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ROLE OF NOTCH SIGNALLING IN HUMAN HEPATOCELLULAR CARCINOMA

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SUMMARY

INTRODUCTION

1.1. The Notch Signalling

Notch signalling pathway is one of the pleiotropic mechanisms that underwent evolution and that constitute the basal cellular language, through which cells decide their fate.

The Notch gene was discovered in 1917 in *Drosophila melanogaster* in which partial loss of function determs notches at the wing margin of flies (Morgan T., 1917) and it keep the name from this phenotypic feature. Notch was defined as a dominant, haploinsufficient, X-mutation, that in homozygous females and hemizygous Notch males is lethal for the embryo. Later, Poulson define the link between Notch mutation and correct morphogenesis and in 1983 Notch was found to be responsible of a neurogenic phenotype in which cells that are determed to become epithelial, gives instead rise to neural cells (Lehmann R. et al. 1983). This discovery evidence its function, conserved from *Drosophila* to *Homo sapiens*, that is to establish cell fate in many tissues, during development and in the adult (Artavanis-Tsakonas S. et al. 1999; Borggrefe T. and Oswald F. 2007). Indeed, it has been involved in control of cell proliferation, survival, apoptosis, differentiation and stem cell maintenance. In past 30 years a field about Notch studies developed greatly and let to understand that normal Notch pathway function is central in the most developmental decision in animals and that its dysfunction is implicated in many diseases, including cancer. (Artavanis-Tsakonas et Muskavitch A.T., 2010).

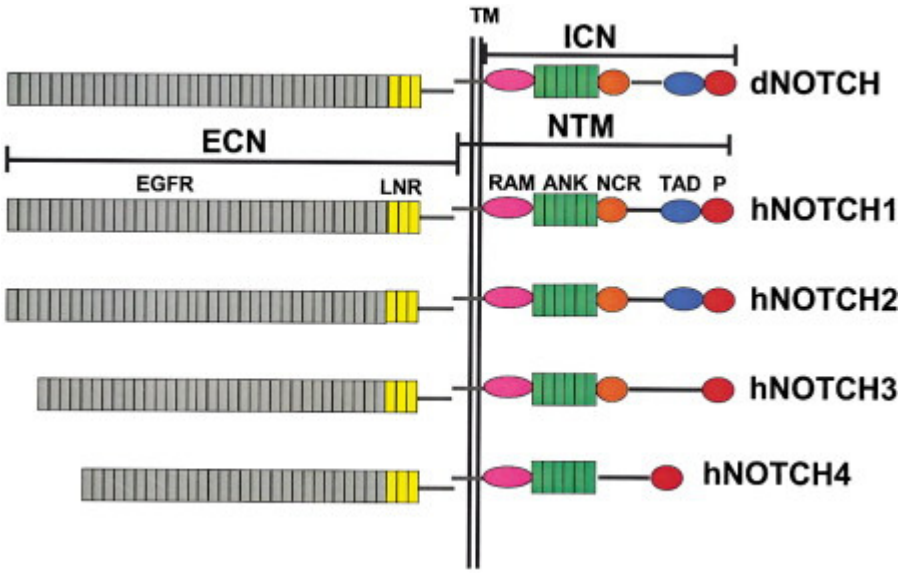
1. Genes and structure of the protein

Notch gene in *Drosophila* code for a 300 kDa single pass transmembrane receptor, composed by many structural and functional domains. The large extracelular domain contains 36 tandem epidermal growth factor (EGF)-like repeats and three cystein rich Notch/LIN-12 repeats. In the intracellular domain six tandem ankyrin repeats, a glutamine-rich domain and a PEST sequence are founded (Artavanis-Tsakonas S. et al. 1999). These structural features are conserved between species. In vertebrate and in human four gene exist, coding for four receptor Notch1, Notch2, Notch3 and Notch4. Despite maintaining common structural and functional domains, genes product are different in protein product size. (EnsmbL, web-source)

	Localization	Exons	Transcript (bp)	Residues (aminacids)
Notch1	9q34.3	34	9371	2555
Notch2	11p12.3	34	11423	2471
Notch3	19p13.12	33	8070	2321
Notch4	6p21.32	30	6740	2003

These paralogs genes display differences in primary sequence, that let to distinguish them from each other and at the same time they show both overlapping and distinct expression profiles and developmental functions (Artavanis-Tsakonas S. et al. 2010). Differeneces in Notch primary structure translate into differential target specificity with the possibility that they could be interchangeable biochemically in some context, whereas in other they show specificity and also antagonist function, as reported for Notch1 and Notch3 (Beatus et al. 1999; 2001).

Despite differences in primary structures, the four human Notch receptors (hNotch) receptors showed a high structural conservation in functional domains each others and with the *Drosophila*'s Notch. They are type I single pass transmembrane receptors. The full-length proteins are expressed on the cell surface as heterodimers composed of noncovalently associated extracellular (ECN) and transmembrane subunits (NTM). All Notch receptors contain epidermal growth factor-like repeats (EGFR), Lin12 Notch repeats (LNR), a RAM23 domain (RAM), Ankyrin repeats (ANK), and PEST (P) sequences. The highest degree of homology between Notch receptors is in the ankyrin repeats, whereas the C-terminal sequences show the greatest degree of divergence. Human Notches1–3 (hNotch) contain sequences immediately C-terminal of the ankyrin repeats (NCR) that regulate functional activity. Further C-terminally, hNotch1 and hNotch2 contain strong and weak, respectively, C-terminal transcriptional activation domains (TAD); a similar domain is also present in dNOTCH (Allman D. et al. 2002)



Structural representation of dNotch and hNotch receptors. From Allman D. et al. 2002, Cell

The ECN (extracellular Notch) is composed by a number of EGF-like repeats that vary from Notch1 and Notch2, that have both 36 and that mantain the higher level of homology also in the intracellular portion, to Notch3 which has 34 EGF-like repeats and Notch4 that has only 29. This external portion is necessary for the productive interaction with the ligands in the membranes of neighbouring cells that occurs through repeats 11-12, whereas of the cis-inhibition by ligands expressed in the same cell depends on 24-29 repeats The three Lin 12/Notch repeats, conserved in all receptors, restrain inappropriate, ligand-independent receptor activation (Kopan R. et al. 2009).

The NTM includes a short extracellular domain, a single transmembrane domain, and a large intracellular domain. The single transmembrane domain ends with a C- terminal stop translocation signal, composed by 3-4 arginine/lysine residues. The intracellular domain comprises a RAM sequence (RBPjκ association module) a high affinity binding module that mediate the protein-protein interaction with the effector of the pathway in mammals (RBP-JK); seven iterated cdc10/ankyrin-like repeats, the ANK domain, two nuclear localization signals (NLS) given by polyglutammines trait, and a C-terminal PEST sequence. In addition, mammalian Notch 1, 2 and 3 contain cytokine response (NCR) regions, and Notch 1 and 2 have C-terminal transcriptional activation domains (TAD). The ANK domain is responsible of interaction with component of the transctriptional apparatus and it seems critical for activity regulation (Beatus P. et al. 2001). The PEST sequence (Pro-Glu-Ser-Thr) is involved

in regulation of the stability of the receptor, as it is harbour a degradation signal. (Kopan R. et al. 2009; Allenspach et al. 2002).

Genes are transcribed in a unique transcript that is translate and processed in the Golgi. Here, a furin-O-protease and Rumi enzyme glycosilate and processe the protein, giving a functional receptor in which the polypeptides are divided in two non-covalently interacting portions, NEC and NTM.

Also Notch ligands are themselves type I transmembrane proteins. The largest class of Notch ligands is characterized by three related structural motifs: 1) a N-terminal DSL (Delta/Serrate/LAG-2) motif, 2) specialized tandem EGF repeats called the DOS (Delta and OSM-11-like proteins) domain (Komatsu H. et al., 2008), 3) and EGF like repeats (both calcium binding and non-calcium binding). DSL ligands can be classified on the basis of the presence or absence of a cysteine-rich domain (Jagged/Serrate or Delta, respectively) and the presence or absence of a DOS domain, both necessary for receptor binding. So, in mammals and in human there are four Delta and two Jagged ligands.

Additional proteins lacking DSL and DOS domains have been reported to act as noncanonical ligands for Notch receptors in the central nervous system and in cultured cells, but these molecules and their physiological functions have been unexpleored for these proteins in the Notch pathway remain to be elucidated (Kopan R. et al. 2009).

1.2 Activation of the pathway

The activation of the pathway occurs when ligand and receptors bind each other with a cell-to cell interaction, between cellular membranes of neighbouring cells. This event activates a series of proteolitical cleavages, that are the key of pathway activation. Ligand binding leads to the cleavage of Notch by ADAM (a disintegrin and metalloprotease) proteases at site 2 (S2), located 12 amino acids before the transmembrane domain and deeply buried within the negative regulatory region. Site 2 cleavage is a key regulatory step in Notch activation. Two enzymes, ADAM17/TACE (tumor necrosis factor α converting enzyme) and Kuzbanian/ADAM10/Sup-17 metalloprotease seems able to mediate S2 cleavage, with partially redundant roles (Brou C. et al. 2000). This cleavage causes the shedding of the Notch ectodomain which creates a membrane-tethered intermediate called Notch extracellular truncation (NEXT) that is a substrate for γ-secretase, a multicomponent member of intramembrane cleaving proteases family (I-CLiPs), enzyme complex that contains presenilin, nicastrin, PEN2 and APH1. γ-Secretase cleaves NEXT within the transmembrane domain, starting near the inner plasma membrane leaflet at site 3 (S3) and ending near the middle of

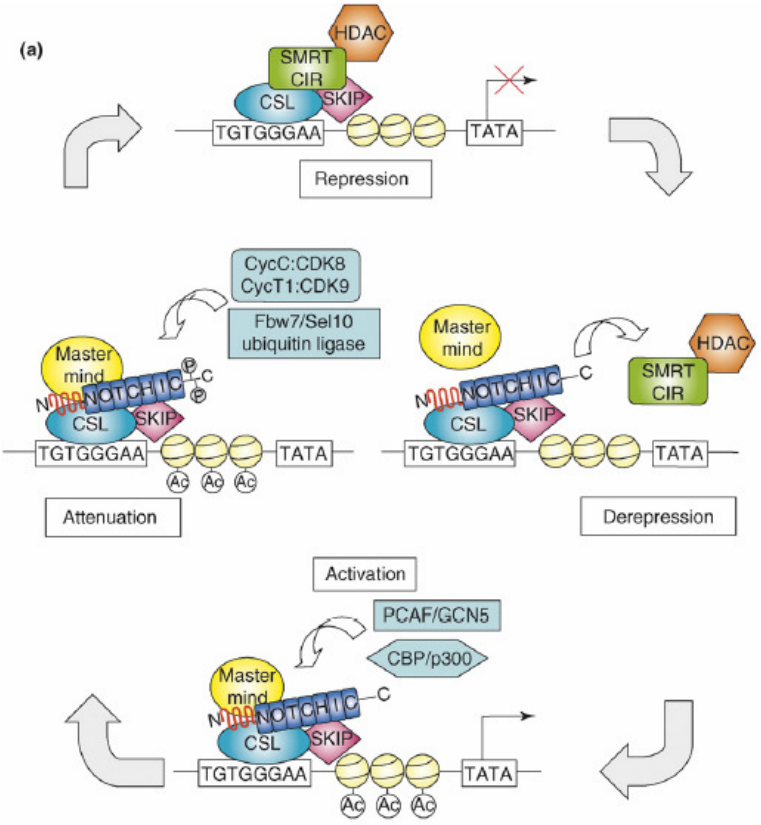
the transmembrane domain at site 4 (S4). Interestingly, γ-secretase ubiquitylation on a juxtamembrane lysine residue was necessary for S3 cleavage in mammalian cell assays, indicating that cleavage occurred after Notch endocytosis (Gupta-Rossi N. et al. 2004). Only after γ-secretase cleavage the Notch intracellular domain, NICD, is released and it can translocate to the nucleus where it activates transcription of target genes (Kopan R. et al. 2009; Bray S.J., 2006). Here the NICD cooperates with the DNA-binding protein CSL (named after CBF1, Su(H) and LAG-1) and its coactivator Mastermind (Mam) to promote transcription of its target genes. These DNA-binding proteins have been highly conserved throughout evolution (for example, there is 84% identity between human and *D. melanogaster* proteins)(Fortini and tsakonas 1994) In mammals, CSL is called RBP-Jk (recombination signal sequence-binding protein Jk) and bind the DNA at the regulatory region of genes that contain RBP-JK binding sites. Because the NICD could not bind directly the DNA, it acts as transcriptional coactivator, heterodymerizing with RBP-Jk and activating the transcription of its target genes. The RBP-Jk converts extracellular and cytoplasmic Notch signals into transcriptional outputs.

RBP-Jk /CSL represses transcription of Notch target genes by interacting with the basal transcription machinery and recruiting ubiquitous corepressor proteins to form multiprotein transcriptional repressor complexes (Lai EC. 2003). Indeed, RBP-Jk was identified as a transcriptional repressor that bind a repressive 10bp DNA motif (Dou S. et al. 1994) and then as transcriptional activator. Indeed, the repressor or activation activity is dependent by the binding with co-repressors or co-activators that is induced by NICD (Borggrefe T and Oswald F). Normally, RBP-Jk binds specific cis-elements in the promoter of Notch target genes, heterodimerizes with co-repressors, such as the SMRT (silencing mediator of retinoid and thyroid receptors)/N-CoR (nuclear receptor corepressor), CIR (CBF1-interacting corepressor), Hairless and SPEN (also known as SHARP, SMRT/HDAC-1-associated protein) and it forms a transcriptional repressive complex. Indeed, the binding of corepressors lets CSL/RBP-Jk to recruit histone deacetylase complexes (HDACs), which convert the local chromatin into a transcriptionally silent form. The sequence recognized by CSL/RBP-Jk is 5'-CGTGGGAA-3' in the promoter of Notch target genes and it is conserved from *Drosophila* to Human. (Borggrefe T et.) Then, after translocation to nucleus, the binding of NICD causes a swhich from a repressor complex to an active transcriptional one, by displacing negative co-factors and binding transcriptional co-activators. The transcriptional coregulator SKIP (Ski-interacting protein) bridges interactions between CSL/RBP-Jk and either NICD or corepressors, but not both simultaneously, and is thought to be present in the complex during

both transcriptional activation and repression (Zhou et al. 2000;). The foundamental event after the displacement of the co-repressor induced by NICD binding is the formation of a ternary complex between NICD-CSL/ RBP-Jk and Mastermind, a transcriptional co-activator protein that is necessary to activate the transcription. Indeed, dominant negative form could completely block Notch signalling activation. Activation of transcription occurs by recruitment of the general transcription factors PCAF/GCN5 and CBP/p300 to the CSL–NotchIC–Mastermind ternary complex. PCAF/GCN5 and p300 are hystone acetyltransferases that let to swich to a transcriptional permissive complex (Kurooka H, Honjo T. 2000; Oswald F.et al. 2001). NICD translocates to the nucleus thanks to RAM23 domain containing the nuclear localization sequence and it interacts with RBP-Jk through the ankyrin repeats.(Tamura K. et al. 1995). The transactivator domain (TAD) participate in activate transcription, whereas the S4 region (WSSSSP) at the C-termnal of NICD is required for its turnover. (Borggrefe) Despite the transcriptional activation occurs as consequence of co-factors binding to the ternary complex, the level of transcriptional activation is dependent on the promoter and cell type, as well as on which NICD engages CSL/RBP-Jk. Transcriptional activation is then blocked by proteolytic turnover of NICD, in which Mastermind and SKIP recruit specific kinases that hyperphosphorylate it and this phosphorylation is translated in a ubiquitin-ligase-mediated degradation through proteasome (Fryer CJ. Et al. 2004 Mol cell biol). The recruited kinase is cyclin-dependent kinase 8 (CDK8), that phosphorylates different sites and this leads to recognition of the NICD by the E-3 ubiquitin ligase Sel-10. Ubiquitylation induces proteasome degradation of the NICD. So, CSL/RBP-Jk is the pivotal element of the transcriptional switch, functioning as a hub for protein–protein interactions and being the crucial mediator of the Notch signalling. Indeed, it binds transcriptional co-repressor factors, then it binds NICD that induces the swich of the complex, recruits transcriptional co-activators and negative regulators of the signals, such as the kinases that targets NICD to induce its degradation. (Kovall R.A. 2007). This aspect become more important considering the fact that the Notch signalling is not amplified by second messenger, so the level of activation is directly dependent by nuclear levels of NICD that is the result of the balance between pathway activation and NICD degradation. Moreover, an addictive level of complexitiy is given by the fact that NICD functions in combination with tissues specific factors. Therefore, this binding contributes to robust target-gene expression, and can explain the specificity of Notch responses in different cell types. In some cases, the precise arrangement of binding sites influences the cooperation between Notch and other DNA-bound activators, in others modification as sumoylation might also regulate the activity

of key nuclear components. Interestingly, Notch activity is highly sensitive to chromatin modifications and histone rearrangements that could contribute to target gene specificity. Furthermore, overexpression of two Polycomb group epigenetic silencers enhances Notch-induced overproliferation and also causes hypermethylation of a tumour suppressor gene (Ferres-Marco D. et al. 2006). This indicates further mechanisms such as epigenetic modifications that regulate the accessibility of enhancers and cooperate with Notch to confer different programmes of gene expression (Bray S. 2006).

A model scheme of the transcriptional complex and its dynamic organization is reported in the following figure.



Notch signalling transcriptional complex. From Kovall R. A., 2007

1.3 Pathway regulations

Notch receptors have broad expression patterns in many tissues, but analyses of where cleavage occurs or where target genes are expressed reveal a limited profile of activation. Furthermore, given that each Notch molecule undergoes proteolysis to generate a signal, it can generate it only once. For that both ligand and receptor availability at the cell surface are way to controlling Notch activation, together with spatial and temporal restriction in expression. Neverthless, these strategies are not enough to fully regulate pathway activation, so ligands and receptors must also be regulated through post-transcriptional mechanisms

(Kopan R. et al. 2009; Bray S.J., 2006). These mechanisms interest both receptors and ligands. Between cellular processes that acts as receptor regulators and are not directly linked to pathway activation, are the following.

Role of glycosylation: Notch extracellular multiple EGF-like repeats are sites for glycosylation. The enzyme *O*-fucosyl transferase (*O*-Fut) adds the first fucose and was thought to be essential for the generation of a functional receptor. Later, it has been demonstrated that also receptors that were non fucosylated were able to bind to ligands and to transduce signals. Not only is the enzymatic activity important, *O*-Fut also functions as a chaperone to promote the folding and transport of Notch from the endoplasmic reticulum to the cell membrane. Moreover, fucosylation appears to be essential for signal events that require regulation by Fringe glycosyltransferase, that extend fucolsyl modification adding more sugars and these modifications can determ which ligand can bind to activate the receptor. In *Drosophila m.,* Fringe-dependent modification of the Notch extracellular domain renders Notch more sensitive to Delta and less sensitive to Serrate in the dorsal compartment leading to elevated Notch activation in the stripe of dorsal cells; conversely, the adjacent stripe of ventral cells also undergoes increased Notch activation, due to its lack of Fringe and relatively higher responsiveness to Serrate expressed by the neighboring dorsal cells. This is an example of how the pathway defines its outcome through post transcriptional modifications of receptors , that determ differents outcomes in pathway activity. In mammals, Fringe protein are Lunatic, Radical, and Manic Fringe, they are expressed in restricted patterns and modulate Notch signaling in analogous patterning events. Neverthless, a wider and more complex range of possible effects resulted by *in vitro* and *in vivo* studies, suggesting that a higher number of ligands, receptors and Fringe proteins, make these modifications more difficult to understand. (Fortini M. E., 2009 ;Kopan R. et al. 2009).
Also N- glycosamintransferases have a crucial role in modifying receptor and establish its ligand specificity (Kopan R. et al. 2009; Bray S.J., 2006).

Endocytosis and trafficking**:** Despite its physiological transmembrane localization**,** a substantial amount of Notch is targeted for degradation and a large fraction of receptor is detected in the cytoplasm in compartments of the endocytic pathway. Neverthless, this Notch cytoplasmic accumulation in endocytic structures does not affect activity of the pathway. Conversely, mutations affecting sorting of ubiquitylated membrane proteins, cause strong impairment of the pathway. (Bray S.J., 2006). Indeed, genetic studies on dynamin mutants

reveal that endocytosis is essential for productive Notch signaling (Parks et al., 2000; Seugnet et al., 1997). These studies, together with molecular analyses of the sequential Notch cleavages in mammalian cells, led to the idea that the dynamic forces of membrane invagination during endocytosis of Notch and its ligands might facilitate ligand-induced ectodomain removal and exposure of the ADAM10/TACE cleavage site (Brou et al., 2000; Mumm et al., 2000; Parks et al., 2000). Interestingly also endocytosis of DSL ligands within the signal-sending cell is also necessary for Notch signaling in Drosophila and mammals. Blocking dynamin-dependent endocytosis in flies has nonautonomous as well as autonomous effects on Notch signaling, implying a requirement for endocytosis in the signal-sending cells that express the ligand Delta (Seugnet et al., 1997). Moreover, DSL ligands are detected in intracellular vesicles in Drosophila and mammalian cells (Itoh et al., 2003; Kooh et al., 1993). The endocytic pathway seems to be a mechanism of pathway regulation, both regulating receptors and ligands disponibility and localization in cell membranes. Moreover, for the receptors activation, it is possible that some endocytic components favour receptor shedding and so the activation of the pathway during the membrane invagination(Gupta-Rossi, N. et al. 2004).

Vescicle trafficking is necessary for the activation of the pathway, both in sending cells, that express the ligand, and also in receiving cells, that express the ligand. Both these aspects are relevant for the signalling in term of functions and cellular outcome.

Ubiquitylation. A surprising discovery was the ubiquitylation as a crucial layer of regulation of Notch signalling, both for receptor stability and ligands modification, leading to a relevant pathway's regulation. Entry into the endosomal and multivesicular-body-sorting pathway is thought to be linked with ubiquitylation of transmembrane proteins. Several E3 ligases that target Notch have been identified, the Itch/ NEDD4/Su(dx) family of HECT domain E3 ligases are predominantly negative regulators of signalling targeting it for degradation in the RAM-Ankyrin repeat region. (Lai E. C. et al. 2002). By modulating receptor turnover, Itch/NEDD4/Su(dx) could regulate the amount of Notch that is available to interact with ligands. (Bray S.J., 2006)

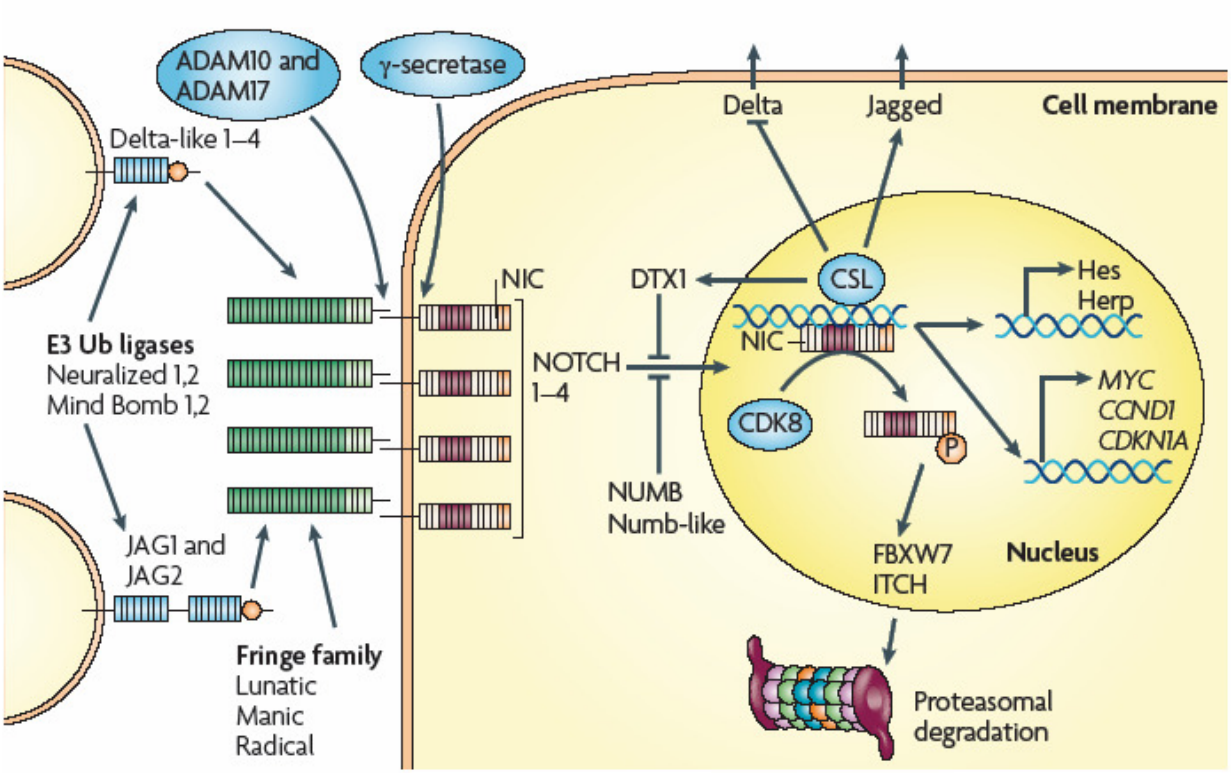
A second E3 ligase that binds to NICD in the ankyrin repeats is the RING finger protein Deltex (Matsuno K. et al. 1995). Deltex regulates accumulation in endocytic vescicles and can antagonize Notch activation in mammal cells (Itoh H. et al 2003; Sestan N. et al. 1999). The balance of different E3-ligase activities dictates the outcome on Notch localization and activity. Ubiquitin modifications could potentially influence the length of time that the

receptor is located on the surface, its accessibility to ligands, or its capability to interact with γ-secretase (Gupta-Rossi, N. et al. 2004; Bray S.J., 2006).

