the importance of Notch3 in T-ALL progression (15), confiming the same role observed also in solid tumors (56, 57), we can speculate that *Juglone*-dependent Notch3 inhibition could be useful also for tumors that do not depend on Notch3 at their onset but that could recur and become more aggressive subsequently, due to a selective growth advantage represented by the Notch3-dependent UPR maintenance. In this view, further studies are required in order to potentially identify and extend the subpopulation of Notch3-overexpressing tumors where the aggravation of ER stress plus Notch3 depletion, *Juglone*-induced, could be particularly beneficial.

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Author Contributions

SC, MVG, GD and EG designed and performed research, analyzed data and wrote the manuscript; GF, LDM, MGP, LT performed research and analyzed data; ZMB, GP, RP, GC, CT, GD, DB analyzed data; MVG, EG, CN performed animal experiments; IS designed research, analyzed data and wrote the manuscript.

Conflict of Interest

The authors declare no competing financial interests.

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Figures Legends

Figure 1. Notch3 silencing favours the response to ER stress in T-ALL

A) Relative cell survival of selected T-ALL cells treated with increasing doses of Tunicamycin for 24 hours.

B-F) Relative cell survival of Notch3-silenced (siN3) or Notch1-silenced (siN1) TALL-1 (B-C), DND41 (D-E) or Molt3 (F-G) cells, treated with increasing doses of Tunicamycin compared with control cells (siCTR) for 24 h.

Data information: Results are shown as the means average deviations of at least three independent experiments and P-values were calculated using Student's T-test at each single dose (i.e. ns, not significant; *P≤0.05; **P≤0.01; ***P≤0.001).

Figure 2. Notch3 modulation in Tunicamycin-treated TALL-1 cells induces UPR defect by decreasing IRE1α expression

A) Western blot analysis of Notch3-silenced TALL-1 cells treated with Tunicamycin (Tun) for 24 hours showed that drugs synergized in increasing GRP78/Bip expression but not in IRE1α expression.

B) Control or anti-IRE1α immunoprecipates from the same cells used in (A) were probes with an anti-Ubiquitin (Ub) and anti-IRE1α antibodies to detect the Ubiquitination *status* of IRE1α and IRE1α immunoprecipitated protein levels, respectively. Proteasomal inhibition with MG132 (30μM for 5 hours) was used.

C) Control or anti-GRP78/Bip immunoprecipates from TALL-1 cells were subjected to western blot and probes with anti-IRE1α and anti-$N3_{IC}$ antibodies to analyse the endogenous GRP78/Bip-IRE1α and GRP78/Bip-Notch3-IC interactions, respectively. The blot with anti-GRP78/Bip antibody was used to detect the GRP78/Bip immunoprecipitated protein levels. The input lanes indicated in all the western blot of the panels (B) and (C) show 5% of total lysate.

Data information: All data are representative of at least three independent experiments, each in triplicate.

D) Graphs showing correlation between Notch3 and IRE1α gene expression levels obtained by an *in silico* analysis described in Material and Methods Section, in human T-ALL cell lines (left) and in a cohort of 53 T-ALL patients (right).

Data information: In both graphs, each dot corresponds to one cell/patient and the expression value of Notch3 and IRE1α is given in log2 scale after normalizing data of T-ALL patients with the MAS5.0 algorithm and after custom normalization of T-ALL cell lines data. The X-Y axis represent Notch3 and IRE1α expression levels, respectively. The index Pearson R indicated express the linear relation between paired samples and P-values were calculated using Student's T-test.

Figure 3. *Juglone* induces cytotoxic effects in Notch3-overexpressing TALL-1 cells via apoptosis, ER-associated

A) Cell count of TALL-1 cells treated with increasing doses of *Juglone* for 24 hours (h) (IC_{50}: 1,8).

B) Cell count of TALL-1 cells treated with a fixed dose (2,5μM) of *Juglone* for the times indicated.

C) Flow cytometric analysis of Annexin V-APC/PI-stained TALL-1 cells treated with a fixed dose (2,5μM) of *Juglone* for the times indicated (h). The percentages of early apoptotic cells (Annexin V-APC+/PI-, bottom right quadrant) and late apoptotic/necrotic cells (Annexin V-APC+/PI+, top right quadrant) are indicated. ctr, untreated cells.

D-E) Relative mRNA expression (D) and nuclear protein expression (E) of CHOP after *Juglone* treatment of TALL-1 cells for the time indicated. Anti-LaminB was used as a nuclear fraction marker.

Data information: All data are representative of at least three independent experiments, each in triplicate.

Figure 4. *Juglone* treatment modulates ER stress/UPR signaling by recapitulating the combined Notch3 downregulation under ER stress conditions

A) Western blot analysis showing the time-dependent modulation of Notch3-IC ($N3_{IC}$), GRP78/Bip and IRE1α protein expression after *Juglone* treatment (2,5μM) of TALL-1 cells.

B) RT-PCR analysis of XBP1 mRNA derived from TALL-1 cells described in A). u, unspliced XBP1; s, spliced XBP1; pos ctr: positive control, cells treated with Thapsigargin for 24 hours.

C) Control or anti-GRP78/Bip (Bip) immunoprecipates from TALL-1 cells treated or not with *Juglone* were subjected to western blot and probes with anti-IRE1α and anti-GRP78/Bip antibody to detect endogenous GRP78/Bip-IRE1α and GRP78/Bip immunoprecipitated protein levels, respectively.

D) Control or anti-IRE1α immunoprecipates from the same cells used in c) were probes with an anti-Ubiquitin (Ub) and anti-IRE1α antibodies to detect the Ubiquitination *status* of IRE1α and IRE1α immunoprecipitated protein levels, respectively. In both panels C) and D), proteasomal inhibition with MG132 (30μM for 5 hours) was used. The input lanes indicated in all the western blot of the panels (C) and (D) show 5% of total lysate. In all panels (A), (C) and (D) the anti–β-actin was used as a loading control.

Data information: All data are representative of at least three independent experiments, each in triplicate.

Figure 5. Notch3 silencing contributes to amplify *Juglone*-dependent ER-stress associated apoptosis

A) Flow cytometric analysis of Annexin V-APC/PI-stained TALL-1 cells, Notch3-silenced for 72 hours and treated for the last 24 hours with *Juglone* showed that the combined treatment was more effective in inducing apoptosis than single treatments. The percentages of early apoptotic cells (Annexin V-APC+/PI-, bottom right quadrant) and late apoptotic/necrotic cells (Annexin V-APC+/PI+, top right quadrant) are indicated. siCTR, cells treated with scramble siRNA; siN3,

Notch3-silenced cells; Jug, *Juglone*; siN3+Jug: combined samples. Data shown are representative of three independent experiments performed in triplicate.

B) Relative cell survival of TALL-1 cells derived from the experiments described in A) with Notch3 silencing prolonged for 96 hours.

C) Western blot analysis of Notch3-silenced TALL-1 cells treated for the last 24 hours with *Juglone* showed that the Notch3 silencing synergized both in increasing GRP78/Bip expression and in decreasing IRE1α expression. Anti–β-actin was used as a loading control.

D) Relative CHOP mRNA expression derived from TALL-1 cells described in C).

Data information: For panels (B) and (D) results are shown as the means average deviations of at least three separate experiments and P-values were calculated using Student's T-test (i.e., ns, not significant; **P≤0.01; ***P≤0.001).

Figure 6. *Juglone* reduces tumor burden in mice bearing TALL-1_luc cells

A) Outline of treatment with *Juglone* in NSG mice bearing TALL1_luc cells subcutaneously injected at day 0. Mice received two intravenous injections (i.v.) of *Juglone* (1mg/kg) or vehicle (CTR) at days 21^{th} and 23^{th}. Mice were sacrificed at day 25^{th}.

B) Tumors were then harvested, fixed in formalin and analyzed by immunohistochemical staining with the antibodies for CD45 (upper panels), Notch3 (middle panels) and CHOP (lower panels).

C) Percentages of positive cells for the indicated stainings described in B). Results are shown as the means average deviations of three separate experiments and P-values were calculated using Student's T-test (i.e., ns not significant P>0,05; *P≤0.05; ***P≤0.001).

D) Outline of treatment with *Juglone* in NSG mice bearing TALL1_luc cells intravenously injected (i.v.) at day 0. Mice received intraperitoneal injections (i.p.) of *Juglone* (1mg/kg) or vehicle (CTR) every two days, starting from day 16^{th} until day 38^{th}. Mice were sacrified at day 40.

E-F) Tumor size were monitored with the Xenogen *in vivo* imaging system, as reported in Material and Methods section. Representative images (E) and quantitative analysis (F) of luciferase activity

in mice treated with *Juglone* or vehicle alone (CTR) at days 15th, 24th and 38th from TALL-1_luc injection are shown. Statistically significant differences in average radiance in the two groups of samples are indicated at day 38th. P-values were calculated using Student's T-test (i.e., ***P≤0.001).

Figure 7. Schematic diagram to summarize our working model

Under ER stress conditions, IRE1α can be activated through the release of the known inhibitory GRP78/Bip-IRE1α binding, thus leading to the UPR activation (i.e. increased expression of the master gene GRP78/Bip), which can result in tumor survival or tumor death.

A) A tumor cell overexpressing Notch3 under ER stress conditions: Notch3 interacts with GRP78/Bip thereby sustaining IRE1α constitutive activation which contributes to favour a pro-survival UPR, finally resulting in maintaining T-ALL cell growth.

B) A tumor cell treated with Notch3 blocking agents under ER stress conditions (i.e. *Juglone*): the absence of Notch3 lets the overexpressed GRP78/Bip free to interact with IRE1α, thereby promoting IRE1α ubiquitination and inactivation, thus contributing to the switch to a pro-apoptotic UPR through increasing CHOP levels and finally resulting in T-ALL cell death.