Other E3 ubiquitin ligases targets ligands, Neuralized (Neur) and Mindbomb (Mib), that interact directly with Notch ligands and are required for ligand activation (Chitnis, A. et al. 2006; Le Borgne et al. 2005). These two enzymes differ each other for expression pattern and probably for capability to interact with different ligands. Interestingly, Notch trafficking results to be completely impaired when Neuralized and Mindbomb are not expressed, because ligands localize in membranes, but they are inactive. Moreover, altered ligands ubiquitylation impairs endocytosis. These seems to be dependent by the capability of ubiquitilation to generate a 'pulling force' on a bound receptor that causes a conformational change in the juxtamembrane region, whereas another possibility is that ubiquitylation promotes ligand clustering or enter in the endocytotic trafficking, leading to a ligand modification and specific cell membrane re-localization. Neverthless, also DSL ligand secreted are ubiquitilated, suggesting that ligand ubiquitilation is a regulatory mechanism independent by strictly membrane behaviour (Bray S.J., 2006).

Moreover, ubiquitylation is foundamental to turn off the signal. The transcriptional activation mediated by NICD is blocked through hyperphosphorylation of the NICD by kinases recruited by CSL/RBP-Jk, but this modification leads to an E3-ubiquitin ligase reaction by Sel10 enzyme and consequent NICD degradation in the proteasome (Fiuza and Arias, 2007).

Numb: Numb is membrane-associated protein that acts as inhibitor of Notch signalling and it is crucial in determining cell fate because it segregates asymmetrically in one of two daughther cells after cell division, leading to determine their cell fate.(Uemura T. et al. 1989). The cells differ for bringing Numb, that acting as inhibitor of Notch signalling, it could drive cell determination. Inhibition of the signalling mediated by Numb involves endocytosis. It interacts with the ear domain of α-adaptin, a component of the endocytic machinery, and with Notch, recruiting it directly into endocytic vesicles. Sequestering full receptor, membrane tethered Notch or NICD in endocytic pathway, it acts as negative regulator of the signalling. Furthermore, mammalian Numb promotes Notch ubiquitylation. Indeed, with its PTB domain it can bind ubiquitin ligases like Itch and at the same time it recruits Notch, acting as scaffold protein for Notch ubyquitylation and consequent degradation. (Gulino A. et al. 2010).



Notch signalling pathway. From Dotto G.P., 9:587-595, 2009, Nature Reviews Cancer

1.4 Target genes

The peculiar aspect of the Notch signalling is the direct activation of the transcriptional activity by the main component of the pathway, without the presence of signal amplification downstream of receptor activation. Activation and arrest of transcription are regulated by NICD levels in the nucleus and interaction with RBP-JK that binding cis-element in the DNA, mediate the physical recogniction and activation of Notch on its target genes. So, the activation of the transcription is regulated by NICD release, due to pathway activation on a side and its degradation on the other. Neverthless, low levels of NICD, behind detection limits, are enough to activate transcription, because of it functions as a transcriptional coactivator on the RBP-Jk factor constantly bound at the promoter of target genes (Schroeter J. A. 1998). Cell type specific activation of a target depends on the presence of coactivators, their modification and accessibility of the chromatin. Transcriptional activation is dependent from interaction with HDAC and acetyltrasferases in the regulatory complex, moreover to epigenetic modification that in some condition could modify and alter Notch mediated activation of transcription. Another aspect that overcomes the absence of signal amplification is the nature of target genes. Indeed, the products of genes activated by Notch are transcription factors that regulate again other genes, giving a signal amplification in the

possibility to acts contemporary on many aspects of the gene expression and so of the cellular functions.

The response to Notch differs greatly between cell types. The capability to elicit different responses might partly arise from crosstalk with other pathways. It also depends on the enhancers that are responsive to Notch regulation in a given cell. (Broggrefe; Bray; Iso)

Among Notch targets, the best characterized are HES in mammals, and E(spl) (for Enhancer of Split) in Drosophila (HES/E(spl) (Egan et al., 1998; Greenwald, 1998; Artavanis-Tsakonas et al., 1999). The human homologues of Drosphila's gene are the three family of transcriptional factor HES, HEY and HERP. (Allenspach. Et al. 2002).

HES

The HES/E(spl) family is a basic helix-loop-helix (bHLH) type trancriptional repressor and acts as Notch effectors by negatively regulating expression of downstream target genes such as tissue-specific transcription factors. (Ohsako et al., 1994; Van Doren et al., 1994; Ishibashi et al., 1995; Chen et al., 1997). The bHLH proteins bind specific DNA sequences as a dimer. DNA binding is mediated by a contact between each basic domain of a dimer and a specific half-site of consensus DNA sequences. The HLH domains are characterized by hydrophobic residues that allow them to form a homo- or hetero-dimer (Murre et al., 1994, Iso t et al. 2003).They are classified by the structure in: - Class A proteins are transcriptional activators such as MyoD and Mash1, and bind class A sites (CANCTG). - Class B proteins are bHLH lucine zipper type proteins such as Myc and Max. Both class A and B sites (CANGTG) are subtypes of the E box (CANNTG). -Class C proteins are transcriptional repressors such as HES in mammals, and hairy and Enhancer of Split in Drosophila, and are characterized by an invariant proline residue at a specific site of the basic domain. Class C proteins bind class C sites (CACGNG) as well as N-box sequences (CACNAG) and are also known to bind class B sites but not class A sites (Ohsako et al., 1994; Van Doren et al., 1994; Fisher and Caudy, 1998; Jennings et al., 1999). (Iso.T.2003).

In mammals are known 7 HES members, all acting as transcriptional repressors, except for HES6 that acts as inhibitor of HES1, resulting in a derepression effect. (Bae et al. 2000).

Both the HES and HERP families have been reported to act as transcriptional repressors except HES6, which antagonizes the function of HES1, resulting in derepression (Bae et al., 2000; Koyano-Nakagawa et al., 2000).

Three model have been identified as adopted by HES factors to repress transcription:

1. Active repression: through the homodimerization of HES factors in a C or N box, the recruitment of a corepressor TLE through the conserved orange domain (helix3-helix4) at the

C.terminal of the HES protein. This cofactor binding to HDAC (hystone deacethylase) induced local eterochromatinization and transcriptional repression. (Sasai et al., 1992;Tietze et al., 1992; Oellers et al., 1994; Ohsako et al., 1994; Van Doren et al., 1994).

2. Passive repression or negative dominance: HES heterodimerizes on its consensus sites with other transcriptional factors. This event prevents the formation of an active HES dimmer. The interacting factors are pother elements of the bHLH family, such as Myo D and Mash 1. This strategy is important in signalling modulation, because frequently, heterodimerizing factors are tissue specifics elements that mediate the specificity and the outcome of Notch activation. (Sasai et al., 1992; Hirata et al., 2000)

3. Orange domain mediated regulation: It is based on the Orange domain (helix3-helix4) and its capability to bind to an unknown elements, acting as and/or the stabilization or regulation of the WRPW-mediated repression function through intra or intermolecular interaction. The Orange domain is essential to repress transcription of its own (HES1) promoter as well as the p21WAF promoter. (Castella et al., 2000).

The best characterized Notch signalling target in mammals, into the HES family, is HES1 (Sasai Y. et al. 1992). It regulates the transcription of many genes, included itself. It binds a N-box on its gene promoter region, activating a negative feed back regulatory loop (Takebayashi et al., 1994). Other HES1 targets are tissue specific genes, frequently involved in differentiation, such as Mash1, a proneural factor, negatively regulating neurogenesis (Ishibashi et al., 1995; Kim and Siu, 1998). Also the CD4 gene, involved in differentiation of T cell lineage, is negatively regulated by HES1 (Kim and Siu, 1998). Morover, some cyclin-dependent kinase inhibitors, involved in cell cycle exit and in activation of differentiative programs, were shown to be regulated by Hes1. Indeed, CDKN1B/p27kip1 and CDKN1C/p57kip2 have been demonstrated to be directly inhibited in transcription by HES1 in mouse intestinal progenitors cells (Riccio O. et al. 2008). Hes1 mediates the stem maintenance effect of Notch signalling activation, maintaining the proliferativo conditions of progenitor cells, regulating inhibitors of cell cycle progression and regulators of terminal differentiation programs. (Bilodeau S. et al. 2009). Also CDKN1A/p21WAF has been proposed as a candidate target gene for HES1, because of HES1 repression effect in CDKN1A/p21WAF transcription, even if the presence of HES1 binding site in the CDKN1A/p21WAF gene promoter has not been confirmed (Castella et al., 2000). Moreover, HES1 gene mediates developmental processes controlled by Notch signalling, such as somitogenesis, myogenesis and gliogenesis, repressing (Iso T. et al. 2003). Other

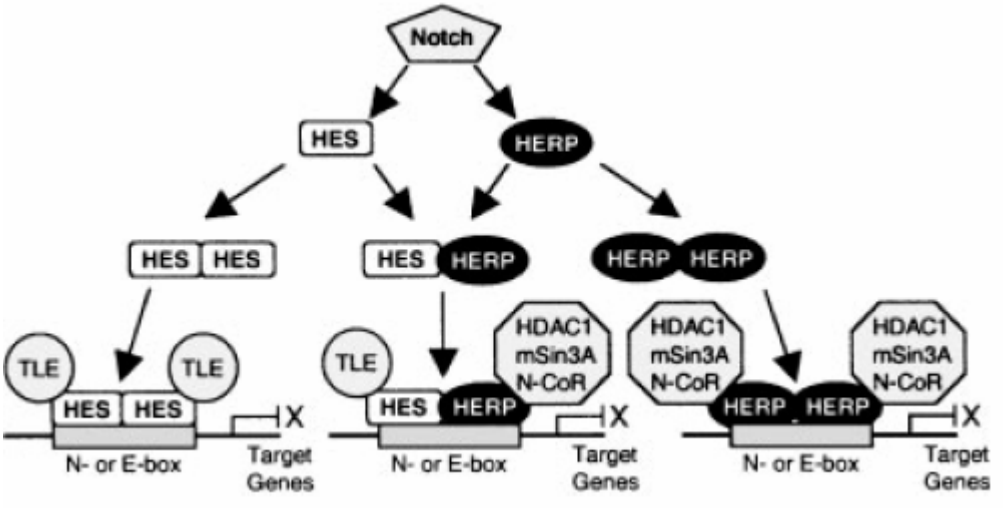
demonstrated target of Notch signalling are Hes5 and HES7. (Otsuka et al. 1999; Bhesso Y. et al. 2001).

HERP

A phylogenetic tree shows that HERP form a distinct subgroup in a large bHLH protein family indicating that HERPs are closely related to the HES family belonging to class C protein, but it forms a distinct subgroup (Vasiliauskas and Stern, 2000; Bessho et al., 2001a; Davis and Turner, 2001; Teramoto et al., 2001). Both HES and HERP function as transcriptional repressors and they present a conserved glycin residue in basic domain, instead of the prolin of HES family. They have a conserved YRPW motif that mediates the interaction with the hystone deacetylase (HDAC), in order to perform the active repression. (Iso T. et al. 2001 a novel partner ecc.). They acts prevalently as dominant repressors, occupying not only class B and C sites, but also class E and A boxes, inhibiting the transcriptional activation mediated by other bHLH transcriptional factors through these sites. (Nakagawa et al. 2000).

HERP1 and HERP2 have been demonstrated to be target of Notch signalling indirectly. Herp transcription is indeed activate after Notch activation and partially mediating its cell fate decisions effect. Indeed, tissue distribution of HERP1 and HERP2 are often observed in a strikingly complementary fashion within single organs. HERP can dimerize with HES factors and together they bind HES1 target sites, inhibiting gene transcription. (Iso T-et al.2003). These indirect evidences however suggest that they are Notch signalling target.

A model of HES-HERP cooperation downstream the Notch signalling showed as different repressive mechanisms may be activated downstream the signalling, mediating a fine tuning modulation of the targets and leading to control differentiation mechanisms with a higher specificity.



Model of HES-HERP cooperation. Iso T. et al. Journal of Cell Physiology, 194:237–255, 2003

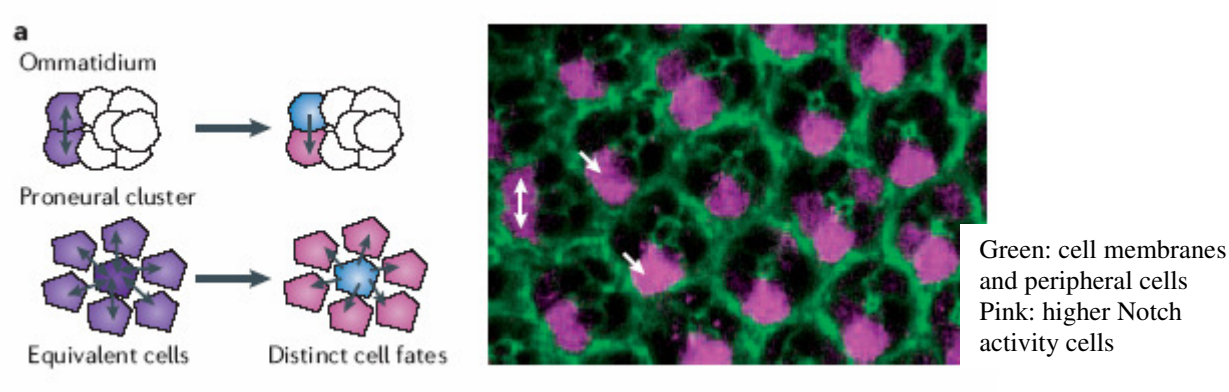
Despite these two family of transcriptional repressors are pivotal targets that mediates the Notch signalling activation, being ubiquitously expresses, also other Notch target genes have been identified. They belong to cell cycle and proliferation regulators, such as MYC, CCND1 (CyclinD1) (Ronchini and Capobianco, 2001) and CDKN1A/p21 (Kabos. P.et al. 2002) are induced in a cell type specific manner (Dotto G.P: 2009). These diverse targets illustrate that the transcriptional consequences of Notch signaling vary depending on cell type and stage of differentiation, despite the stereotyped nature of the central Notch/CSL signaling axis. Major challenges in Notch biology are to identify the relevant targets of Notch signaling in particular contexts, and to understand the basis for divergent effects in the different contexts. (Allenspach 2002).

1.5 Functions in Development

The intrinsic nature of the pathway, that is a cell to cell interaction between molecules in the cellular membrane, leads to functions that are based on spatial definition of cell fates during developmental processes. The processes regulated by Notch signalling are the follow:

1) Lateral inhibition (a)

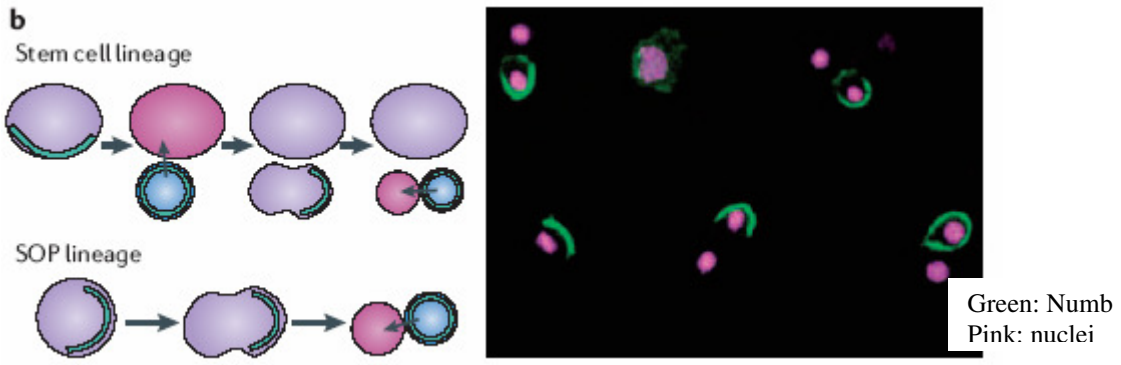
The activation of the signalling amplifies small or weak differences within equivalent populations of cells, deciding which cells from a progenitor population will differentiate in a lineage or in another. So, in a cellular population composed by equal precursors, differences in ligand or receptor expression were amplified, giving rise to two subpopulations of high and low Notch expressing cells. This is the consequence of the mechanism of intrinsic inactivation of ligands or receptors through internalization of one of them and the exposition of the others. (Katsube and Sakamoto, 2005). The activation or not of the pathway in a cells will determ its differentiative program, activating a program and inhibiting the other possible lineage retained by the precursor cell. In this way, the neural precursor can differentiate in neural or epithelial differentiated cells, depending on the fact they are Notch high or Notch low cells. (Allenspach. et al. 2002)



Upper- Lateral inhibition in the development of ommatidia in Drosophila's eye.
Lower- Neural precursor (Bray S., 2006, Nature Reviews, 7: 678-689, 2006)

2) Lineage decisions

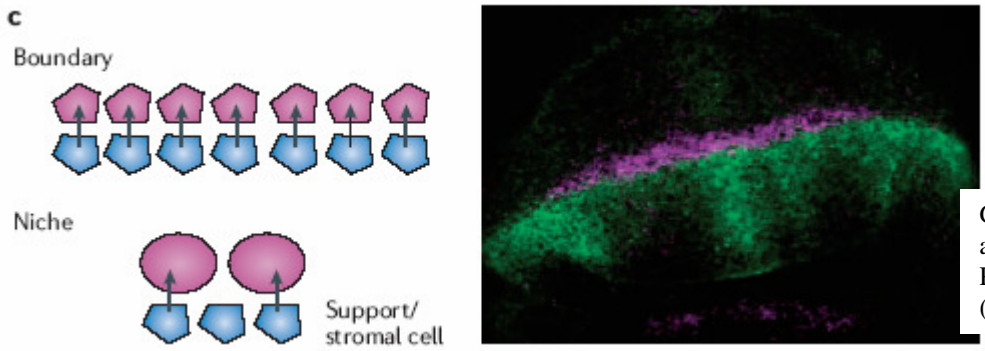
Notch signalling between two daughter cells is dependent on asymmetrical inheritance of Notch regulators (for example, Numb). The principle is the same of inducing high Notch or low Notch expressing cells, but it occurs in cells that originate from the same divided cells for differential distribution of regulatory factors in daughter cells. The best example is the segregation of regulators in progeny from a hypothetical stem cell lineage and the *Drosophila melanogaster* sensory organ precursors (SOP) lineage. The asymmetric division produces a cell bringing Numb and Neuralized, whereas the other cell does not. The cell bringing Numb and Neuralized will become a sending cell, because of Notch signalling inhibition by Numb and ligand activation by Neuralized. The other cells will be a receiving cells, acquiring high Notch activity. Subsequent asymmetric assignations will produce all required cell types (Fiuza and Arias, 2006; Le Borgne, 2006; BrayS: et al. 2006). The same let to maintain population of stem cells (Nickoloff, et al. 2007).



Upper- Lineage decision stem cell lineage. Lower- Lineage decision in SOP development of Drosophila. (Bray S., 2006, Nature Reviews, 7: 678-689, 2006)

3) Boundaries formation or lateral induction

Notch signalling occurs between two populations of cells and can establish an organizer and/or segregate the two groups. This mechanism interests two different neighbouring cell belonging to two different cell types. Cells expressing high Notch or high receptor will form two different lineage, activating or repressing a fate program in a way depedent on being Notch or ligand expressing cells. This process let to form boundaries in dorsoventral wing formation in Drosophila or in mammalian somitogenesis. (Allenspach et al. 2002; BrayS: et al. 2006)



Upper-Boundary formation. Lower-Stromal and progenitor cells (Bray S., 2006, Nature Reviews, 7: 678-689, 2006)

1.6 Mammalian Notch: structural and functional differences

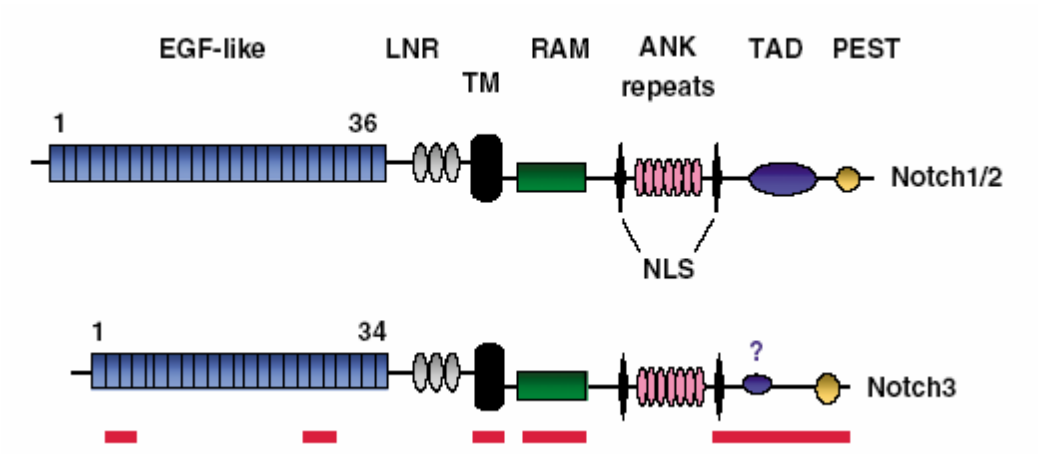
Despite the expression in many tissues, during development but also in adult organisms, Notch signalling shows an high context specificity. Indeed, even if the core pathway of activation is always the same, many layers of positive and negative regulations are present. Moreover, structural differencies between the receptors, together with the interaction with tissue or context specific factors, define and modify the outcome of the signalling. Notch is expressed ubiquitously, mediating a wide range of processes, such as increased proliferation or growth arrest, commitment or blockage of differentiation, stem cell maintenance and survival. The effect of Notch in each individual cell is highly dependent to signal dose and to the context. (Roy M. et al. 2007).

The stoichiometric character of this pathway that lacks enzymatic amplification suggests that regulations are important in defining how and in which measeure the signalling is active. (artavanis 2010). The signal dose is the result of the many events, the most evident the activation of the signalling against the degradation or recruitment of the NICD by negative regulators.This will determ NICD levels in the nucleus, regulating the transcription of targets. Moreover, general cofactor disponibility and presence of cell specific element modify Notch signalling.

In mammals, an additional element is due to the structural specificity of the four receptors, that regulates some targets and processes in a redundant way, whereas other functions are specific. Moreover, tissue distribution is both overlapping and distinct, suggesting a complex and interconnected aspect of the signalling activation. (Wu and Bresnick 2007).

Principal differencies are in the intracellular domain, as together with the three conserved domains: RAM, (Rbp-Jk associated molecule) domain, Ankyrin domain and the C-terminal PEST sequence, only Notch1 and Notch2 present a complete transactivating domain TAD.(Beatus et al 2001).

The main difference is between Notch1 and Notch3, whereas Notch1 and Notch2 show the higher similarity in sequence. Notch3 have a shorter TAD region and a significantly lower aminoacidic identity in the RAM sequence and in the RE/AC region (repression/activation). This supports differential capability in recruitment of co-repressor and co-activator or in conformational changes.(Beatus et al. 2001; Bellavia). Moreover, the differences in RAM domain that is responsible of the interaction of NICD with RBP-Jk might explain different capability to activate transcription in vitro for some promoter regions, comparing Notch1 to Notch3 (Beatus et al 2001). This interaction is crucial to induces allosteric changes and derepression, together with activation of transcription. (Barrick and Kopan 2006). Moreover, ANK domain that recruits Mastermind and thestructure of the TAD domain maight strongly influence transcription and specificity in binding sites, such as increased affinity of Notch1 for paired RBP-Jk binding sites and an higher capability of Notch3 to bind single sites, with others *cis*-elements. (Bellavia2008). This is just an example of how differences in structure might explain differential regulations.