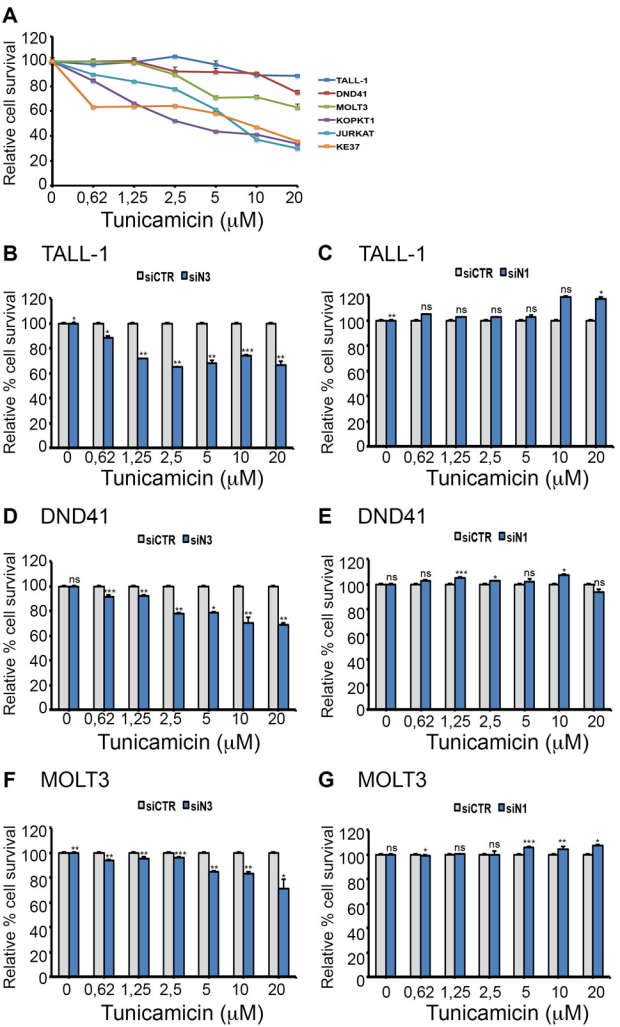


Figure 1 Giuli et al

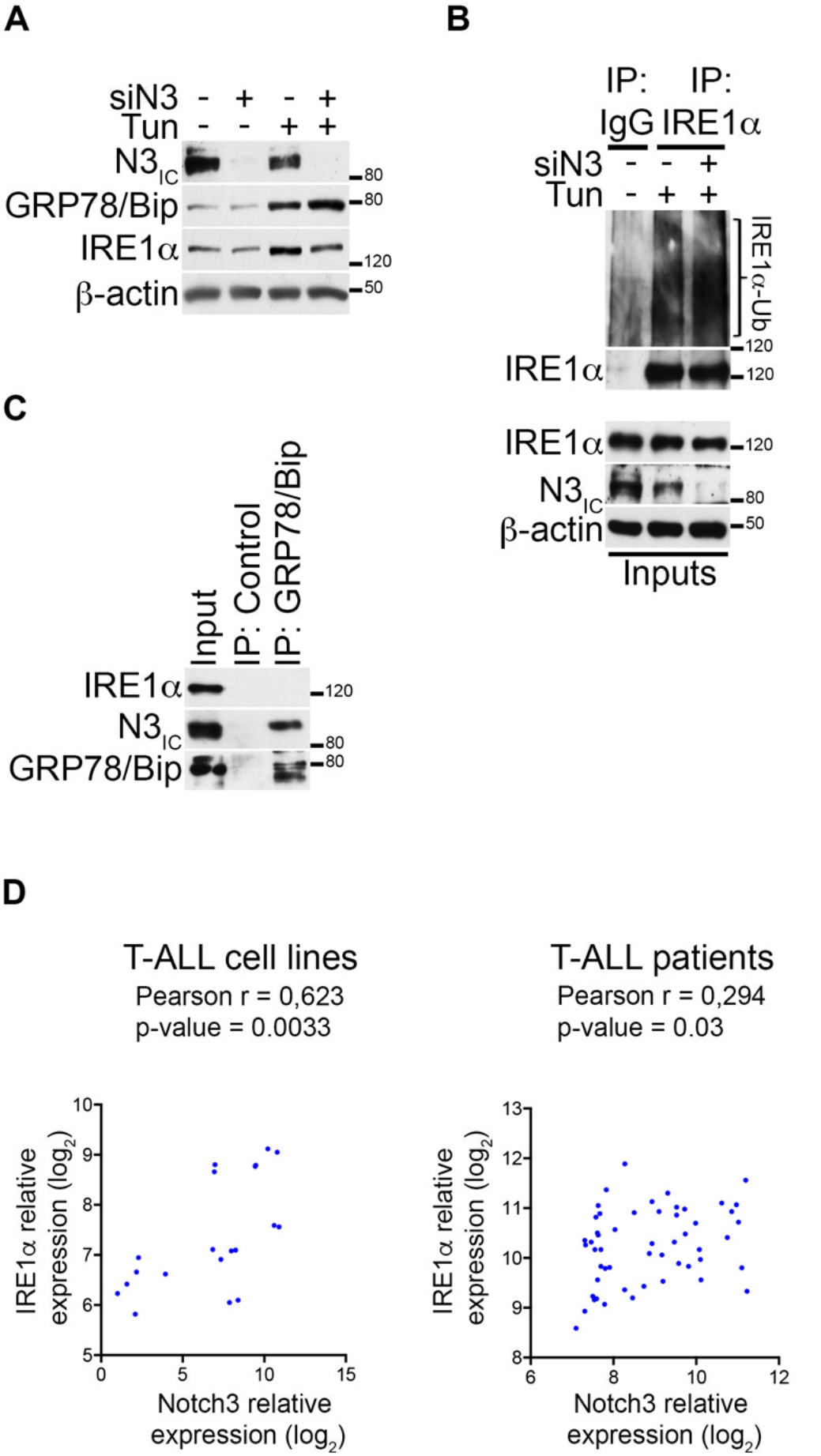


Figure 2 Giuli et al

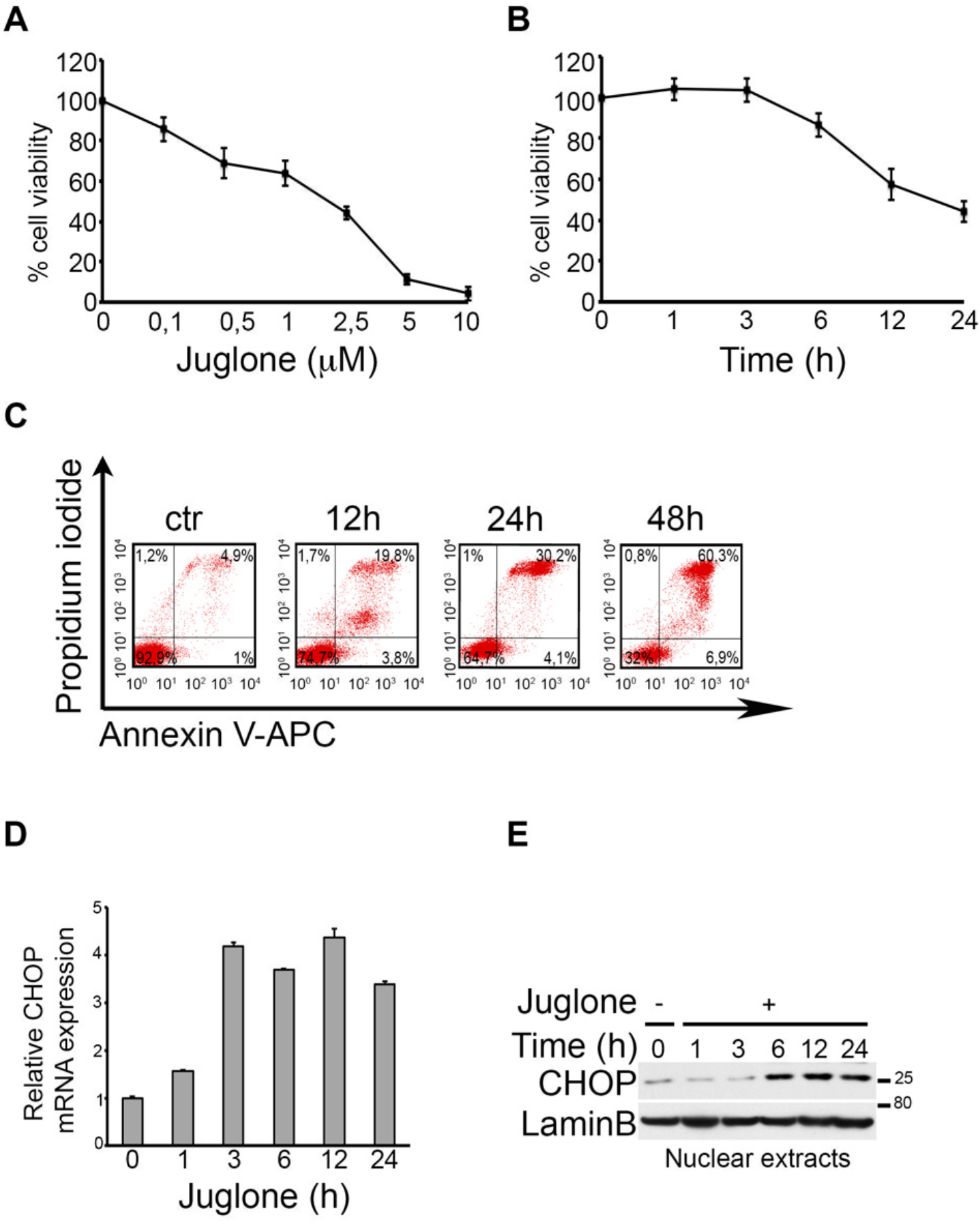


Figure 3 Giuli et al

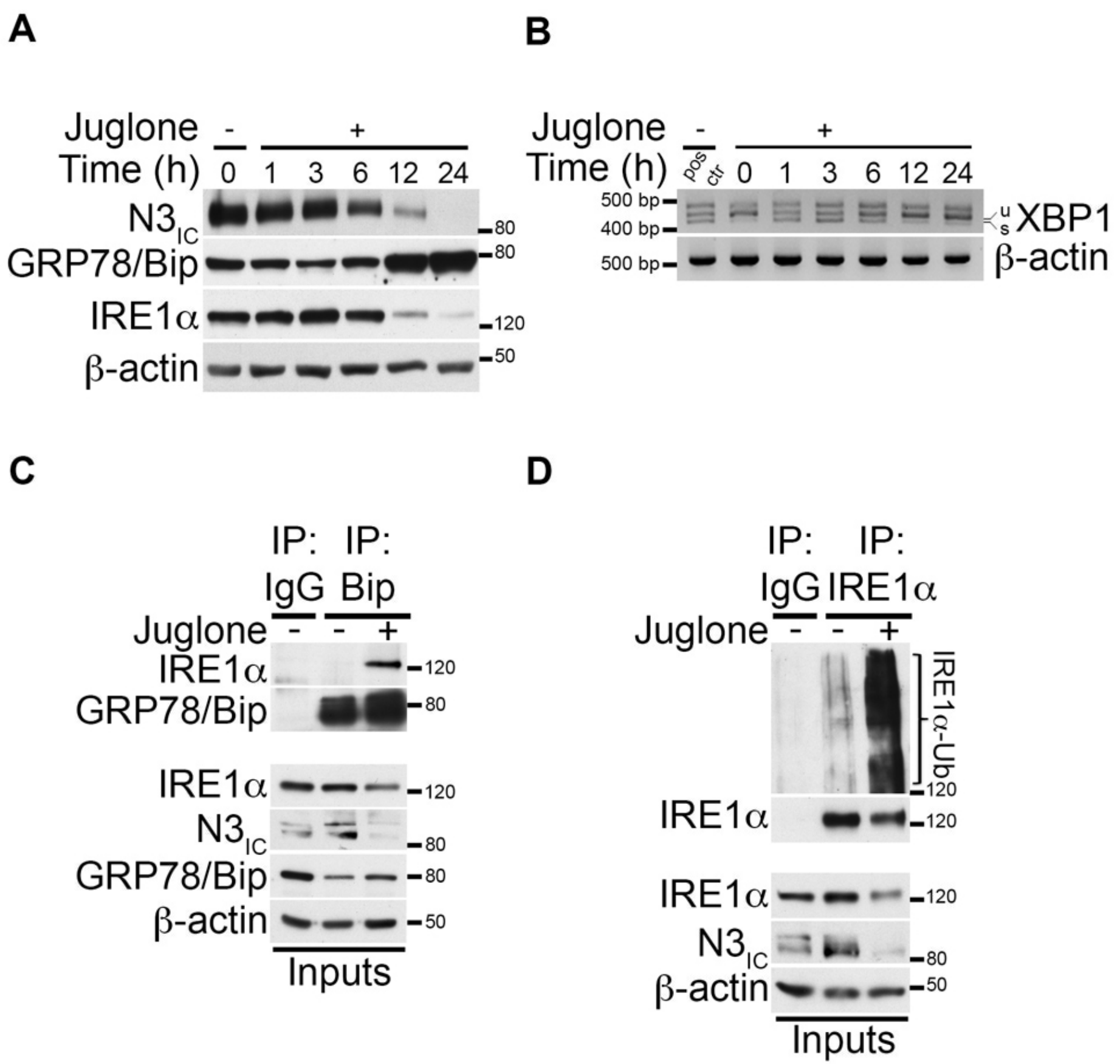


Figure 4 Giuli et al

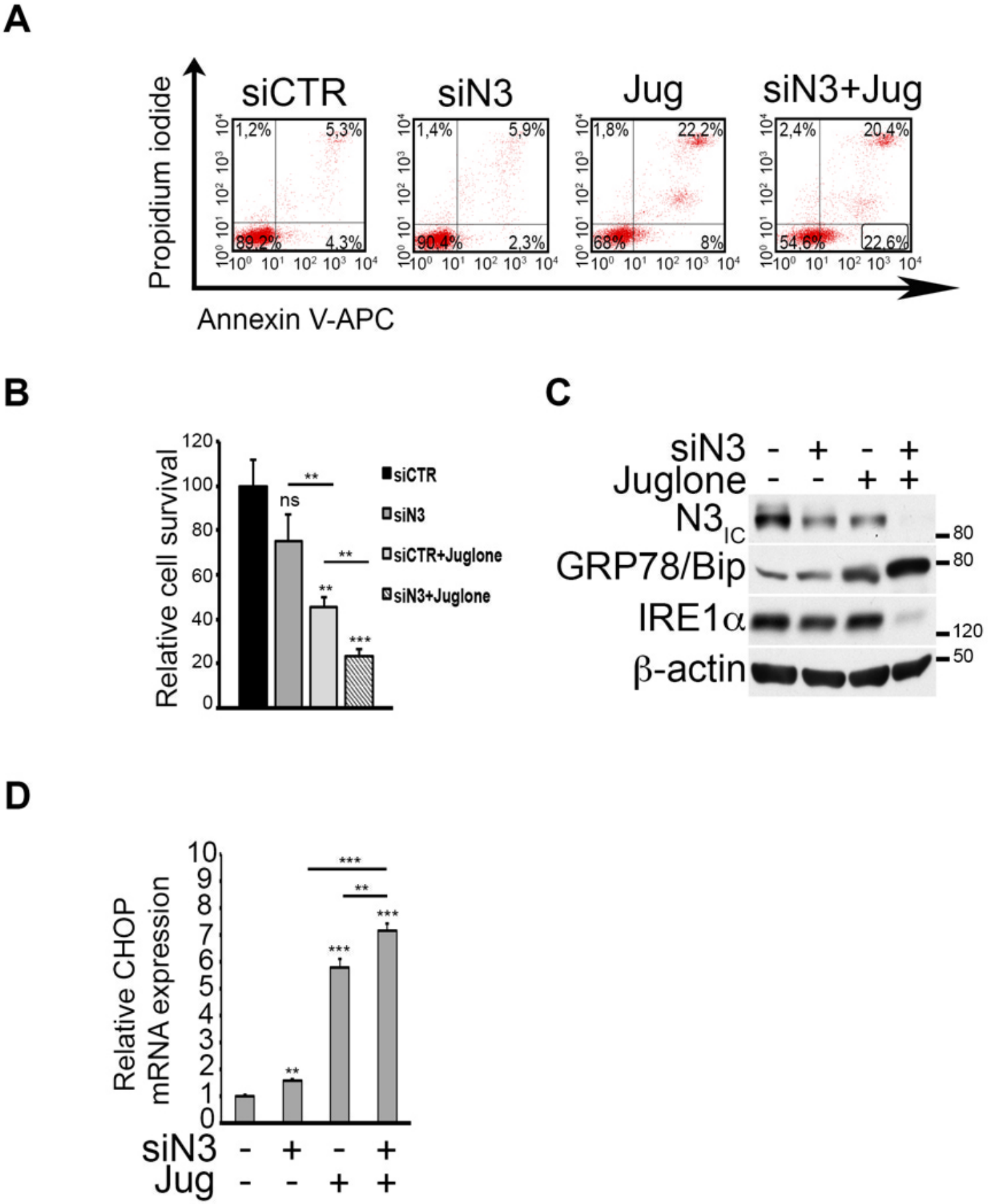
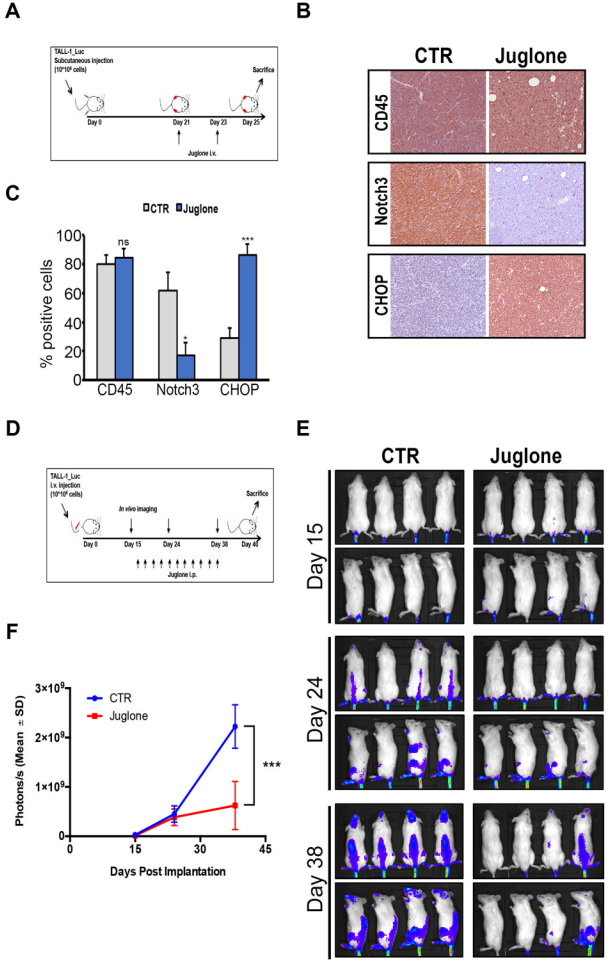


Figure 5 Giuli et al



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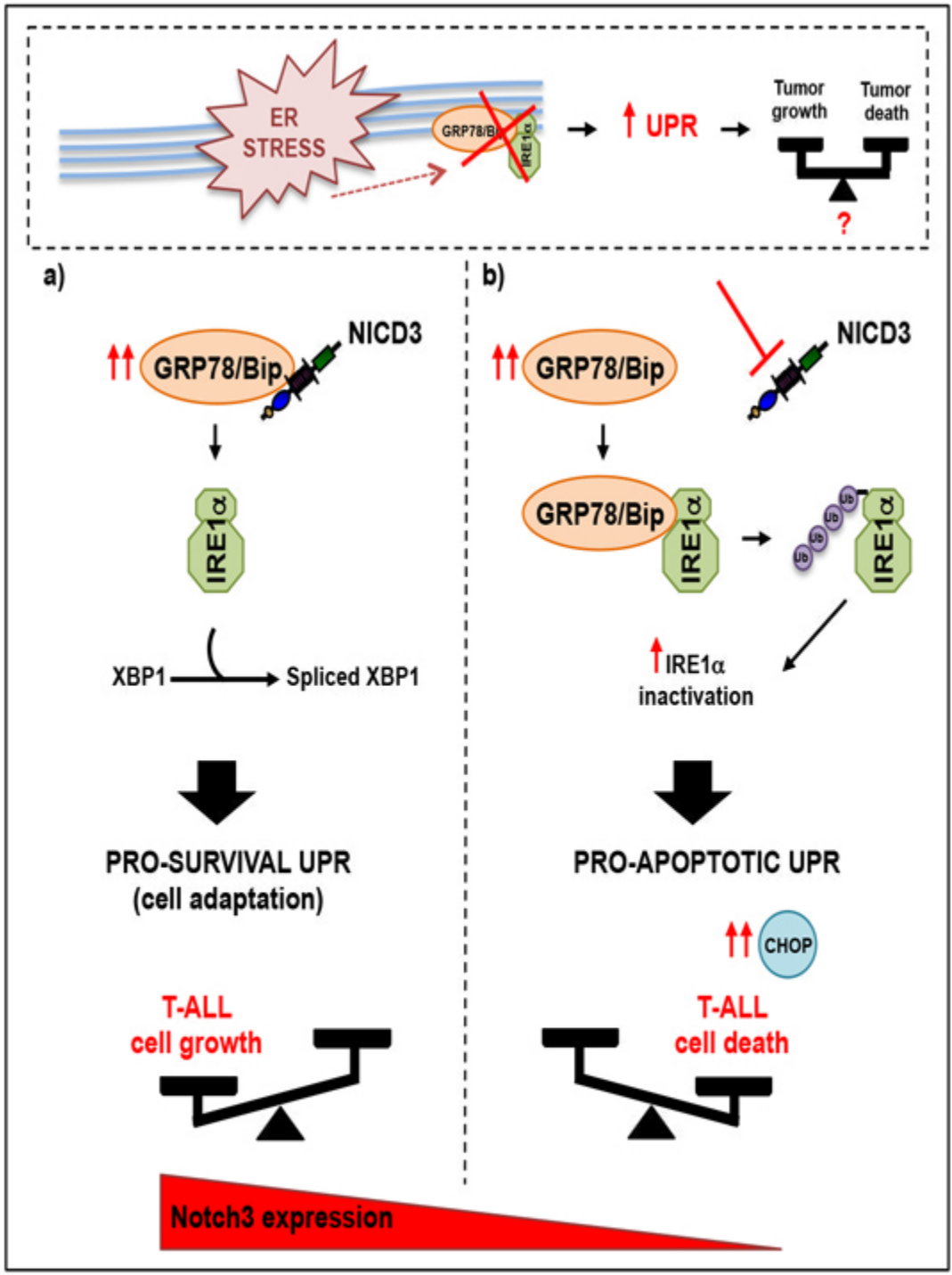


Figure 7 Giuli et al

In the Supplementary Informations there are included the following files:

- Supplementary Table S1 and Legend
- Supplementary Table S2 and Legend
- Supplementary Figure S1
- Supplementary Figure S2
- Supplementary Figure S3
- Supplementary Figure S4
- Supplementary Figures Legends
- Supplementary Materials and Methods

Table S1

Gene (human)	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
β-actin	CTACAATGAGCTGCGTGTGG	CGGTGAGGATCTTCATGAGG
CHOP	TGGAAATGAAGAGGAAGAATCAAAA	CAGCCAAGCCAGAGAAGCA
GAPDH	TGCACCACCAACTGCTTAG	GAGGCAGGGATGATGTTC
GRP78/Bip	CAATCAAGGTCTATGAAGGTGAAAGA	CACATCTATCTCAAAGGTGACTTCAATC
XBP1	CCTTGTAGTTGAGAACCAGG	GGGGCTTGGTATATATGTGG

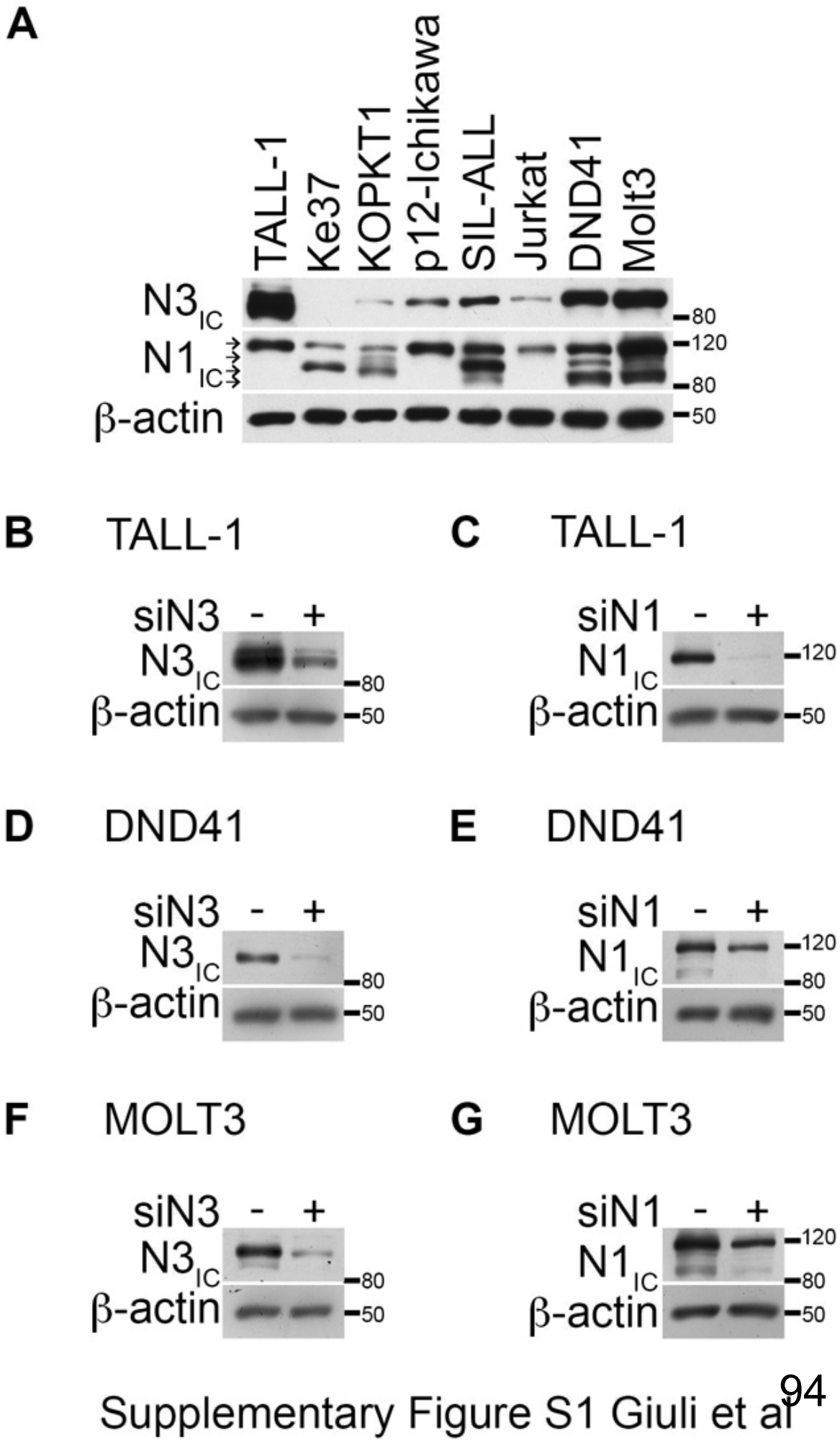
Supplementary Table S1. Primers sequences used for *mRNA* analysis of the indicated gene expression levels.

Table S2

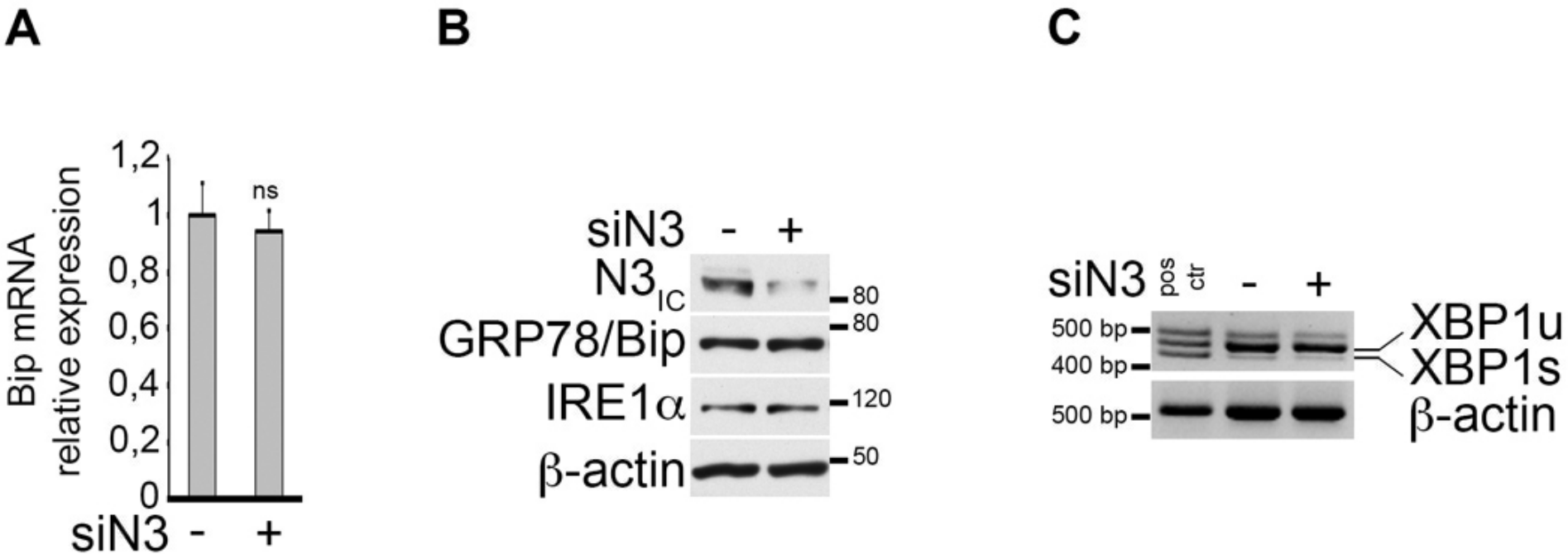
Cell Lines of Van Vlierberghe _Cellines dataset
SIL-ALL
ccrf-cem
ctv1
cutll1
DND41
hpball
KOPKT1
loucy
rpmi8402
TALL-1

Supplementary Table S2. T-ALL cell lines used for *in silico* analysis of Notch3 and IRE1α gene expression levels.

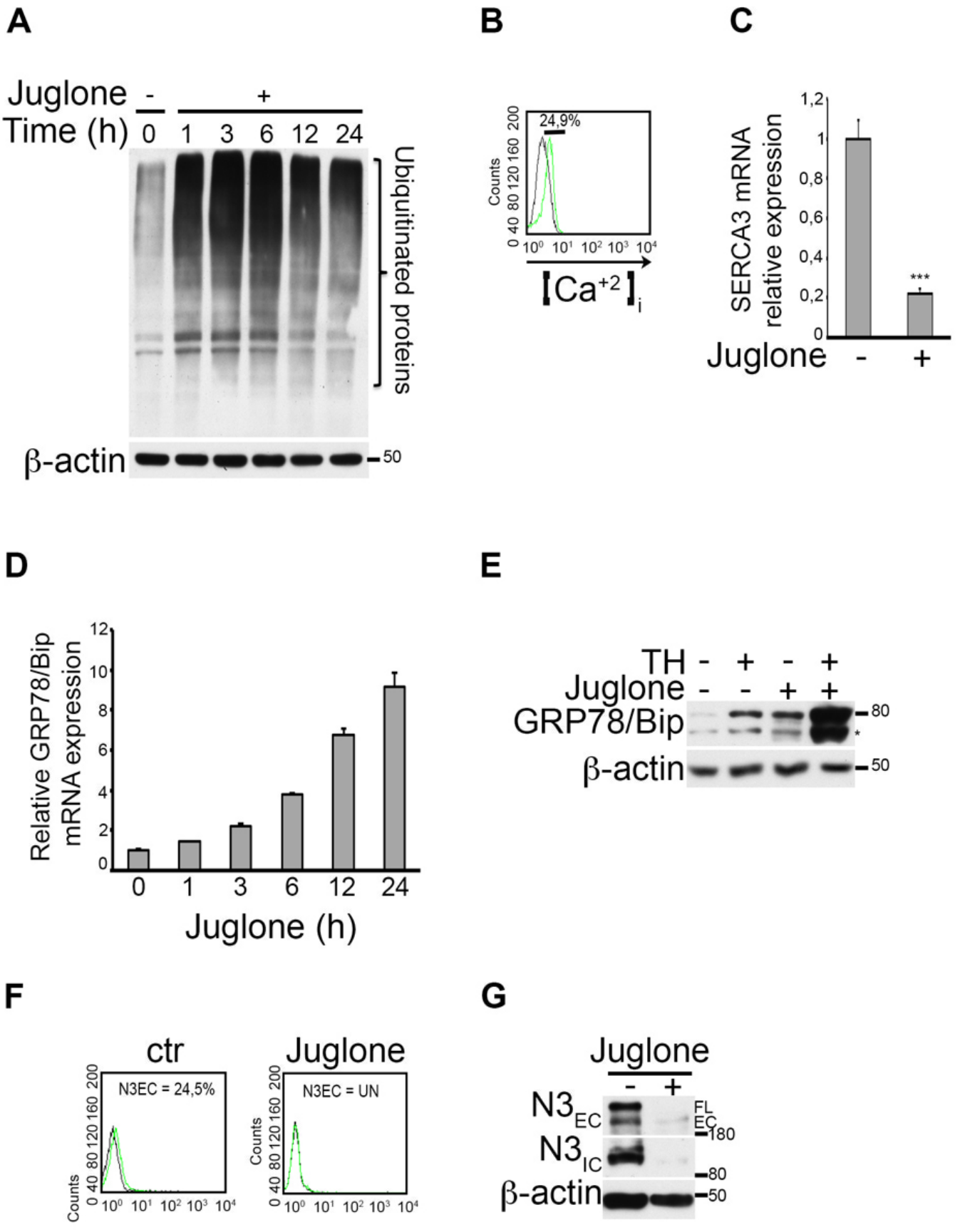
Table shows the T-ALL cell lines (dataset from Van Vlierberghe P. et al, ref#28 of the manuscript) used for *in silico* analysis shown in the Figure 2D (left panel).