Nocth1 and Notch3 receptors. From Oncogene 2008 27:5092-5098

Moreover, different pattern of expression sustain specific and non redundant roles for the four Notch receptors. Indeed, with an almost ubiquitous expression of Notch1 and Notch2, Notch3

and Notch4 have a more restricted distribution. In addition, Notch1 and Notch2 knock out mice gives to embryonic lethality, whereas knock out of Notch3 does not lead to lethality during development, despite impairing correct vascular differentiation. (Domenga et al. 2004; Swiatek 1994; Hamada et al 1999). The high diffusion of Notch1 and Notch2 imply involvement in many cellular process, with redundant function, but also peculiar aspect, as dependent by the same lethal phenotype caused by their knock out. Neverthless, amino acid sequences of a large region of the Notch intracellular domain are not highly conserved and thus let them to be able to interact with distinct transcription factors and mediate the expression of different sets of genes. But despite differences in aminoacidic sequence, Notch1 and replace Notch2 domain and activate transcription. (Kraman and mccricght) Notch4 appears to be evolutionary the most distant from other Notch members. It has fewer EGF repeats, a significantly shorter IC, no transactivating domain, and no cytokine-responsive elements as compared to other Notch receptors. Notch4 also differs from the other Notch receptors by its expression pattern in the mouse, which is strongest in endothelial cells and male germ cells. Notch4 has known effects on embryonic endothelial development, and active forms of Notch4 inhibit angiogenesis (Vercauteren S. M. and Sutherland H. J., 2004).

1.7 Notch and cancer: Oncogene and Tumour suppressor

As Notch is a pivotal pathway in development and cancer could be considered a developmental disease par excellence, it is not surprising that Notch signalling deregulation is involved in cancer. Indeed, Notch defines the balance between proliferation, differentiation, apoptosis and stemness, then the altered control of this cellular processes could lead to cancer insurgence. At first, the strong capability to induce proliferation constitutes a great advantage for cell that highly express Notch and this could give an additive selective advantage for cancer cells. (Artavanis Tsakonas and Musckavitch 2010). Despite confirmed functions in control of proliferation, Notch signalling is involved in tumorigenesis in a more complex way. Indeed, Notch signalling could act both as oncogenic pathway, but also as tumour suppressor. This double effect depends by the high repertoire of genes activated by Notch targets genes that controls cell processes in a cell specific fashion. Moreover, the crosstalk with other pathway makes the outcome of Notch signalling activation or deregulation more difficult to predict.

On the basis of developmental functions controlled by Notch, its involvement in cancer is principally linked with 1) the maintenance of a stem cell like phenotype altering the balance

between progenitors and differentiated cells.(Lewis J. 1996; 1998); 2) Partecipation in cell fate decision; 3) induction of terminal differentiation. It is evident that the deregulation of cellular processes linked to proliferation, cell cycle exit and differentiation could be crucial in cancer setting. (Radtke and Raj).

Notch was firstly identified as oncogene in T-ALL leukemia. In this neoplasia a translocation t(7;9) causes the expression of a truncated form of Notch1, corresponding to the NICD-1, causing the constitutive activation of the pathway and altering the process of differentiation.(Reynolds 1987).

Then evidences of its involvement in solid tumours, comes from the identification of int3 site. The *int-3* gene was originally identified on the basis of its oncogenic effects in the mouse mammary gland. *int-3* is a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (Gallahan and Callahan, 1987; Robbins et al., 1992; Sarkar et al., 1994). The site of integration corresponds to Notch4 (Int-3) locus and this event causes constitutively active NICD expression, in a truncated form. After Notch4 oncogenic activity was detected also in human breast, lung, and colon carcinoma cell lines. (Vercauteren S. M. and Sutherland H. J., 2004).

After this first evidences, the oncogenic role of Notch in other cancers has been identified in epithelial cancer, such as colorectal and pancreatic cancers. In this contest, Notch signalling aberrant activation maintains cells in a proliferative and undifferentiated status, leading to aberrant proliferation. Moreover, also mechanisms involving altered apoptotic response or resistance to anoikis are features given to cancer cells by Notch signalling deregulation. In this context, Notch alters cell cycle increasing pro-survival factors, inactivating cell cycle checkpoints and leading to uncontrolled proliferation and apoptotic resistance. (Rangarajan a. et al 2001 virology, (Radtke and Raj; Koch and Radtke 2007)

The tumour suppressive role has been identified in keratynocytes, in which Notch1 and Notch2 are involved in cell cycle exit. The loss of function of Notch in this context give rise to a tumour favourable condition, with hyperprolyferation of the skin.(Riccio et al.2008)

Moreover, Notch interacts with other pathways. Crosstalk are an interesting aspect linked to the nature of the pathway is the high interconnection and crosstalck with other pathways. This is due to the high number of factors that intervene in the activation of the pathway, together with the localization of Notch in all cell compartment (membrane, cytoplasm and nucleus) leading it an higher capability to interact with many molecules. This feature might also explain the strong context specificity in the activation of the signalling.

Numb interplay with p53 pathway, controlling its tumour suppressor activity (Colaluca I.N. et al. 2008). Due to the negative regulatory role of Numb, the balance between Nocth/Numb defines the fate between proliferation and differentiation, in development and in cell homeostasis. As regulator of Notch pathway, loss of Numb has been associated with increased oncogenic effect. Then, the multifaceted role of Notch in cancer depends not only by the context, but also by the crosstalk with other pathway and cellualar processes.

Epithelial to mesenchymal transition (EMT)

The epithelial to mesenchymal transition (EMT) is a foundamental mechanism in development, playing a crucial role in the formation of body plan and in the differentiations of tissues and organs during embryo formation. EMT is also crucial in diseases because its role in tissue homeostasis: even if it contributes to tissues repair, EMT can cause fibrosis and promote cancer progression (Cell.139-2009). EMT determs migratory, invasive and stem cell properties, prevents apoptosis and senescence and contribute to immunosuppresion.

During development, tissue and organ formation arise from a series of conversion of epithelial cells to mesenchymal cells throught epithelial to mesenchymal transition (EMT) and the inverse process, the mesenchymal to epithelial transition (MET). Epithelia are characterized by close contacts between cells, apicobasal polarity and they are separated from adjacent tissues by a basal lamina. Conversely, mesenchymal cells have less organized features, comprising the extracellular matrix and connective tissues adjacent to epithelia. For these functional and morphological differencies, the conversion from ones to others involve a wide series of phenotypical changes, as loss of cell to cell adhesion and cell polarity and the acquisition of migratory and invasive properties.

Conversions occurs in gastrulation and in the formation of neural crest of vertebrates, being crucial for tissue formation and distribution and determination of three-dimensional structure of internal organs. A lot of signalling pathways that drive cell fate specification and morphogenetic movements controls them activating the EMT-MET program, regulating transcriptional patterns that are highly conserved in species. A transcriptional factor that resulted important and conserved in gastrulation is Snail, SNAI1 and SNAI2 in human. It is a transcriptional repressor of E-Cadherin and it results necessary for gastrulation and EMT, because knock out mice fail to gastrulate, being unable to downregulate E- Cadherin in mesodermal cells and to loss cell to cell contact to migrate (Carver et. al. 2001, Nieto et al.

1994). Moreover Snail contributes to basal membrane degradation by activating metalloproteases and repressing the expression of some components. (Jorda et al. 2005).

The determination of the neural crest also includes EMT events, but these are regulated by a higher number of pathways that show strong plasticity and redundancy, as FGF, Wnt and Notch signalling. These are involved in define territory of neural crest, otherwise Wnt controls migration during neural crest formation. (De Calisto et al. 2005; Carmona-Fontaine et al. 2008). Also Cadherin mediated adhesion is crucial in neural crest EMT and it is obtained by downreglation of more adhesive cadherins as well as the expression of less adhesive type II cadherins. Moreover, ADAM 10 protease and γ- secretase activity are crucial to let delamination of cells because cleavage of N-Cadherin and its migration to the nucleus with β catenin induces cell cycle progression, prerequisite for cell migration of crest cell. Interestingly, the role of Snail in this process is to block S phase exit and synchronize the cell cycle of all these cell, giving a timing. (Shoval et al.; Tanehill et al. 2007). Even if the pathway involved in gastrulation and neural crest migration are common, in this last mechanism there is a higher cooperation and plasticity to assure a correct neural crest migration, suggesting that EMT have a high grade of conservation in development in order to drive particular biological processes.

After the primayr EMT a MET process let to formation of epithelial structures, but successive EMT events permit the formation of mesenchymal cells with a more restricted potential and the formation of precursors for cellular subtypes of future organs. In particular, actively migrating myoblast let to hepatocyte formation, through hepatocyte growth factor /scatter factor (HGF/SF) activation of PI3K and Src pathways, mediated by the HGF receptor c- Met. (Main et al. 2001). So, these factors result to be crucial for liver formation, together with the EMT that occurs during endodermal derivatives development of the future liver. (Tanimizu and Miyajima). EMT is a crucial process that drives development at differents levels and its impairment cause development in embryo and in organ formation.

EMT is also involved in wound healing, as physiological response to unjury. It has been demonstrated that keratinocytes in wound healing recapitulate part of EMT and also that the metastable state they reach let them to migrate maintaining contacts each other and migrating together and not as individual cells. (Arnoux et. al. 2008) This particular state of EMT process is important in cancer dissemination of metastasis, leading migration of a group of cells instead of a singular cell. The importance of EMT is not only for cancer progression, but also for other pathologies as organ degenerative ones, like fibrosis. In fact, pathological activation of EMT is similar to the physiological one, but activated in contest and timing that are

incorrect and arrecate damage. Even it has thought that fibrosis originate from activation of interstitial fibroblast, a part of the epithelial cells convert to a mesenchymal state by EMT. Also hepatocytes produce fibrosis through this reversion and this capability is particular interesting because in cyrrothic hepatocytes has been observed EMT, giving strong implication in progression of these cells to hepatocellular carcinoma (Nitta et al .2008). Foundamental is the role of EMT in cancer progression, even if difficulties in demonstrating EMT occurance in vivo have been an obstacle in considering its importance in cancer progression. Indeed, the presence of mesenchymal cells derived from epithelia is difficult to identify them in comparison with physiolocal mesenchymal cell type, as stromal cell and fibroblasts. Neverthless, in vivo studies have demonstrated the presence of cellular cords and aggregates detaching from the front of tumours, supporting the idea that cancer fronts makes EMT. Instead, the loss of E- Cadherin, the activation of WNT pathway and the consequent detach from the membrane basement let to In vivo imaging has confirmed EMT in tumour front and its involvement in cancer progression. The activation of EMT at the tumour front reflects the balance between cancer mass growth pressure and the free edge of tumour periphery. At the same time also EMT independent invasion occur in this area of the tumour, through cytoskeletal remodelling. Expression of EMT effectors, as Snail is associated with tumour stage and poor prognosis in a wide range of carcinomas and EMT suppression could sensitize cells to EGFR target therapy in hepatoma and pancreatic cancer, suggesting that EMT identification could be a tool not only to make a prognostic evaluation of cancer, but also for define efficacy of treatments (Thiery J. P. 2009).

MATERIALS AND METHODS

Hepatocarcinoma cell lines and culture conditions

HepG2, Hep3B, SNU398, Snu449 HCC cell lines were from the American Type Culture Collection (ATCC, Rockville, MD, USA). HepG2 and Hep3B cells were cultured in Eagle's Minimum Essential Media (MEM), and SNU398 and Snu449 in RPMI 1640 (Gibco, Invitrogen). Both medium were supplemented with 10% fetal bovine serum (FBS), 100 U/ml of Penicillin, and 100 mg/ml Streptomycin at 37°C in a 5% CO_2 incubator. (Gibco, Invitrogen)

Induced cell cycle arrest and treatments

Artificially induced cell cycle arrest was obtained by high-density cell culture or serum deprivation. In first case, cells were plated at high density whereas for starvation they were plated at low density and serum deprivation was performed 24h after seeding. Demethylation treatment was carried out culturing cells with 1μM or 2 μM of the hypomethylatig agent 5-aza-2'-deoxycitidine (Sigma-Aldrich, Saint Louis, MO, USA) Gene expression was evaluated 24h, 48h and 72h after treatment.

Treatment with Lithium Cloride (LiCl, Sigma-Aldrich, Saint Louis, MO, USA) was performed at final concentration of 20 uM adding the salt in water solution in cell medium, 24h after cell seeding. Cells were collected 2-5-8-11-24h after the treatment.

Retroviral transduction of shRNAs for Notch1 and Notch3

Short hairpin oligonucleotides (shOligos) complementary to shRNA sequences were inserted into the pSuper.puro expression vector according to the manufacturer's instructions (OligoEngine, Seattle, WA). (Brummelkamp TR, Science 2002)

ShOligos targeted to different Notch3 and Notch1 exons were as follows: Notch3 (N3), 5'-ctcccctcaccacctaataaa-3'; Notch3 (N3-A), 50-gggggacctgccgccgtggctata-30; Notch1 (N1), 50-ggccgtcatctccgacttca-30; and Notch1 (N1-A), 50-gcctcttcgacggctttga-30. Cells were stably transduced with a GL2 luciferase specific shRNA as a negative control (Elbashir SM, 2001). Retroviruses were produced by transient transfection of pSuper.puro retrovectors into Phoenix A packaging cells (kindly provided by Dr. Gary Nolan, Stanford University) (Pears WS, PNAS 1993). The efficiency of transfection was assessed through co- transfection of HSG lentiviral vector coding for GFP (kindly provided by Prof. K. B. Marcu, Stony Brook

University). The HSG vector was unable to be packaged by retroviral enzyme in virion particles, in order to exclude the production of retroviral particle bringing HSG DNA instead of the desired one. Retroviral particles released by Phoenix A packaging cells were collected at 48h and 72h post transfection and filtered with a 45uM filter. HCC cells were transduced using spinoculation protocol, plating them the day before of the infection to have a confluence of 40% the next day and putting viruses in contact with them in presence of gravitational force, 2000 rpm at 32°C for 1.5 hours. Polybrene (Sigma Aldrich, St Louis,MO, USA) was used at concentration of 8 ug/ml to increase transduction efficiency. After 6 h of incubation at 32°C the viral solution was removed from cells, which begin selection 48h after infection. Stable retroviral transduced populations of cells were selected in growth media supplement with Puromycin (Life Tecnologies,UK) at the sub-killing concentration determed for each cell line.

Retroviral transduction of shRNAs for Hes1, CDKN1C and CDKN1C expressing vector

ShOligos targeted to different Hes1 and CDKN1C/p57 exons were purchased from Origene (OriGene Technologies Inc., Rockville, MD) For Hes1, pRS vectors, containing ampicillin resistance and pGFP-V-RS Stable, retroviral transduced populations were obtained as described for Notch1 and Notch3. Cells were selected in growth media supplemented with Puromycin or Blasticidin (Life Tecnologies,UK) respectively for cell infected with Hes1 and CDKN1C/p57 short hairpin RNA vectors. A full-length human CDKN1C/p57 cDNA was excised from the pCMV6-XL4-p57 plasmid (OriGene Technologies Inc) and inserted into the multiple cloning site of pFB-Neo Retroviral vector (#217523, Agilent Technologies, USA). The pFB-Neo vector containing CDKN1C/p57 was used for stable infection as previously described and transduced cells were selected using Geneticin selection (G418, Life Tecnologies,UK) at the sub-killing concentration determed for each cell line.

Transient transfections with siRNAs and DNA plasmidic vector

Hes1 siRNA

HepG2 and SNU398 cells were seeded into 6 well plates and grown to ~40% confluence prior to Lipofectamine 2000 (InVitrogen) transfection with 40 nM of human Hes1 specific siRNAs or scrambled (NC) siRNAs, whereas for CyclinG1 and GSK3β siRNA 20 nM were transfected.

Transfection efficiencies were greater than 90% as determined by co-transfection with a fluorescein-labelled siRNA (InVitrogen). Cells silenced for Hes1 were harvested 48h and 72h after transfection and pellets used for protein and RNA extractions.

siRNA sequences: Hes1-233 5'-cggacauucuggaaaugacagugaa -3', Hes1-233 3'-uucacugucauuuccagaauguccg-5', Hes1-245 5'- aaaugacagugaagcaccuccggaa-3'; Hes1-245 3'-uuccggaggugcuucacugucauuu-5'obtained by InVitrogen as Stealth siRNAs; E-Cadherin (sc-35242) and GSK3β (sc-35527) obtaianed as human siRNAs pool of three different RNA against human messenger (Santa Cruz Biotechnology Inc, Santa Cruz, CA); CyclinG1 5'-agcauagcuacuacagaauaacuca-3', 3'-cuucguaucgaugaugucuuauugagu-5' obtained as Dicer-substrates siRNA (DsiRNA) from IDT (Integrated DNA Technologies Inc., Coralville, Iowa, USA).

Validated p53 siRNAs pool TP53-VHS40367 was purchased from Invitrogen, 5'-uuccgucccaguagauuaccacugg-3', 3'-ccagugguaaucuacugggacggaa-5'.

For plasmid transfection cells were seeded into 6 well plates and transfected 0,2 μg of pCMV6-XL4-p57kip2 plasmid, containing the full length human CDKN1C/p57kip2 cDNA, or empty vector pCMV6-XL4 (OriGene Technologies Inc., Rockville, MD) using Lipofectamine 2000. Analyses of genes and proteins expression were performed at 48h and 72h.

NICD3 vector was tagged using Label IT Tracker ™ Fluorescein Kit (Mirus Bio LLC, Madison, WI, USA)

In the same way 1 μg of tagged NICD3 plasmid was transfected in the same way. As negative control was used HSG vector, containing the GFP. Cells were collected 24h after transfection and sorted using FACS. Cells after sorting were put again in culture for 24h to let recovery dividing the subpopulations of effective transfect and not. Analyses of genes and proteins expression were performed at 24h and 48h for NICD3.

E-Cadherin overexpressing vector pCMV6-AC-GFP containing the ORF of human E-Cadherin gene or empty vectors (OriGene Technologies Inc., Rockville, MD) was transfected using Lipofectamine 2000. 1 μg of each vector was transfected for each well and the effective overexpression was confirmed by GFP expression. Cells for invasion assay proteins were detached 24h after transfection, whereas cells for protein expression analysis were collected 48h after transfection.

Bacterial transformation and plasmidic DNA extraction

Escherichia Coli JM109 competent strain (Promega, Madison, WI) was used for plasmid amplification and DNA extraction. Bacteries were transformed with Heat shock for 1 minute at 42°C and left for 2minutes on ice before adding SOC medium and being incubated at 37°C, 225rpm for 1h,30 minutes. Transformed bacteries were selected plating the preculture at two different concentrations, whereas on LB- agar plate cointaining the prokaryotic selection brought by the transformed plasmid and incubated overnight at 37°C. Selections were Kanamycin (25ug/ml) for and Ampicillin (100ug/ml) for all others vectors. (pFB- Neo, The following day, a single colony was picked and grown in liquid LB in presence of selective antibody, overnight at 37°C, shaking at 225 rpm. Plasmidic DNA was extracted using a mini prep protocol or a maxi prep QUIAGEN extraction tips. In both cases, bacterial pellet was lysed with 50mM Tris-HCl pH 8.0, 10mM EDTA and in presence of RNAase A 100ug/mL. Cell lysis and extraction was obtained using 200mM NaOH and 1% SDS, followed by 3M Potassium acetate. QUIAGEN-tips were used to separate bacterial genomic DNA, whereas plasmidic one was precipitated with isopropanol and washed in ethanol 70% in ultrapure water. DNA extracted was resuspended in TE buffer and quantified with Nanodrop.

Cloning

The human CDKN1C/p57 sequence was excised by digestion from pCMV6-XL4-p57kip2 plasmid containing the human cDNA ORF of CDKN1C/p57. (OriGene, Technologies Inc., Rockville, MD) The insert were introduced in the purchased pCMV6-XL4 vector via *Not I* restriction. Digestion was conducted using *Not I* enzyme (Invitrogen) in order to excise the complete ORF from the dono r plasmid, including the Klenow sequence. *Not I* was used also to open the accepting vector pFB-Neo, using the unique restriction site in its Myltiple Clonong Sites. pCMV6-XL4 digestion was performed in a mix containing 10X Buffer H, 0,01%BSA; 0,01% Triton X-100, 1 ug of DNA , 2uL of *NotI* (InVitrogen) for 2h at 37°C. The digestion was stopped charging the digestion mix on a 0,8% agarose gel containing ethiudium bromide and the excised fragment of 1.7 kb was extracted from gel, using the extraction kit Wizard SV Gel extraction kit (Promega, Madison, WI, USA). The band was excised by the gel with a scalpel and the add of a UV lamp and then incubated in provided Membrane binding solution for 10' at 50°C, in order to dissolve the gel. The DNA is extracted using a chromatographic column and washed many times, until it is eluted in water. A part of the obtained DNA is verified for quality and quantity on a agarose gel in TAE1X and 0,01% of Ethidium. Restriction o pFBNeo was conducted as already described with

adding Calf Intestinal Alkaline Phosphatase (CIAP) 1U/uL to maintain the vector linearized. The reaction solution was incubated 5' at 50°C, then the CIAP inactivated using EDTA pH8.0and incubating the raction at 65°C for 15'. Then the ligation reaction of the insert in pFB-Neo was conducted with T4 DNA ligase (Promega, Madison, WI, USA) for 1,30h at RT. Then, *E. Coli* was transformed with the product of the ligase reaction and a mini prep was conducted on 12 picked single colonies, with the same method described in the previous section, but using a small of LB for the culture. Digesteion of DNA extracted from different clones was digested with *Apa I* that has a unique site into the pFB- Neo vector, producing two fragments with different length on the basis of the direction of insertion of the insert. A clone with the insert in the open reading frame was selected for maxi prep and DNA extraction. NICD3 overexpressing vector was obtained by cloning the human ORF into topoTA cloning vector, using TA cloning strategy.

Luciferase assay

Luciferase assay was performed in HepG2 cells, transfected with pPTA vector or pp53PTA vector with the p53 responsive element controlling transcription of Luciferase gene. The assay was performed using Dual Lucferase Assay (Promega). After 24h form transfection, cells were lysed in passive Lysis buffer for 15' and LARII substrate were added, the first lecture made and then Stop&Glo reagent was added. The second lecture was performed. Luciferase and Renilla activity were deteted with Luminometer GLOMAX (Promega) and data analysed as ratio of Luciferase/Renilla activity.

FACS analysis and Cell sorting

HepG2 and Snu398 cells shRNA for Notch1 and Notch3 were analysed for cell cycle distribution after puromycin selection was concluded, plated in the same number and collected 24h later. Forty-eight hours and seventy-two hours after p57kip2 transfection cells were collected. Cells were washed twice with PBS, fixed with 70% cold ethanol at -20°C, resuspended in 500µl of PBS containing 10µg/ml propidium iodide and 50µg/ml RNase A and incubated for 30 min at room temperature. The cells were then centrifuged at 1200 rpm for 5 min at room temperature, resuspended in 500 ul of PBS and analyzed with Fluorescent-Activated Cell Sorter BD FACSaria (BD Bioscences, San Jose, CA, USA).

Apoptosis was investigated in p57kip2 transfected cells by using the Annexin V-FITC apoptosis detection kit (Bender Medsystems, Vienna, Austria) staining via FACS. After

p57kip2 transfection cells were collected, fixed in 70% ethanol and analyzed according to the manufacture instructions.

Protein extraction

Cells were collected thought trypsinization or cell scraping and centrifuged at 1800 rpm at 4°C for 5 minutes. Then the pellet was resuspended in cold PBS and again centrifuged at 1800 rpm at 4°C for 5 minutes, followed by another resuspension in cold PBS and centrifuged at 7500 rpm at 4°C for 2 minutes. After having taken out the supernatant, the pellet was frozen in liquid nitrogen and conserved at -80°C until protein extraction. The pellets were lysed in buffer composed by 10 mM Tris-Hcl, pH 7.6, 100 mM NaCl, 10 mM EDTA pH8.0, 0.1% Triton X100, 1 mM DTT, 1mM PMSF, 1 mM Na3VO4, 1X of a cocktail of protein inhibitors. Lysates were obtained by vortexing every 10 minutes for three times and maintained on ice for all the time. For tissue extraction instead, frozen liver sample were homogenized in the same buffer, but extraction was performed using a syringe to have a stroger mechanical lysis. Centrifugation at 13000 rpm, for 20 minutes at 4°C leads to obtain protein lysate without residual cellular debris. Quantification of the protein concentration was measured using Lowry Quantification kit (BioRad,) using as reference a standard curve made by Albumin Serum Bovine protein at known concentration. The absorbance of the solutions was measured at 500 nm using a spectrophotometer.

SDS-PAGE and Western blot analysis

For each protein extract different amount of protein were used for SDS-PAGE, depending on the relative expression of the target protein and the total amount, dependent on the number of cells in the originating pellet. Generally, 10-15 ug of total extract were boiled at 95°C for 10 minutes with 2X Laemmli buffer containing : 65mM Tris-HCl, pH 7.5, 65mM 2-βmercaptoetanol, 1% SDS, 10% glicerol e 0.003% bromofenol blue and then separated in reducing condition on a polyacrilammide gel containing SDS. Gels were blotted on nitrocellulose membrane (Amersham) and then incubated in blocking solution of 5% milk in PBS and then probed with the following antibody.

Primary antibody used were in Table 2. Protein bands were revealed with the EnVision dextran polymer visualization system (Dako Cytomation) or using secondary antibody coniugated with horseradish peroxidase (Amersham) and quantitated with Densitometric analysis was performed using Quantity-one software (Bio-Rad, Hercules, CA, USA).

Immunofluorescence

Cells were grown to ~50% confluence on coverslips, fixed with 70% cold ethanol at -20°C for 10 min and rinsed with PBS. Aspecific antigenic sites were blocked with goat serum and then incubated with p57kip2 antibody at 4°C for 16h. After incubation, cells were washed three times for 20 min in PBS- and incubated for 1h with Texas Red goat anti-rabbit antibody (Molecular Probes, Eugene OR) at room temperature. Glasses were mounted with the Prolong Gold reagent (Invitrogen) containing DAPI and cells were visualized using an epifluorescent microscope.

Gene expression analysis

Total RNAs was extracted from the cell line, HCC tissue samples and surrounding tissue using TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, and 1 μg of RNA was retro-transcribed using SuperScript II (Invitrogen, Carlsbad, CA) or ImProm-II™ Reverse transcription System (Promega, Madison, WI, USA). Relative genes expression was determined by semiquantitative PCR with the primer sequences in Table 1. PCR products were visualised on 2% agarose gel, stained with Ethidium Bromide. Densitometric analysis was performed using Quantity-one software (Bio-Rad, Hercules, CA, USA).

Real time PCR for p53 was performed using SyberGreen (Bio-Rad, Hercules, CA, USA) on cDNA retrotrascribed as already indicated. Each sample was analyzed in triplicate and a pool of HCC not treated cells was used for standard curve. The level of p53 expression was measured using *C*t (threshold cycle) and ΔΔ*C*t method for relative quantitation of gene expression was used to determine expression levels. The Δ*C*t was calculated by subtracting the *C*t of β actin from the *C*t of the p53.

Real time PCR for miRNA-221 and for miRNA-122: The expression of mature miRNAs was assayed using the Taqman MicroRNA Assays (Applied Biosystems). Each sample was analyzed in triplicate. Reverse transcription reaction was done starting from 10 ng of total RNA and using the looped primers. Real-time PCR was done using the standard Taqman MicroRNA Assays protocol on the iCycler iQ Real-Time PCR Detection System (Bio-Rad). The 20 μL PCR included 1.33 μL reverse transcription product, 1× Taqman Universal PCR Master Mix, No AmpErase UNG (P/N 4324018; Applied Biosystems), 0.2 μmol/L Taqman probe, 1.5 μmol/L forward primer, and 0.7 μmol/L reverse primer. The reactions were incubated in a 96-well plate at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and

60°C for 1 min. The level of miRNA expression was measured using *C*t (threshold cycle). The ΔΔ*C*t method for relative quantitation of gene expression was used to determine miRNA expression levels. The Δ*C*t was calculated by subtracting the *C*t of *U6* RNA from the *C*t of the miRNA of interest. The ΔΔ*C*t was calculated by subtracting the Δ*C*t of the reference sample from the Δ*C*t of each sample. Fold change was generated using the equation $2^{-\Delta\Delta Ct}$. A pool of three normal livers was used for the standard curve calculation and as reference sample for the ΔΔ*C*t. The Taqman MicroRNA Assays for *U6* RNA) was used to normalize the relative abundance of miRNA.

Senescence assay

Senescence analysis was performed in HepG2 and Snu398 cells 96 hours post p57kip2 transfection. Senescence analysis in HepG2 and Snu398 cells shRNA for Hes1 and both Hes1 and p57kip2 shRNA were performed 7 after infection, as the same way of stable overespressing CDKN1C/p57 cells. As described previously, senescence-associated β-galactosidase (SA-β-Gal) (Dimri, Lee et al. 1995) is a specific biomarker for cellular senescence. Neverthless, to avoid the possible artificial effect due to high cell density culture for long time (Severino J. et al. 2000), cells were cultured in high subconfluent conditions and analysed at once at the end of the selection. SA-β-Gal activity was measured using a standard senescence detection kit (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions. Cells were fixed and stained in presence of X-gal substrate overnight, in the dark,at 37°C. The percentage of blue cells versus total cells was measured in 10 random microscopic fields.

Animals

Male Wistar rats were used for all in vivo experiments. They were obtained from Harlan Ital (Udine, Italy) and were maintained in an animal facility at the Department of Experimental Pathology (Bologna), receiving humane care in accordance with the European Legislation. The protocols of the experiments were approved by the Ethical Committee of the University of Bologna. Animals were maintained at a temperature of 20–22 °C and fed a standard pellet diet ad libitum.

HCCs were induced in 12 animals (weighing 125–150 g), which received DENA given in their drinking water (100 mg/l) for 8 weeks (Rajewsky et al., 1966). Four weeks after the last day of DENA administration, animals underwent an echographic examination of the liver (Di

Stefano et al., 2008) to verify the appearance of tumor nodules, which were detected in all rats. Animals were sacrificed under anesthesia and livers were taken for analysis.

Patients and HCC samples

HCC tissues were obtained from 34 consecutive patients (29 males and 5 females) undergoing liver resection for HCC at the Department of Surgery of the University of Bologna from January 2004 to December 2006, after obtaining their informed consent. Tissue samples were collected at surgery, and divided in two parts: the first one was immediately frozen in liquid nitrogen and stored at –80°C until RNA and protein extraction; the second one was fixed in 10% formalin and paraffin-embedded for histopathology examination. Exclusion criteria were a previous history of local or systemic treatments for HCC. For second project, 40 HCC choosen with the same criteria were selected and tissues analysed in western blot. For the third project 35 sample were analysed.

Statistical analysis

Differences between groups were analyzed using a double-sided Student t-test, when two groups were present. Experimental data are expressed as the mean ± SE from two independent experiment. Pearson's correlation was used to explore the relationships between Hes1 and CDKN1C/p57kip2 mRNA expression in HCC tissues, Notch1 and E- Cadherin protein expression and Nothch3 and CyclinG1 or GSK3β. Pearson's correlation was also used to assay any relationship between Hes1, AFP serum levels and tumor size. T-test was used to explore significant difference in Hes1 expression between low-grade (Edmondson and Steiner's grade 1 and 2) and high grade (Edmondson and Steiner's grade 2 and 3) HCCs ANOVA was used to explore any difference in Hes1 expression among different etiological groups. The Kaplan-Meyer survival analysis was used to compare patients survival and time to recurrence (TTR) based on different Hes1 and CDKN1C/p57kip2 mRNA expression levels (the cut-off values were chosen on the basis of the median values) and statistical p value was generated by the Cox-Mantel log-rank test. In the same way were evaluated Notch1 and E-Caherin association with the recurrence. Survival analysis was performed considering only cancer-related deaths whereas events related with other causes, including liver failure, were excluded. P-values less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 8.0.

Wound healing assay

The assay was performed after Notch1 depletion in Snu398 and Snu449 cell lines. Cells plated at high confluence were treated at the time of complete growth in monolayer. Scratch was performed with a 10uL tip and medium changed after the scratch. Notch1 depleted SNU449 showed a reduced capability to repare the wound compared to negative controls that at T2 results repaired. Photographies were taken at: T0, the moment of the scratch; T1= 8 hours after; T2= 16h after for Snu449 cells, whereas for Snu398 cells, T0, the moment of the scratch; T1= 24hours after; T2= 36h

Invasion assay

Invasion assay was performed in boyden chamber using a 8um pores filter, coated with Matrigel (SigmaAldrich).Cells were plated in absence of serum and medium with 10% bovine albumin serum was added in lower chamber. After 24hours invaded cells were evaluated for controls and both two shRNA vector for each cell lines. Cells invaded were evaluated counting them at the microscopy after GIEMSA staining. The meaning of invaded cells for field was calculated counting 10 random fields and obtaining the meaning. (Albini A. et al. 2007)

Zymography

The method let to detect in native condition the activity of gelatinases Metalloprotease 2 and Metalloprotease 9 in presence of gelatine, their substrate. Proteins presents in the supernatant medium of cells were separated in reducing but not denaturing condition on a polyacrilammide gel. Cells were incubated in presence of medium without serum for 18h and medium collected, centrifuged at 1800rpm for 5' at 4°C to remove cell debris and protein concentration was determed using Lowry Standard Method. Acrylammide gel containing gelatin at 0.1% was used to separate protein in electrophoresis. Gel was prepared without SDS and supernatant protein loading only contains SDS to charge proteins. After the run, the gel was washed with a solution of Triton X-100 al 2,5% in H20 for 5'. Then, the gel was incubated in the Zymography buffer at 37°C for 18h and digested bands couloured with Comassie blue.

Secretome analysis: conditioned medium and LC/MS analysis

HepG2 and Snu449 cells, SRGL2 and Notch1 shRNA N1/5724 clones were plated in full medium, respectively MEM and RPMI. 5x 10^6 HepG2 and Snu449 cells were plated in T75 flask, in order to have a 60% of confluence cell after 24h. The following day medium was taken out and 4 washes with Dulbecco's PBS were performed, in order to eliminate all serum residues. Conditioned medium was added at the cell and collected after 24h. Supernatants were centrifuged 5' at 1200rpm at 4°C to eliminate cell debris and frozen at -80°C until analysis.

Proteomic Analysis. Thawed secretomes were normalized to a total protein concentration of 12μg, reduced with DTT, alkylated with 4-vinyl pyridine and digested with trypsin before undergoing shotgun RP-HPLC-MS/MS analysis (three technical replicates); resulting mzData.xml raw data were searched against human SwissProt database with Mascot v. 2.3: search results were re-scored with Mascot Percolator[35] percolator score $\geq$ 50, at least two significantly scoring peptides/protein at 1% false discovery rate (detailed MIAPE-compliant methods are provided as supplemental methods). Proteins MS/MS identifications were cross-related to the corresponding MS1 data for label-free quantification through the software IDEAL-Q, which performs a peptide search hit-driven alignment of retention times and a hit to MS-scan match semi-automatic validation before peak area integration(Tsou CC. et al 2010). Peaks area of significant hits (p-value<0.05, protein ratio>4) were manually integrated and reprocessed.

Serum analysis

Serum was obtained by collecting blood in Vacuette Z serum beads clot activator (Greiner Bio-One, Kremsmunster, Austria), centrifuging at 2000 rcf for 10 minutes at 4°C and stored at -80°C until the use. Sera were collected by patients followed up for cirrhosis and hepatocellular carcinoma in Department of Clinical Medicine, University of Bologna, University-Hospital S. Orsola- Malpighi, Bologna, after their consensus agreement. Patients were stratified based on the advancement of the pathology in 1) cirrhotic, 2) early hepatocellular carcinoma and 3) advanced hepatocellular carcinoma, in agreement with indication of Barcellona. Serum were depleted for albumin presence using ProteoExtract Albumin Removal Kit (Calbiochem, Merck KgA, Germany) belonging to producer instruction. Albumin depleted serum was precipitated with 3 volums of Acetone at -20°C overnight, centrifuged centrifuged at 18800 rcf for 20 minutes at 4°C and the pellet dried in

Speed Vacuum for 30 minutes at room temperature. Pellets were reconstituted with Urea 8M and protease inhibitor 1X (Promega, Madison, WI, USA). The same procedure was performed on negative control, made by a pool of sera given by healthy volunteer donors. Proteins extracted by serum were quantified and analysed in western blot and normalization was performed on the total amount of charged proteins evaluated with ponceau staining of the membranes, measured in optical density. Membranes were probed with antibodies after blocking in milk 5% in PBS- Tween 0,1% ,overnight at 4°C. Antibodies specificities and conditions are reported in Table 2

Table 1. Antibodies used for W.B., IHC, IF,

Antigen	Dilution	Use	Clone	IsoType	Company
Albumin	1:2000	IHC	A0001	Rabbit	Dako Cytomation
Alpha feto protein	1:2000	WB	A0008	Rabbit	Dako Cytomation
Alpha SMA	1:500	WB	1A4	Mouse	Dako Cytomation
p-Akt (Ser 473)	1:500	WB	C31E5E	Rabbit	Cell Signalling
Beta actin	1:1000	WB		Mouse	Santa Cruz Biotech
CK19	1:700	WB	3F8	Mouse	Novus Biologicals
Cyclin D1	1:200	WB	DCS-6	Mouse	Novocastra
c-Met	1.1000	WB	25H2	Mouse	Cell Signalling
E- Cadherin	1:200	IHC, WB		Mouse	Dako Cytomation
p-ERK (Tyr204)	1.500	WB	E-4 sc7383	Mouse	Santa Cruz Biotech
GSK3 alpha/beta	1:500	WB	0011-A	Mouse	Santa Cruz Biotech
GSK3 beta	1:1000	WB	27C10	Rabbit	Cell Signalling
P-Gsk3beta (Ser9)	1:1000	WB	5B3	Rabbit	Cell Signalling
P-Gsk3beta (Ser9)	1:500	WB, IHC	PAB12625	Rabbit	Abnova
Hes1	1:500	WB	PA1-17102	Rabbit	Affinity Bioreagents
HMGA2	1:1000	WB	S0816	Rabbit	Epitomics
ICAM-5	1:1000	WB	Ab85191	Rabbit	Abcam
Mdm2	1:1000	WB	N-20	Rabbit	Santa Cruz Biotech
Mdm2 (Thr216)	1:500	WB	SMP14	Mouse	Santa Cruz Biotech
p- Mdm2 Ser 166	1:800	WB	# 3521	Rabbit	Cell Signalling
MMP-9	1:200	WB	Ab1	Mouse	Calbiochem
Notch1	1:1000	WB	mN1A	Mouse	BioLegend
Notch3	1:1000	WB	M-134	Rabbit	Santa Cruz Biotech
PAI-3	1:200	WB	H-62	Rabbit	Santa Cruz Biotech
P57kip2	1:200	WB, IF	C-20	Rabbit	Santa Cruz Biotech
P53	1:500	WB-Hum	DO-7	Mouse	Dako Cytomation
P53	1:500	WB-Rat	C-11	Mouse	Santa Cruz Biotech
p-p53 ser 20	1:200	WB	hSer 20	Rabbit	Santa Cruz Biotech
p-p53 ser15	1:500	WB		Rabbit	Cell Signalling
Snail	1:500	WB		Rabbit	Cell Signalling
mTOR	1:700	WB	7C10	Rabbit	Cell Signalling
Thrombospondin-1	1:100	WB	3F357	Mouse	Santa Cruz Biotech

Thrombospondin-2	1:200	WB	4 sc136283	Mouse	Santa Cruz Biotech

Gene	Primers sequence (5'-3')	Annealing T (°C)	Cycle n°	Product size (bp)*
β ACTIN F	GAGGCACTCTTCCAGCCTTC	55	26	189
β ACTIN R	GGATGTCCACGTCACACTTC			
BAX F	ACAGGGTTTCACCAGGATC	60	29	363
BAX R	GCTGCCACCCGCAAGAAGAC			
BNIP3 F	TGGACGGAGTAGCTCCAAGAGC	56	29	465
BNIP3 R	AGAAGCCCTGTTGGTATCTTGTG			
CDKN1C/p57 F	AGAGATCAGCGCCTGAGAAG	60	35	220
CDKN1C/p57 R	GGGCTCTTTGGGCTCTAAAC			
CYCLIN G1 F	AATGAAGGTACAGCCCAAGCA	63	27	197
CYCLIN G1 R	GCTTTGACTTTCCAACACACC			
E-CAD F	TCCTGGGCAGAGTGAATTTTGA	56	29	550
E-CAD R	GCGTGAGAGAAGAGAGTGTATGTGG			
HES1 F	CTCTCTTCCCTCCGGACTCT	60	31	186
HES1 R	AGGCGCAATCCAATATGAAC			

Vimentin	1:1000	WB	5G3F10	Mouse	Cell Signalling

MDM2 F	GAGCCTCCAATGAGAGCAAC	61	31	87
MDM2 R	AGGCTGCCATGTGACCTAAG			
MMP-9 F	ACGCCGCTCACCTTCACTC	65	33	180
MMP-9 R	GGACCACAACTCGTCATCGTC			
N-CAD F	GACAATGCCCCTCAAGTGTT	58	29	179
N-CAD R	CCATTAAGCCGAGTGATGGT			
NOXA F	AGCAGAGCTGGAAGTCGAGTGTG	65	29	490
NOXA R	CCAGCCGCCCAGTCTAATCA			
P16 F	GATGAACTGGTTGCCAAGGT	60	28	221
P16 R	CGAGCTGAGTTCCCACTCTC			
P53 F	GGCCCACTTCACCGTACTAA	57	29	150
P53 R	GTGGTTTCAAGGCCAGATGT			
PUMA F	TCCTCAGCCCTCGCTCTC	62	29	285
PUMA R	GGATGTCCACGTCACACTTC			
SNAIL F	ATCCAGAGTTTACCTTCCAGCAG	65	33	145
SNAILR	CCAGGACAGAGTCCCAGATG			
TWIST F	GAGACCTAGATGTCATTGTTTCCAG	65	33	104
TWIST R	CACGCCCTGTTTCTTTGAAT			
VIMENTIN F	GAGAACTTTGCCGTTGAAGC	65	29	170
VIMENTIN R	TCCAGCAGCTTCCTGTAGGT			

Table 2: RT-PCR Primers

AIM OF THE STUDY

Notch signalling is involved in many cellular functions: differentiation, proliferation, self-renewal and survival, that are all involved in cell fate determination, in embryo and in differentiated tissues. Due to its importance in development, deregulation of the pathway has been associated with cancer, presenting a double role. Notch could acts as oncogene but also be a tumour suppressor, with a role dependent on the function that it has in normal tissue (Radtke F. and Raj K., 2003). Notch signalling deregulation could affect different aspect such of cancer, tumour suppressive mechanisms, oncogenesis, progression or drug resistance (Ranganathan P. et al. 2011). It crosstalks with other pathways, so the outcomes of Notch deregulation in cancer context, depends not only on its tissue specific role, but also on biochemicals properties and cellular contest. For that, it is possible a double role of Notch signalling also in the same cancer. (Radtke F. and Raj K., 2003) Moreover, despite of the high grade of homology of these receptors between species, mammalian Notch shows different structural and functional features. Notch3 shows structural differencies in all extracellular, transmembrane and intracellular domain, in particular with a different transactivation domain (TAD), that leads to activation of different target genes (Bellavia D. et al. 2008). For that and for its tissue specificity, Notch3 has not a redundant function compared with Notch1. In addition, even if expressed in the same tissue, both redundant and specific functions for four paralogue receptors exist (Kopan 137 Cell). Moreover, crosstalk with other pathways suggests that the transcriptional activation alone could not reflect the full function of the signalling (Talora et al. 2003; Zhang et al. 2007).

Considering these elements and starting from observation of a previous work that evidence how Notch1 and Notch3 were aberrantly expressed in HCC, (Giovannini C. et al. 2009) the aim of this study was to define the role of Notch1 and Notch3 in tumorigenesis or progression of human hepatocellular carcinoma, dissecting common and un-common pathway through which they exert their role in this cancer. Particular attention was given on specific contests, in order to establish a deeper understanding of cellular mechanisms involved by Notch signalling activation and to identify prognostic or therapeutic targets dependent by Notch signalling. The existence of redundant and not redundant functions between paralogue receptors suggests the existence of both common and peculiar functions for Notch1 and Notch3. So the steps through which we tried to dissect these aspects may be summarized as follow:

1) Common pathway: Both Notch3 and Notch1 showed control of proliferation and modification of cell cycle distribution, but they seem to activate both common and non-common pathways. The first step was to define their common regulation on Hes1 target gene and to dissect the effect on cell cycle.

2) Notch1: In order to establish if exist a specific function of Notch1 in HCC either than the common regulation of cell behaviour through Hes1, the study will focus on analysis of phenotypic features and functional aspects determed by Notch1 in HCC. Due to Notch1 role in inducing Epithelial to Mesenchymal transition (EMT) in other epithelial cancers (Saad S. et al. 2010), we would like to investigate if Notch1 has a role in this aspect of HCC.

3) Notch3 : Starting from theorical structural differencies between Notch1 and Notch3 proteins that sustain specific and not redundant downstream pathway, together with functional evidences on exclusively Notch3 regulation of p53, the second aim of the study was to define the nature of Notch3 regulation of p53 levels that is responsible of Notch3 chemoresistance in HCC (Giovannini C. et al. 2009). Moreover, due to high frequence of functional p53 in HCC, understanding alternative way of inactivation is interesting for carcinogenesis understanding and therapy.

RESULTS AND DISCUSSIONS

BACKGROUND RESULTS

Notch1 and Notch3 result to be aberrantly overexpressed in tumour tissues of 60 HCC samples, analysed by immunohistochemestry (Giovannini C. et al. 2009). In order to define the functional role of Notch1 and Notch3 receptors in HCC, an in vitro model of short interfering RNA was established. HepG2 and Snu398 cell lines derived by hepatocellular carcinoma were silenced for both Notch1 and Notch3 expression with two specific shRNA, in order to exclude off- target effect. After control of silencing by western blot, the best effective clone for each cell line was chosen to perform analysis (Fig.1A-B). For both cell lines, knocked down cells showed a reduced proliferation, after evaluation of the growth rate, with a stronger effect for Notch3 silenced cell that underwent to proliferation arrest after two weeks in culture. Analysis of cell cycle distribution with FACS showed a reduction of cell proliferation as enrichment of G1 cell phase in both Notch3 and Notch1 knocked down cells (Fig1B). Despite this similar phenotype Notch3 silenced cells showed an induced expression of p53, whereas Notch1 does not influence p53 expression (Giovannini C. et al. 2009). p53 is the main regulator of cell fate and it coordinates cell cycle through its target p21/Cip1 (Kastan M. B. et al. 1992; el-Diery W.S. et al.1993; Xiong Y. at al. 1993), but its involvement in mediate Notch3 cell cycle regulation in our model has been excluded, as p21/Cip1 expression was abrogated by HES1 silencing. HES1, a target of Notch signalling positively regulates p21/Cip1 expression and its abrogation due to Notch3 silencing leads to absence of p21/Cip1 expression. (Yu X. et al. 2006; Giovannini C. et al. 2009).

So, both Notch1 and Notch3 control cell cycle progression of HCC cells through a molecular way that seems to be at least in part p53 independent and completely p21/Cip21 independent. Neverthless, it remains to be understood if they really activate the same molecular pathway and which are molecular pathways activated by them commonly and in specific ways.