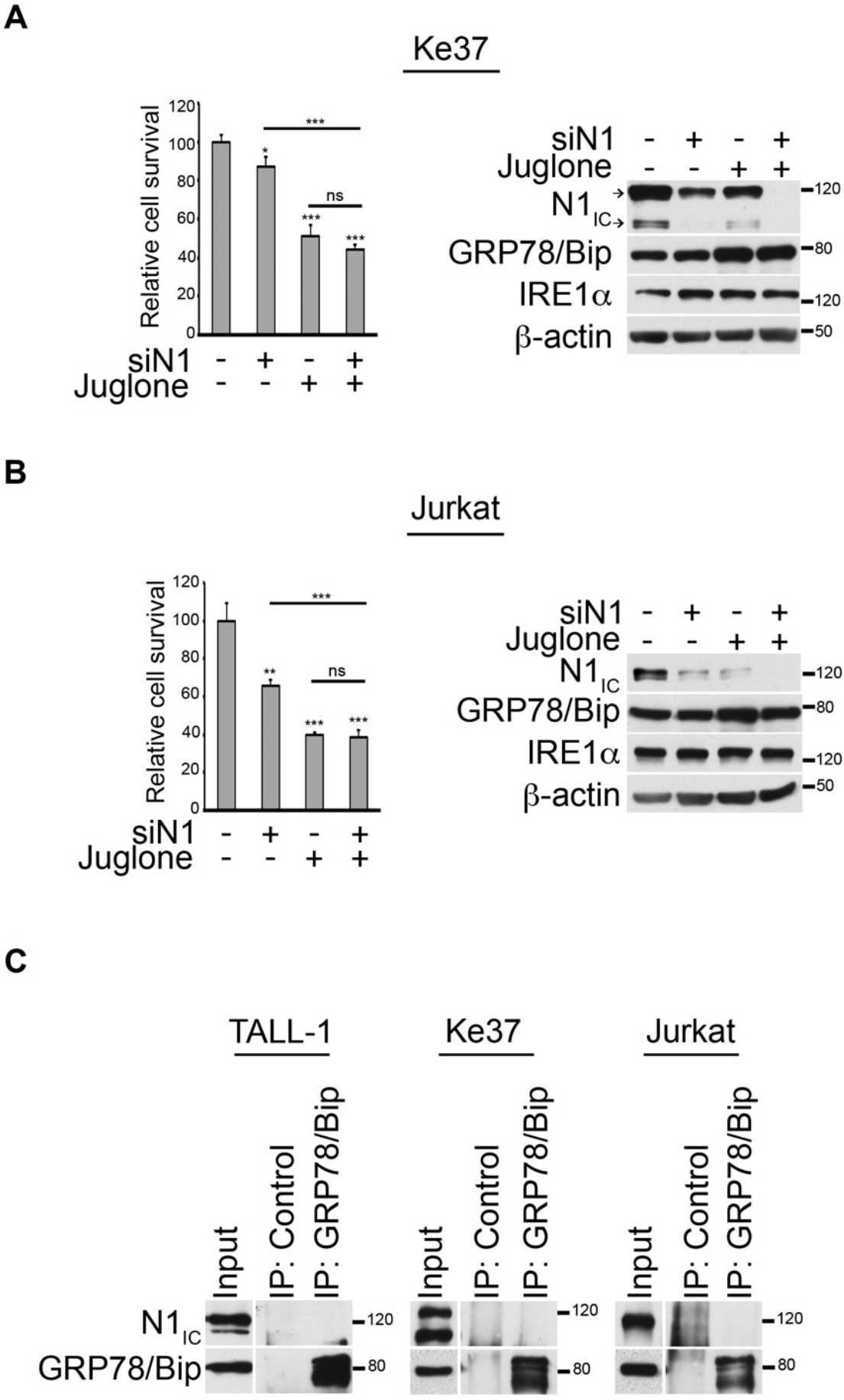
Supplementary Figure S1 Giuli et al



Supplementary Figure S2 Giuli et al



Supplementary Figure S3 Giuli et al



Supplementary Figure S4 Giuli et al

Supplementary Figure S1. Differential Notch1 and Notch3 expression in T-ALL cell lines

A) Western blot analysis of Notch3 ($N3_{IC}$) and Notch1 ($N1_{IC}$) on a subset of T-ALL selected cells (TALL-1; Ke37; KOPKT1; p12-Ichikawa; SIL-ALL; Jurkat; DND41; Molt3).

B-G) Representative Western blot of Notch3 ($N3_{IC}$) and Notch1 ($N1_{IC}$) on TALL-1 (B,C), DND41 (D,E) and Molt3 (F,G) cells after 20μM Tunicamycin treatment shown in the Figures 1B-1G, to control the quality of Notch silencing.

Supplementary Figure S2. Notch3 silencing alone does not influence the ER stress/UPR markers expression

A-C) With respect to control cells, Notch3-silenced TALL-1 cells (siN3) showed A) similar mRNA expression levels of GRP78/Bip, B) similar protein expression of GRP78/Bip and IRE1α, C) similar splicing of XBP1 mRNA. u, unspliced XBP1; s, spliced XBP1; pos ctr: positive control, cells treated with Thapsigargin for 24 hours.

Data information: For panel (A) results are shown as the means average deviations of at least three separate experiments and P-values were calculated using Student's T-test (i.e., ns, not significant).

Supplementary Figure S3. *Juglone* simultaneously induces ER stress and downregulates Notch3 through different mechanisms

A) Time course analysis of *Juglone* treatment (2,5μM) on TALL-1 cells showing the fast increase of poly-ubiquitinated proteins.

B-C) *Juglone* treatment (2,5μM) of TALL-1 cells and analysis of B) intracellular calcium concentration, $[Ca^{+2}]_i$ and C) mRNA levels of SERCA3, as described in the Material and Methods section.

D) Relative mRNA expression of GRP78/Bip after *Juglone* treatment of TALL-1 cells for the time indicated (h).

E) Western blot analysis of TALL-1 cells treated for 24 hours with *Juglone* and Thapsigargin (TH), either alone or in combination, showed that the combined treatment synergized in increasing GRP78/Bip. Anti–β-actin was used as a loading control. * a-specific band?

F) Cytofluorimetric analysis of Notch3 extracellular expression ($N3_{EC}$) from TALL-1 cells after treatment with *Juglone*, indicated as percentages inside each quadrant. The black curve represent the isotypic control. Ctr, untreated cells.

G) Western blot analysis of extracts from the same TALL-1 cells described in F) and probed with anti-Notch3-EC ($N3_{EC}$) and anti-Notch3-IC ($N3_{IC}$) antibodies to evaluate the Notch3 protein expression after drug treatment. The β-actin expression was used as loading control. FL: Notch3 full-lenght (280Kda); EC: Notch3 extracellular (220Kda).

Data information: All data are representative of at least three independent experiments, each in triplicate. Results are shown as the means average deviations and P-values were calculated using Student's T-test (i.e. ***P≤0.001).

Supplementary Figure S4. Combined Notch1 silencing during *Juglone* treatment does not influence the *Juglone*-dependent ER-stress associated apoptosis

A-B) Notch1-silenced Ke37 (A) and Jurkat (B) cells treated treated for the last 24 hours with *Juglone* showed that the combined treatment was not more effective in inducing cell death than single *Juglone* treatments (left panels, relative cell survival analysis) and in increasing GRP78/Bip expression and in decreasing IRE1α expression (right panels, western blot analysis). Anti–β-actin was used as a loading control.

Data information: For left panels (A) and (B) results are shown as the means average deviations of at least three separate experiments and P-values were calculated using Student's T-test (i.e. ns, not significant; *P≤0.05; **P≤0.01; ***P≤0.001).

C) Control or anti-GRP78/Bip immunoprecipates from TALL-1 (left panels), Ke37 (middle panels) and Jurkat (right panels) cells were subjected to western blot and probes with anti-$N1_{IC}$ antibody to analyse the endogenous GRP78/Bip-Notch1-IC interaction. The blot with anti-GRP78/Bip antibody

was used to detect the GRP78/Bip immunoprecipitated protein levels. The input lanes show 5% of total lysate.

Data information: All data are representative of at least three independent experiments, each in triplicate.

Supplementary Materials and Methods

Intracellular Ca^{+2} measurement. To measure the intracellular Ca^{2+} levels after 24 hours of *Juglone* treatment, cells were stained with Fluo-3-acetoxymethyl ester (Fluo-3/AM; LifeTechnologies) through two subsequent steps: loading and de-esterification. At the end of treatment, cells were harvested, counted and re-suspended at a density of 10^6 cells/ml in 10 μM Fluo-3/AM solution, then incubated at 37 °C for 30 minutes (to allow the ester loading). Then, cells were centrifuged, washed twice in PBS, and incubated at 37°C for further 30 minutes in PBS alone (to allow-de-esterification). Cells were then analysed and fluorescence was measured by FACSCalibur flow cytometer and the CellQuest Software (Becton-Dickinson).

***In silico* analysis of T-ALL cell lines and patients deposited data.** Bone marrow lymphoblast samples from a group of 20 human T-ALL cell lines [Van Vlierberghe, 2011, ref#28 of the manuscript] and 53 T-ALL patients (Van Vlierberghe, 2013, ref#1 of the manuscript) were analyzed for the correlation between Notch3 and IRE1a using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). The expression values of Notch3 and IRE1α were filtered in each analysis utilizing the expression probe sets ILMN_1658926 for T-ALL patients and 203238_s_at for T-ALL cell lines representing Notch3 and the expression probe sets ILMN_1698404 for T-ALL patients and 227755_at for T-ALL cell lines representing IRE1a. The expression value of Notch3 and IRE1α is given in $\log_2$ scale after normalizing data of T-ALL patients with the MAS5.0 algorithm and after custom normalization of T-ALL cell lines data. The index Pearson R expresses the linear relation between paired samples and was calculated with the use of GraphPad 6 (La Jolla, California). P-values were calculated using Student's T-test.

3.2 POST-TRANSLATIONAL MODIFICATIONS OF NOTCH3

3.2.1 Introduction

Globally, ovarian cancer (OC) is one of the most commonly diagnosed cancer in women worldwide **(Siegel RL et al., 2016)**. It is considered the most lethal gynecologic malignancy because OC patients are initially asymptomatic and they are ultimately diagnosed when cancer metastases are already highly distributed **(Matulonis UA et al., 2016).** In the main, patients with OC primarily undergo cytoreductive surgery followed by platinum- and taxane-based chemotherapy **(Armstrong DK et al., 2006).** In spite of the promising initial response rates, OC is characterized by high incidence of recurrence after the first-line chemotherapy, thus contributing to decrease the five-year survival rate (approximately 40%) **(Armstrong DK et al., 2006).** Therefore, the identification and characterization of targets for novel therapies is urgently required.

Notch signaling is evolutionary conserved in all metazoan and its deregulation has been implicated in the development of several diseases, including cancer **(Aster JC et al., 2017).** Genomic analyses of TCGA data have disclosed that Notch pathway is frequently altered in OC **(Bell D et al., 2011).** Specifically, the over-expression of Notch3 (N3), coincident with gene locus amplification, has been detected in a wide panel of OC **(Park JT et al., 2006)**. Park and colleagues demonstrated that N3-overexpressing OC cell lines are molecularly dependent on N3 for cellular growth and survival **(Park JT et al., 2006)**. In addition, N3 is involved in OC stem cells maintenance tumor resistance **(McAuliffe SM et al., 2012; Park JT et al., 2010)** and recent studies suggested that inhibition of N3 could be exploited to restore chemo-sensitivity **(Li H et al., 2019; Xu Y et al., 2019).** Furthermore, OC is highly aggressive and it is well known that the failure of cancer treatment may be due not only to the development of chemo-resistance but also to the promotion of cancer invasion and metastasis. A growing piece of

evidence strongly suggests that Notch pathway sustains cancer cell seeding to secondary organs, highlighting a novel role in inducing epithelial-mesenchymal transition (EMT), also in ovarian cancer **(Pazos MC et al., 2017).** EMT is a highly conserved cellular program implicated in tumor metastasis and progression **(Culig Z, 2019).** It has been demonstrated that Notch receptors interact with several key factors of EMT, including Snail, Slug **(Espinoza I et al., 2013)**, and MMPs **(Zhou L et al., 2013; Franciosa G et al., 2016).** However, an advanced knowledge of N3 signaling underlying the promotion of EMT is still limited and needs further studies.

To sum up, in the past decades several studies have witnessed that N3 has been playing a pivotal role in the pathogenesis and tumor progression of OC. Therefore, selective targeting of N3 may represent a potential therapeutic approach for OC patients without the known toxicities associated with pan-Notch inhibition (such as gamma-secretase inhibitors, GSIs) **(Shih IeM and Wang TL, 2007)**. In keeping with this observation, specificity should be achieved by using monoclonal antibodies against N3 receptor or by targeting factors that sustain N3 signaling.

In this scenario, we have recently demonstrated that the peptidyl-prolyl isomerase Pin1 positively regulates N3 protein expression and sustains its oncogenic role in T-cell acute lymphoblastic leukemia (T-ALL) aggressiveness and progression **(Franciosa G et al., 2016).**

The isomerase Pin1 is a small and evolutionarily conserved protein **(Lu KP, 1996)** and binds phosphorylated Serines or Threonines residues preceding a Proline (pSer/Thr-Pro), catalyzing the *cis*/*trans* isomerization of peptidyl-prolyl peptide bond **(Schwede T et al., 2003).** Structurally it is possible to distinguish two domains: the amino-terminal WW domain recognizes pSer/Thr-Pro motifs

whereas the carboxyl-terminal prolyl isomerase (PPIase) domain is responsible for the enzymatic reaction **(Lu KP et al., 2002).**

Pin1-catalysed isomerization leads to changes in the conformation of its target proteins, affecting for instance protein-protein interaction and protein stability **(Cheng CW et al., 2016)**. Furthermore, several studies documented that Pin1 is involved in diverse cellular processes, including differentiation, proliferation, and apoptosis **(Zannini A et al., 2019).** Since its pleiotropic role, it is not surprising that an aberrant function has been implicated in tumor initiation and progression **(Zhou XZ and Lu KP, 2016).** Indeed, Pin1 fine-tunes and amplifies several oncogenic pathways, among them Notch signaling **(Rustighi A et al., 2009; Rustighi A et al., 2014; Baik SH et al., 2015).**

Therefore, the major aim of this project is to evaluate whether and how N3 is connected to Pin1 also in ovarian cancer context. Giving new insights of Notch3-Pin1 crosstalk in regulating OC behaviour may support the development of specific targeted therapy and could potentially ameliorate OC current treatments.

3.2.2 Material and Methods

Cells line and treatments

HEK293T (purchased from ATCC), HEK293T-Pin1$^{-/-}$, and HEK293T-Pin1$^{+/+}$ (kindly provided by Dr. Del Sal) were maintained in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% Glutamine. OVCAR3 were cultured in DMEM (Gibco) containing 20% FBS (Gibco), 1% Glutamine (EuroClone, Milan, Italy), 10mM Hepes (EuroClone), 1mM Sodium Pyruvate (Sigma-Aldrich, St. Louis, MO, USA), and 0.001mg/mL Insulin (Sigma-Aldrich). A2780 were maintained in RPMI-1640 (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% Glutamine. SKOV3 were cultured in McCoy's 5A medium (Merck, Kenilworth, NJ, USA) containing 10% FBS (Gibco) and 1% Glutamine (EuroClone). All cell lines are mycoplasma-free.

Cells were treated with different doses (as indicated in some Figures) of CHIR99021 (SML1046-5MG – Sigma-Aldrich), SB-216763 (S3442-5MG - Sigma-Aldrich), All-Trans Retinoic Acid [ATRA (R2625 – Sigma-Aldrich), GSI (565770 - Calbiochem, Darmstadt, Germany), MG132 [Z-L-Leu-D-Leu-L-Leu-al (C2211 – Sigma-Aldrich)], and Cycloheximide (66-81-9 - Sigma-Aldrich) for the times indicated, according to their datasheet's instructions.

Cells were transfected using Lipofectamine 2000 (11668-019 – Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The plasmids used are: pcDNA3-^{FLAG}N3ICD **(Bellavia D et al, 2000)**, pcDNA3-^{HA}GSK-3β (Addgene – Watertown, MA, USA), pcDNA3-^{HA}PIN1$_{wt}$ (**Rustighi A et al, 2009**), pcDNA3-^{MYC}WWP2 (kindly provided by Mary A O'Connell), and pcDNA3-^{HA}Ubiquitin (Addgene). The plasmid pcDNA3-GFP (Addgene) was used to evaluate transfection efficiency.

RNA interference was performed using Lipofectamine RNAiMAX (13778-075 - Invitrogen). The list of siRNAs (Santa Cruz Biotechnology, Dallas, TX, USA) is as

follows: siRNA-Ctrl (sc-37007), siRNA-Notch3 (sc-37135), siRNA-GSK-3β (sc-35527), and siRNA-Pin1 (sc-36230).

Sample preparation and MS-spectrometry analysis

Samples were reduced in 10mM TCEP and alkylated in 20mM CAA before loading them on SDS-PAGE gels. Then, the gels were stained with Colloidal Blue Staining Kit (Invitrogen) according to manufacturer's instructions. After the staining, the bands corresponding to Notch3-FLAG molecular weight were excised, destained, and further in-gel digested with Trypsin (Sigma-Aldrich), Chymotrypsin (Promega, Madison, WI, USA), rAspN (Promega) [1:100 (v/v), 25 mM ammonium bicarbonate, pH 8.0, overnight digestion at 37°C] or Pro-Ala (Promega) [1:50 (v/v), HCl pH 1.5, 2 hours digestion at 37°C]. Protease activity was quenched by acidification with trifluoroacetic acid (TFA) and the resulting peptides mixture were extracted sequentially with increasing % v/v of acetonitrile (ACN). The ACN was removed by vacuum centrifugation for 40 min at 60°C and the peptide mixture was loaded on homemade Stage-Tips (Stage-Tips are ordinary pipette tips containing very small disks made of beads with reversed phase) with C18 disks.

Peptides were eluted off the Stage-Tips with 40% ACN and 0,1% formic acid (FA). Organic solvents were removed by vacuum centrifugation for 15 min at 60°C and peptides were analyzed using on-line nanoflow LC-MS/MS on a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), which was interfaced with an EASY-nLC system (Proxeon, Odense, Denmark).

Raw MS Data Processing and bioinformatics analysis

Raw LC-MS/MS files were processed and analyzed by MaxQuant software version 1.6.5.0 with Andromeda search engine against the human Uniprot database. Two

missed cleavage sites were allowed for Trypsin, Chymotrypsin, and rAspN digestion whereas five missed cleavages sites were allowed for Pro-Ala digestion. Carbamido-methylation of cysteine was specified as fixed modification while protein N-terminal acetylation, oxidation of methionine, and phosphorylation of Serine, Threonine, and Tyrosine residues were set as variable modifications. Before analyzing the samples, we first checked manually the enrichment of N3ICD and its sequence coverage. The bioinformatics analysis has been performed using the Perseus software v1.6.5.0.

Protein extracts preparation, Immunoprecipitations, Western Blot and Antibodies

For total protein extracts cells were lysed in Lysis buffer [50mM Tris HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 0.5% Triton X-100, 10mM NaF, 1mM Na_3VO_4, 1mM PMFS, 1% Protease inhibitors] and clarified at 13.000 × rpm for 15 min.

FLAG immunoprecipitation was performed using anti-flag M2 affinity gel (A2220 – Sigma-Aldrich) according to datasheet's instructions.

Before immunoblotting, samples were added with β-mercaptoethanol (M6250 - Sigma-Aldrich) and boiled for 5 min. For immunoblotting, protein extracts were run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (1620115 – Biorad, Hercules, CA, USA). Blots were then incubated with several primary antibodies. From Cell Signaling Technology (Beverly, MA, USA): anti-Notch3IC (#2889), and anti-GSK-3β(27C10) (#9315S); from Santa Cruz: anti-Pin1 (sc-46660), anti-Ubiquitin (sc-8017), and anti-GFP(B-2) (sc-9996); from Covance (Princeton, NJ, USA): anti-HA (MMS-101P); from Sigma-Aldrich: anti-β-actin (A5441), anti-FLAG (A8592), and anti-c-MYC (M4439). The blots were then incubated with secondary antibodies HRP conjugated: anti-rabbit (A120-108P - Bethyl Laboratories, Montgomery, TX, USA) or anti-mouse (A90-116P - Bethyl

Laboratories). Bound antibodies were detected with enhanced chemiluminescence (Clarity™ Western ECL kit - Biorad).

RNA Extraction, Reverse Transcription and qPCR analysis

Total RNA was isolated by using TRIzol reagent (15596018 - Invitrogen) according to manufacturer's instructions. Briefly, TRIzol was added to each sample and incubated at room temperature for 5 minutes (min), then 200 μl of chloroform per ml of TRIzol was added to each sample. The samples were then mixed for 30 sec and incubated for further 3-5 min. Samples were centrifuged at 13,000 x rpm for 15 min at 4°C. The upper aqueous phase was carefully collected and equal volume of Isopropyl alcohol was added to it, after what samples were vortexed for 30 sec, and incubated for 10 min at Room Temperature. The samples were centrifuged at 13,000 x rpm for 10 min. The supernatant was discarded out and 1 ml of 70% ethanol was added. The samples were centrifuged at 13,000 x rpm for 5 mins. Supernatant was discarded, after what the same wash was repeated. The samples were dried and RNA pellet was re-suspended in RNase-free water. cDNA was synthetized with SensiFAST™ cDNA Synthesis Kit (BIO-65054 – Bioline, Boston, MA, USA) according to the manufacturer's protocol. TaqMan™ Universal Master Mix (4440040) and Taqman Gene Expression Assays for NOTCH3 (Hs00166432_m1), MMP9 (Hs00957562_m1), MMP2 (Hs01548727_m1), SNAIL1 (Hs00195591_m1), SNAIL2 (Hs00161904_m1), and GAPDH (Hs02758991_g1) were purchased from Applied Biosystems (Foster City, CA, USA). The samples were run on the StepOnePlus Real-Time PCR System (Applied Biosystems). Relative quantification was performed using the comparative CT method ($2^{(-\Delta\Delta CT)}$) method. GAPDH was used for normalization of mRNA levels. Measurements were performed in technical triplicates and figures show the averages ± SEM of at least 2 biological replicates.

Lentiviral transduction

Lentiviral construct encoding the entire human N3ICD fragment [hNICD3 (3xFLAG)-pCDF1-MCS2-EF1-copGFP] and the empty relative control vector have been purchased from Addgene. Viral supernatants were produced by transient transfection of lentiviral vectors and packaging plasmids into HEK293T by Lipofectamine 2000 according to the manufacturer's instructions. 48 hours after transfection, conditioned medium containing lentiviral particles was collected and immediately used for transduction assay. SKOV3 cells to be infected were plated the day before at 1x10^6 per 60mm plate in 4mL of complete medium. 4mL of the conditioned medium was used for each 60mm plate in the presence of 8μg/mL Polybrene (TR-1003-G – Sigma-Aldrich) for two days at 37°C and then it was replaced by fresh medium. After infection, cells were collected and GFP-transduced cells were subjected to Fluorescence-Activated Cell Sorting.

Fluorescence-Activated Cell Sorting

SKOV3 transduced cells were sorted based on GFP expression using a FACSAriaIII (BD Biosciences, Franklin Lakes, NJ, USA) equipped with a 488nm laser and FACSDiva software (BD Biosciences version 6.1.3). Briefly, cells were first gated based on forward and side scatter area (FSC-A and SSC-A), detected in the green fluorescence channel for GFP expression, and isolated based on high GFP levels. Upon sorting an aliquot of the collected cells was checked for purity (purity > 99%).