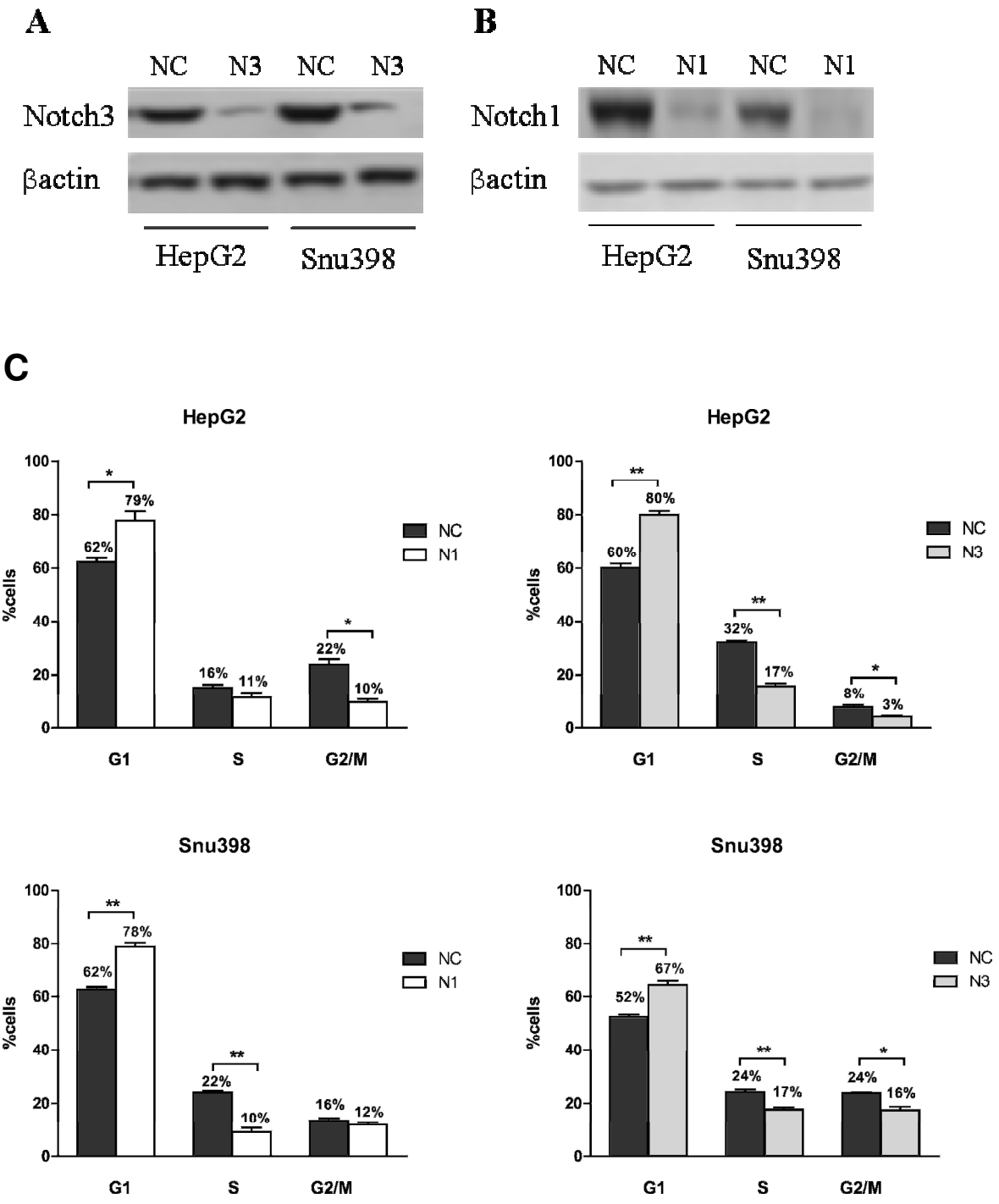


Figura 1. Notch3 and Notch1 depletion and effects on cell cycle distribution. A-B) Western blot analysis for Notch1 and Notch3 in HepG2 and Snu398 silenced cells, to verify effective shRNA. **C)** Histogram representing the growth rate of HepG2 and SNU398 cells evaluated by propidium iodide staining and flow cytometry two weeks post- infection with Notch1 or Notch3 shRNA. When compared to negative controls Notch infected cells showed and increase of the G1 cell population. Results are expressed as the means of three independent experiments (+/- S.E.). NC: GL2 negative control shRNA, N3; Notch3 shRNA, N1; Notch1 shRNA.

RESULTS 1

Notch1 and Notch3 mediated inhibition of p57/CDKN1C expression involves HES1 signalling in HCC cell lines.

In order to define the Notch1 and Notch3 effect on cell proliferation in HCC cell lines, we verified if in our cell lines they mediate their effect by activating HES1 (8), that is a potential targets of both NICD1 and NICD3. Indeed, Hes1 has been demonstrated to repress CDKN1C/p57 transcription in pancreatic progenitor mouse cells and in murine intestinal crypt progenitors. (Georgia S. et al. 2006; Riccio O. et al. 2008). CDKN1C/p57 is an inhibitor of cyclin dependent kinases complexes that is involved in checkpoint at G1/S cell cycle transition and it has been identified as tumour suppressor gene, frequently functionally impaired in HCC (Matsuoka S. et al. 1995) .So, we aimed at investigating the existence of a relationship between CDKN1C/p57 mRNA and HES1 protein accumulation in human hepatocellular carcinoma. Since the promoter of p57/CDKN1C human gene is frequently hypermethylated in cancer, and this mechanism has been linked to reduction of p57/CDKN1C mRNA expression (Schwienbacher C. et al., 2003), we first analyzed CDKN1C/p57 promoter methylation status in two HCC cell lines: HepG2 and SNU398, characterized by low levels of miR-221. Indeed, miR-221 could regulate CDKN1C/p57protein expression in HCC model cell lines, as it has been previously identified as modulator of CDKN1C/p57protein expression, targeting its mRNA and being upregulated in HCC (Fornari F. et al. 2008). Choosing cell lines with lower miR-221, we aimed to exclude or at least strongly reduced this post transcriptional regulation. Moreover, the expression pattern of CDKN1C/p57 does not correlate with the methylation status in these cell lines as assessed by high density cell culture, serum deprivation and treatment with the hypomethylating agent 5-aza-2'-deoxycytidine (Figure 2 A-B), suggesting that in HepG2 and SNU398 cells CDKN1C/p57 mRNA expression is regulated by different mechanisms than promoter silencing. (Kuang S-Q. et al. 2007 oncogene).

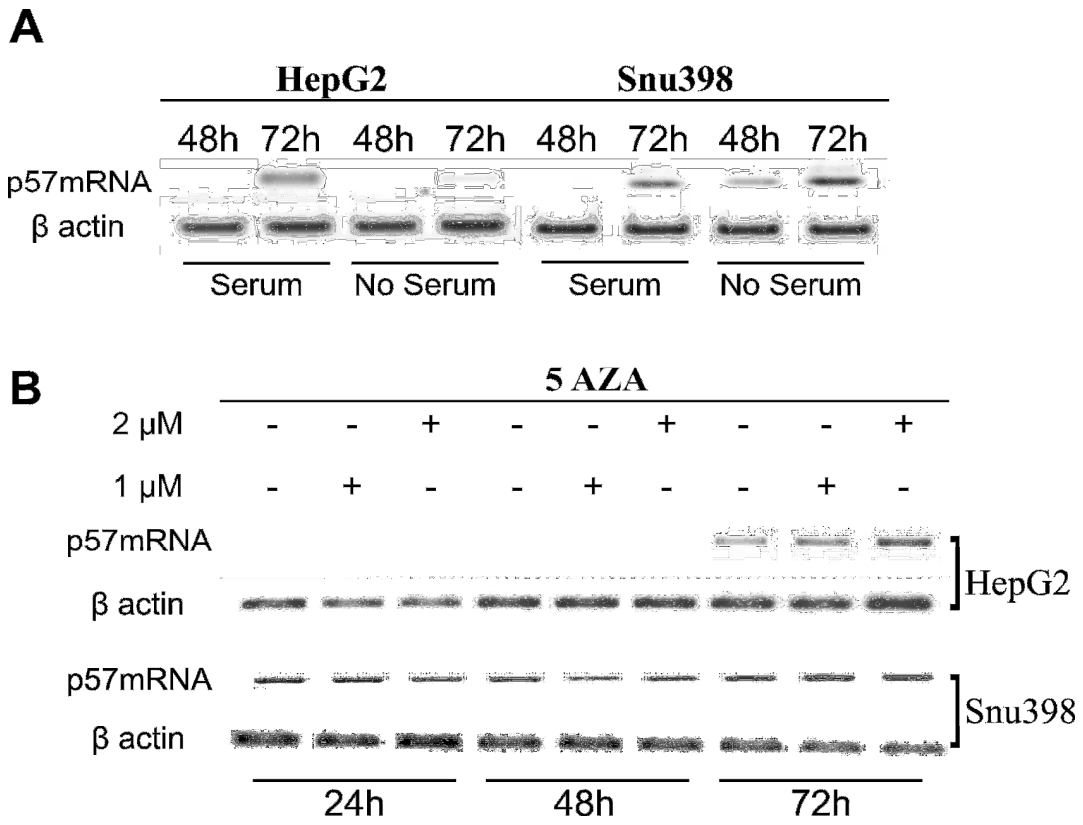


Figure 2. CDKN1C/p57 expression is regulated by Notch1 and Notch3 receptors. (A) CDKN1C/p57 mRNA is up-regulated by cell cycle arrest induced by high density cell culture or serum withdrawal in HepG2 and SNU398 cell as evaluated by semi-quantitative RT-PCR. β Actin serves as a reference control for mRNA levels. (B) HCC cells were cultured in the presence of different concentration of 5'-aza-2'-deoxycytidine (5'AZA) for 24h, 48h and 72h. CDKN1C/p57 mRNA expression was evaluated by RT-PCR.

After having excluded epigenetic regulation, we investigated the reciprocal regulation of Notch1 and Notch3 on their target gene HES1. HepG2 and SNU398 cells stable knocked-down for Notch3 and Notch1 expression by retroviral transduction of specific short hairpin RNAs (shRNA) (Figure 1A-B), both showed down-regulation of Hes1 target gene (Figure 3A) together with an up regulation of CDKN1C/p57 mRNA and protein levels (Figure 3B-C).

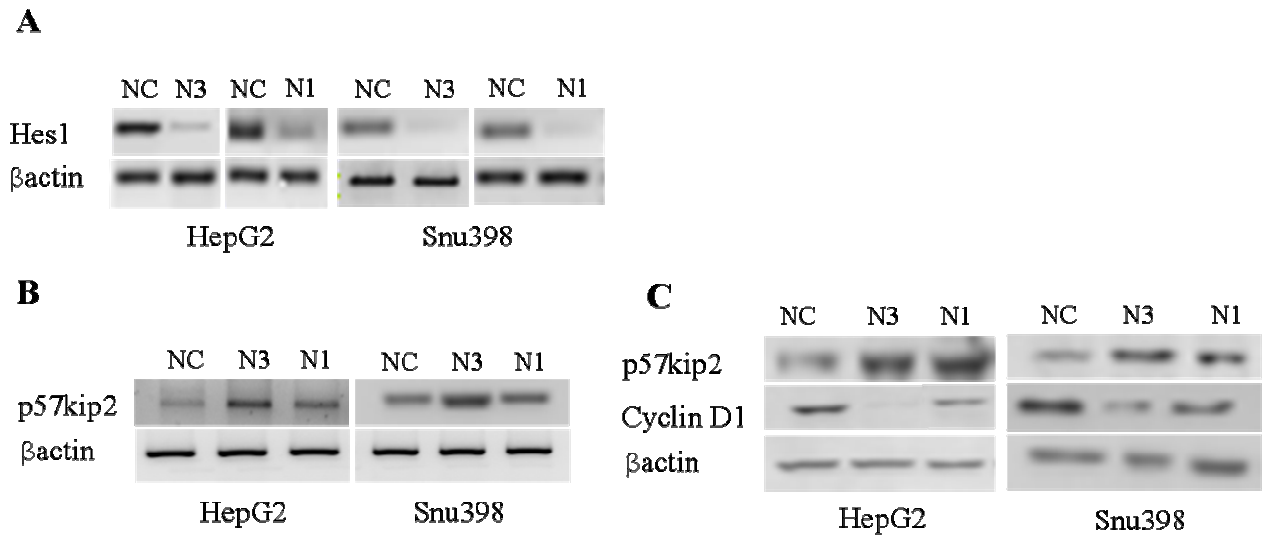


Figure 3. CDKN1C/p57 expression is regulated by Notch1 and Notch3 receptors. (A-B)RT-PCR expression analyses of Hes1 and CDKN1C/p57 in Notch silenced cells. (C) Western blots of CDKN1C/p57 and Cyclin D1 in Notch silenced cells. An increased in CDKN1C/p57 mRNA and protein levels were observed in both Notch1 and Notch3 silenced cells.NC: GL2 negative controls hRNA, N3; Notch3 shRNA, N1; Notch1 shRNA. β Actin was used as a reference control for both mRNA and protein levels.

By immunofluorescence analysis, CDKN1C/p57 was found to be expressed in the nucleus of silenced cells suggesting its role as an active cell cycle inhibitor (Figure 4) and excluding other possible functions associated with cytoplasmic localization, such as cytoskeleton organization (Yokoo T. et al. 2003). According with this observation and the reduced proliferation rate already highlightened in Notch1 and Notch3 silenced cells (Fig. 1), Cyclin D1, the cyclin which levels are regulated by many proliferatives stimuli and that mediate the passage through the R point in cell cycle progression, results downregulated (Figure 3C), in agreement with the reduced proliferation assessed by FACS analysis (Figure 1C). Cell cycle distribution shows an arrest of cell cycle progression as accumulation of cell in G1 phase after Notch1 and Notch3 silencing, with an increase of 17% and 16% in Notch1 knockdown cells, HepG2 and Snu398 respectively and with 20% and 15% of increase in HepG2 and Snu398 silenced for Notch3. Indeed, the increase in G1 phase was statistical significant in all cell lines, together with the consequent reduction of S and G2/M populations after both Notch1 and Nothc3 silencing, with higher significance in Notch3 silenced cell lines than Notch1 in both cell lines.

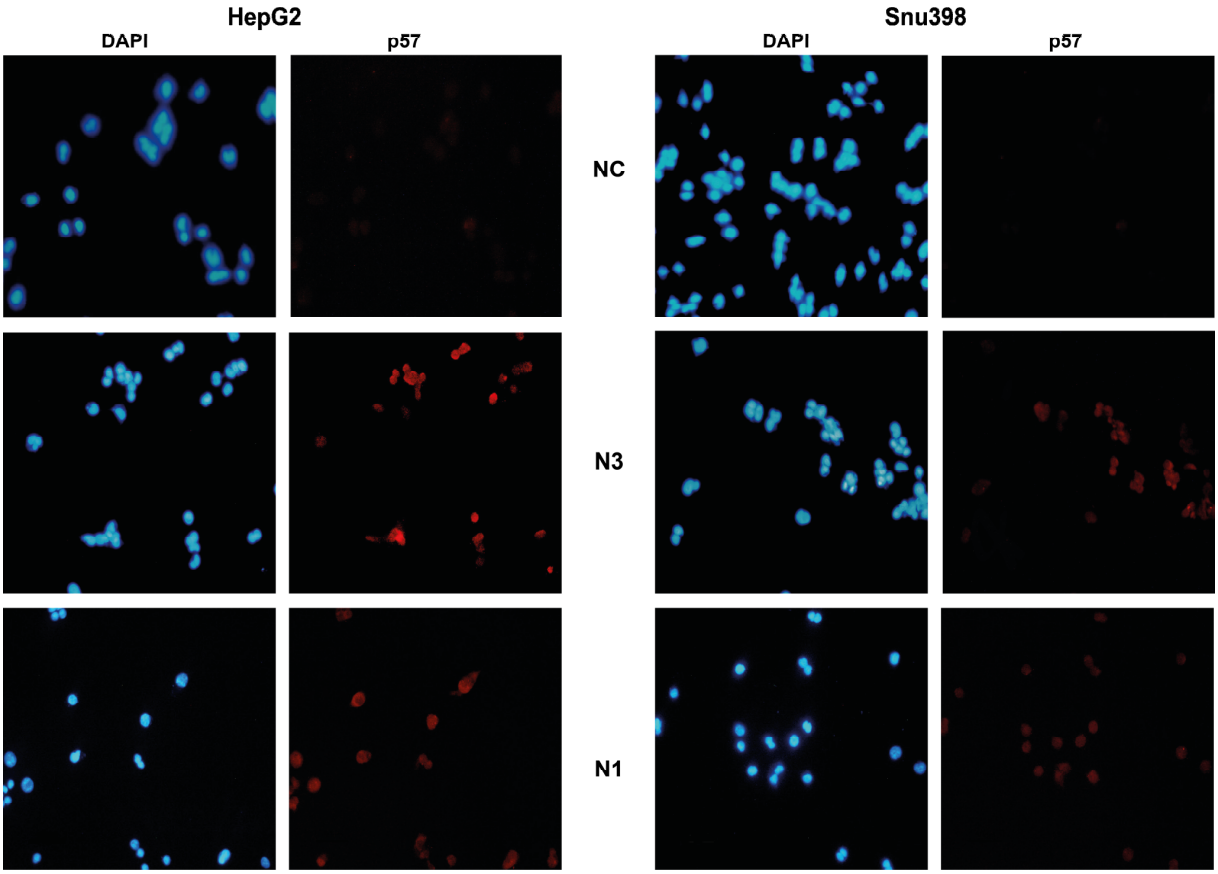


Figure 4. Immunoflurescence analysis of CDKN1C/p57 expression in Notch1 and Notch3 silenced cells.
CDKN1C/p57 was found to be expressed in the nucleus of Notch1 and Notch3 silenced cells (red). Cells were counterstained with DAPI (4,6-Diamidino-2-phenylindole). NC: GL2 negative control shRNA, N3; Notch3 shRNA, N1; Notch1 shRNA.

To confirm whether the down-regulation of Hes1 in Notch1 and Notch3 silenced cells was the real determinant of CDKN1C/p57 upregulation, Hes1 was silenced both in HepG2 and SNU398 cells by using specific siRNAs. Down-regulation of Hes1 significantly increased CDKN1C/p57 mRNA and protein levels in both analyzed cell lines (Figure 5A-B). In line with Notch1 and Notch3 knockdown, Hes1 silencing caused cyclin D1 down-regulation (Figure 5B) suggesting that Hes1 is one of the major effectors of Notch signaling in this setting and that, regulating CDKN1C/p57, it mediate the proliferation reduction observed in Notch silenced cells.

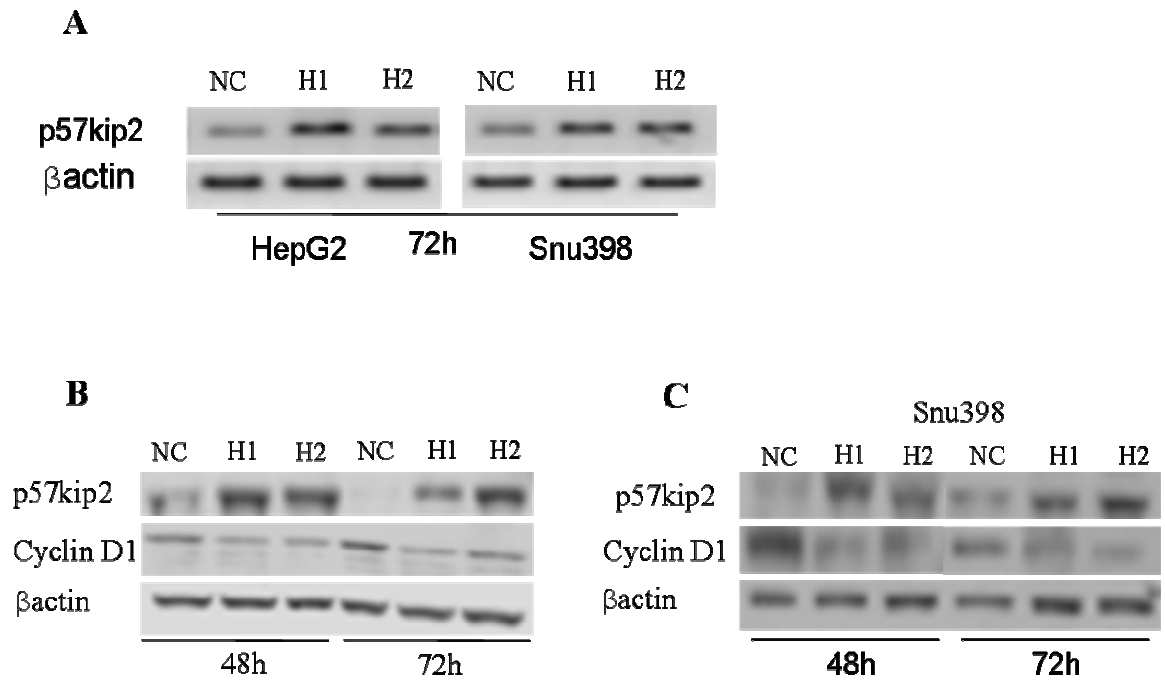


Figure 5: Hes1 regulates CDKN1C/p57 expression.
A: HepG2 and SNU398 cells were transfected with Hes1 siRNA or scrambled RNA. Hes1 andCDKN1C/p57 mRNA levels were evaluated by RT-PCR. B: CDKN1C/p57 and Cyclin D1 protein expression were evaluated in Hes1 silenced cells by western blot .NC: scrambled siRNAs; H1 and H2: two different Hes1 siRNAs. β Actin was used as a reference control for both mRNA and protein levels.

CDKN1C/p57 inhibits in vitro growth of human HCC.

In order to confirm that the reduced proliferation and the cell cycle arrest in G1 were dependent by CDKN1C/p57, HCC-derived cancer cells were transfected with human CDKN1C/p57 expression vector or empty vector and CDKN1C/p57 protein expression was assessed by Western blotting 48h and 72h post-transfection (Figure 6A). To investigate whether induction of exogenous CDKN1C/p57 can reduce cell growth we performed a proliferation assay and FACS analysis. Induction of CDKN1C/p57 reduced proliferation of HepG2 and SNU398 HCC cells as demonstrated by growth curve analysis (Figure 6 B-C). FACS analysis revealed that CDKN1C/p57 transfected cells accumulate in G1 phase of cell cycle, with a variation in G1 that is statistical significant with a percentage of increase of 14,05% (p=0,007) and 19,5% (p=0,003) respectively at 48h and 72h in HepG2 and a percentage of 22,7% (p=0,002) and 14,8% (p=0,005) for Snu398 (Figure 6C). These results confirm that CDKN1C/p57 contributes to G1 cell cycle arrest and they suggest that the higher expression of CDKN1C/p57 was responsible, at least in part, of the G1 arrest of Notch

knocked down cells. In agreement with these observations Cyclin D1 downregulation was also observed in CDKN1C/p57 transfected cells compared with negative control (Figure 6A).

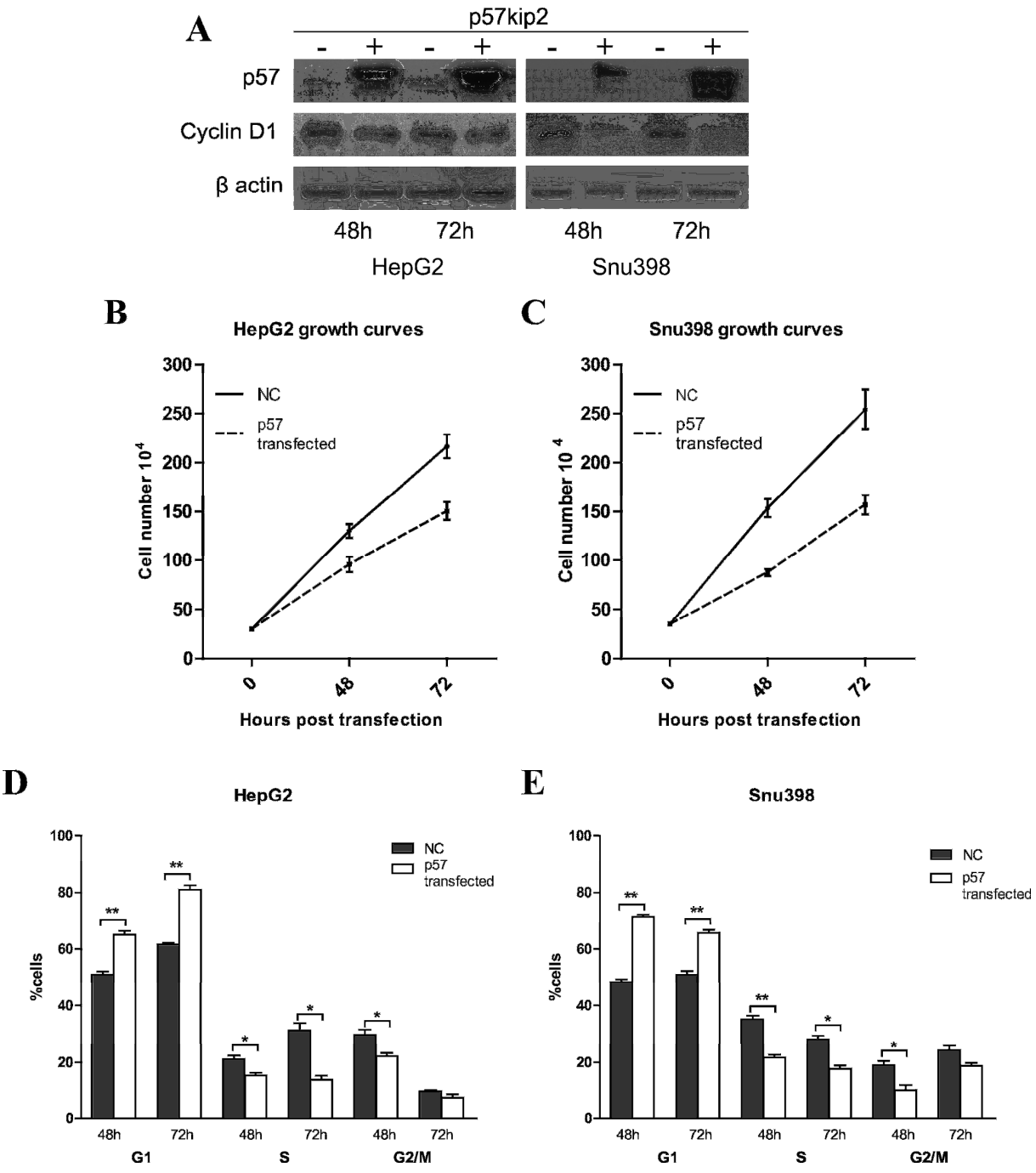


Figure 6. CDKN1C/p57 regulates cell growth of hepatocellular carcinoma (HCC) derived cell lines. (A) Western blot analysis of CDKN1C/p57 and Cyclin D1 in HepG2 and SNU38 cellstransfected with CDKN1C/p57 cDNA. β Actin was used as a reference control for protein levels. (B-C) Transfection of p57 caused a proliferative block in HCC cells as assessed by growth curves. CDKN1C/p57 over-expression caused a 1.5 and 1.6 fold reduction in HepG2 and SNU398 cell growth respectively in comparison to negative control (P=0,025 at t-test for both cell lines at 72h after transfection). (D) Histogram representing the growth rate of HepG2 and SNU398 cells evaluated by propidium iodide staining and flow cytometric

analysis. When compared to negative controls CDKN1C/p57 transfected cells showed and increase of the G1 cell population. Cell cycle distribution was as follows: HepG2; NC 48h (G1 52,1%; S 20,9%; G2/M 27%), p57 48h (G1 64,2%; S 13,7%; G2 22,1 %), NC 72h (G1 61,7%; S 28,1%; G2 10,2%), p57 72h (G1 79,2%; S 14,3%; 6,5%). SNU398; NC 48h (G1 47,1,1%; S 34,3%; G2/M 18,6%), p57 48h (G1 69,5%; S 19,2%; G2 11,3 %), NC 72h (G1 49,3%; S 26,9%; G2 23,8%), p57 72h (G1 62,9%; S 17,2%; 19,9%). Results are expressed as the means of three independent experiments (+/- S.E.). NC: cells transfected with empty vector; p57/Kip2: cells transfected with CDKN1C/p57 cDNA.