Matrigel invasion assay

For the invasion assay, 1x10^5 SKOV3-$N3^-$ and SKOV3-$N3^+$ cells were resuspended in McCoy's 5A medium without serum and seeded in the top chamber onto the Matrigel-coated membrane [24-well insert, pore size 8μM; 354480 (Corning Life

Sciences, Corning, NY, USA)] while McCoy's 5A medium (Merck) supplemented with 10% FBS (Gibco) was used for the lower chamber. Then cells were incubated for 24 hours. Cells on the lower surface of the membrane of the top chamber were fixed in 4% paraformaldehyde for 5 minutes at Room Temperature, permeabilized with 100% methanol for 20 minutes at Room Temperature, and stained with Crystal Violet for 2 minutes at Room Temperature. The number of invading cells was counted under a light microscope (magnification, x10; four random field per well).

***In silico* analysis of OC patients deposited data**

Tissue samples from 126 OC patients were analysed for the correlation between Notch3 and Pin1 using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl).

Tissue samples from 527 OC patients were further analysed for the correlation between Pin1 and Notch3 gene expression levels with respect to the phenotypic differentiation of tumor stage.

In all graphs each dot corresponds to one patient and the expression value of Notch3 and Pin1 is given in log2 scale after normalizing data with justRMA algorithm normalization. The X-Y axis represents Pin1 and Notch3 expression levels, respectively. The index Pearson R indicated expresses the linear relation between paired samples and was calculated with the use of GraphPad 6 (La Jolla, CA, USA). P-values were calculated using Student's T-test.

Computational analysis of the amino acid sequence of the intracellular domain of Notch3

All computational analysis were performed by using KinomeXplorer: an integrated platform for kinome biology studies **(Horn H et al., 2014)**.

Statistical Analysis

For all experiments, P-values were determined using Student's t-test and statistical significance was set at $P \leq 0.05$. Results are expressed as mean ± SD from an appropriate number of experiments (at least three biological replicates). Values significance: $*P \leq 0.05$, $**P \leq 0.001$, $***P \leq 0.0001$.

3.2.3 Results

Pin1 affects Notch3 intracellular domain (N3ICD) protein expression in ovarian cancer

We have previously demonstrated that Pin1 increases Notch3 (N3) processing by γ-secretases complex and it is also involved in sustaining N3 intracellular domain (N3ICD) protein stability in T-ALL **(Franciosa G et al., 2016).**

In order to dig deep into the underlying molecular mechanism by which Pin1 regulates N3ICD, we first analyzed ^{FLAG}N3ICD half-life in HEK293T wild type (HEK293T-Pin1$^{+/+}$) and Pin1 knock-out (HEK293T-Pin1$^{-/-}$) in the presence of the protein synthesis inhibitor Cycloheximide (CHX), for 0, 2 and 4 hours. As shown in **Figure 1a,** the absence of Pin1 seemed to destabilize ^{FLAG}N3ICD expression, thus confirming our previous studies. Indeed, the half-life of ^{FLAG}N3ICD was reduced in HEK293T-Pin1$^{-/-}$ cells when compared with HEK293T-Pin1$^{+/+}$.

We would perform rescue experiments in HEK293T-Pin1$^{-/-}$ to evaluate the stability of ^{FLAG}N3ICD in the presence of increasing doses of Pin1.

Subsequently, we decided to translate our studies in an endogenous system by using the ovarian cancer (OC) context, as N3 plays a pivotal role in many hallmarks of this type of cancer **(Park JT et al., 2006; McAuliffe SM et al., 2012; Park JT et al., 2010; Li H et al., 2019; Xu Y et al., 2019).** We first assessed the endogenous N3 mRNA and protein expression levels in three OC cell lines (SKOV3, OVCAR3 and A2780): it is possible to evidence that SKOV3 are N3-negative (N3^{-}) whereas A2780 and OVCAR3 are N3-positive (N3^{+}) (**Figure 1b**).

Among N3^{+} OC cell lines, to easily evaluate a potential modulation of N3ICD, further studies were conducted using OVCAR3 cells which displayed lower expression of N3ICD with respect to A2780 cells.

First of all, we evaluated whether Pin1 affects the endogenous N3ICD protein expression by transient transfection of exogenous Pin1 (^{HA}Pin1) in OVCAR3 cells,

further treated with γ-secretases inhibitor (GSI) for 72 hours to prevent N3 processing which may increase the basal levels of N3ICD. As shown in **Figure 1c**, Pin1 over-expression increased the protein levels of N3ICD. To confirm this data, we next evaluated the effects of endogenous Pin1 knock-down on N3ICD in the presence of GSI for 0, 2 and 4 hours. We obtained unexpected and interesting results: in control cells we observed a differential mobility of the band recognized by the antibody anti-N3ICD. We supposed that it was probably due to the accumulation of post-translational modifications (PTMs) that are involved in its proteasomal degradation, as the use of the proteasome inhibitor, MG132, led to a more evident up-shift. The absence of Pin1 amplified the observed trend, thus suggesting that Pin1 may prevent N3ICD degradation (**Figure 1d**).

Since protein phosphorylation most frequently causes a similar protein mobility up-shift **(Wegener AD and Jones LR, 1984)**, we sought to determine if phosphorylation plays a key role for the N3ICD's mobility shift observed. Our hypothesis is that, starting from an initial phosphorylation event, multiple and subsequent modifications may occur on N3ICD, thus triggering it to proteasomal degradation. We obtained promising results but further investigations are needed to confirm these data.

GSK3-β phosphorylates N3ICD and reduces its protein stability

We have previously demonstrated that N3ICD harbors several Pin1 consensus motifs (phosphorylated Serine or Threonine residue preciding Proline - pSer/pThr-Pro) and that Pin1 is able to directly interact with N3ICD **(Franciosa G et al., 2016)**. Very preliminary co-immunoprecipitation studies conducted using several N3ICD deletion mutants, lacking for one or more regions, showed the Pin1-N3ICD binding involves three different domains of Notch3 protein (ANK, RE/AC, and TAD) (data not shown).

Computational analysis revealed that there are 7 putative Pin1 consensus motifs in these regions (**Figure 2a**). To unravel which Pin1 consensus motifs on N3ICD are phosphorylated, we over-expressed ^{FLAG}N3ICD in HEK293T for 24 hours followed by quantitative Mass Spectrometry (MS) analysis (which I performed during my internship in the laboratory of Dr. Jesper Velgaard Olsen at Novo Nordisk Foundation in Copenaghen), by using 3 biological replicates per each enzyme used (Trypsin, Chimotrypsin, rAspN, and Pro-Ala). FLAG immunoprecipitation assay and in-gel digestion have been performed to reduce the complexity of the samples prior to MS analysis. MS allowed us to analyze the phosphorylation status of N3ICD and various enzymes to ensure the coverage of each site have been thoroughly tested. As shown in **Figure 2a** and **Table1**, we found 4 out of 7 phosphorylated residues.

Therefore, we believed that these 4 residues are involved in the Pin1-N3ICD interaction and we are planning to confirm these data by performing co-immunoprecipitation assays on HEK293T cells co-transfected with ^{HA}Pin1 and different ^{FLAG}N3ICD-mutants that we previously generated by using insertional mutagenesis (Serine residues substitution with Alanine residues), thus impeding phosphorylation and consequently Pin1 binding.

Given that the absence of Pin1 may led to the accumulation of a hypothetical N3ICD phosphorylation form, we performed computational analysis to find whether Pin1 consensus motifs may overlap with kinase consensus motifs. Among all the inspected kinases, the one which mostly recurs in the 4 Pin1 sites previously identified is the GSK3-β kinase.

GSK3-β substrates require a priming phosphorylated residue (Serine or Threonine) at +4 amino acids (C-terminal) to be phosphorylated by GSK3-β kinase in specific Serine/Threonine residues at the N-terminal region, Ser/ThrXXXpSer/pThr (Ser = Serine; Thr = Threonine; X = variable amino acid) **(Xu**

C et al., 2009). As shown in **Figure 2b**, 3 out of 4 Pin1 consensus motifs overlapped with GSK3-β ones.

To investigate whether GSK3-β is able to phosphorylate N3ICD, we co-transfected ^{HA}GSK3-β and ^{FLAG}N3ICD vectors in HEK293T cells for 24 hours, and we treated them with two GSK3-β-inhibitors (CHIR99021 and SB-216763). We used 4 biological replicates per condition and we then performed quantitative MS analysis using the same workflow of the above-described MS analysis. We then evaluated whether there are more phosphorylated Serine or Threonine residues in the presence of GSK3-β and less phosphorylated in the other conditions: as shown in **Figure 2c** we found one specific Serine residue, S367 (if we consider only N3ICD; S2033 if we consider the entire N3 receptor), that is certainly phosphorylated in the presence of GSK3-β kinase.

The aforementioned experiment was conducted digesting overnight the samples with trypsin but we it has been already planned to use the other enzymes listed in **Table1** to cover each sites to eventually find other GSK3-β-dependent phosphorylated residues.

Since it has been demonstrated that GSK3-β kinase is able to regulate N3ICD both in a positive and negative manner **(Hermida MA et al., 2017; Guha S et al., 2011; Kashikar ND et al., 2011),** we performed some experiments to investigate the functional role of GSK3-β on N3ICD.

First, ^{FLAG}N3ICD was co-transfected with increasing amount of ^{HA}GSK3-β for 24 hours in HEK293T cells and we observed a strong decrease of N3ICD levels (**Figure 3a**) that was rescued by the use of proteasome inhibitor, MG132, thus suggesting that GSK3-β is involved in the N3ICD proteasomal degradation (**Figure 3b**).

We then repeated the same experiment which led to the results described in **Figure 3a** but using a time course analysis (0-2-4 hours) with CHX prior to whole

cell protein extraction, in order to focus our attention on the N3ICD stability. As shown in **Figure 3c - left panel**, N3ICD half-life is shorter in the presence of GSK3-β, in a dose-dependent manner. **Figure 3c – right panel** shows the relative quantification as determined by optical densitometry (OD) only for the highest dose that we decided to choose for the following experiments.

Indeed, to confirm the obtained results, we performed the same time course analysis with CHX on HEK293T cells co-transfected with both ^{FLAG}N3ICD and ^{HA}GSK3-β plasmids for 24 hours and treated for 1 hour with the same GSK3-β-inhibitors previously mentioned for MS studies (CHIR99021 and SB-216763). As expected, ^{HA}GSK3-β decreased ^{FLAG}N3ICD stability that was rescued in the presence of both GSK3-β-inhibitors (**Figure 3d**). The efficiency of the treatments was evaluated by analyzing the protein expression levels of β-catenin, a known target of GSK3-β (data not shown).

Thus, our *in vitro* studies performed by using exogenous systems revealed that the GSK3-β kinase is able to phosphorylate N3ICD and to negatively regulate its expression.

Subsequently, we decided to translate our studies in the same ovarian cancer context used before **(Figures 1b-d**). OVCAR3 cells were subjected to RNA interference for GSK3-β kinase (siGSK) for 72 hours: the absence of GSK3-β led to increased N3ICD protein expression levels (**Figure 3f**) and a longer own half-life with respect to cells interfered by a negative control (siCTR) (**Figure 3e**).

Therefore, these preliminary results documented that GSK3-β kinase is able to negatively regulate N3ICD also in ovarian cancer.

WWP2 E3-ligase is involved in the GSK3-β-dependent proteasomal degradation of N3ICD

Recently, it has been demonstrated that E3-ligase WWP2 is a negative regulator of N3ICD in ovarian cancer (**Jung JG et al., 2014**) and we hypothesized that it could be involved in GSK3-β-dependent proteasomal degradation.

We first assessed the interaction between ^{FLAG}N3ICD and ^{MYC}WWP2 proteins by bi-directional co-immunoprecipitation assay on HEK293T cells previously co-transfected with the relative plasmids (**Figure 4a, Upper panel: IP Flag, blot Myc; Lower panel: IP Myc, blot Flag**).

We then evaluated whether ^{MYC}WWP2 is functionally active on ^{FLAG}N3ICD: as shown in **Figure 4b**, WWP2 is able to induce the poly-ubiquitination of N3ICD thus sustaining its involvement in N3ICD degradation.

Therefore, we tested if the presence of WWP2 could amplify the previously described negative regulation of GSK3-β on N3ICD (**Figure 3**). As shown in **Figure 4c**, co-transfection of ^{HA}GSK3-β and ^{MYC}WWP2 plasmids in HEK293T cells induced higher decrease of ^{FLAG}N3ICD protein expression with respect to the presence of the ^{HA}GSK3-β alone. In addition, we also demonstrated that the presence of GSK3-β increased the interaction between ^{MYC}WWP2 and ^{FLAG}N3ICD (**Figure 4d**). This observation is consistent with an increase of N3ICD poly-ubiquitination occurred upon the combined presence of WWP2 and GSK3-β proteins with respect to the presence of the kinase alone (**Figure 4e**).

Taken together, these findings strongly supported that the E3-ligase WWP2 is implicated in the GSK3-β-dependent proteasomal degradation of N3ICD.

Pin1 and GSK3-β proteins acts on N3ICD but with different outcome

The obtained results strongly suggested the possibility of an antagonistic effect of Pin1 and GSK3-β proteins on N3ICD, acting as positive (Pin1) (**Figure 1**) or negative regulator (GSK3-β) (**Figure 3**), respectively.

In order to further validate this hypothetical antagonism, we first analyzed the effects of the over-expression of ^{HA}GSK3-β with the combined endogenous Pin1 knock-down on ^{FLAG}N3ICD expression levels in HEK293T cells. As shown in **Figure 5a**, the protein expression of ^{FLAG}N3ICD was reduced in both conditions and their combination led to a sharper decrease which was rescued by using MG132, thus confirming that Pin1 and GSK3-β may really share the same substrate.

Based on the showed functional overlapping of their consensus motifs on N3ICD (**Figure 2b**), we hypothesized that Pin1 and GSK3-β proteins may compete for the N3ICD binding.

We are planning to investigate this interesting hypothesis by co-immunoprecipitation of ^{FLAG}N3ICD and ^{HA}GSK3-β in the presence of increasing amount of exogenous Pin1 (^{HA}Pin1) in HEK293T-Pin1$^{-/-}$ cells.

Subsequently, we decided to verify our hypothesis also in ovarian cancer context. We first co-transfected ^{FLAG}N3ICD, ^{HA}Pin1 and ^{HA}GSK3-β vectors in Notch3-negative OC SKOV3 cells (**Figure 5b**). As expected, the over-expression of ^{HA}GSK3-β negatively affected ^{FLAG}N3ICD (**Figure 5b** - **third lane**). Interestingly, the presence of ^{HA}Pin1 restored partially the ^{FLAG}N3ICD protein levels (**Figure 5b – forth lane**).

We then examined the effects of both proteins on endogenous N3ICD: OVCAR3 cells were subjected to RNA interference for GSK3-β kinase (siGSK) for 72 hours in the presence or absence of ^{HA}Pin1 (**Figure 5c**). In keeping with our previous results, the higher activity of Pin1, due to over-expression of the exogenous Pin1,

led to increased N3ICD stability (**Figure 1c**). Moreover, GSK3-β knock-down amplified Pin1 activity (**Figure 5c – forth lane**).

Therefore, the results obtained in the ovarian cancer cell lines are consistent with our hypothesis of a functional antagonism on N3ICD but further studies on the cross-talk between Pin1 and GSK3-β are needed.

Pin1 inhibition leads to N3-dependent down-regulation of MMP9 mRNA expression

Since our results suggested that Pin1 is able to sustain N3 signaling in OC cell lines by impairing its GSK3-β-dependent degradation (**Figure 5**), we investigated the functional role of the Notch3-Pin1 crosstalk in OC in order to identify therapeutic options based on Pin1 inhibition aimed at targeting Notch3.

In silico analysis highlighted a significant direct correlation (R=0,76; p=0,0001) between Pin1 and Notch3 gene expression levels in a cohort of 126 ovarian cancer patients (**Figure 6a**). Interestingly, this correlation appeared significantly linked with the phenotypic differentiation stage of the tumor [n. 467 OC patients III/IV stages: R=0,089; p=0,05 (**Figure 6c**) *vs* n.39 OC patients I/II stages: R=0,023; p=0,89 (**Figure 6b**)], thus confirming that Notch3-Pin1 axis might be involved in the acquisition of an aggressive phenotype also in OC context, in addition to T-ALL context **(Franciosa G et al., 2016).**

A growing piece of evidence supports that Epithelial-to-Mesenchymal transition (EMT) is involved in tumor invasion and metastatic spread **(Yeung KT and Yang J, 2017)**. Even if it has been reported the role of Notch signaling in the EMT regulation in ovarian cancer **(Pazos MC et al., 2017)**, the molecular mechanism of the observed regulation has not yet been fully understood.

Therefore, we transduced SKOV3 cells with a lentiviral plasmid which encodes ^{FLAG}N3ICD. After infection, parental SKOV3 and SKOV3-^{FLAG}N3ICD were tested

for the expression of ^{FLAG}N3ICD (**Figure 7a**). As shown in **Figure 7b**, the over-expression of ^{FLAG}N3ICD drove SKOV3 to acquire fibroblastoid-like features, thus supporting a specific role of N3 in EMT.

We then focused our attention on understanding whether the observed morphological change was consistent with higher invasive properties. By using matrigel-coated invasion chambers to simulate extracellular matrix (ECM), known to be degraded during tumor dissemination **(Vacca A et al., 2000)** we observed a significant increase in the invasion of SKOV3-^{FLAG}N3ICD cells when compared to parental ones (**Figure 7c**).

Notably, this increase correlated with significant up-regulation of the pro-invasive matrix metalloproteinase MMP9 mRNA levels (**Figure 7d**), which is known to play a role in tumor progression by inducing ECM degradation **(Lin LI et al., 2002; Redondo-Munoz J et al., 2010).**

Intriguingly, N3 knock-down in OVCAR3 cells (**Figure 7e – right panel**) significantly impaired pro-invasive MMP9 expression levels (**Figure 7e – left panel**), further strengthening that N3 may contribute to OC aggressiveness through MMP9 regulation.

Since the up- or down-regulation of MMP9 levels was clearly dependent on N3, we hypothesized that MMP9 could be a novel direct transcriptional target of N3. Moreover, several potential Notch-CSL binding sites in MMP9 promoter region have been found. Therefore, in order to investigate in detail this aspect we have already planned to perform several experiments including dual-luciferase reporter assay to measure luciferase activity of the available MMP-9-pGL2 reporter gene vector in OC cells in the presence of increasing amount of N3ICD.

Given the observed correlation between N3 and MMP9, we decided to pharmacologically inhibit Pin1 in order to target N3ICD thus focusing our attention on MMP9 mRNA levels as a read-out of the treatments.

Among Pin1 inhibitors, it has been recently demonstrated that All-Trans Retinoic Acid (ATRA) is able to bind the active site of Pin1 promoting its degradation **(Wei S et al., 2015).** As shown in **Figure 7f**, ATRA treatment reduced pro-invasive MMP9 expression levels only in SKOV3-^{FLAG}N3ICD, thus suggesting that ATRA-mediated down-modulation of MMP9 depends on the presence of N3. Moreover, ATRA treatment reduced Pin1 protein levels and consequently N3ICD ones (**Figure 7g – right panel**) in OVCAR3 cells, which was consistent with a significant MMP9 down-modulation (**Figure 7g – left panel**).

Taken together, these observations support that Pin1 inhibition could be a promising therapeutic strategy to treat N3-overexpressing OC-bearing patients.

3.2.4 Discussion

Ovarian cancer is highly metastatic, rendering the current treatments ineffective **(Pazos MC et al., 2017).** As a result, elucidating which pathways are involved in ovarian cancer progression becomes a paramount issue in order to develop appropriate approaches directed against those effectors.

In this scenario, several studies underpinned the correlation between Notch3 signaling and the promotion of EMT in ovarian cancer **(Xu Y et al., 2019; Xu Y et al., 2018).** Here we report that the activation of Notch3 in SKOV3 cells leads to a spindle and fibroblast-like morphology (**Figure 7b**) confirming what Gupta and colleagues obtained when they stably transduced OVCA429 cells with retroviral vector encoding the intracellular domain of Notch3 (N3ICD) **(Gupta N et al., 2013).**

Furthermore, it is known that Notch signaling pathway contributes to tumor invasion by the up-regulation of matrix metalloproteinase 2 (MMP2) and 9 (MMP9) **(Zhou L et al., 2012; Groeneweg JW et al., 2014)**, which are critically involved in the promotion of tumor cell invasion and metastasis **(Curran S and Murray GI, 2000)**. Interestingly, we observed a significant increase of MMP9 mRNA expression in SKOV3-^{FLAG}N3ICD cells (**Figure 7d**), which was consistent with their higher motility when compared to the parental SKOV3 (**Figure 7c**). Moreover, we hypothesized that MMP9 is a direct transcriptional target of Notch3 in keeping with our previous studies and others highlighting the Notch3-MMP9 correlation in different tumor contexts **(Franciosa G et al., 2016; Zhang M et al., 2016).**

Given the contribution of the Notch3 signaling pathway in ovarian cancer progression, understanding the bases of its post-translational regulation might be exploited to pave the way for Notch3-targeted therapies. It is widely acknowledged that protein activity, localization and stability within a cell are

partially affected by post-translational modifications (PTMs) **(Walsh CT, 2005).** In this scenario, several studies are oriented towards the characterization of the PTMs of Notch receptors to deepen the understanding of PTM-driven Notch signaling fine-tuning **(Antfolk D et al., 2019)**.

We previously demonstrated that the phosphorylation of N3ICD in Serines or Threonines residues preceding a Proline (pSer/Thr-Pro) motifs leads to increased N3ICD stability following interaction with Pin1 in T-cell acute lymphoblastic leukemia **(Franciosa G et al., 2016)**.

Since we did not explain in detail how Pin1 positively regulates N3ICD, a further investigation on the underlying molecular mechanism was clearly warranted. First of all, we found four potential pSer/Thr-Pro motifs that could responsible for Pin1 recognition and binding (**Figure 2a**) but further studies are needed to validate them. Moreover, here we demonstrate that the Pin1 binding to N3ICD is able to prevent it from the GSK3-β kinase-dependent proteasomal degradation in OC cell lines (**Figure 5**). These data are in keeping with a recent study conducted in breast cancer cell lines where the interaction of Pin1 with N1ICD and N4ICD impaired FBXW7-mediated poly-ubiquitination **(Rustighi A et al., 2014)**.

Up to the present time, contradictory results were obtained concerning the role of GSK3-β kinase on N3ICD in cancer **(Guha S et al., 2011; Kashikar ND et al., 2011; Li C et al., 2011)**. Nevertheless, in this study we individuate one residue phosphorylated by GSK3-β (**Figure 2c**) at least and we document that the GSK3-β-mediated phosphorylation of N3ICD triggers its poly-ubiquitination by E3 ubiquitin-protein ligase WWP2 (**Figure 4**), which has been recently identified as a negative regulator of Notch3 signaling specifically in ovarian cancer context **(Jung JC et al., 2014).**

To sum up, our results strongly suggest that Pin1 is a positive regulator (**Figure 1**) whereas GSK3-β is a negative regulator (**Figure 3**) of N3ICD in OC.

Moreover, since they partially share their consensus motifs (**Figure 2b**), we hypothesize that Pin1 and GSK3-β may compete for the N3ICD binding, thus resulting in one effect (N3ICD stabilization) or in another one (N3ICD degradation) respectively, depending on the balance between Pin1 and GSK3-β proteins within the cells.

Since our *in silico* analysis revealed a significant direct correlation between Pin1 and Notch3 gene expression levels in a cohort of ovarian cancer patients (**Figure 6**), Pin1 inhibitors might be useful in therapy in order to tip the balance in favor of N3ICD proteasomal degradation.

In addition, considering the gastrointestinal toxicity and side effects related to the use of pan-Notch inhibitors, such as γ-secretase inhibitors (GSIs) **(Shih IeM and Wang TL, 2007)**, treatment with Pin1 inhibitors may represent a more selective therapeutic strategy to decrease the oncogenic potential of N3ICD.

Recently, Wei and colleagues demonstrated that All-Trans Retinoic Acid (ATRA) inhibits and degrades active Pin1 in acute promielocytic leukemia (APL) and breast cancer **(Wei S et al., 2015).** Moreover, ATRA is used clinically for APL-bearing patients **(Lo-Coco F et al., 2013)** and this compound is under investigation for the treatment of solid cancers due to its low toxicity **(Schenk T, 2014; Centritto F et al., 2015)**.

In this study we report that ATRA decreases Pin1 and consequently N3ICD protein expression in ovarian cancer cell lines (**Figure 7g – right panel**). Nevertheless, since it has been demonstrated that ATRA reduced the mRNA levels of Notch1 **(Zanetti A et al., 2015)**, it is quite expected that ATRA could affect Notch3 also in a Pin1-independent manner and this aspect certainly requires further investigation. Moreover, our preliminary data suggested that ATRA is more effective in regulating MMP9 mRNA levels in Notch3-positive OC cells (OVCAR3 and SKOV3-^{FLAG}N3ICD) with respect to Notch3-negative ones (SKOV3)

(**Figure 7f** and **Figure 7g – left panel**), thus indicating that ATRA has promising potential to inhibit the motility and EMT phenotype of ovarian cancer.

These findings concur with recent studies in which ATRA was tested on a wide range of OC cells, resulting effective on A2780 **(Kim D et al., 2018)** and OVCAR-3 **(Lockman NA et al., 2019)** whereas the SKOV3 appeared resistant **(Ravikumar S et al., 2007)**, thus finally suggesting that sensitivity to ATRA may depend on the presence of Notch3.

3.2.5 Conclusion

We demonstrate that Pin1 amplifies Notch3 signaling in ovarian cancer by preventing its GSK3-β-dependent proteasomal degradation.

All in all, our findings might be useful for ameliorating ovarian cancer treatment because they provide a rationale for the use of ATRA as a novel Pin1 inhibitor, aimed at reducing the malignant progression of Notch3-overexpressing ovarian cancers.

Figure & Figure Legends

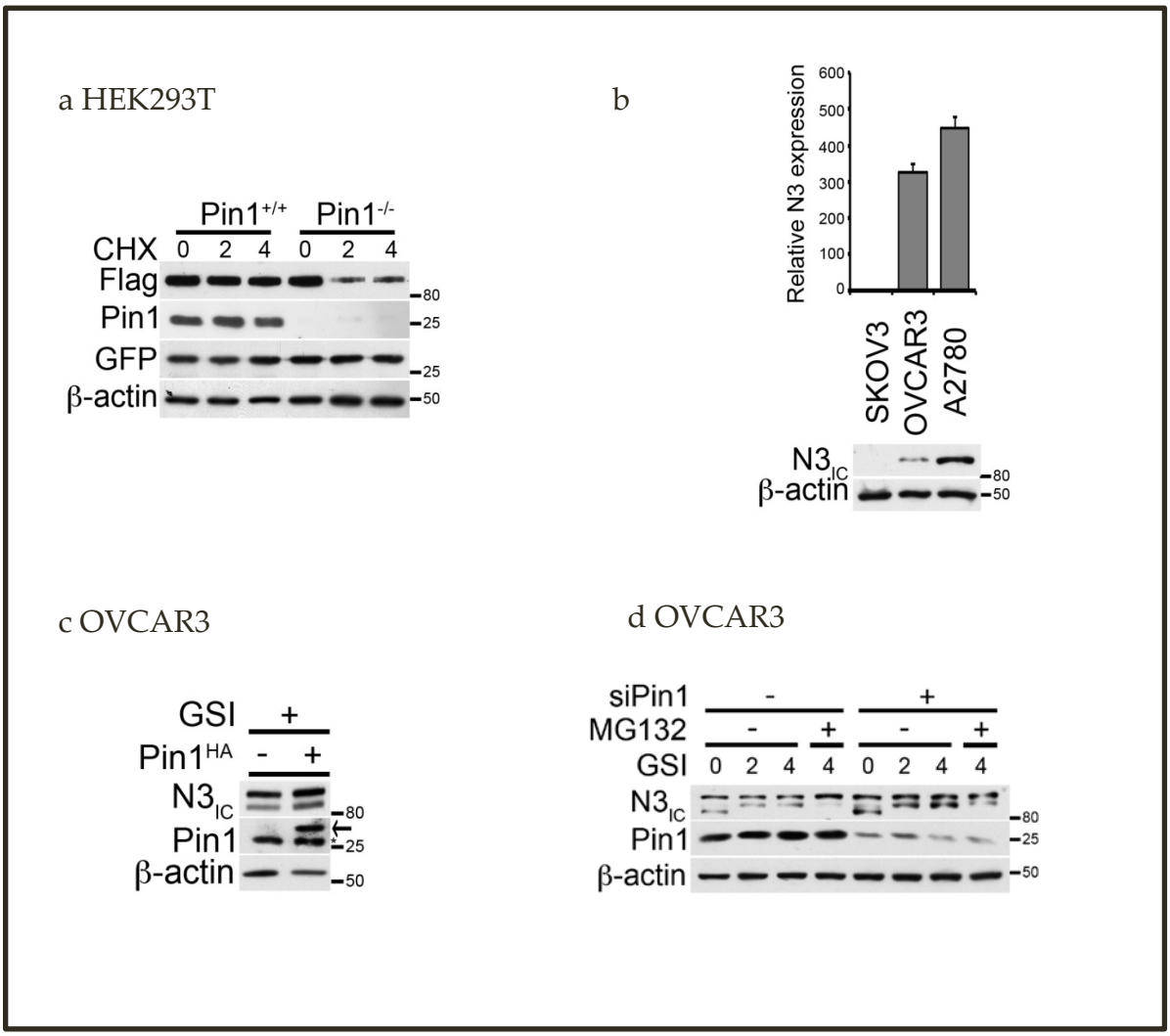


Figure 1. Pin1 affects N3ICD protein expression in ovarian cancer.

(a) Western Blot showing ^{FLAG}N3ICD half-life in HEK293T Pin1 wild type (Pin1$^{+/+}$) and knock-out (Pin1$^{-/-}$) in the presence of 10μg/mL Cycloheximide (CHX) for 0-2-4 hours. Anti-FLAG antibody was used to detect N3ICD expression, anti-Pin1 antibody was used to detect the endogenous level of Pin1, anti-GFP antibody was used to evaluate transfection efficiency and anti-β-actin antibody was used as a loading control.

(b) qPCR (**Upper panel**) and Western blot (**Lower panel**) analysis of N3ICD RNA and protein expression levels, respectively, on a subset of Ovarian Cancer (OC) selected cells (SKOV3, OVCAR3 and A2780).

(c) Western Blot analysis of N3ICD protein expression in OVCAR3 after transient transfection of exogenous ^{HA}Pin1 in the presence of 20μM γ-secretase inhibitor (GSI). Anti-N3$_{IC}$ antibody was used to detect endogenous N3ICD, anti-Pin1 antibody was used to detect Pin1 levels, both the endogenous (lower band) and the exogenous (upper band indicated by the arrow), and anti-β-actin antibody was used as a loading control.

(d) Western Blot showing N3ICD half-life in OVCAR3 after Pin1 knock-down (siPin1) in the presence of 20μM GSI for 0-2-4 hours and of 30μM proteasome inhibitor (MG132) in the indicated samples. Anti-N3$_{IC}$ antibody was used to detect endogenous N3ICD, anti-Pin1 antibody was used to detect the endogenous level of Pin1 and anti-β-actin antibody was used as a loading control.

a

-178

GETALHLAARYARADAAKRLLDAGADTNAQDHSGRTPLHTAVTADAQGVFQI
LIRNRSTDLDARMADGSTALILAARLAVEGMVEELIASHADVNAVDELGKSALH
WAAAVNNVEATLALLKNGANKDMQDSKEETPLFLAAREGSYEAAKLLLDHLA
NREITDHLDRLPRDVAQERLHQDIVRLL-363

-364 Site 3

DQPSGPRSPSGPHGLGPLLCPPGAFLPGLKAVQSGTKKSRRPPGKTGLGPQGTRG
RGKKLTLACPGPLADSSVTLSPVDSLDSPRPFSGPPASPGGFPLEGPYAT-468

Site 4 Site 6

-469

TATAVSLAQLGASRAGPLGRQPPGGCVLSFGLLNPVAVPLDWARLPPPAPPGPSF
LLPLAPGPQLLNPGAPVSPQERPPPYLAAPGHGEEYPAAGT-563

Site 7

b

-364

DQP**SGPRSP**SGPHGLGPLLCPPGAFLPGLKAVQSGTKKSRRPPGKTGLGPQGTRG
RGKKLTLACPGPLADS**SVTLSP**VDSLDSPRPF**SGPPASP**GGFPLEGPYAT-468

c

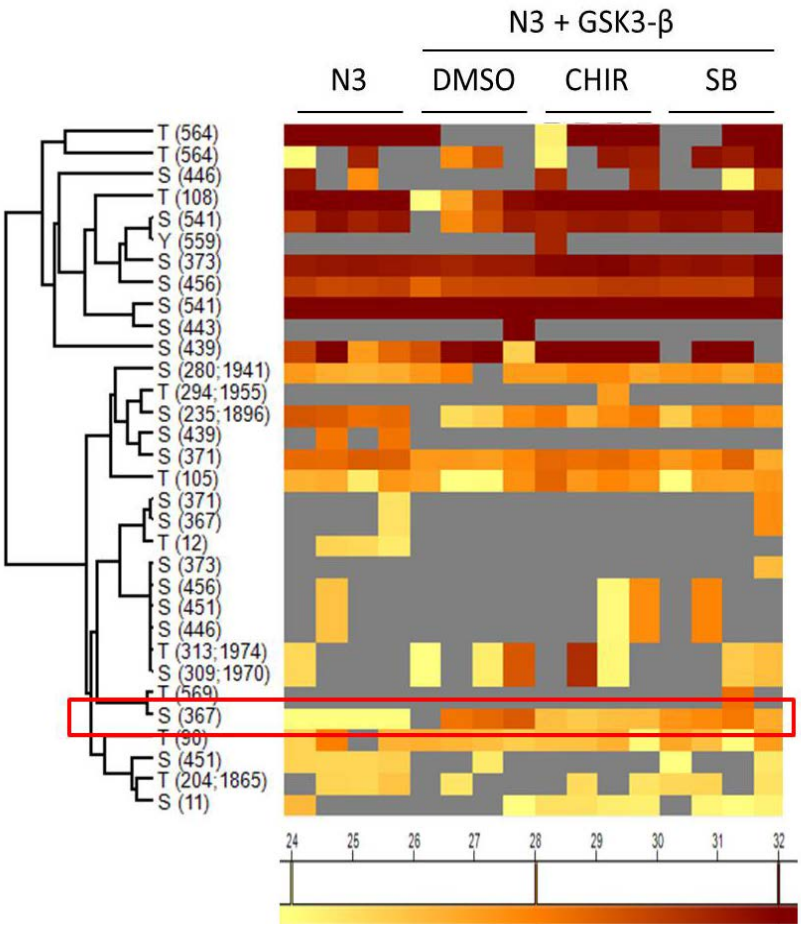


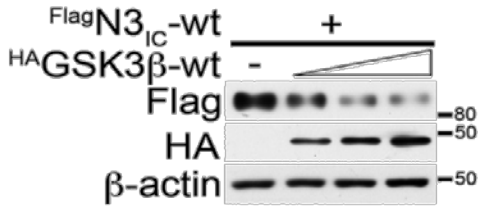
Figure 2. GSK3-β phosphorylates N3ICD.

(a) Representation of the amino acid sequence of three domains of N3ICD (Green: ANK; Dark Blue: RE/AC; Light Blue: TAD) [Underlined: Pin1 consensus motifs; red: phosphorylated Serine (S) residues found by MS analysis].

(b) Representation of the amino acid sequence of the RE/AC domain showing the overlapping between Pin1 and GSK3-β consensus motifs (Underlined: Pin1 consensus motifs; bold: GSK3-β consensus motifs).

(c) Heatmap of the Pearson's correlation of N3ICD phosphorylated Serine (S) and Threonine (T) residues identified by MS analysis in HEK293T following transient co-transfection of ^{FLAG}N3ICD and ^{HA}GSK3-β plasmids. In the last 1 hour of co-transfection cells were treated with two GSK3-β-inhibitors (CHIR99021 and SB-216763) at a final concentration of 5μM each. Different colors relate to different degree of phosphorylation (grey: absent; yellow: low; orange: medium; red: high).

a HEK293T



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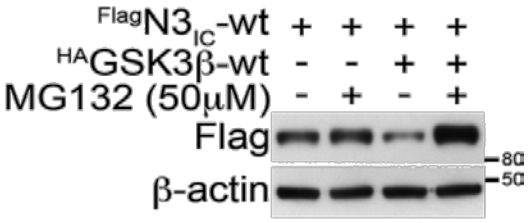
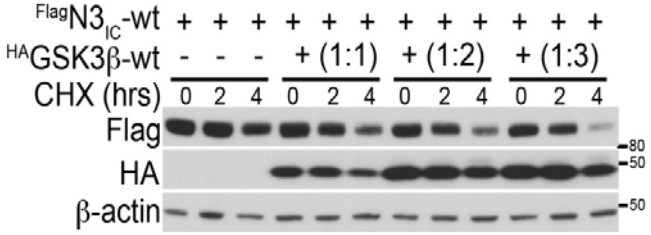
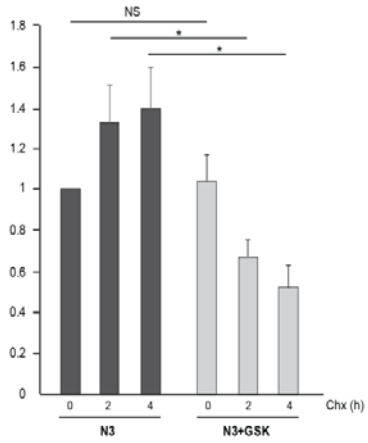
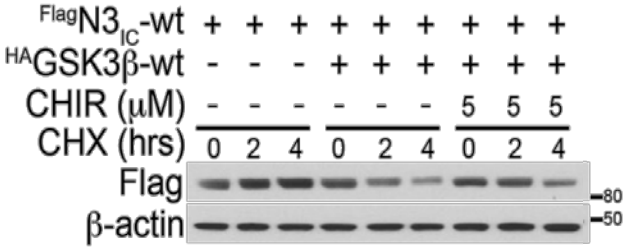


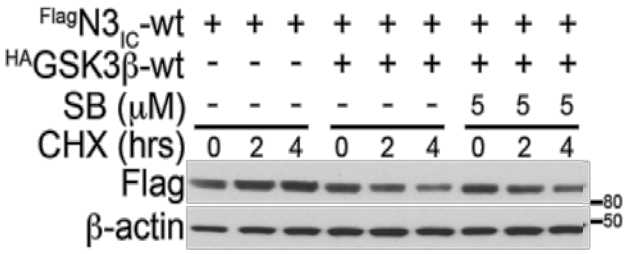
c HEK293T



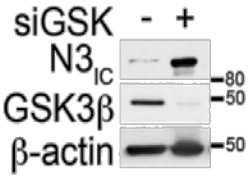


d HEK293T





e OVCAR3



f OVCAR3

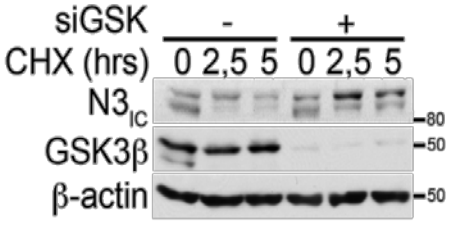


Figure 3. GSK3-β decreases N3ICD stability.

(a) Western Blot showing the ^{FLAG}N3ICD protein expression levels in the presence of increasing amount of ^{HA}GSK3-β plasmid.

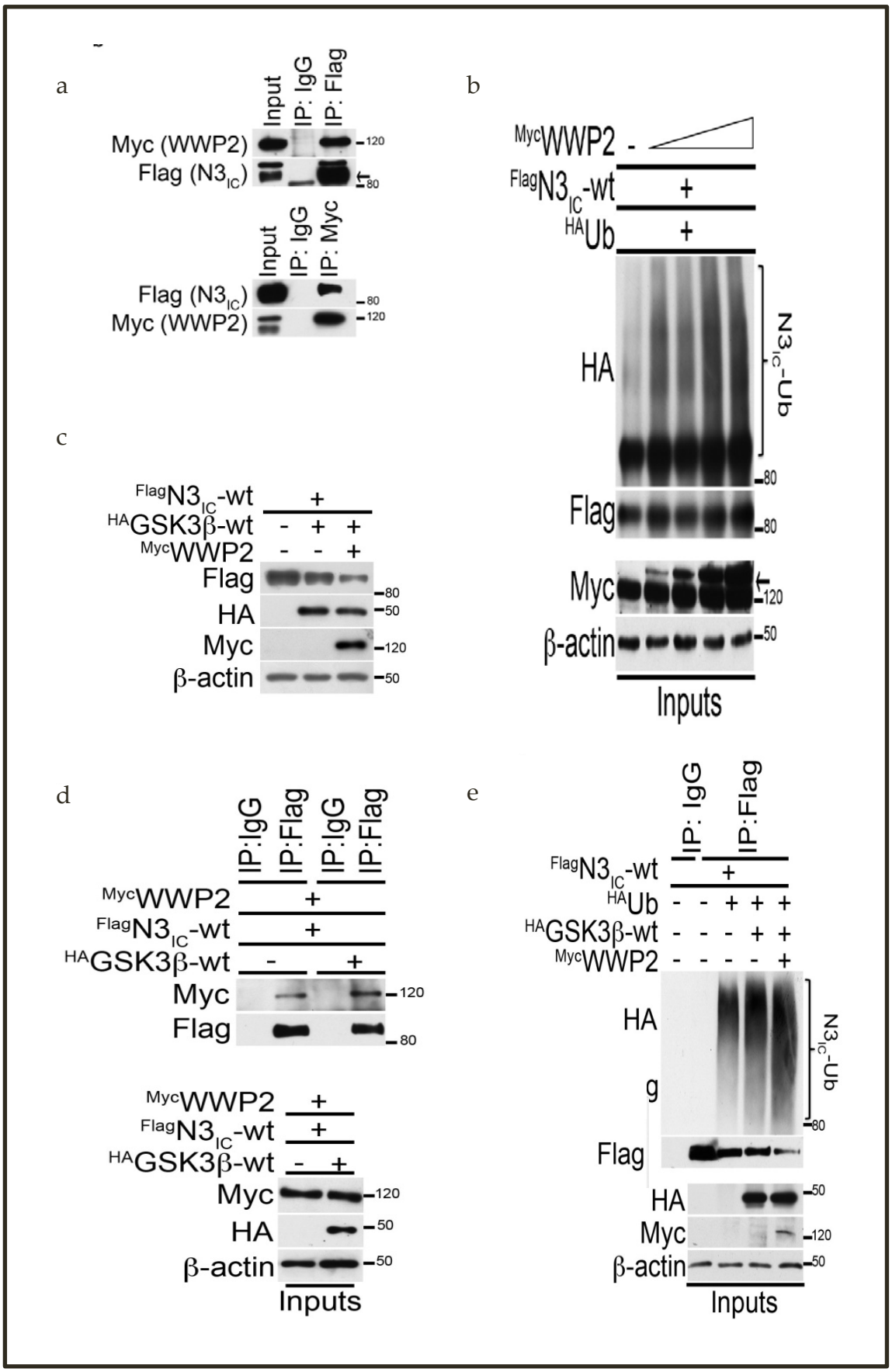
(b) Western Blot showing the ^{FLAG}N3ICD protein expression levels in the presence of the highest dose of ^{HA}GSK3-β (1:3) plasmid following MG132 treatment at a final concentration of 50μM for 4 hours.

(c – left panel) Western Blot showing N3ICD half-life in the presence of increasing amount of ^{HA}GSK3-β plasmid; **(c – right panel)** graphs showing the relative quantification as determined by optical densitometry (OD) only for the highest dose of GSK3-β (1:3). The results are expressed as the means average deviations of five separate experiments and P-values were calculated using Student's T-test (i.e., ns: not significant P>0,05; *P<0.05).

(d) Western Blot showing N3ICD half-life in the presence of two GSK3-β-inhibitors CHIR99021 **(Upper panel)** and SB-216763 **(Lower panel)** at a final concentration of 5μM each.

(a-d) All the experiments were performed in HEK293T. Anti-FLAG antibody was used to detect N3ICD levels, anti-HA antibody was used to detect GSK3-β and anti-β-actin was used as a loading control.
(c)-(d) In the last 4 hours of co-transfection, HEK293T were subjected for 0-2-4 hours to 10μg/mL CHX.

(e-f) Western Blots showing endogenous N3ICD protein expression levels **(e)** and half-life **(f)** after GSK3-β knock-down (siGSK) in OVACR3 OC cells. Anti-N3$_{IC}$ antibody was used to detect N3ICD levels, anti- GSK3-β antibody was used to detect GSK3-β and anti-β-actin antibody was used as a loading control. In **(f)** cells were subjected for 0-2.5-5 hours to 10μg/mL CHX.


a
Input
IP: IgG
IP: Flag
Myc (WWP2)
Flag (N3IC)
120
80
Input
IP: IgG
IP: Myc
Flag (N3IC)
Myc (WWP2)
80
120
b
MycWWP2 -
FlagN3IC-wt +
HAUb +
HA
N3IC-Ub
80
Flag
80
Myc
120
β-actin
50
Inputs
c
FlagN3IC-wt +
HAGSK3β-wt - + +
MycWWP2 - - +
Flag
80
HA
50
Myc
120
β-actin
50
d
IP:IgG
IP:Flag
IP:IgG
IP:Flag
MycWWP2 +
FlagN3IC-wt +
HAGSK3β-wt - +
Myc
120
Flag
80
MycWWP2 +
FlagN3IC-wt +
HAGSK3β-wt - +
Myc
120
HA
50
β-actin
50
Inputs
e
IP: IgG
IP:Flag
FlagN3IC-wt +
HAUb - - + + +
HAGSK3β-wt - - - + +
MycWWP2 - - - - +
HA
N3IC-Ub
g
80
Flag
HA
50
Myc
120
β-actin
50
Inputs

133

Figure 4. WWP2 E3-ligase is involved in the GSK3-β-dependent proteasomal degradation of N3ICD.