CDKN1C/p57 induction does not affect apoptosis

Previous studies reported that CDKN1C/p57 interplays with the apoptotic cell death machinery in cancer cells, activating the intrinsic apoptotic pathway and p73β mediated apoptosis (Vlachos P. et al. 2007; Gonzalez S. et al. 2005). To rule out the possibility that the cell growth inhibition observed in CDKN1C/p57 transfected cells could be due to increased cell death, apoptosis related genes like Bax, BNIP3, NOXA, PUMA and caspase-3 were analyzed (Howard R.M. and Harris A.L., 2007). No differences were observed in the expression of all these genes (Figure 7A-B) following CDKN1C/p57 enforced expression. The evidence that CDKN1C/p57 was not involved in apoptosis in HCC was confirmed by Annexin V staining which showed no differences between CDKN1C/p57 transfected cells and negative controls (Data not shown).

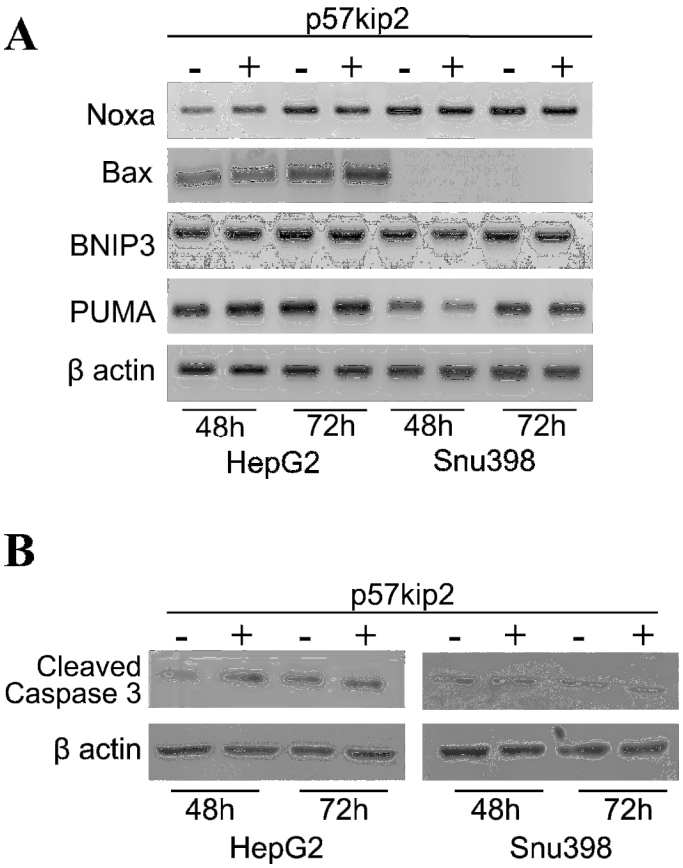


Figura 7. Effect of CDKN1C/p57 on apoptosis.
(A) Expression levels of several pro-apoptotic genes in HepG2 and SNU398 evaluated 48h and 72h post CDKN1C/p57 transfection. β Actin was used as a reference control for mRNA levels (B) Total cellular proteins were resolved by polyacrylamide gel electrophoresis followed by immunoblotting with monoclonal anti-Cleaved Caspase3 and anti-β actin as a protein reference control.

Over-expression of CDKN1C/p57 induces senescence in HCC cells

After having observed cells overexpressing p57kip2, we observed that seventy-two hours post CDKN1C/p57 transfection cells began to change morphology becoming enlarged and flattened and adopting a senescent like phenotype when compared to vector-only cells (Figure 8A). Replicative senescence is thought to be a major barrier to tumour formation as it limits the replicative potential of cells and preserves genomic integrity (Campisi J., 2005). On the other hand, recent studies show that senescence is involved in tumour regrowth and disease recurrence (Gewirtz D.A.,2009) suggesting that the role of senescence depends on cell type and cellular context. To demonstrate that CDKN1C/p57 plays a role in the induction of senescence in HCC, two well known senescence markers were analyzed: senescence-associated β-galactosidase (SA-β-gal) and p16INK4A (Dimri G. et al. 1995; Pitto L. et al. 2009). Senescent status is also characterized by resistance to apoptosis (Jackson JG et al., 2006) meaning that the expression levels of several genes involved in apoptosis are reduced

during senescence. Because high expression levels of CDKN1C/p57 for both cell lines could be maintained no longer than 96 hours, and the expression of apoptotic genes didn't change between 48h and 72h post-transfection (Figure 7), SA-β-gal and p16 were evaluated 96h after CDKN1C/p57 induction. According with the senescent phenotype, induction of CDKN1C/p57 in HepG2 and SNU398 HCC cells led to the identification of SA-β-gal positive cells (Figure 8B). An increase in p16 protein levels was also observed in HepG2 cells (Figure 7C). P16 protein does not appear to be expressed in SNU398, nevertheless as p16 is frequently subject to epigenetic control in HCC, we verified that the loss of p16 does not correlate with p16 promoter hyper-methylation in our experimental setting (Figure 8D) (Matsuda Y. 2008).

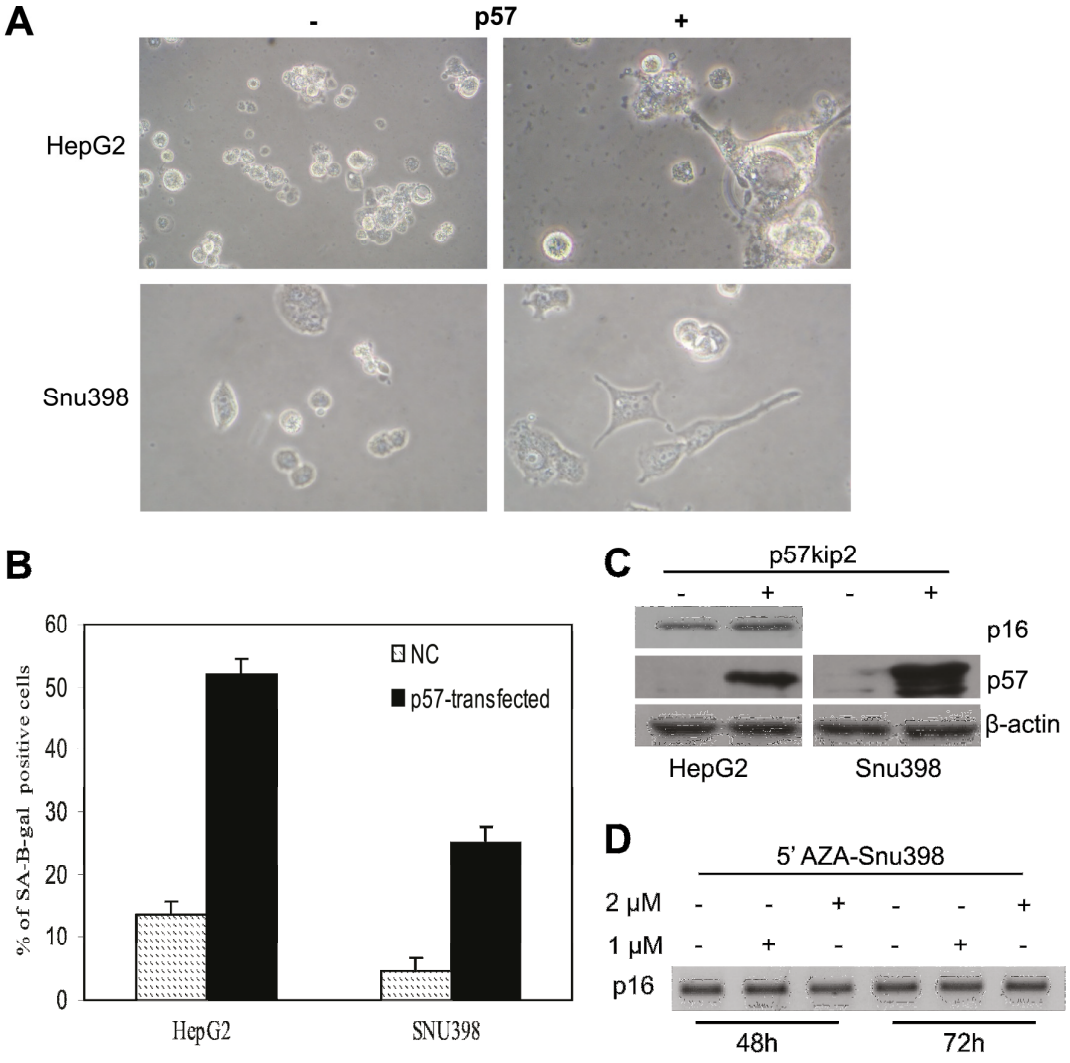


Figure 8. CDKN1C/p57 induces cell senescence in HepG2 and SNU398 cells.
(A) Morphological alterations in human HCC cell lines after p57 transfection, day 3. Cells expressing p57 appeared flattened and enlarged compared to vector only (B). SA-β-gal evaluation 96h post p57-transfection. * $p<0,05$, ** $p<0,01$.(C) Upper panel: p16 protein expression evaluated in HepG2 and SNU398 cell lines 96h post p57 transfection by western

blot. β Actin was used as reeference control for protein levels. P16 doesn't appear to be express in SNU398 cells. Lowerpanel: SNU398 cells were cultured in the presence of different concentration of 5'-aza-2'-deoxycytidine (5'AZA) for 48h and 72h. and p16 mRNA expression was evaluated by RT-PCR.

CDKN1C/p57 mRNA expression inversely correlates with Hes1 protein in human HCC.

To assess whether our in-vitro findings reflect the biology of human HCC, we analyzed the expression of CDKN1C/p57 mRNA and Hes1 protein in 34 surgically resected HCCs. We found a significant inverse correlation between CDKN1C/p57 mRNA and Hes1 protein accumulation (Pearson's test: $p<0.0001$ Figure 9A) suggesting that Hes1 participates in the control of CDKN1C/p57 mRNA transcription in human hepatocellular carcinoma. Possible correlations between CDKN1C/p57 mRNA and Hes1 expression with different clinical-pathological features of HCCs including size, focality, grading, AFP serum levels and aetiology of the underlying liver disease were investigated, however no correlation was found. Low Hes1 levels in HCC tissue were associated with a shorter time to recurrence (TTR) (Figure 9B) while they did not achieve a statistical significance for the overall survival. Conversely, CDKN1C/p57 mRNA expression was not associated with different survival or recurrence rates. We next investigated the relationship between CDKN1C/p57 mRNA and protein expression in human HCC. Since our group previously proved that the cyclin-dependent kinase inhibitor CDKN1C/p57 is a direct target of miR-221, CDKN1C/p57 protein expression was analyzed only in primary tumours with low miR-221 expression (lower than median miR-221 values) (Fornari F. et al. 2008). Based on this exclusion criteria CDKN1C/p57 was evaluated in 17 of 34 HCCs; a positive correlation between CDKN1C/p57 mRNA and protein expression was found in the analyzed samples (Pearson's correlation: $p=0.037$). In addition, high levels of CDKN1C/p57 protein resulted to be associated with tumours recurrence (Figure 9C).

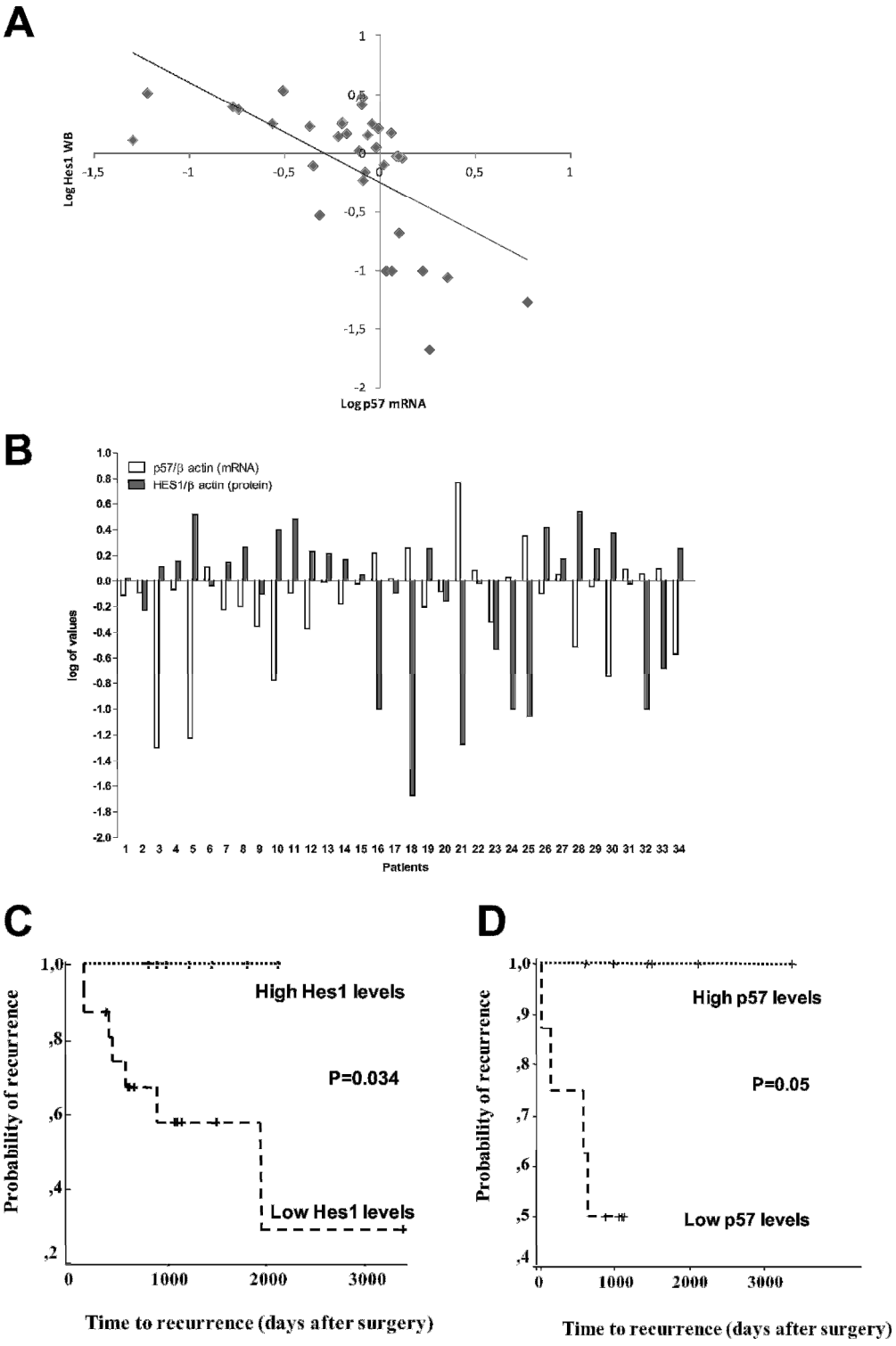


Figure 9. Hes1 regulates CDKN1C/p57 in vivo and predicts HCC time to recurrence (TTR).(A) Inverse Correlation between Hes1 protein and p57 mRNA expression levels of 34 studied human HCC. Expression values are plotted on a log scale. A statistical significant correlation between CDKN1C/p57 mRNA and Hes1 protein levels (p<0,0001) was observed by using two tailed Pearson's test. (B-C) Kaplan Meier Analysis showing the pattern of disease-free survival relative to HES1 protein expression and to CDKN1C/p57 protein expression.

Senescent state in Hes1 depleted cells depends on the accumulation of CDKN1C/p57

To evaluate more critically the role of Hes1 and CDKN1C/p57 in senescence we used retrovirus to stably suppress Hes1 and CDKN1C/p57 protein expression or to mediate stable over-expression of CDKN1C/p57 in HepG2 and SNU398 cell lines (Figure 10). The generation of Sa-β-gal positive cells was evaluated 7 days after infection. The Hes1 shRNAs induced cellular senescence as shown in Figure 10A, supporting that it was the effect of CDKN1C/p57expression consequent to Hes1 silencing. Then, to analyse the effective role of CDKN1C/p57 in cellular senescence, avoiding alternative mechanisms associated with Hes1 inhibition, cells were co-infected with p57shRNAs and Hes1shRNA. CDKN1C/p57 inhibition associated with Hes1 silencing reversed the senescent phenotype suggesting that Hes1 silenced cells senesced primarily due to CDKN1C/p57 up-regulation (Figure 10B). As Hes1 has been demonstrate to be involved in the commitment between quiescence and senescence in fibroblasts and also in tumorigenic settings, avoiding cells going to senescence, we want to exclude a CDKN1C/p57 unspecific role due to Hes1 silencing. (Sang L. et al. 2008). Next we performed stable over-expression of CDKN1C/p57 (single copy retroviral integration). As expected, CDKN1C/p57 retroinfected cells arrested growth with a senescent morphology and SA-β-gal expression (Figure 11 C). The percentage of senescent cells resulted higher in CDKN1C/p57 over-expressing cells than in Hes1 silenced cells probably because ectopic protein expression, by using a retrovirus, is not affected by factors that potentially control transcription of the endogenous protein.

Finally, in order to confirm that Notch1 and Notch3 silenced cells phenotype is due to CDKN1C/p57 dependent senescence, we evaluated the senescent state in both Notch3 and Notch1 silenced cells (Figure 11 D) and we found that the increase in SA-β-gal expression reflects both the accumulation in G1 phase of the cell cycle observed in Figure 1C and SA-β-gal expression induced by Hes1 silencing (Figure 11A). Taken together these findings outline the role of CDKN1C/p57 in the induction of senescence in HCC. This event was confirmed in the long-term culture and it was not overcome by compensatory mechanisms inducing cell proliferation.

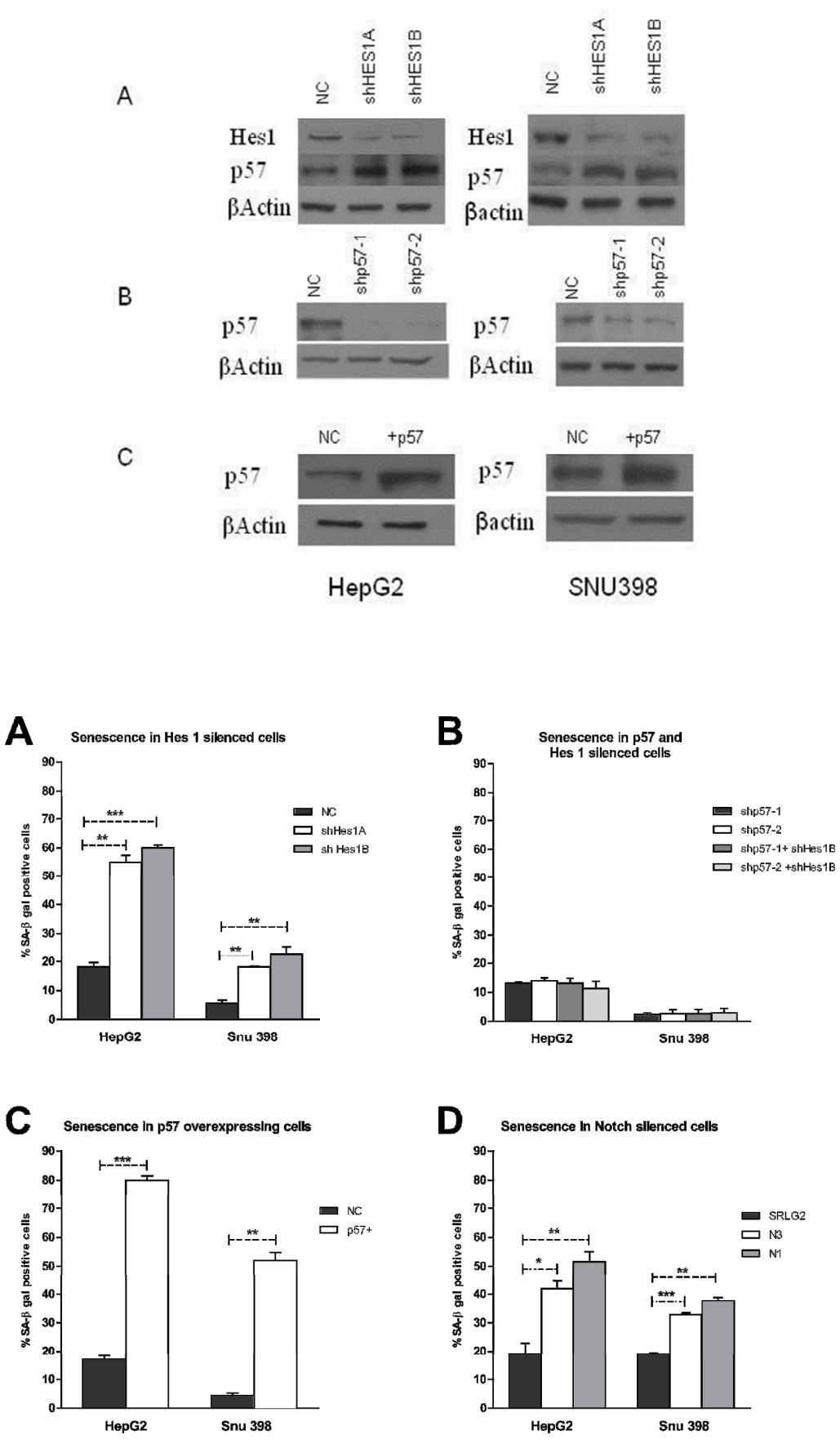


Figure 11. Generation of SA-β gal positive cells in different cellular context.
SA-β gal was evaluated after Hes1 silencing (A), Hes1 and CDKN1C/p57 silencing (B),

CDKN1C/p57 over-expression (C) and in Notch silenced cells (D). Results are expressed as the means of three independent experiments (+/- S.E.). NC: cells infected with empty vector; shHES1A and shHES1B: cells infected with different Hes1 specific shRNAs retrovirus; shp57-1 and shp57-2: cells infected with two different CDKN1C/p57 specific shRNAs retrovirus; p57+: cells infected with retrovirus over-expressing p57: SRGL2: GL2 negative control shRNA, N3; Notch3 shRNA, N1; Notch1 shRNA.

DISCUSSION 1

The four known Notch receptors mediate signalling from the cell surface to the nucleus regulating proliferation, differentiation and apoptosis at all stages of development (Artavanis-Tsakonas S. et al. 1999). Notch activation is generally believed to promote cancer development, whereas it can also play an opposite role, acting as tumour suppressor, in a dependent way from each tissue and context, moreover with a dose effect of the Notch pathway activation (Roy M. et al. 2007). Biologically, this is not surprising, in view of the opposite role that Notch can play in enhancing stem cell potential and suppressing differentiation along certain lineages, while promoting commitment to others (Artavanis-Tsakonas S. et al. 1999). From this study, the involvement of Notch signalling in terminal differentiation mechanisms is confirmed by the regulation of CDKN1C/p57 cell cycle inhibitor, responsible of cell cycle exit, linked to gain of quiescent and non proliferative status during terminal differentiation in development (Bilodeau S. et al. 2009), but to which have been attributed novel functions (Pateras I. S. et al. 2009). Indeed, it has been shown that CDKN1C/p57 is involved in hepatocyte growth arrest at two distinct points during liver development, the perinatal period and the postnatal transition to a quiescent adult hepatocyte phenotype (Awad M.M., 2000). Moreover, the CDKN1C/p57 gene belongs to the Cip/Kip family of cyclin-dependent kinase (CDKs) inhibitors and has been suggested to be a tumor suppressor gene inactivated in various type of human cancers, but its over-expression has been involved not only in cell cycle arrest in G1 phase, but also in cell differentiation, cytoskeleoton organization, apoptosis and senescence (Tsugu A. et al. 2000; Vlachos P. et al. 2007; Yokoo T. 2003). The participation of CDKN1C/p57 in the control of hepatocytes proliferation at developmental level, together with evidences of novel specific CDKN1C/p57 functions, raise the possibility that it takes a role in disorders associated with hepatocyte aberrant proliferation. Indeed, it seems to have a key role in hepatocellular carcinoma however little was known about CDKN1C/p57 function and regulation in human HCC even if, as for the majority of malignant tumours, also in HCC it has been demonstrated the deregulation of different components of the cell cycle machinery including CDKN1C/p57 (Mann C. D. et al. 2007). So, these results about CDKN1C/p57 novel function in liver cancer confirm the linkage between developmental mechanism and their deregulation in cancer and the persistence of molecular actors in cancer compared to development of a specific tissue.