(a) Western Blot showing bi-directional co-immunoprecipitation assay on HEK293T transiently transfected with ^{FLAG}N3ICD and ^{MYC}WWP2 pasmids; **(Upper panel)** Control or anti-FLAG antibody immunoprecipitates from HEK293T were probed with anti-MYC antibody. **(Lower panel)** Control or anti-MYC antibody immunoprecipitates from HEK293T were probed with anti-FLAG antibody.

(b) Control or anti-FLAG immunoprecipitates from HEK293T transiently co-transfected with ^{HA}Ub, ^{FLAG}N3ICD, and ^{MYC}WWP2 plasmids were probed with an anti-HA antibody to detect the Ubiquitination status of ^{FLAG}N3ICD in the presence of increasing amount of ^{MYC}WWP2. Proteasomal inhibition with MG132 (50μM for 4 hours) was performed.

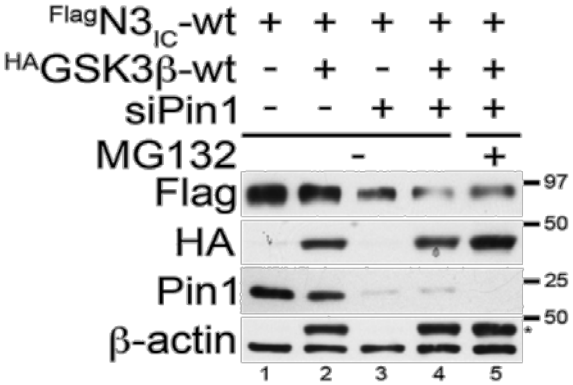
(c) Western Blot showing ^{FLAG}N3ICD protein expression in the presence of ^{HA}GSK3-β and ^{MYC}WWP2 plasmids.

(d) Control or anti-FLAG immunoprecipitates from the same cells used in **(c)** were probed with anti-MYC antibody to detect the N3ICD-WWP2 interaction.

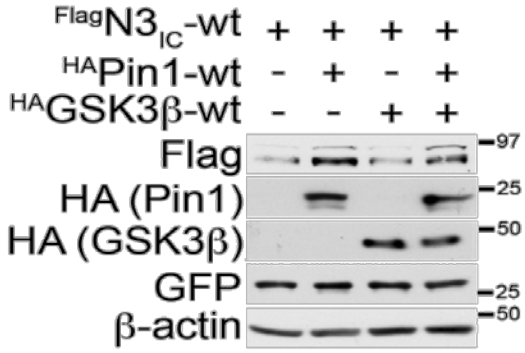
(e) Control or anti-FLAG immunoprecipitates from HEK293T transiently co-transfected with ^{HA}Ub, ^{FLAG}N3ICD, ^{HA}GSK3-β, and ^{MYC}WWP2 plasmids were probed with anti-HA antibody to detect the Ubiquitination status of ^{FLAG}N3ICD. Proteasomal inhibition with MG132 (50μM for 4 hours) was performed.

(a-e) Anti-FLAG antibody was used to detect N3ICD, anti-HA antibody to detect Ubiquitin and GSK3-β, anti-MYC antibody to detect WWP2 and anti-β-actin antibody as a loading control.

a HEK293T



b SKOV3



c OVCAR3

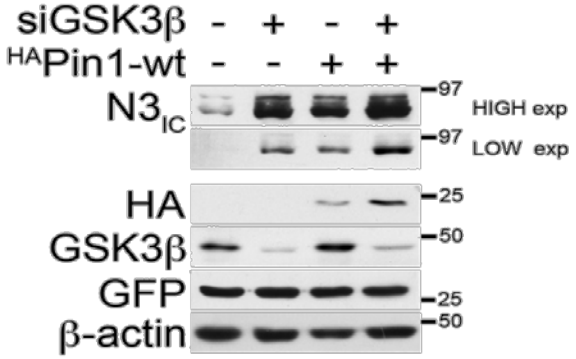


Figure 5. Pin1 and GSK3-β proteins acts on N3ICD but with different outcome.

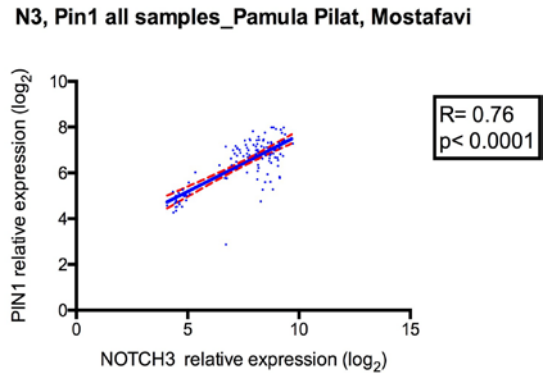
(a) Western Blot analysis of ^{FLAG}N3ICD protein expression levels in HEK293T transiently transfected with ^{HA}GSK3-β plasmid and silenced for Pin1 (siPin1) for 48 hours. In the indicated samples cells were also subjected to MG132 (50μM) for 4 hours.

(b) Western Blot showing ^{FLAG}N3ICD protein expression levels in SKOV3 OC cells following transient co-transfection of ^{HA}GSK3-β and ^{HA}Pin1 plasmids.

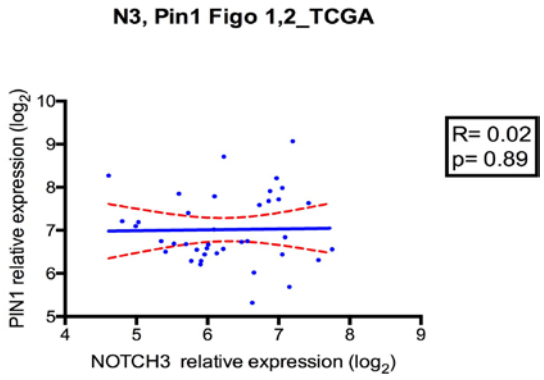
(c) Western Blot analysis of the endogenous N3ICD protein expression levels in OVCAR3 OC cells transiently transfected with ^{HA}Pin1 plasmid and silenced for GSK3-β (siGSK3-β) for 48 hours.

(a-c) In all the experiments anti-FLAG antibody was used to detect exogenous N3ICD levels, anti-HA to detect exogenous GSK3-β and Pin1 plasmids. Anti-N3$_{IC}$, anti-GSK3-β and anti-Pin1 antibodies were used to detect the relative endogenous proteins. Anti-β-actin antibody was used as a loading control and anti-GFP antibody to evaluate transfection efficiency.

a


N3, Pin1 all samples_Pamula Pilat, Mostafavi
PIN1 relative expression (log2)
NOTCH3 relative expression (log2)
R= 0.76
p< 0.0001

b


N3, Pin1 Figo 1,2_TCGA
PIN1 relative expression (log2)
NOTCH3 relative expression (log2)
R= 0.02
p= 0.89

c

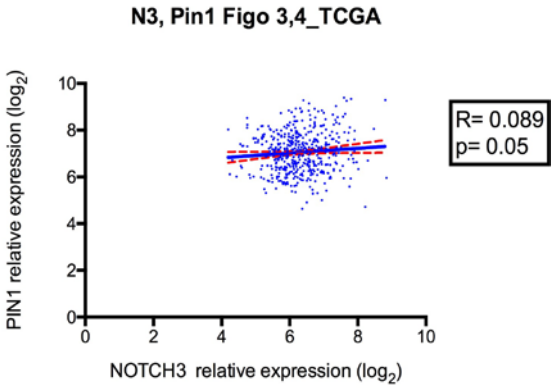

N3, Pin1 Figo 3,4_TCGA
PIN1 relative expression (log2)
NOTCH3 relative expression (log2)
R= 0.089
p= 0.05

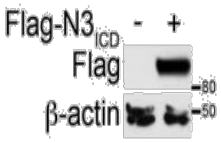
Figure 6. Notch3 and Pin1 levels correlate in OC patients.

(a) Graph showing correlation between Pin1 and Notch3 gene expression levels obtained by an *in silico* analysis in a cohort of 126 OC patients.

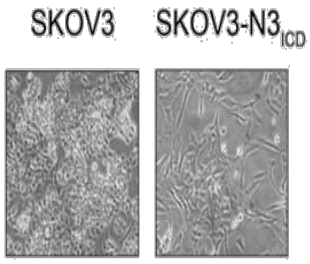
(b-c) Graphs showing correlation between Pin1 and Notch3 gene expression levels after phenotypic differentiation of tumor stage obtained from a cohort of 527 patients. Graphs **(b)** and **(c)** show data belonging to 39 and 467 patients, respectively.

(a-c) In all graphs each dot corresponds to one patient and the expression value of Notch3 and Pin1 is given in log2 scale after normalizing data with justRMA algorithm normalization. The X-Y axis represents Pin1 and Notch3 expression levels, respectively. The index Pearson R indicated expresses the linear relation between paired samples. P-values were calculated using Student's T-test.

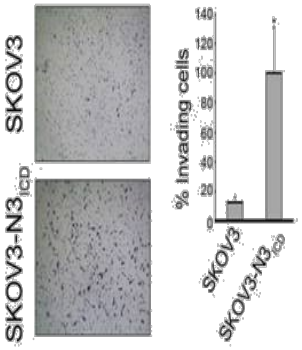
a SKOV3



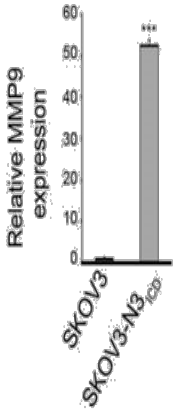
b SKOV3



c SKOV3



d SKOV3



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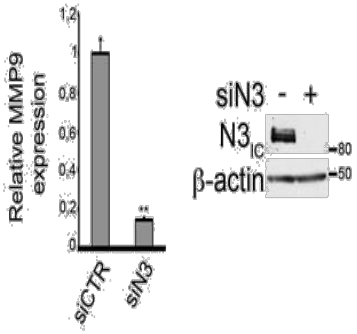
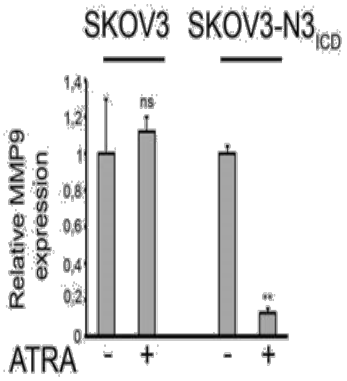


f SKOV3



g OVCAR3

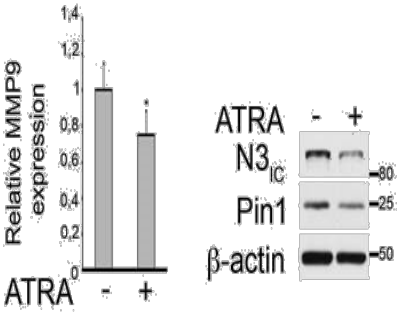


Figure 7. Pin1 inhibition leads to N3-dependent down-regulation of MMP9 mRNA expression.

(a-d) SKOV3 were stably transduced with lentiviral plasmid which encodes ^{FLAG}N3ICD protein. **(a)** Anti-FLAG antibody was used to evaluate the efficiency of lentiviral transduction in SKOV3-^{FLAG}N3$_{ICD}$. **(b)** Representative images showing the differential morphology between parental SKOV3 and SKOV3-^{FLAG}N3$_{ICD}$. **(c)** SKOV3 and SKOV3-^{FLAG}N3$_{ICD}$ were subjected to matrigel invasion assay: **(left panel)** representative cell images; **(right panel)** relative percentage of cell invasiveness. **(d)** qPCR showing up-regulation of MMP9 mRNA expression in SKOV3-^{FLAG}N3$_{ICD}$ with respect to the parental SKOV3.

(e) OVCAR3 OC cells were silenced for N3 (siN3) for 72 hours: **(left panel)** qPCR showing down-modulation of MMP9 mRNA expression in N3- silenced cells compared to negative control (siCTR); **(right panel)** Western Blot analysis of N3 knock-down efficiency.

(f-g) Treatment with 5μ All-Trans Retinoic Acid (ATRA) for 72 hours of SKOV3, SKOV3-^{FLAG}N3$_{ICD}$ **(f)** and OVCAR3 **(g)** OC cells. Evaluation of MMP9 mRNA expression levels **(f) – (g – left panel)** and analysis of efficiency of ATRA treatment on Pin1 and its effect on N3ICD **(g – right panel).**

In panels **(a), (e)** and **(g)** anti-FLAG and anti-N3$_{IC}$ antibodies were used to detect exogenous and endogenous N3ICD, respectively, and anti-β-actin antibody as a loading control. In panel **(g)** the anti Pin1 antibody was used to detect endogenous Pin1.

In all the graphs, results are expressed as the means average deviations of five separate experiments and P-values were calculated using Student's T-test (i.e., ns: not significant P>0,05; *P<0.05; **P<0.01; ***P<0.001).

Enzyme	Putative Pin1 consensus motifs on N3ICD						
	Site 1	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Site 6**	**Site 7**
Trypsin	-	-	0.998	0.956	-	0.937	1
Pro-Ala	-	-	0.993	1	-	1	1
rAspN	-	-	-	1	-	0.993	-
Chymotrypsin	-	-	-	-	-	0.726	-

Table 1.

Pin1 consensus motifs found phosphorylated by MS analysis after FLAG (^{FLAG}N3ICD) immunoprecipitation and in-gel digestion with different enzymes: Trypsin (it cleaves C-terminal to Arginine and Lysine residues), Pro-Ala (it cleaves C-terminal to Proline and Alanine residues), rAspN (it cleaves N-terminal to Asparagine residues), and Chymotrypsin (it cleaves Tryptophan, Tyrosine and Phenilalanine residues). All phosphorylated sites have a localization probability ≥ 0.70.

4. CONCLUSION

In keeping with a longer life expectancy, the number of patients affected by cancer is dramatically increased.

Decades of studies have raised the consciousness that cancer cells continuously evolve and it is necessary to keep pace with them. Moreover, cancer cells always find strategies to overcome any attempt to destroy them. In addition, another consideration that should be taken into account is the problem of heterogeneity. Not only the inter-tumoral heterogeneity but also the intra-tumoral one. Indeed, it is becoming increasingly evident that each tumor is different, rendering unsuccessful most of the proposed therapeutic strategies. Therefore, there is a renewed fervor for personalized medicine and we are moving towards targeted therapies.

In the last decade the research paradigm is "from bench-to-bedside" which means that a lot of effort should be put for translational studies moving from results obtained in the laboratories to the development of novel strategies to treat patients.

Given the problem of heterogeneity, it is paramount to encourage basic scientific research. Indeed, digging deep into the involved pathways and dissecting the molecular mechanisms implicated in tumor initiation and progression is indispensable to combat this life-threatening disease.

All in all, it is undoubtedly only the "tip of the iceberg" and there is a long way to go but the characterization of novel targets are needed to overcome heterogeneity.

In this scenario, since aberrant Notch signaling has been implicated in cancer, increasing evidence suggests that therapeutic strategies that effectively target Notch signaling could have a major impact on cancer patient survival.

Nowadays, pan-Notch inhibition based on the use of γ-secretase inhibitors (GSIs) represents the current clinical approach **(Takebe N et al., 2014)**. Beyond GSIs, γ-secretases activity could be neutralized also by using A5226A, a monoclonal

antibody that it has been recently developed against the extracellular domain of nicastrin, a component of the γ-secretases complex **(Hayashi H et al., 2012)**. An additional approach to target all Notch isoforms is based on the use of sarco(endo)plasmic reticulum Ca+2 ATPase (SERCA) pump inhibitors **(Roti G et al., 2013)**. Indeed, SERCA pump is responsible for maintaining the proper intracellular Ca+2 concentration which is required for membrane protein trafficking, including Notch receptors **(Chemaly ER et al., 2018)**.

However, given the known toxicity associated with pan-Notch inhibition **(Shih IeM and Wang TL, 2007)** and the notion that different Notch receptors could play an opposite role in the same tumor context **(Aster JC et al., 2017)**, individual Notch targeting seems to be fundamental.

In this PhD thesis, we focused our attention on Notch3 receptor in order to gain further knowledge about its function in cancer, finally aimed at understanding whether its modulation could be fruitful and beneficial to cancer-bearing patients.

Here we demonstrate that Notch3 is crucially implicated in tumor progression.

Indeed, in each project a specific role for Notch3 was described: Notch3 is involved in the tyrosine kinase inhibitors (TKI)-resistance in the most aggressive breast cancer subtype, the Triple-Negative Breast Cancer (TNBC) (**Project n°1**); it sustains T-cell acute lymphoblastic leukemia (T-ALL) growth by regulating the Unfolded Protein Response (UPR) (**Project n°2**), and it promotes the aggressive phenotype of Ovarian Cancer (OC) through the regulation of the Epithelial-to-Mesenchymal Transition (EMT) (**Project n°3**).

Therefore, our findings suggest that Notch3 pathway may be considered such a critical drug target for therapeutic interventions in both hematological and solid tumors which both rely on Notch3 for their survival and cancer behavior.

Specifically, since we documented that Notch3 cross-talks with EGFR (**Project n°1**) and UPR (**Project n°2**) pathways, we proposed combined therapies of anti-EGFR

or ER stress inducer, respectively, with Notch3-specific suppression. Therefore, thanks to the results obtained in both **Project n°1** and **Project n°2,** we strongly support the combination therapies as a promising option to develop novel Notch3-targeting strategies in the clinical application. However, how these alternative approaches could be clinically achieved is still an open question and it needs to be further investigated.

One option could be represented by the use of monoclonal antibody against Notch3, which represents the main object of an increasing number of preclinical studies **(Li K et al., 2008; Choy L et al, 2017)**. Moreover, it has been recently developed a Notch2/3 blocking monoclonal antibody named tarextumab (OMP-59R5) that was successfully tested on patient-derived epithelial tumor xenograft models and it is currently under clinical trial investigation for solid tumors treatment such as pancreatic and small cell lung cancers **(Yen WC et al., 2015)**.

An indirect option could be the modulation of proteins able to positively affect Notch3. Indeed, the apparently endless discovery of factors that have a regulatory effect on Notch receptors underscores the significance of post-translational modification (PTMs) in the control of their function **(Palermo R et al., 2014)**. However, to date little is known about the PTMs of Notch3. For this reason, understanding the bases of Notch3 post-translational regulation and the involved proteins is urgently needed (**Project n°3**) as it might be exploited to pave the way for specific and alternative Notch3-targeted therapies.

In summary, our findings provide evidence for the usefulness of Notch3-targeting in the treatment of Notch3-dependent cancer and suggest a way to make it possible, in order to try to go beyond the "emerged iceberg surface".

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APPENDIX I

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Review Article

Notch Signaling Activation as a Hallmark for Triple-Negative Breast Cancer Subtype

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Triple-negative breast cancer (TNBC) is a subgroup of 15%-20% of diagnosed breast cancer patients. It is generally considered to be the most difficult breast cancer subtype to deal with, due to the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), which usually direct targeted therapies. In this scenario, the current treatments of TNBC-affected patients rely on tumor excision and conventional chemotherapy. As a result, the prognosis is overall poor. Thus, the identification and characterization of targets for novel therapies are urgently required. The Notch signaling pathway has emerged to act in the pathogenesis and tumor progression of TNBCs. Firstly, Notch receptors are associated with the regulation of tumor-initiating cells (TICs) behavior, as well as with the aetiology of TNBCs. Secondly, there is a strong evidence that Notch pathway is a relevant player in mammary cancer stem cells maintenance and expansion. Finally, Notch receptors expression and activation strongly correlate with the aggressive clinicopathological and biological phenotypes of breast cancer (e.g., invasiveness and chemoresistance), which are relevant characteristics of TNBC subtype. The purpose of this up-to-date review is to provide a detailed overview of the specific role of all four Notch receptors (Notch1, Notch2, Notch3, and Notch4) in TNBCs, thus identifying the Notch signaling pathway deregulation/activation as a pathognomonic feature of this breast cancer subtype. Furthermore, this review will also discuss recent information associated with different therapeutic options related to the four Notch receptors, which may be useful to evaluate prognostic or predictive indicators as well as to develop new therapies aimed at improving the clinical outcome of TNBC patients.

1. Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide [1, 2]. The presence or absence of estrogen receptors (ERs), progesterone receptors (PRs), and the human epidermal growth factor receptor 2 (HER2/neu) classifies breast cancer in different subtypes [3]. Hormone receptor positive breast cancers represent 60% of all breast cancers [4], while the lack of expression of ER, PR, and HER2 characterizes TNBC subtype [5, 6], which accounts for 15-20% of breast cancer cases.

TNBCs predominantly affect younger patients (< 40 years) and are more frequent in African-American women, where they are associated with BRCA gene mutations [7, 8]. They are heterogeneous tumors with aggressive phenotype and higher relapse rate. Moreover, compared to other BC subtypes, TNBCs are less differentiated [8, 9] and prone to metastasize within 5 years of diagnosis [8]. Furthermore, TNBC-bearing patients have a shorter overall survival when compared to other BC subtypes [7, 10]. The intertumoral and intratumoral heterogeneity represent one of the major challenges for the efficacy of the treatment of this cancer. Lehmann and colleagues classified TNBC into six different subtypes by analyzing their gene expression profiles: the basal-like (BL1 and BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), and luminal androgen receptor (LAR)-enriched tumors [9]. Since TNBCs patients are characterized by this molecular heterogeneity, chemotherapy (anthracycline and taxane-based treatments also with platinum agents addiction) represents the primary systemic treatment. Moreover, although combination therapies have ameliorated the response rates, this improvement

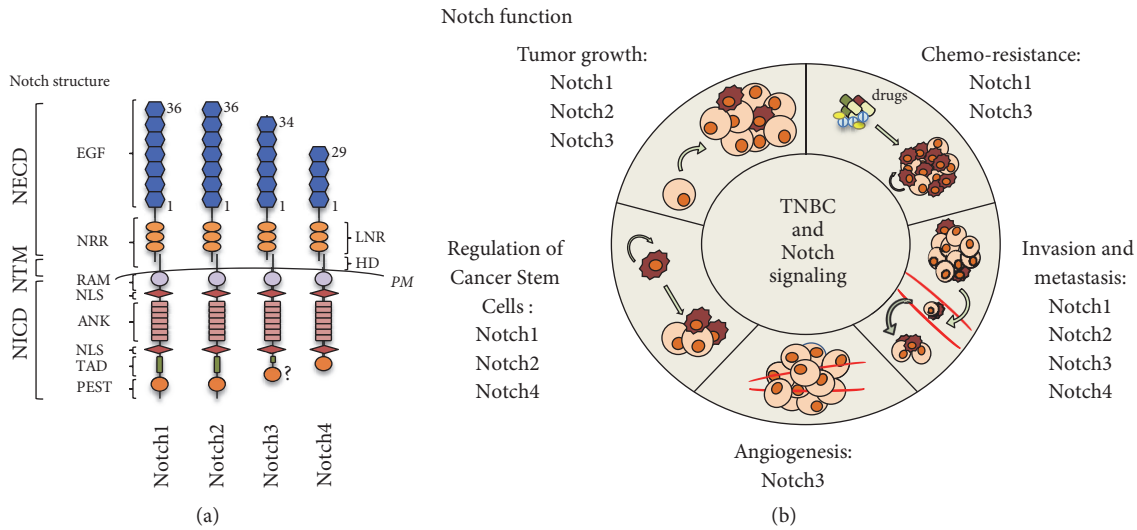

Figure 1: *Role of Notch signaling in TNBC.* (a) Schematic representation of the Notch receptors structure. Abbreviations. NECD: Notch extracellular domain; NTM: Notch transmembrane; NICD: Notch intracellular domain; EGF: epidermal growth factor-like repeats; NRR: negative regulatory region; LNR: Lin12/Notch repeats; HD: heterodimerization domain; *PM*: plasmatic membrane; RAM: RBP-j associated molecule; NLS: nuclear localization signal; ANK: ankyrin repeats; PEST: proline (P), glutamic acid (E), serine (S), and threonine (T). (b) The cartoon schematically depicts the involvement of each Notch receptor on TNBC initiation and progression.

leads to increased toxicity and multidrug resistance. On the basis of the stratification of TNBCs into subtypes, many preclinical and clinical trials are allowing the development of new targeted therapies to treat the 60–70% of patients who do not respond to chemotherapy [11]. These alternative approaches include the use of PARP and tyrosine kinase receptor inhibitors, the targeting of Wnt/β-catenin or PI3K/AKT/mTOR pathway, the emerging immunotherapy, and the use of epigenetic drugs and androgen receptor (AR) antagonists [12], as described below in more detail.

In this scenario, since it has been demonstrated that Notch signaling plays an important role in breast cancer cell growth, migration, invasion, and metastasis, and its aberrant activation is associated with a poor prognosis, resistance to treatments, and relapse [13], here we discuss the therapeutic potential of targeting Notch signaling in breast cancer treatment, focusing on the TNBC field. Until now, a lot of effort has been made to find the optimal pharmacological Notch inhibition, as the typical approach to target Notch pathway is mainly based on γ-secretase inhibitors (GSIs) use [14], which however represents a pan-Notch inhibitor drug strongly associated with severe gastrointestinal toxicity [15]. Inhibition of a specific receptor alone may reduce or avoid toxicity, thus showing a clear advantage over pan-Notch inhibitors. Although the Notch signaling pathway has been widely studied, the specific role of the individual Notch receptor in cancer is still unclear.

In this review we summarize (and discuss) the current knowledge of the role of each individual Notch receptor in TNBC (Figure 1), in order to suggest the identification of drugs targeting specific Notch(s) with an effective anticancer potential and low toxicity, trying to direct future directions in this challenging field (Table 1).

2. Notch Signaling Overview in TNBC

2.1. Notch Structure and Function. Juxtacrine signaling is pivotal in several developmental processes and relies on communication between one cell and a neighboring cell through the interaction of transmembrane receptors and ligands [16]. The Notch signaling pathway is an example of this short-range cell-cell communication and plays an essential role in metazoan development [17]. The Notch receptor is a single-pass transmembrane protein expressed on the plasmatic membrane as a processed heterodimer after the cleavage by furin-like protein convertase in the Golgi compartment [18]. It was discovered in Drosophila melanogaster. The fly genome encodes only one Notch protein while two receptors, which have redundant roles, were identified in Caenorhabditis elegans [19]. In contrast, mammals have four Notch paralogs that only partly share the same functions [20] and this is due to their variable structural homology [21].

Regarding the structural organization of the Notch receptors (Figure 1(a)), they share a three-domain structure: an extracellular domain (NECD), a transmembrane region (NTM), and an intracellular domain (NICD) which translocates to the nucleus after two sequential proteolytic cleavages triggered by ADAM metalloproteases and a γ-secretase complex, respectively. According to the canonical Notch signaling model, these events are due to the interaction between the receptors and their ligands [21], expressed on neighboring cells.

The NECD contains 29 to 36 epidermal growth factor-like (EGF-like) repeats which are responsible for the ligands binding [22], the negative regulatory region (NRR), consisting of three cysteine-rich LNR Notch repeats, and the heterodimerization domain (HD), which prevents receptor activation in the absence of ligands [23]. The NTM region contains

Table 1: Summary of notch receptors-related processes and treatments in TNBC.

Notch receptor	Process	Refs	Treatment	Studies	Refs
Notch1	Tumor growth	[59–61]			
	Mitochondrial metabolism	[60, 80]	mAbs	Preclinical	[156]
	Regulation of cancer stem cells	[64, 65, 68]	mAbs (+chemotherapeutic agents)	Preclinical	[64, 66, 156, 159]
	Drug resistance	[67–70, 73, 75]	GSI + chemotherapeutic agents	Preclinical and clinical	[74, 151–153]
	Invasion and metastasis	[59, 71, 73]			
Notch2	Tumor growth	[87, 88]			
	Regulation of cancer stem cells	[84, 85]	mAbs	Preclinical	[157]
	Invasion and metastasis	[84, 85]			
Notch3	Tumor growth	[92, 93]			
	Angiogenesis	[97, 98]	mAbs	Preclinical and clinical	[93, 157, 161]
	Drug resistance	[110, 111]			
	Invasion and metastasis	[103, 104, 107]			
Notch4	Regulation of cancer stem cells	[125–128]	mAbs	Preclinical	[126]
	Invasion and metastasis	[117, 118]	GSI	Preclinical	[118]

Abbreviations. mAbs: monoclonal antibodies; GSI: γ-secretase inhibitor.

a γ-secretase cleavage site which is critical for signal activation [24]. The NICD consists of a RAM (RBP-j-Associated Molecule) domain, ankyrin (ANK) repeats flanked by two nuclear localization signals (NLS), a transcriptional activation domain (TAD), and a C-terminal Pro-Glu-Ser-Thr (PEST) domain which is the substrate of ubiquitin ligases that target the NICD for proteasomal degradation [25]. Both RAM ad ANK domains are necessary to recruit transcriptional coactivators within the nucleus [26] (Figure 1(a)).

In mammals, the five Notch ligands, Dll1, Dll3, and Dll4 (members of the Delta family of ligands) and Jagged1 and Jagged2 (members of the Serrate family of ligands), are single-pass transmembrane proteins [27]. Dll3 gene encodes a decoy receptor and, as a result, it is not able to activate Notch receptors in-trans [28].

Notch signaling has pleiotropic effects during development and in adult tissues, in spite of the simplicity of the core pathway [29]. As a matter of fact, Notch activity affects both proliferation and cell death and drives differentiation and acquisition of specific cell fates. Furthermore, it is involved in the maintenance of stem cells [30].

Since the Notch receptors is central for these processes, its deregulation has been implicated in the development of congenital diseases [31] or cancer, as either oncogenes or tumor suppressors [32, 33].

Specifically, Notch signaling pathway is involved in mammary development and homeostasis as well as in the promotion of breast cancer when dysregulated [34]. Indeed, accumulating evidence sustains the importance of Notch pathway in mammary stem cells (MaSCs) generation and maintenance during mammary gland development [35]. This process normally takes place over a period of rapid growth during puberty and, subsequently, it undergoes cycles of expansion and regression with each estrous cycle, pregnancy, lactation, and involution until menopause [36]. In this scenario, it has been demonstrated that Notch pathway plays a fundamental role in regulating both self-renewal [37] and differentiation of MaSCs [38, 39], thus allowing mammary gland homeostasis. Thus, the aberrant activation of Notch signaling has been shown to be an early event in breast cancer development [37]. A TCGA breast cancer data was analyzed for mutations in Notch receptors genes [40]. Among the 956 breast tumor samples analyzed, there were 42 mutations in Notch genes: 25 of them are clustered in the HD or lead to a PEST domain disruption, finally leading to NICD overexpression [40, 41]. In addition, compared to normal tissues, a lower expression of known Notch negative regulators in breast cancer was frequently found [42, 43]. In particular, FBXW7 mutations were significantly more frequent in TNBC compared to other breast tumor subtypes [44] and these determine an increased NICD stability, thus correlating with poorer prognosis of breast cancer-bearing patients [45]. Moreover, a novel molecular mechanism that correlates low NUMB expression with high Notch activity in the regulation of breast tumor EMT, especially in TNBCs, was found [46].

In keeping with these findings, the role of Notch signaling in breast cancer initiation and progression has been extensively studied and most of the reported data highlights its oncogenic role in breast cancer [47].

2.2. Role of Notch Paralogs in TNBC

2.2.1. Notch1. The first demonstration that Notch receptors are oncogenes also in regard to breast cancer derives from studies on murine models. In particular, the Notch1 gene was identified as a novel target for mouse mammary tumor virus (MMTV) insertional activation, thus leading to the overexpression of Notch1 mutated forms, finally involved in mammary tumor formation [48]. Compared with normal tissues, Notch1 is fairly expressed in human breast cancer and its elevated expression represents an early event during carcinogenesis, as it has been demonstrated that the enforced expression of ectopic N1ICD contributes to the incidence and development of breast cancer [49], being predictive of poorest

overall patient survival [50–52]. Several studies have related Notch1 signaling to TNBCs [53]. In particular, the basal-like 1 (BL1) and mesenchymal stem-like (MSL) subtypes are characterized by the high expression of this receptor [54, 55], strongly correlated with ominous outcomes of tumor [56].

Notch pathway is aberrantly activated via multiple mutational mechanisms and it is liable of TNBC tumor development. Although PEST domain mutations in Notch1 protein mainly regard oncogenic events in T-ALL [57], around 13% of TNBC exhibits in-frame deletions of Notch1 exons 21-27, which disrupt the NRR and HD domains, thus leading to upregulation of its pathway caused by either ligand-independent receptor activation or N1ICD half-life extension [40]. As a consequence, Notch1-mutated-TNBCs show a strong overexpression of Notch1 target genes, like NOTCH3, HES1, HEY2, MYC, CCND1, HES4, NRARP, and NOTCH1 itself, in comparison with Notch1 wild-type tumors, thus resulting in oncogenic phenotype of TNBCs [40]. In addition, a correlation has been found between the expression of Notch1 protein and known prognostic factors in breast cancer, analyzed by IHC assay in 115 breast cancer tissues [58]. The presence of Notch1 in tumor tissue was significantly associated with TNBC subtype (P=0.041), high metastasis rate (P=0.035), tumor-node-metastasis (TNM) stages, and ALDH1 *status*, a known marker of cancer stem cells (CSCs).

Furthermore, a significant positive correlation was observed between Notch1 protein and both AKT and NF-κB proteins activation in preclinical models, thus finally promoting TNBC cell growth, migration, and invasion [59]. Interestingly, more recently Hossain and colleagues described in detail noncanonical mechanisms downstream of Jagged-1-mediated Notch1 activation that trigger AKT phosphorylation, NF-κB activation, and mitochondrial metabolism, thus leading to the transcription of survival genes in TNBC cells [60]. In agreement with these data, it has been demonstrated that *Genistein*, a phytochemical originally isolated from soybean, by inhibiting Notch1, affected MDA-MB-231 TNBC growth through modulating NF-κB activity [61].

It is well demonstrated that CSCs are involved in initiation, progression, and chemotherapy resistance of cancers [62, 63].

Notch1 appears to be in part responsible for maintaining CSC stemness in TNBCs, and the specific inhibition of its signaling has a remarkable inhibitory effect on this cancer subtype, thus increasing the sensitivity of TNBC to chemotherapeutic reagents [64]. It is reported that in HCC70, SUM149, and MDA-MB-231 TNBC cell lines, the c-Jun N-terminal kinase (JNK) protein promotes CSC self-renewal and maintenance via transcription of Notch1, whose activation affects migration and invasion of tumor cells [65]. In accordance with these findings, both JNK and Notch1 knockdown significantly reduced mammosphere formation in TNBC cells [65]. Moreover, Mittal and colleagues, by using a novel monoclonal antibody to inhibit Notch1 (MAb602.101), observed a significant reduction in tumor growth and in the number and sizes of mammospheres compared to controls, thus resulting in the depletion of the putative cancer stem-like cell subpopulation [66]. Furthermore, Bhola and colleagues demonstrated that resistance to TORC1/2 inhibition in TNBC is driven by Notch1 activation whose expression is increased in response to treatment. In consequence, genetic and pharmacological blockade of Notch1 is able to revert the increase in CSC markers expression, mammosphere formation, and tumor-initiating ability, all induced during TORC1/2 inhibition treatment [67]. All these studies sustained an important correlation between Notch1 inhibition and the restoration of the sensitivity to drug treatments, thus showing interesting findings which would improve the efficacy of conventional therapies by directly targeting the CSC niche [64, 68]. In addition, significant upregulated Notch-1 protein levels are found in Doxorubicin resistant MCF-7 cells compared to parental sensitive MCF-7 cells [69]. In keeping with these data, Notch1 inhibition enhanced the antitumor effects of Paclitaxel, the first-line chemotherapeutic drug for clinical treatment of TNBC, in both MDA-MB-231 and MCF-7 chemoresistant cells [70].

Emerging evidence demonstrated the involvement of Notch1 also in the invasion and migration steps which characterize the epithelial-to-mesenchymal transition (EMT) process in TNBC [71]. The authors observed that Notch1 is negatively regulated by miR-3178, which is significantly lower in TNBCs when compared to the other subtypes: the lower levels of miR-3178 lead to increased Notch1 activity followed by increased Snail1 expression, which finally contributes to EMT regulation [71]. Indeed, the inhibition of Slug/Notch1 signaling axis, by regulating EMT process, seems to be sufficient to decrease tumor-initiating cells (TICs) number, tumor induction, and metastasis [72]. In keeping with these data, Notch1 expression is higher in Cisplatin-resistant MDA-MB-231 TNBC cells, compared to the parental cells, and this helped to induce chemoresistance via activating AKT pathway and promoting EMT [73]. Furthermore, it has been demonstrated that the combined treatment with Doxorubicin plus GSIs of the same resistant cells, besides downregulating Notch-1, is also able to decrease both Cyclin D1 and antiapoptotic protein Bcl-2 while upregulating PTEN and proapoptotic proteins, finally leading to synergistic antitumor effects *in vitro* and *in vivo* TNBC xenografts models [74].

More recently, Lee and colleagues demonstrated that Notch1 inactivation, obtained as a consequence of the knockdown of Tribbles Homolog 3 (TRIB3) protein in MDA-MB-231 and AS-B244 radio-resistant TNBC cells, correlated with a cell resensitization toward radiation therapy [75]. Interestingly, some studies showed a Notch1 involvement in metabolic alterations of cancer cells. Abnormal mitochondrial fission is implicated in the development and progression of many human cancers [76] and Notch signaling has been reported to be closely related to mitochondrial network and function in different cellular contexts [77–79]. Dynamin related protein (Drp1) is involved in mitochondrial fission while Mitofusin-1 (Mfn1) is a mitochondrial membrane protein that participates in mitochondrial fusion, thus contributing to the maintenance of the mitochondrial network. Perumalsamy and colleagues identified the N1ICD-Akt-Mfn signaling cascade as a novel pathway regulating cell survival, in a way independent of the canonical functions associated with N1ICD activity, thus demonstrating the Notch1

involvement in mitochondrial network and apoptotic resistance in HeLa cells [79]. More recently, it was demonstrated that the Notch1/Mfn2 pathway was able to favor the protective effect of melatonin on myocardial infarction, by using both *in vitro* and *in vivo* models [78]. In TNBC context, Chen and colleagues demonstrated that the observed increase in the mitochondrial fission, characterized by the combined upregulation of Drp1 and downregulation of Mfn1, was due to a positive feedback loop closely dependent on Notch1 protein: mitochondrial fission contributes to activation of Notch1, which in turn promotes and amplifies the mitochondrial fission through the maintenance of both Drp1 and Mfn1 altered expression. This process strongly correlated with TNBC progression and a poorer overall survival of TNBC-bearing patients [80].

All these studies suggest that activation of the Notch1 pathway is a key event in TNBC etiology and it contributes to the development and progression of malignant phenotype of TNBC subtype.

2.2.2. Notch2. The role of Notch2 in breast cancer is less well characterized with respect to Notch1. Previous studies reported that Notch2 increases tumorigenicity in thymic lymphoma [81] and in embryonal brain tumor cell lines [82]. Conversely, Notch2 signaling causes cells growth arrest in small cell lung cancer [83]. Therefore, the cellular context is important for tumorigenic outcome of Notch2 signaling. Notch2 can play a different role in TNBCs, thus acting as an oncogene or tumor suppressor. Evidence for its oncogenic role came from studies on cultured breast cancer cells where knockdown of Notch2 leads to the inhibition of cell migration and cancer stem cell survival [84, 85]. In particular, Kim and colleagues revealed that treatment of MCF-7, MDA-MB-231, and SUM159 human breast cancer cells with Benzyl isothiocyanate (BITC), a constituent of cruciferous vegetables, increases levels of the active form of Notch1, Notch2, and Notch4 in both cultured and xenografted cells. In this scenario, only Notch2 activation is able to impede inhibitory effect of treatment on cell migration [85]. In keeping with these data, the proapoptotic effect of Zerumbone (ZER), a sesquiterpene isolated from subtropical ginger, on TNBC cells was counteracted by Notch2 activation and significantly increased upon its knockdown [86].

Analysis of Notch2 expression in normal mammary tissue and breast tumors, in association with clinical data, also sustained a tumor suppressor function for this receptor. The most convincing evidence for this Notch2 capability is provided by O'Neill and colleagues [87]. They reported that overexpression of N2ICD in MDA-MB-231 cells is potently able to suppress tumor growth both *in vitro* and *in vivo* in xenografts. Therefore, Notch2 plays a role in the inhibition of mammary adenocarcinoma growth, mostly in comparison with Notch4 ICD in the same context. Another study revealed that the *in vivo* growth of MDA-MB-231 and SUM159 xenografted cells is enhanced by stable knockdown of Notch2 [88]. Notably, this increased *in vivo* tumor growth is determined by the increase in cytokines secretion and Notch1 activation, thus suggesting a compensatory response of cancer cell [88]. More interestingly, numerous studies suggested that Notch2 overexpression is related to a greater chance of survival of breast cancer patients [89]. Parr and colleagues analyzed Notch-1 and Notch-2 mRNA and protein expression levels in normal and breast cancer tissues also in association whit clinicopathological parameters [89]. The results showed that high level of either Notch1 mRNA or protein is associated with a poorer outcome for patients while a high expression of Notch2 is correlated with a better prognosis. In addition, the authors demonstrated an opposite expression of Notch1 and Notch2 proteins during tumor development, related to its differentiation state. Regarding Notch2 gene mutational pattern in TNBC samples, many focal amplifications were also found in its PEST or HD domain: in particular, the PEST domain showed six mutations, three of them leading to a gain of function, while the HD domain exhibited two missense mutations, finally leading to Notch2 overexpression [40].

All these data suggest that Notch-2 role remains ambiguous in TNBC. However, to date there is much more evidence to support the view that it should have a tumor suppressive role rather than an oncogenic role.

2.2.3. Notch3. As we have previously described, TNBCs are genetically unstable and they are usually characterized by a complex pattern of genetic aberrations such as focal amplifications. On the basis of the evidence that Notch3 is highly expressed in TNBCs [51], Turner and colleagues subjected a wide subset of TNBCs to high resolution microarray-based comparative genomic hybridization and to genome-wide gene expression analysis in order to model mutational signatures of Notch3 gene. The obtained results highlighted that Notch3 gene amplification is quite recurrent and it is significantly overexpressed when amplified [90]. Furthermore, a broad spectrum of activating mutations that disrupt both HD and PEST domains, thus favoring N3ICD expression, were discovered in Notch3 gene [40].

In keeping with these findings, the presence of activating mutations, coincident with gene amplification and overexpression, lends genetic weight to the idea that there is a selective pressure to increase Notch3 activity for TNBCs initiation and progression. Indeed, the correlation between Notch3 signaling and TNBCs is corroborated by several studies.

First of all, it is already ascertained that Notch3 has transforming potentials *in vivo*, since transgenic mice overexpressing the intracellular domain of Notch3 (N3ICD) developed breast cancer [91]. In addition, Notch3 pivotal role in the proliferation of ErbB2-negative breast cancer cell lines has been demonstrated [92].

More recently, it was shown that Notch3 altered expression activates an oncogenic program in a panel of TNBCs. Selective Notch3 inhibition impairs tumor growth, whereas Notch3 agonism correlates with a malignant phenotype and increased proliferation. Indeed, transcriptomic analyses showed a Notch signature that includes overexpression of the c-Myc oncogene [93].

As occurred for cancers in general, TNBC malignancy correlates with tumor angiogenesis [94–96]. Reedijk and

colleagues pointed out that Jagged1 and Notch3 are overexpressed in blood vessels of primary breast cancer [97], but little is done to understand whether Jagged1 and Notch3 are closely related to angiogenesis in TNBCs. Recently, Xue and colleagues speculated on the possible crosstalk between VEGF and Notch signaling in TNBCs [98] but further studies are needed. In addition, they showed that Jagged1 and Notch3 are detected in TNBCs at significantly higher levels than in no-TNBCs and their expression leads to more aggressive clinicopathological characteristics and poorer prognosis, confirming previous studies [56]. Moreover, RNAi-mediated depletion of Jagged1 and Jagged2 proteins in ErbB2-negative breast cancer cell lines inhibited proliferation and induced apoptosis *in vitro*, thus demonstrating an important autocrine/juxtacrine loop between Jagged1/Jagged2 ligands and Notch3 in TNBC context [99], which was then also observed in other tumor contexts [100].

In general, in about 50% of breast cancer patients bone is recognized as the first site of metastasis and TGFβ plays a central role in this process [101]. Increasing evidence suggested that cancer cells interact with the bone microenvironment in order to promote the initiation and progression of bone metastasis [102]. Zhang and colleagues focused their attention on Notch3 and bone metastasis potential relationship in TNBCs: they observed that both osteoblasts and their secretion of TGFβ increased Notch3 expression in TNBC cells that reside in the bone marrow niche. Notably, the inhibition of Notch3 expression is able to reduce osteolytic bone metastasis in xenograft animal models of TNBCs [103].