As already reported, aberrant Notch1 and Notch3 gene expression was observed in HCC and have been associated with modulations of cell growth and response to chemotherapy

(Giovannini C. et al. 2009, Qi R. et al. 2003). Here we show that a mediator of cell cycle exit, CDKN1C/p57, is a target of transcriptional repression by Hes1, a downstream target of Notch signalling, both in vitro and in human HCCs. We demonstrated that CDKN1C/p57 over expression in two HCC cell lines affected their proliferation and morphology without affecting the apoptotic machinery. We proved, for the first time in HCC, that CDKN1C/p57 caused a cell senescent phenotype as determined by SA-β-gal staining and induced p16 expression. Finally we showed that a senescent state in the Notch and Hes1 depleted HCC cells depends on the accumulation of CDKN1C/p57.

Starting from the observed cell cycle arrest in Notch 1 and Notch3 silenced cells, this work demonstrate that they controls cell cycle arrest through Hes1 mediated control of CDKN1C/p57 expression. The co-regulation of Notch1 and Notch3 on Hes1 target it not predictable, as even if Hes family is the most important mechanism to transduce the Notch signalling (Artavanis- Tsakonas s. et al. 1999), it has been demonstrate that they compete for the binding to RBP-Jk factor on HES promoter, suggesting a different capability in Hes activation by two receptors (Beatus P. et al. 1999). Subsequently, the lower Notch3 capability in inducing Hes1 promoter has been explained through two structural different regions in respective NICD functionally linked to transcriptional activation (Beatus P. et al. 2001). Neverthless, both receptors are able to activate Hes1 transcription with a different outcome dependent by the context, as in astrocytic differentiation Notch3 and Notch1 functions in a similar way (Tanigaki K. et al. 2001), furthermore in thymocytes Notch3 ectopic expression induces Hes1 expression (Bellavia D. et al. 2000). Similarly, in our HCC cellular setting, two receptors seem both able to regulate Hes1 promoter activity with comparable function respect to Hes1 expression.

The effect of Notch induced Hes1 expression in HCC cells is translated in a transcriptional repression of CDKN1C/p57 transcription. Indeed, Hes1 is a basic helix-loop-helix (bHLH) type of transcriptional repressor that suppresses expression of downstream target genes such as tissue-specific transcriptional activators (S.E Egan et al. 1998; Greenwald I., 1998). Regulation on the murine promoter of CDKN1C/p57 by Hes1 has been reported both in pancreas than in intestinal crypt progenitors, showing a control of proliferation during differentiation and suggesting Hes1 as a possible general mechanism of regulation (Georgia S. et al. 2006; Riccio O. et al. 2008). Others different mechanisms were already known to be involved in the regulation of CDKN1C/p57 expression, both at transcriptional and post-transcriptional level including DNA methylation status, (Schwienbacher C. et al., 2003), MyoD (Figliola L. et al. 2004), p63 (Beretta C. et al. 2005), p73 β (Balint E. 2002), TGF-β

signalling (Scandura J.M.et al. 2004) and miR-221 (Fornari F.et al. 2008). In agreement with our present and previous findings, Sang and co-authors demonstrated a role of Hes1 in preventing irreversible cell cycle exit in fibroblasts (Sang L. et al 2008), highligtening the involvement of Hes1 in cell fate outcome. Not only in physiological but also in pathological conditions, such as HCC cancer, Hes1 seems to exerts its decisional function, mediating cell cycle arrest and senescence. Indeed, Hes1 role in balancing quiescence and differentiation in cancer has been already highlightened, also as a possible target for therapy (Sang L. et al. 2009). Neverthless, here we have demonstrated that the function of Hes1 in HCC is mediated by CDKN1C/p57, as evident by the reversed phenotype of Hes1 silenced cells when also CDKN1C/p57 is silenced. The effect of CDKN1C/p57 expression consequent to Hes1 silencing is responsible of a senescent phenotype in HCC cells. Other members of cyclin-dependent kinase inhibitors family are demonstrated to mediate senescence in many context, included cancer (Campisi J. and D'Adda di Fagagna F., 2007). But this novel function of CDKN1C/p57 in liver and in hepatocellular carcinoma, it has previously reported only in astrocytoma and prostate cancer cells (Schwarze S.R. et al. 2001). Senescence in the field of cancer, open a wide field of functional considerations. Indeed senescence limits the growth of many tumours including epithelial tumours of the colon, head and neck and thyroid (Wynford- Thomas D., 1997; Edington KG et al. 1995; Paraskeva C. et al 1988) and it has postulated to be a barrier to cancer progression (Campisi J. and D'Adda di Fagagna F., 2007). On the other hand the presence of senescent cells in the tumour bulk is an important biological factor that might have prognostic implications for the disease outcome, as the accumulation of senescent cells can facilitate cancer progression (Campisi J. and D'Adda di Fagagna F., 2007). Senescent tumour cells serve as a reservoir of secreted factors with mitogenic, antiapoptotic and angiogenic activities (Roninson IB, 2003) leading to a worse prognosis. Senescence was not the subject of many studies in HCC, therefore the potential role of senescence in this tumour is poorly understood. However because of repeated episodes of cell necrosis and cell division, the process of chronic hepatitis and liver cirrhosis could represent a model of accelerated replicative senescence. Indeed it has been shown that accumulation of replicative senescent cells in non cancerous liver serves as a predictive marker of hepatocellular carcinoma in the surrounding tissue (Paradis V. et al. 2001). Thus, senescent cells can alter their microenvironment for as long as they persist, being metabolically actives and showing to increase the secretion of some factors, called "senescence associated secretory phenotype" (SASP) that are growth factors, chemokynes, cytokines and proteases (Freund A. et al. 2010).

Different genes act as positive regulators of senescence in tumour cells like p21, p53 and p16 but they are not absolutely required for this response suggesting that other genes are likely to play a role in inducing senescence of tumours cells (Campisi J. et d'Adda di Fagagna, 2007). In the present study we showed that another gene involved in HCC senescence is CDKN1C/p57 which results to be negatively regulated by Hes1. The analysis of primary HCC samples did not show any relationship between Hes1 or CDKN1C/p57 mRNA expression and size, focality, grading, aetiology and AFP serum levels of HCC patients. Conversely, time to recurrence (TTR) was shorter in patients with lower Hes1 expression whereas CDKN1C/p57 mRNA was significantly associated neither with TTR nor to survival rate. That's not surprising because mRNA expression values are almost only correlative rather than causative (Lichtinghagen R.et al. 2002). In HCC, CDKN1C/p57 down-regulation was found to be associated with high PCNA expression indicating an increased proliferating activity (Nan KJ et al. 2005). Nevertheless cell proliferation is not always associated to tumour recurrence suggesting that tumours with low proliferation rate could be more invasive and aggressive (Mastronardi L. 2001; Schultz IJ et al. 2006). Moreover, the nature of pro inflammatory composition of the "senescence associated secretory phenotype" observed in senescent cells and that has been demonstrated to induce invasion and angiogenesis in cancer progression (Freund A. et al. 2010), might explain our *ex vivo* data. In line with these last observations our results showed that TTR was shorter in patients with higher CDKN1C/p57 protein expression even though this protein reduces cell proliferation in vitro. Since CDKN1C/p57 protein has been described to associate with different complexes (Reynaud EG et al. 2000), immunohystochemistry (IHC) results more accurate than western blot for p57 evaluation (Ito Y. et al. 2001).

Future studies will be directed towards dissecting the mechanism for this novel Hes1-CDKN1C/p57 crosstalk in a well selected cohort of HCC by analyzing CDKN1C/P57 and others factors known to be involved in cells senescence including senescence associated heterochromatic foci (SAHFs) components and HMGA2 expression levels. Moreover, in order to confirm the direct regulation of Hes1 on CDKN1C/p57 promoter also in human, a promoter study and chromatin immunoprecipitation for Hes1 will be performed. Indeed, Hes1 promoter binding and transcription activation, it has demonstrated only in mouse, (Georgia S. et al. 2006; Riccio O. et al. 2008)) even if the regulatory regions seem to be conserved in human.

Thus far we can conclude that our in vitro and ex-vivo results suggest that Hes1 safeguards against irreversible cell cycle exit by modulating CDKN1C/p57 expression in human HCC. In

our restricted series of surgically resected HCCs, Hes1 expression identifies patients with different risk of recurrence. The lack of correlation between CDKN1C/p57 or Hes1 expression and survival is not surprising in surgically-treated HCC due to the impact of subsequent treatments and liver failure on survival.

On the basis of our experimental evidence, caution should be used in suggesting treatments with single general inhibitors of Notch activity (such as γ-secretase inhibitors) for HCC. More promising approaches may be provided instead by selective inhibitors that can suppress the Notch pro-survival function while leaving intact or enhancing its ability to repress invasion, or by a combination therapy based on Notch inhibitors in conjunction with chemotherapeutic compounds with proapoptotic activity as we previously showed (Giovannini C. et al. 2009).

RESULTS 2

Notch1 silencing induces changes in morphology and reduces migratory properties of HCC cell lines

HepG2, Snu398 and Snu449 HCC cell lines were depleted for Notch1 expression with shRNA , using two specific shRNA sequences against Notch1 mRNA (N1/5724 and N1/4543) and as negative control a shRNA for luciferase (NC). The effective silencing of Notch1 expression was evaluated by western blot in all three cell lines, and the more effective clone for each cell line was chosen for further analysis. They were respectively N1/5724 for HepG2 and Snu449 and N1/4543 for Snu398 cells (Fig12A). Cells steadily silenced for Notch1 expression show changes in morphology, appearing more epithelial and differentiated, with a less fibroblastoid aspect respect to control cells. In particular, Snu398 cells after Notch1 depletion loss the phenotype of quasi-suspension to gain a more adhesive phenotype, growing in clusters. (Fig12B) As Notch1 was involved in control of epithelial to mesenchymal transition in development and in progression of some carcinomas (Thiery JP et al., Cell 2009), it was hypothesized if the modified morphology could reflect a different migratory and invasive capacity of these cells. Wound healing assays demonstrate that cells silenced for Notch1 expression have a reduced capability to migrate and close the scratch in the culture compared to controls cells, which migrate rapidly in the wound. The wound healing assays was performed in Snu398 and Snu449 cells that shows a monolayer phenotype. Both cell lines show a reduced time of closure of the scratch when Notch1 was not expressed, leading to reduced motility (Fig 13). Conversely, the assay was not performed in HepG2 cell line because it does not grow in monolayer but forming three-dimensional clusters and it was not possible to evaluate the motility in agreement with the reference protocol (Chun-Chi Liang et al, Nature, 2007). The invasive properties of Notch1 silenced cells were evaluated through boyden chamber assay in presence of Matrigel, in order to evaluate not only the migratory features of the cells, but also the ability to degrade the extracellular matrix and to invade. All cells lines demonstrate a statistical significative reduction in number of invaded cells in Notch1 knock down cells compared to control. In Snu398 and Snu449 cells the invasion was almost abolished (percentuali), in HepG2 the reduction in invasion was less dramatic (Fig.14). The modified migratory and invasive phenotype linked to Notch1 expression suggests the involvement of metalloproteases. Metalloparotease-2 (MMP2) and Metalloprotease-9 (MMP9) activity were evaluated with zymography in Notch1sh and control cell supernatants and an evident reduced activation of MMP9 emerged. MMP9 activity results completely abolished in Notch1 depleted cells, as was evident from the disappearance of the

band corresponding to the active form of the enzyme that was instead evident in controls (Fig 15A). In addition, MMP-9 total protein expression was reduced in cells that loose Notch1, supporting the reduced level of proenzymatic form of the enzyme found in the supernantant of them (Fig. 15A). These results support the hypothesis that Notch1 could regulate the invasive and metastatic potential of HCC cells with a double regulation on MMP9 expression and activation.

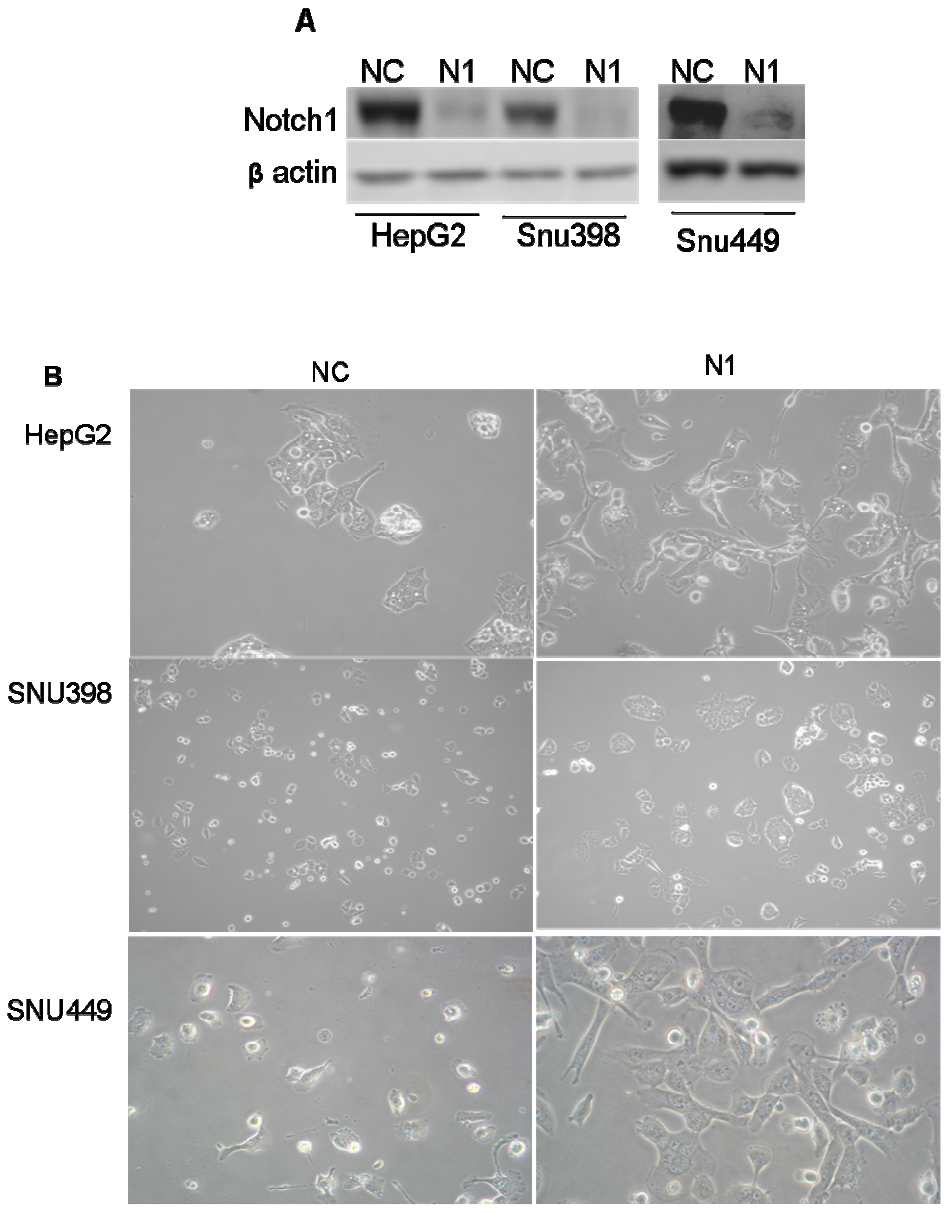


Figure 12: Notch1 silencing through short hairpin RNA induced changes in hepatocellular carcinoma cells. A) Western blot showing the effective silencing of Notch3 protein in HCC cells lines. Beta actin was used as housekeeping gene. B) Cells were photographed after 7 day after the end of the selection. Selection was maintened and cells were plated 48h before the acquisition. Photographies were done using a camera on a Inverted Optic Microscopy. NC: Negative control of shRNA; N1: Notch1 shRNA, the best silencing clone between the two tested for each cell line.

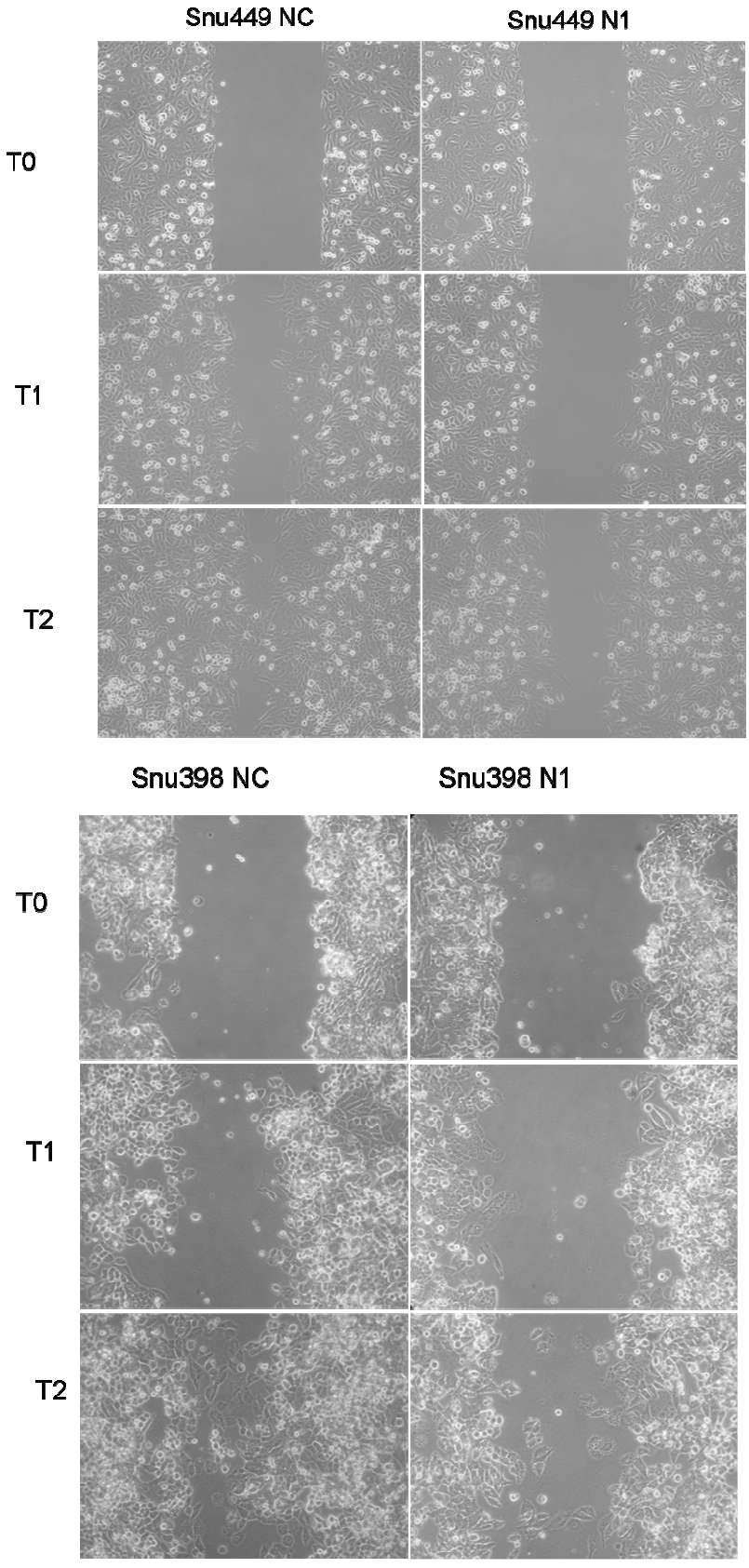


Figure. 13 Wound healing assay Snu449 cells. Upprer: Wound healing assay in Snu449 cells silenced for Notch1. T0, the moment of the scratch; T1= 8 hours after; T2= 16h after. NC: Negative control of shRNA; N1: Notch1 shRNA; Lower: Wound healing assay in Snu449 cells silenced for Notch1.

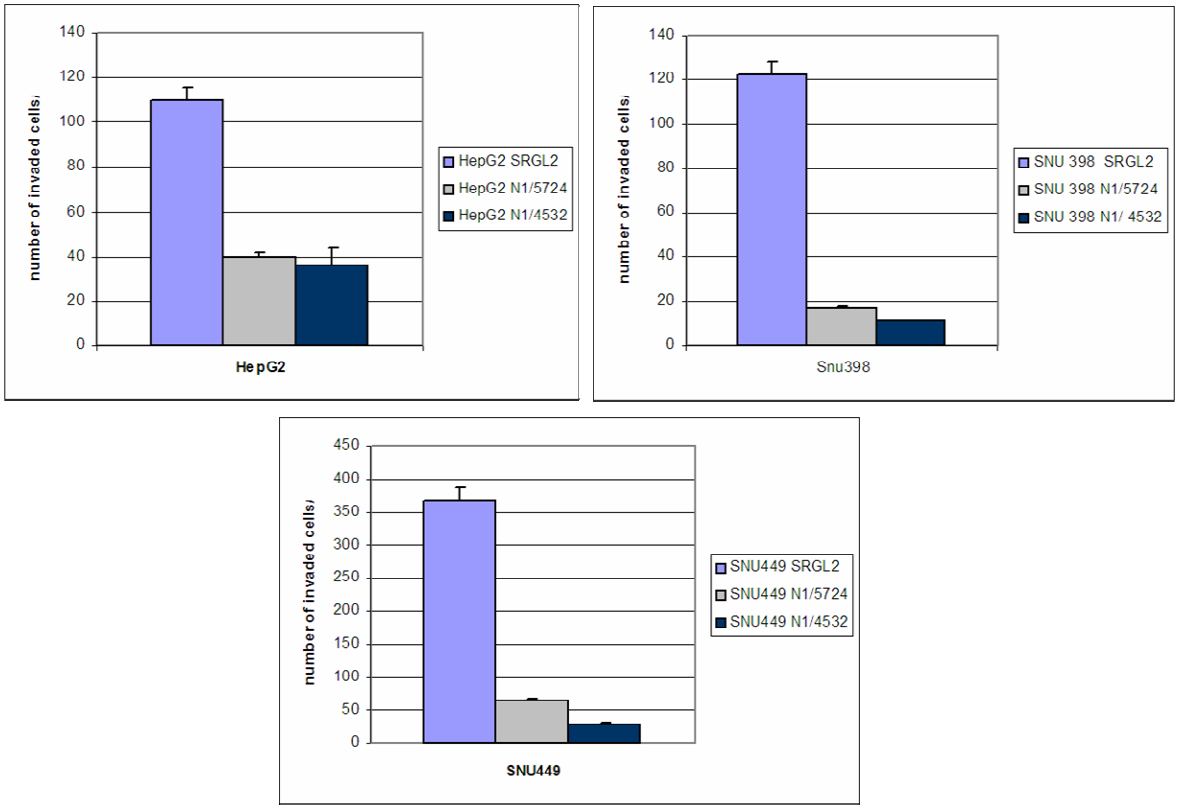


Figure 3: Cells lacking Notch1 have a reduced invasive capability in matrigel. Invasion assay in boyden chamber using a 8um pores filter, coated with Matrigel shows a decreased in invaded cells after the silencing of Notch1. Both clones for each cells line were analysed after 24hours form the chamber preparation. Invaded cells were evaluated counting them at the microscopy after GIEMSA staining. The meaning of invaded cells for field was calculated counting 10 random fields and obtaining the meaning. The reduction in invasive capability of Notch1 silenced cells was obtained by t test between two indipendent experiments. Columns: meaning of counted cells for field; Bars: standard deviation.SRGL2: Negative control of ShRNA; N1/5724: Nocth1 shRNA stable clone; N1/4532: Nocth1shRNA stable clone

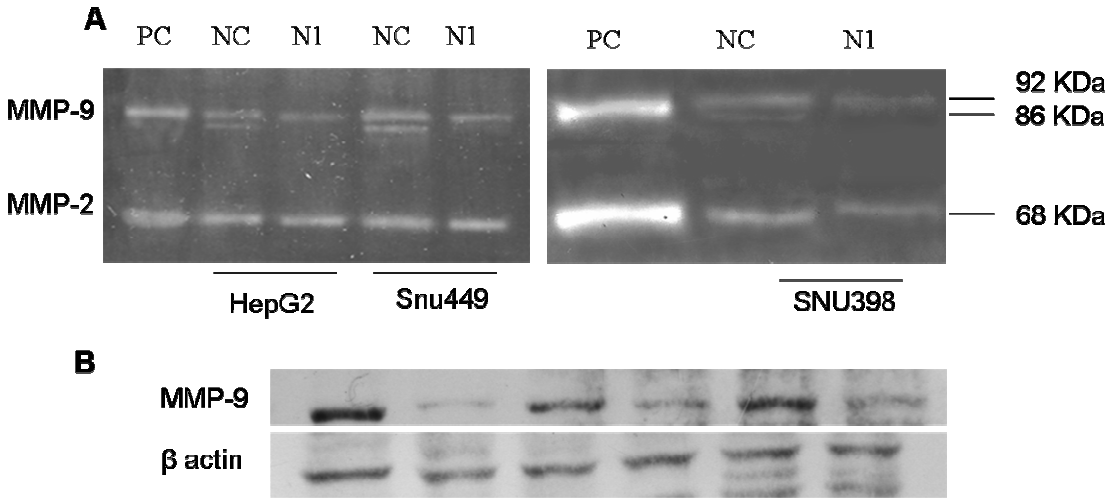


Figure 15: Zymography and Western blot for MMP-9: Notch1 regulates extracellular metalloprotease activity. A) Zymography assay on culture medium of HCC cells showed presence of MMP-9 and MMP-2 enzyme. For MMP-9 is detectable the proenzymatic form (92KDa) and the active one (86kDa) only in negative control, whereas Notch1 silenced cells did have the active form. MMP-2 results present in the supernatants of both NC and N!, without differences. PC: Positive control, HT1080 cells that highly express MMP-9 and MMP-2; NC, Negative control of shRNA; N1, Notch1 shRNA. **B)** Western blot for MMP-9 protein expression in Notch1 silenced cells. Expression was evaluated in HepG2, Snu398 and Snu449 cells. Beta actin was used as housekeeping.