All these data supported the hypothesis of Notch3 involvement in promoting TNBC invasiveness and cancer cell seeding to secondary organs, thus being able to influence the acquirement of the metastatic phenotype and to complete the invasion-metastasis cascade. In this view, Leontovich and colleagues demonstrated that the MDA-MB-231 LM cells, isolated from experimental lung metastasis (LM), showed higher self-renewal capacity with respect to parental cells thanks to the upregulation of Notch3 reprogramming network. *In vitro* inhibition of Notch3 impaired the invasive capacity of MDA-MB-231 LM cells and interfered with late stages of the invasion-metastasis cascade. Interestingly, the pivotal role of Notch3 in determining an invasive phenotype and worst outcome was corroborated in unique TNBC cells resulting from a patient-derived brain metastasis [104].

Recently, some studies reported different molecular mechanism by which Notch3 seems to inhibit EMT in breast cancer [105, 106], including TNBCs [107], but overall high transcript levels of Notch3 were associated with less distant metastasis and better prognosis only in ER+ breast cancer [105, 106, 108].

Currently, several groups focus on the understanding of how the tumor microenvironment dictates treatment response. For instance, stromal cells sustain cancer cell survival after genotoxic and targeted therapy through paracrine and juxtacrine signaling [109]. In particular, it was demonstrated that stromal cells expressing Jagged1 on their surface were able to activate Notch3 on TNBC cells, thus promoting the expansion of cells resistant to chemotherapy and reinitiating tumor growth [110]. Therefore, these data supported the Notch3 role in chemoresistance of TNBCs.

Furthermore, Notch3 seems to be also involved in the resistance to targeted treatments, such as tyrosine kinase inhibitors (TKIs) against EGFR [111]. Targeting EGFR may be a promising approach to treat TNBCs since it is commonly overexpressed in this breast cancer subtype [112], but several clinical trials failed due to intrinsic and acquired resistance. In this scenario, the authors demonstrated a novel role of Notch3 in promoting resistance to TKI-gefitinib through regulating EGFR localization, thus rendering it targetable by TKI-gefitinib [111].

Overall, these studies suggested that Notch3 is strictly associated with pathogenesis of TNBCs and it is responsible for their aggressive phenotype.

2.2.4. Notch4. The first evidence that Notch4 could function as a protooncogene was associated with mouse mammary tumors which showed integration of the mouse mammary tumors virus (MMTV) into the Notch4 locus [113]. The major consequence of this integration is the production of a truncated protein which is constitutively activated. Therefore, aberrant expression of Notch4 leads to mammary epithelial dysplasia and impaired differentiation, finally resulting in mammary tumorigenesis in mice [114].

Several studies documented a correlation between TNBCs and high expression of Notch4. Speiser and colleagues analyzed 29 TNBC-bearing patients and Notch4 was widely expressed in 73% of the cases [53], in agreement with a previous study [115]. Moreover, Wang and colleagues analyzed a wider panel of breast cancers (98 samples) in which TNBCs exhibited the highest Notch4 expression [116], thus suggesting a pivotal role of Notch4 receptor in this subtype. This was further confirmed from genome-wide analysis of TNBC human samples in which Notch4 was found commonly mutated in patients with progression free survival (PFS) less than 3 months [41]. Notch4 seems to be associated with metastatic TNBCs: Lawson and colleagues, by analyzing the transcriptomic signature of TBNC patient-derived xenografts, detected high levels of Notch4 in metastatic cells [117]. In accordance with these findings, the expression of Notch4 correlated with overall poor prognosis and experimental evidence indicates that Notch4 contributed to tumor invasion and metastasis by sustaining EMT at the invasive front of primary tumors [118]. Castro and colleagues performed *in vivo* experiments on mice that established spontaneous lung metastasis from JygMC(A) cells. The authors state that Notch4 promoted tumor growth and metastasis through the finding of Notch4 nuclear localization in both primary tumors and lung metastasis. The treatment with an orally active GSI inhibitor (RO4929097) reverted the phenotype, thus inhibiting primary tumor growth, reducing the number of metastatic lung nodules, and finally confirming the contribution of Notch4 during mammary tumor progression [118]. More recently, Castro and colleagues tested Sulforaphane (SFN) in both human and murine TNBC cells and they observed that the same JygMC(A) cells were more resistant to SFN. Molecularly,

the authors demonstrated that SNF is able to reduce the promoter activity of Cripto1, a known positive regulator of Notch receptor maturation and signaling [119], thus linking the Cripto-mediated Notch4 signaling impairment with the observed inhibition of the proliferation of breast CSCs [120]. As previously mentioned, CSCs are associated with high-grade breast cancer and distant metastasis [121, 122] and contribute to intratumor heterogeneity [123]. Therefore, the understanding of signaling networks that regulate CSCs is urgently required. Since stem cells and cancer stem cells are usually characterized by the activation of the same pathways and Notch4 has been implicated in mammary stem cells [124], during the last decade several studies demonstrated that Notch4 activity strongly correlated with self-renewal and chemoresistance of breast cancer stem cells (BCSCs). Harrison and colleagues isolated BCSCs from breast cancer cell lines and primary breast cancer samples. They compared the activation of Notch1 and Notch4 in BCSC-enriched population to differentiated cells and they found that Notch1 and Notch4 are differentially expressed: Notch1 promotes the proliferation of progenitor cells and sustains their differentiation whereas Notch4 plays a role in the commitment of BCSCs to progenitor cells. Interestingly, decreased levels of Notch4 (but not of Notch1), obtained by both RNA interference or pharmacological treatment, significantly reduced mammosphere formation *in vitro* and reduced tumor formation *in vivo*, thus suggesting a specific role of Notch4 in regulating this subpopulation [125]. These results were consistent with a previous study in which Notch4-neutralizing antibody is able to inhibit cancer stem cell activity *in vitro* [126].

In keeping with these data, Rustighi and colleagues found that Notch1/4 is involved in the maintenance of breast stem cell self-renewal. The authors pinpointed the role of the prolyl-isomerase Pin1 in sustaining high levels and transcriptional activity of Notch1/4 through preventing their E3-ligase FBXW7-dependent proteasomal degradation [127, 128]. More interestingly, the authors demonstrated that the Notch1/4 suppression, Pin1-dependent, correlated with a sensitization of BCSCs to chemotherapy *in vitro* and *in vivo* [128].

All together these results suggest that high Notch4 levels are crucial to promote mesenchymal signature and to keep pro-stemness signaling constant during tumor progression of TNBC.

3. Notch-Targeting Approaches and Clinical Perspectives in TNBC

Chemotherapy is the current primary therapy for TNBCs in the neoadjuvant, adjuvant, and metastatic settings [129]. Although there is a small subgroup of patients with TNBC for whom chemotherapy may be effective, the heterogeneity of these tumors requires the development of most promising new targets and associated therapies that may improve the outcome of TNBC-bearing patients. The deregulation of various signaling pathways has been confirmed in patients suffering from TNBC and has recently come under development as a novel treatment option [130]. Among them, ADP ribose polymerase (PARP) inhibitors named PARPi (olaparib, veliparib, rucaparib, niraparib, talazoparib, and CEP-9722) have been evaluated on TNBC patients as mono- or combination therapies. Interestingly, BRCA mutated tumor cells are more sensitive to PARPi for combined loss of PARP and homolog recombination repair [131]. Tyrosine kinase receptors targeted by therapy include epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and vascular endothelial growth factor receptor (VEGFR) [90]. Expression of EGFR has been reported in up to 89% of TNBC patients, particularly for BL2-subtype tumors [132], which depend on EGFR for proliferation and represent the major candidates for anti-EGFR therapies [133]. Unfortunately, only limited benefit has been reported in clinical trials using anti-EGFR agents, such as monoclonal antibodies (Cetuximab or Panitumumab), in combination with chemotherapy [134, 135]. Defect of Wnt/β-catenin pathway has been identified as an alternative therapeutic approach [136] and PI3K/AKT/mTOR pathway is also emerging as a promising target. It has been reported that inhibition of the PI3K pathway enhanced sensitivity to PARPi in TNBC cell lines [137]. Moreover, Yunokawa et al. reported positive effects of Everolimus, an mTOR inhibitor [138]. For years, TNBC was not considered sensitive to immunotherapy, but now this option is emerging as an exciting treatment [139], because of the immunogenic nature of TNBC compared with other breast cancer subtypes [140]. However, these strategies are effective in less than 20% of cancer patients or are useful only for certain TN cancer subgroups [141]. Therefore, further therapeutic strategies are urgently needed.

In this scenario, targeted therapy focused on modulating aberrant Notch signaling is emerging as a possible treatment approach for patients with TNBC (Table 1). Novel opportunities arise from the discovery of Notch crosstalk with many oncogenic signaling which suggested that Notch pathway may be considered such a multitarget drugs' candidate [13, 142–144]. To date, several clinical studies involved targeting of Notch pathway with either γ-secretase inhibitors (GSIs) or monoclonal antibodies (mAbs) against Notch receptors [145], which represent the major therapeutic targets of Notch signaling pathway.

3.1. γ-Secretase Inhibitors (GSIs) in TNBC. GSIs act by preventing the cleavage of the active form of all Notch receptors, thus inhibiting their transcriptional activity [146, 147]. It is demonstrated that GSIs interfere with cell cycle, lead to apoptosis in both luminal and TNBC cell lines [14], and, in particular, reduce the growth and dissemination of MDA-MB-231 TNBC xenografts [148]. It is shown that GSI treatment upregulates the proapoptotic protein Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), reduces CSC colony formation, and results in apoptosis of human TNBC cell lines [149]. In another study, it is demonstrated that 13% of TNBCs with PEST domain mutations in NOTCH1, NOTCH2, and NOTCH3 receptors and patient-derived xenografts are highly sensitive to the PF-03084014 GSI [40]. These mutations provoke a truncation in the C-terminus of Notch protein,

removing the PEST domain while retaining the γ-secretase cleavage site. These findings suggest that GSI might be promising in treatments of TNBC subset with specific Notch sequence alterations.

Unfortunately, the gastrointestinal negative effects impede the clinical use of GSIs [150], suggesting that much more work is required for having favorable effects after GSI treatments. In this scenario, novel therapeutic strategies will likely come from combinations of GSIs with conventional chemotherapy, in order to reduce the single dose of both treatments, thus limiting either toxicity. Zhi-Lu Li and colleagues demonstrated the feasibility of the combined use of GSIs and Doxorubicin on MDA-MB-231 cells, resulting in encouraging new therapeutic approach in TNBC treatment [74]. Actually, RO-4929097 and MK0752 GSIs are investigated in phase I/II clinical trials and, recently, the combination of RO-4929097 and chemotherapics like Paclitaxel and Carboplatin is in a phase I clinical trial for TNBCs [151]. Moreover, since preclinical studies prompted evaluation of combination of PF-03084014 GSI with docetaxel for the treatment of patients with TNBC [152, 153], Locatelli and colleagues designed a phase I study in order to evaluate safety, tolerability, pharmacokinetics, and antitumor activity of this combination. Preliminary results demonstrated feasibility of the combined GSI-chemotherapy approach, thus promoting further studies in order to use Notch signaling inhibitors in combination with conventional chemotherapy in the treatment of TNBC-bearing patients [154].

3.2. Monoclonal Antibodies (mAbs) in TNBC. Despite these encouraging results on GSI treatment, there is an increasing number of studies based on the use of monoclonal antibodies against Notch members in order to achieve higher specificity. The use of specific monoclonal antibodies is based on their capacity to bind the extracellular regulatory region of the receptor, to mask the cleavage domain of metalloproteinase ADAM, and to induce a conformational change of the receptor into its inactive form [155]. Recently, it has been shown that an antibody against the negative regulatory region (NRR) of Notch1 resulted in reduced proliferation, restricted expression of its targets HES1, HES5, and HEY-L, reduced colony forming ability, and lessened cancer stem-like population in MDA-MB-231 cell lines [156]. As previously mentioned, the inhibition of Notch1 with the novel monoclonal antibody MAb602.101 reduced TNBC cell lines tumor growth and sphere-forming potential, thus directly affecting CSCs niche [66]. In accordance with these results, TNBC patients which display high level of Notch1 expression are characterized by poorer survival, thus suggesting that hyperactivation of Notch1 receptor may be used as a predictive marker for TNBCs [66] and finally pointing out the Notch1 inhibition as a potential novel approach to achieve the outcome of TNBC-bearing patients. Interestingly, it has been also demonstrated that the antibody use can amplify chemotherapy treatments: in a TNBC patient-derived xenograft model, Notch1 monoclonal antibodies exhibited synthetically antitumor efficacy combined with docetaxel via inhibition of CSCs generation and maintenance [64].

Moreover, a Notch2/3 blocking monoclonal antibody named tarextumab (OMP-59R5) was developed: it was successfully tested on patient-derived epithelial tumor xenograft models, including breast, thus showing significant antitumor activity [157]. Recently, Choy and colleagues used a novel monoclonal antibody that selectively targets the Notch3 NRR (anti-N3.A4) [158] to make a comparison between Notch3-specific versus pan-Notch effects for treatment of TNBCs. They documented that both treatments significantly inhibited colony formation *in vitro* and modestly reduced tumor growth *in vivo* to similar extent [93]. Therefore, the authors strongly suggested that the therapeutic targeting of Notch3 could provide therapeutic benefit without the known toxicities associated with pan-Notch inhibition, as GSIs fail to distinguish the particular Notch receptor driving growth [93]. Similar results have been obtained by Farnie and colleagues who demonstrated that Notch4-neutralizing antibody inhibited cancer stem cell activity *in vitro* [126].

Notch ligands targeting could be also a promising strategy to reduce Notch activation. Hoey and colleagues used monoclonal antibody against DLL4 ligand to block its binding to Notch1, thus observing antitumor effects in a wide range of human tumor xenografts from various tumor types, including breast cancer. Specifically, the inhibition of DLL4-Notch1 axis decreased CSC frequency [159]. More recently, a monoclonal antibody against Jagged1 ligand has been developed to be used for the treatment of established bone metastasis that is refractory to chemotherapy [160]. The authors observed that chemotherapy agents were able to induce Jagged1 expression at the cell membrane of osteoblasts and mesenchymal stem cells of bone marrow, which in turn activated Notch signaling, finally promoting chemoresistance [160].

Interestingly, more recently it has been demonstrated that the overexpression of Notch receptors or their ligands at the cell membrane of cancer cells might be also turned to our advantage in order to effectively deliver cytotoxic agents to the tumor sites. In this view, a novel anti-Notch3 antibody-drug conjugate currently named PF-066580808 is now under clinical investigation (phase I) for the treatment of breast cancer, including TNBCs [161]. Besides above described approaches, several natural compounds and their derivatives are showing Notch inhibition and antiproliferative activities in different *in vitro* cancer models, thus suggesting their potential application as additional therapeutic option in Notch-related cancers [68, 162].

Further studies into mechanisms of action of individual Notch receptor in TNBC development and behavior should be addressed in order to ameliorate the understanding of the complexity and mechanisms that underlie TNBCs. In this view, the aforementioned results suggest that the potential targeting of the Notch signaling pathway with different molecules should be studied in more detail to further improve the treatment options for TNBC-bearing patients.

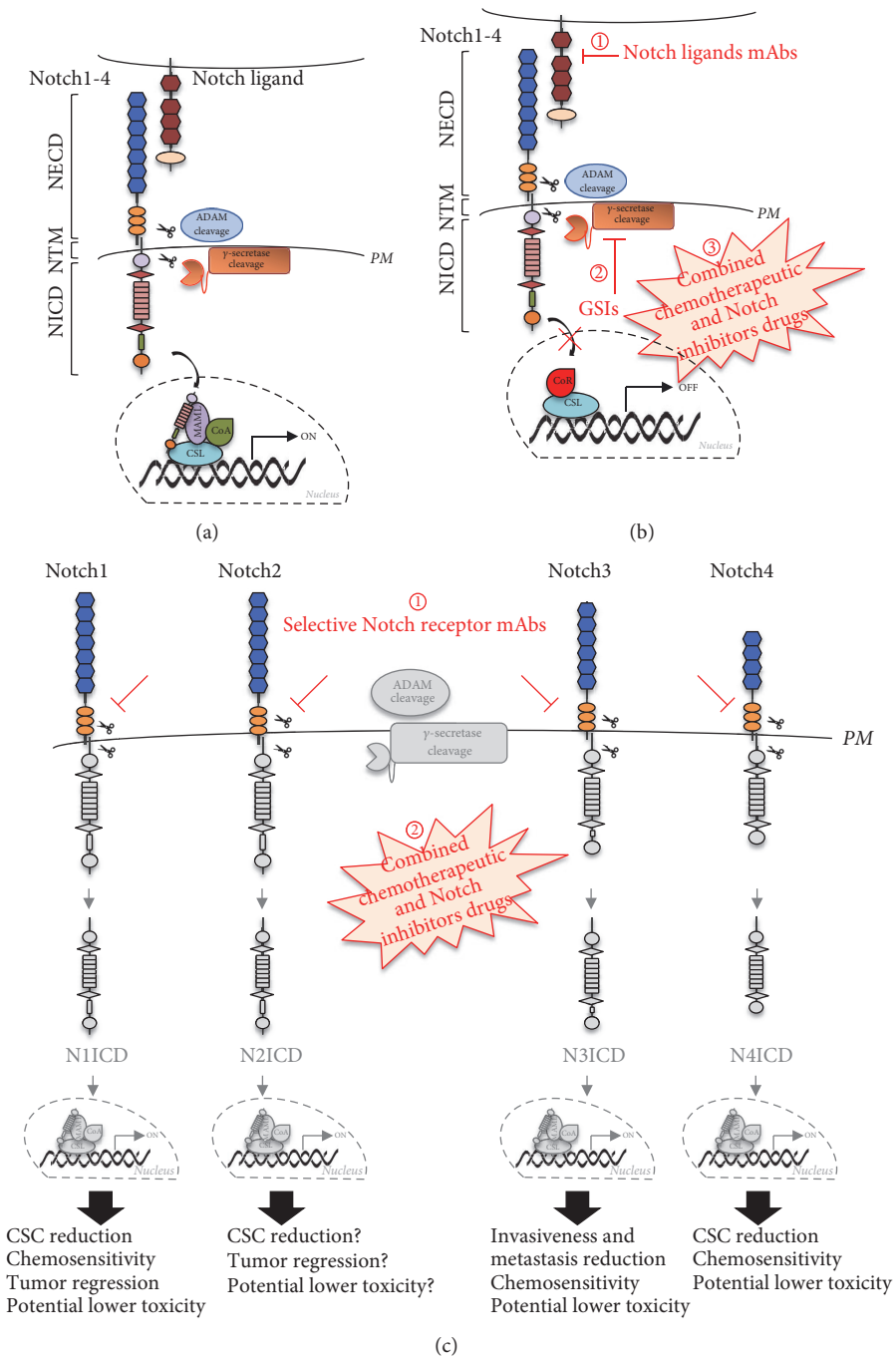

Figure 2: *Notch-targeting therapeutic approaches in TNBCs.* (a) The canonical Notch signaling pathway: ligand binding promotes sequential cleavages of the Notch receptors (Notch1-4) by ADAM enzyme and γ-secretase complex, resulting in the release of NICD, which translocates in the nucleus, interacts with transcriptional regulators to transcriptionally activate the canonical Notch target genes (ON), thus leading to the regulation of TNBC growth and progression. (b) Notch inhibitors with lower or absent selectivity, respectively, include mAbs targeting the Notch ligands and GSIs. (1) mAbs against Notch ligands prevent ligand-receptor interaction and the subsequent Notch cleavages, preventing Notch signaling triggering. Little is known about the specific Notch-ligand relationship in TNBC; thus further studies are needed to consider ligand blocking as a potential alternative selective approach in TNBC treatment. (2) GSIs act as pan-Notch inhibitors since they prevent the cleavage of all Notch receptors, thus avoiding the release of any NICD. This unselective mechanism of action is strongly correlated with a high intestinal toxicity in patients, which significantly impairs their clinical use. (3) Lower doses of GSIs used in combination with chemotherapeutic drugs result in improved clinical outcome and less toxicity, which however must be overcome. (c) A higher selectivity can be obtained by using monoclonal antibodies directed against the extracellular domain of a specific Notch receptor (1): mAbs mask the cleavage domain of ADAM, thus preventing the binding of this enzyme and the subsequent γ-secretase cleavage. The final effect will depend on the specific block of the single Notch receptor, also used in combination with chemotherapeutic drugs (2). Several studies detailed in the text have suggested that a greater selectivity in the Notch inhibition approach for TNBCs treatment is strongly correlated with a higher probability of success in favoring tumor regression, associated with less toxicity and therefore with a potential better prognosis of TNBC-bearing patients. Abbreviations. ADAM: a disintegrin and metalloproteinase; CSL: CBF1/Su(H)/Lag-1; CoA: coactivator; CoR: corepressor; GSIs: γ-secretase inhibitors; mAb: monoclonal antibody; MAML1: mastermind-like 1; NECD: Notch extracellular domain; NICD: Notch intracellular domain; NTM: Notch transmembrane; *PM*: plasmatic membrane.

4. Conclusion

TNBC is an aggressive subgroup of human breast cancer, characterized by high rates of relapse and frequent metastasis. Since unresponsiveness to current treatment is often observed, the development of novel strategies to treat also this form of breast cancer is urgently required.

Several pathways are involved in the pathogenesis of TNBC. Among them, Notch signaling plays a key role in tumor initiation and mainly in tumor progression. Indeed, several experimental studies documented the role of Notch signaling in promoting EMT for cancer cell seeding to secondary organs and in sustaining the maintenance of CSCs which are responsible for chemoresistance. Therefore, inhibition of Notch signaling has been considered as an attractive strategy for the treatment of TNBC. Several pan-Notch inhibitors are currently under clinical trials in combination with chemotherapy [163] but they fail to distinguish individual Notch receptors and cause intestinal toxicity. In addition, since individual Notch receptors can have opposite role in the same cancer, their simultaneous inhibition may have pleiotropic effects possibly resulting in tumor stimulation.

This review covers the roles of individual Notch receptors in TNBC development and progression, thus showing that they only partly share the same functions in TNBC context. As a result, determining the Notch receptor which is specifically involved in different TNBC subtypes might be useful to identify patients who are most likely able to respond to different targeted therapy, paving the way for avoidance or likely reduction of the therapeutic complications associated with nonselective Notch inhibitors. In conclusion, this review will aid further research in identifying a suitable treatment for TNBC, as the specific inhibition of a single Notch receptor or ligand might promote new clinical trials aiming to evaluate more selective and less toxic alternatives for Notch inhibition in the treatment of TNBC-bearing patients (Figure 2).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

M. V. Giuli and E. Giuliani contributed equally to this work.

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