Notch1 regulates EMT transition of HCC cells

In order to investigate the function of Notch1 activation in HCC progression, an analysis of the pleiotropic factors involved in epithelial to mesenchymal transition was performed. The crucial factor in vertebrates EMT is Snail, a transcription factors that represses E-Cadherin expression by binding at the promoter of E-Cadherin gene. (Peinado et al.,2007; Wang et al.2007). Other two indirect repressors of E- Cadherin are involved in EMT induction, Twist and Slug, but only Twist is related to invasion and metastatization of HCC (Yang M-H et al. 2009.) Snail mRNA resulted down-regulated in all Notch1 silenced cells and Twist, expressed only in Snu398 and Snu449 cell lines was also downregulated after Notch1 silencing. E-Cadherin transcript results strongly increased after Notch1 silencing and Snail and Twist reduction, suggesting a switch to a more epithelial phenotype. Interestingly, Snu398 and Snu449 control cell lines do not express E-Cadherin, but they gain mRNA transcript only after Notch1 depletion. On the other hand, the expression of mesenchymal markers N-Cadherin and Vimentin was not significative changed in either cells lines, except for Snu398 in which loss of Nocth1 leads to a reduced expression of vimentin. These mesenchymal

adhesion and cytoskeleton markers were expressed exclusively by Snu cells, suggesting that these two cell lines have a more mesenchymal phenotype than HepG2, that could represent an epithelial cell model of HCC. (Fig. 16A) Also the protein expression level of these mesenchymal markers together with some invasion associated proteins was evaluated. Vimentin expression confirms the mesenchymal features of Snu398 and Snu449 cells, with a reduced expression when Notch1 was abrogated. Alpha-SMA and alpha-feto protein, expressed only by HepG2 cells, resulted reduced after Notch1 abrogation, in agreement with the reduced invasion capacity of these cells. The expression of epithelial specific keratin CK18 shows no significative changes after Notch1 silencing in expressing cell lines, but CK8 was expressed by all three cell lines and upregulated after Notch1 silencing. CK19 expression results only in Hepg2 cell lines with augmented levels in Notch1 silenced cells (Fig 17B-C). In addition, Snail protein expression reflects the mRNA downregulation observed, confirming that Notch1 may control EMT through this factor (S.Saad et al .2010). Surprisingly, together with Snail and Twist reduction and consequent E- Cadherin mRNA increase, protein levels of the complete 120 KDa and of the cleaved 82 kDa forms result both downregulated in HepG2 cells, the only that express the protein. Also in immuno-hystochemestry analysis E-Cadherin confirm reduction in expression and it evidences the functional localization in the cell membrane in order to establish cell to cell contacts (Fig. 5B). Moreover, the expression of c-Met, receptor of the multifunctional liver cytokine Hepatocyte Growth Factor (HGF) and accepted markers of liver invasion, results reduced in Notch1 KD cells, supporting the role of Notch1 in inducing EMT in HCC.(Nakanishi K. et al. 1999; Xie Q. et al.2001). Althought E-Cadherin down-regulation after Notch1 depletion may suggest an unusual role of the protein in promoting invasion of HCC. Moreover, E- Cadherin expression data suggest a possible transcriptional regulation of Notch1 on E-Cadherin through its known repressors and a contemporary opposite effect at post-transcriptional level.

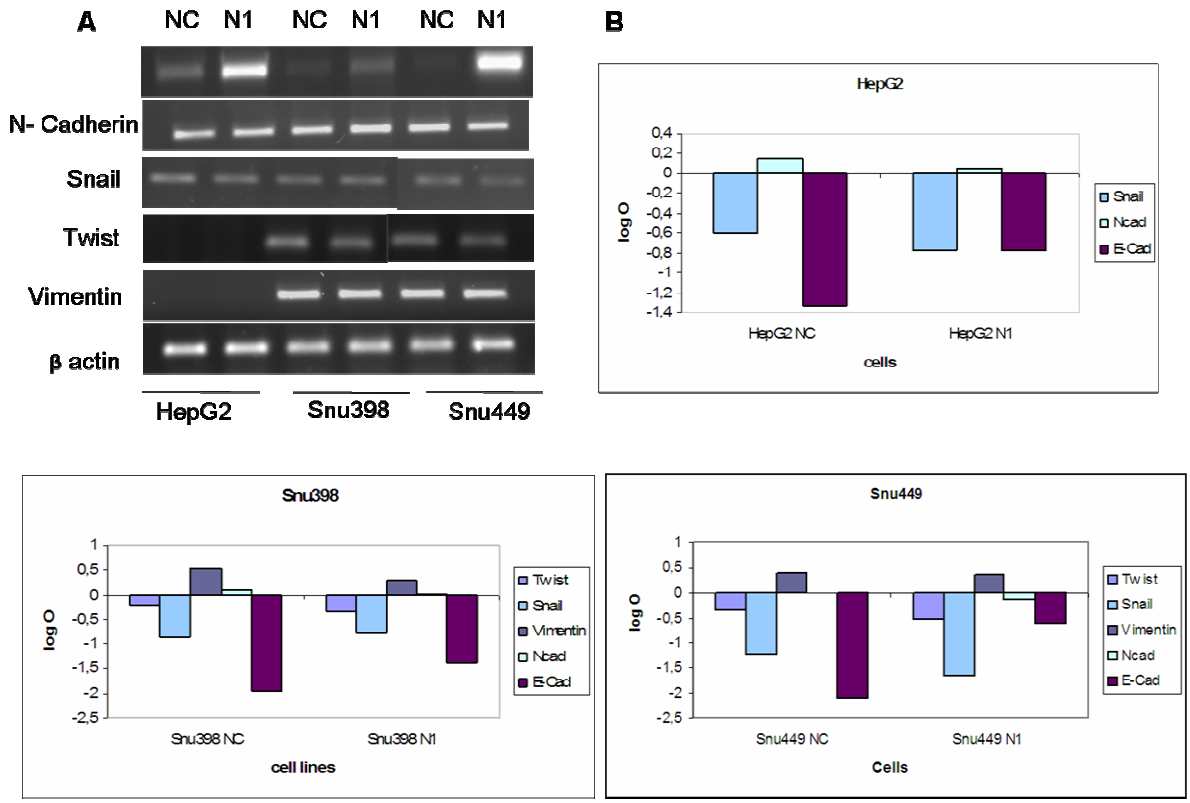


Figure 16: Epithelial to Mesenchymal markers are downregulated in absence of Notch1. A) Semiquantivative RT-PCR for genes involved in EMT in Negative control and shRNA for Notch1 in HepG2, Snu398 and Snu449 cell lines. Quantification of the optical density (OD) of each band was normalized with the beta actin OD. B) Relative quantitations were displayed in logaritmic scale comprising all expressed gene, dividing them for each cell lines. Statistical significance was displayed in N1 bars, indicating the differential expression compared to controls calculated in three indipendent experiments.

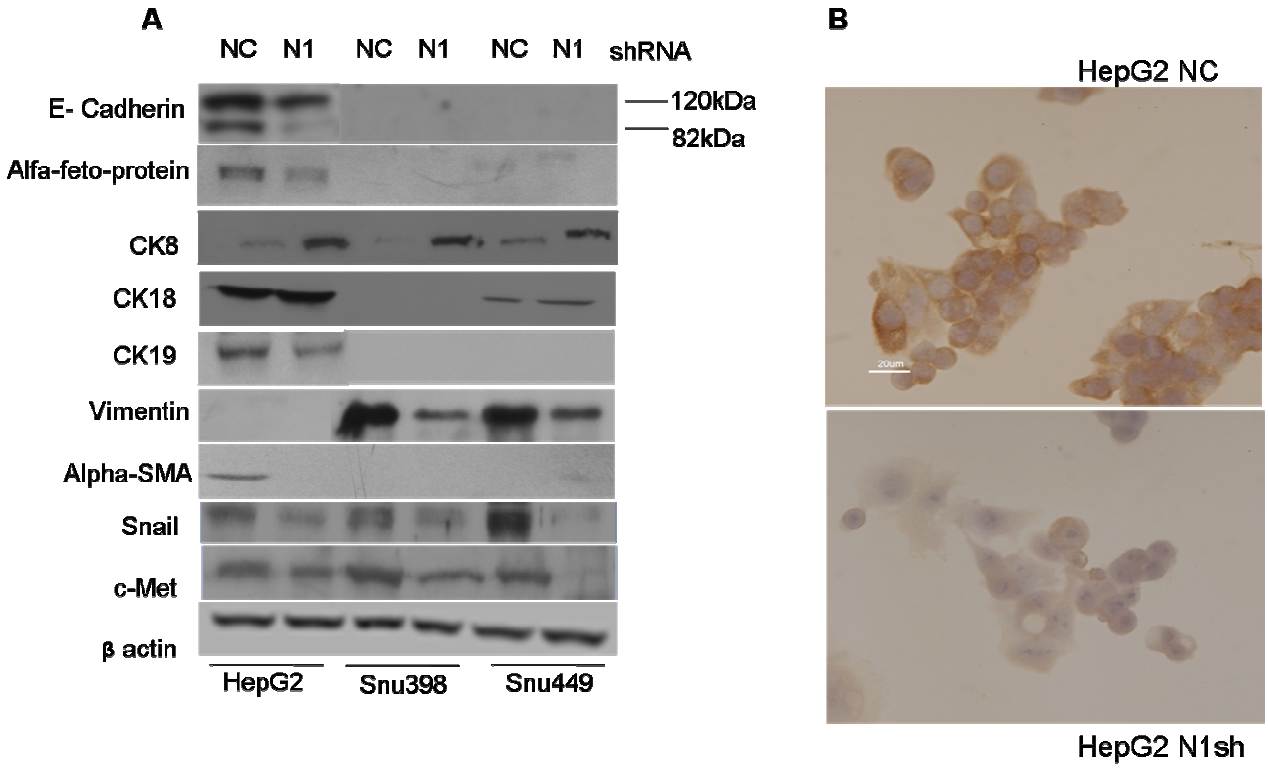


Figure 17: EMT and epithelial markers changes at protein level after Notch1 silencing. A)Western blot on HepG2, Snu398 and Snu449 cells silenced for Notch1. E- Cadherin, Cytokeratin 8(CK8); cytokeratin 18 (CK18) and cytokeratin 19 (CK19) were performed as epitelial markers. Vimentin, Alpha-SMA were performed as mesenchymal markers. Alpha feto protein, c-Met are markers of aggressivity; Snail as EMT inducer and beta actin as load control.B) Immunohystochemestry for E- Cadherin on HepG2 cells,, Negative control (NC) and Notch1 silenced cells (N1).

E- Cadherin expression is linked to a more aggressive phenotype *in vitro*

To asses the hypothesis of E-Cadherin negative role in aggressiveness, HepG2 cells were silenced for E-Cadherin expression through transient siRNA. Both after 24h and 48h the number of invaded cells results reduced compared to controls, instead alpha-feto protein and CK19 markers increase. (Fig17 A-B). The partial loss of E- Cadherin expression in epithelial cells seems to determ EMT and an aggressive phenotype, instead of promoting maintenance or gain of epithelial features. In Snu398 cell line that has not the endogenous protein and that is easily transfectable, the expression of E-Cadherin was obtained by transfecting an E-Cadherin cDNA vector in SRGL2 control and Notch1 KD cells, in order to dissect the relative contribution of E- Cadherin and Notch1 expression to the observed phenotype. At 48h after transfection western blot for some markers and invasion assay were performed. The forced expression of E- Cadherin in HCC cell with a mesenchymal phenotype gives a more invasion capability, both in expressing and not expressing Notch1 cells (Fig 17 C). The effect of both

Notch1 and E-Cadherin seems to be additive in inducing invasion and determining a more aggressive phenotype, as it is evident from invaded cells and expression levels of vimentin, MMP-9 and c- Met after E- Cadherin expression (Fig. 17 C-D) Indeed ectopical, expression of E- Cadherin in Notch1 expressing cells results in an augmented invasion and in a more mesenchymal phenotype, but the loss of Notch cause reversion of invasion capability and aggressive markers expression. Moreover, in these cells E-Cadherin forced expression restore the invasion rate of control cells, suggesting that even if the E-Cadherin solely expression can interfere on invasion, it does not determ completely Notch1 associated phenotype. For that, the higher invasive rate of HepG2 cells compared to Snu398 and Snu449 ones after Noth1 silencing could be referred to the E- Cadherin expression. (Fig.17D). Due to E- Cadherin role in regulation of transcription after proteolitical cleavage by presenilin-1 and migration to the nucleus of its 34kDA intracellular portion, we asked if that could be effective on Notch expression. In order to exclude that the phenotype observed in HepG2 cells could be determed by E- Cadherin mediated induction of Notch1 and to better define the reciprocal regulation and roles of Notch1 and E-Cadherin in our model, level of Notch1 expression was evaluated after ectopic E- Cadherin expression. In Snu398 cells expressing E- Cadherin c-DNA vector Notch1 protein shows no changes in expression, confirming a one sense post transcriptional regulation of Notch1 on E- Cadherin. (Fig. 17)

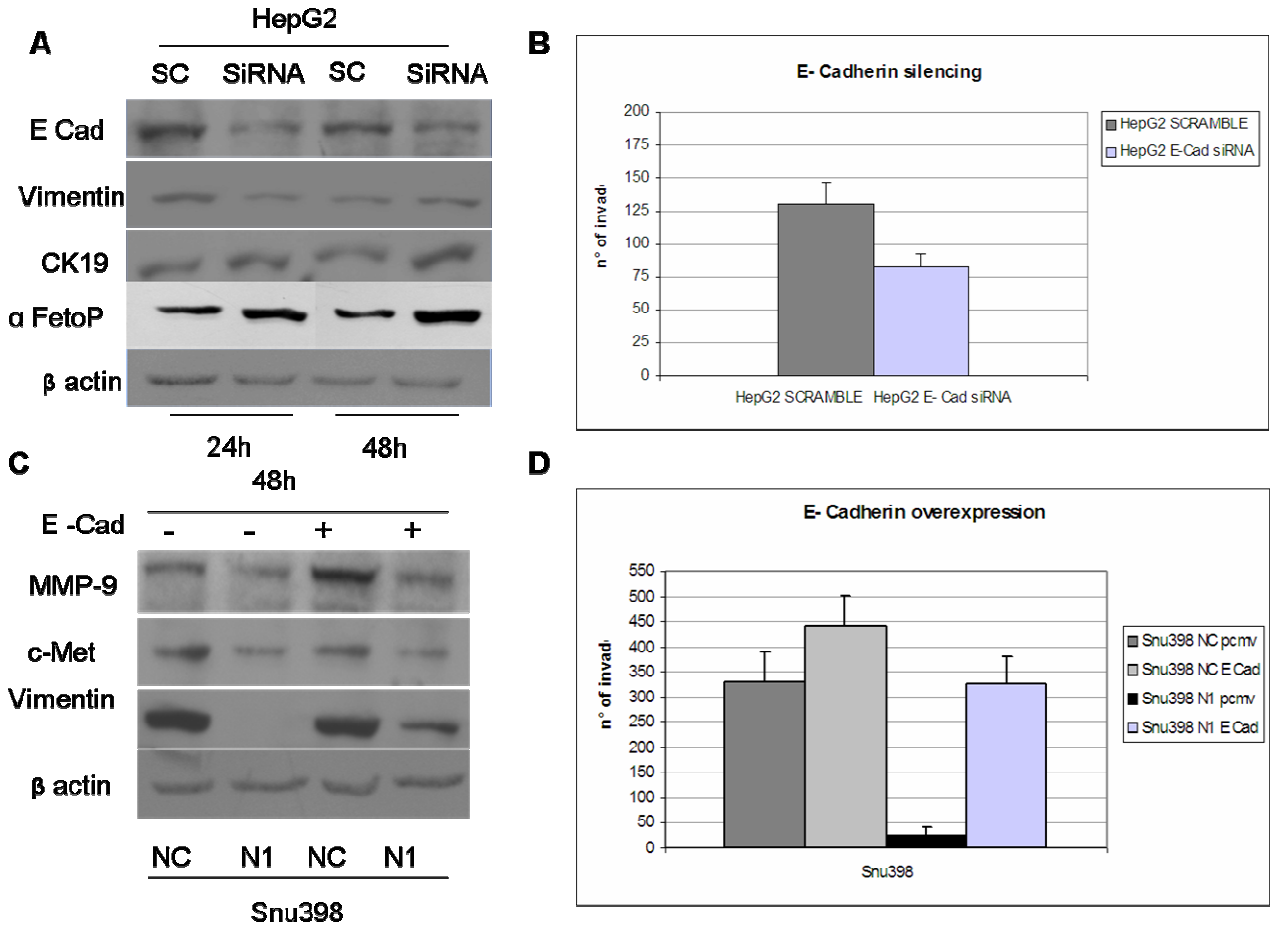


Figure 18: E- Cadherin expression is associated with a more aggressive phenotype in HCC. A)Western blot analysis after E- Cadherin transient silencing in HepG2 at 24 and 48h after transfection. Cells lacking E- Cadherin reduces Vimentin expression, increases Cytokeratin 19 and Alpha pheto ptrotein expression.Beta actin was use as loading control. B) Invasion assay on HepG2 silenced for E- Cadherin at 48h after transfection. Invasion capability is reduced after E- Cadherih silencing. Scramble: negative siRNA ; E-Cad: E-Cadherin siRNA. C) Western blot analysis after E- Cadherin overexpression in Snu398 cells silenced for Notch1 at 48h after transfection. Metalloprotease 9(MMP-9). C-Met and Vimentin were evaluated to define the phenotype. -: Empty pCMV vector transcfection; + : pCMV E- Cadherin overexpressing vector transfection. D) Invasion assay after E- Cadherin overexpression in Snu398 cells silenced for Notch1 at 48h after transfection. Expression of E-Cadherin in presence of Notch1 increases the invasion capability of these cells; Notch1 silencing almost abrogate invasion; E-Cadherin expression in Notch1 silenced cells rescue invasion levels to control cells expressing Notch1 . NC: Negaiv shRNa control; N1: shRNA for Notch1; pcMV: emprty vector; E-Cad: pcmV-Ecadherin expressing vector

Notch1 and E- Cadherin expression correlates in vivo with multifocality and predict worse prognisis in HCC patients

In order to verify these observations in a more adequate model, Notch1 and E-Cadherin expression were evaluated in of 40 HCC samples, in order to establish a possible relation between them and to asses E-Cadherin role in a tissue and microenviromental contest.

Specimens derived from HCC were analysed in western blot and quantified with densitometry. Notch1 and E-Cadherin protein levels show a positive correlation in a Pearson's two tail test p< 0,01 (Fig. 19A). These results not only support the hypothesis of Notch1 role in controlling E- Cadherin, but also the idea of a link between Nocth1 and E-Cadherin expression with invasion and intrahepatic metastatization of HCC *in vivo*. In addiction, analysis of the probability of recurrence at 800 days after surgery with Kaplan Meyers test shows that patients with low levels of Notch1 and E-Cadherin have a better prognosis and a reduced time to recurrence compared to patients with higher expression of two proteins. Pearson's test conduced on these data showed statistical relevance and it evidence predictive values of these factors in recurrence. (Fig. 19B)

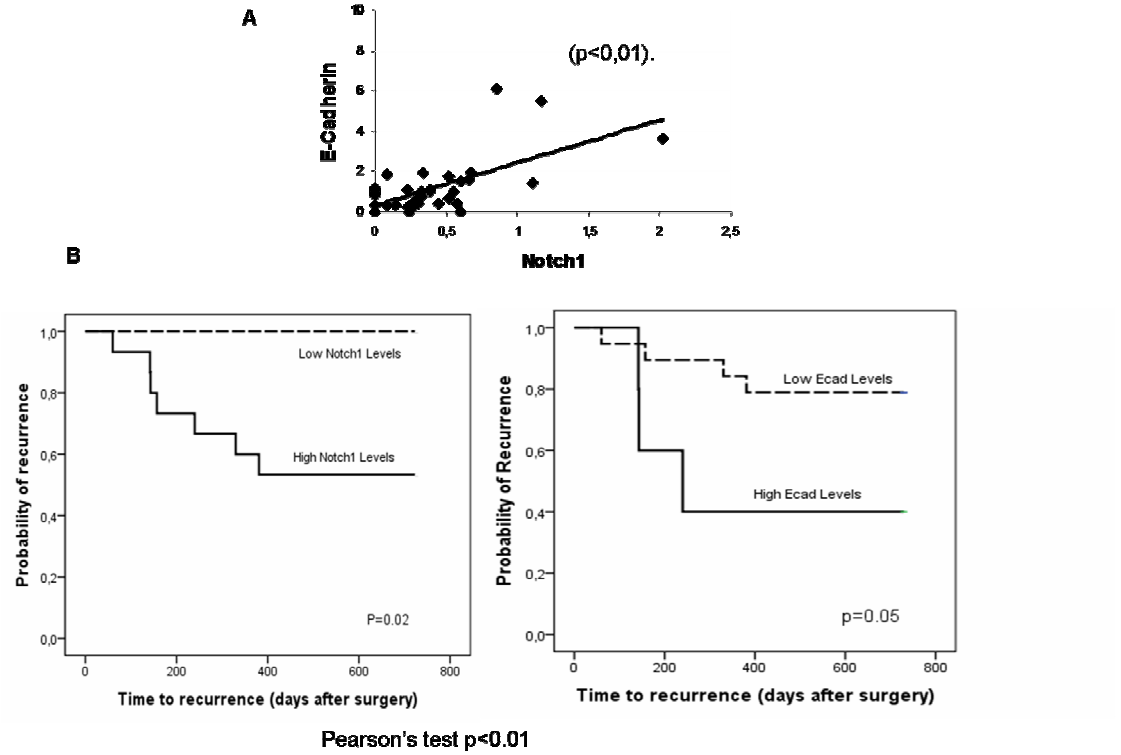


Figure 19 : Analysis of Notch1 and E-Cadherin expression and association with the recurrence of HCC. A) 40 HCC samples for Notch1 and E- Cadherin protein expression shows a significative positive correlation in Pearson's two tail test (p<0,01).B)) Survivval analysis with Kaplan Meyers test, shows that patients with low levels of Notch1 and E-Cad have a better prognosis and a reduced recurrence compared to patients with higher expression of two protein. Pearson's two tail test (p<0,01).

Notch1 regulates dependent cell adhesion, cytoskeletal dynamic and metabolism: a secretome analysis

In order to find instruments to predict the probability of recurrence linked to Notch1 expression and to define more exaustives and easy detectables markers, a proteomic approach was followed on HCC secreted proteins. Secretome includes all proteins released or shed by a cell, a tissue or an organism through different secretion mechanisms and identify molecules involved in processes as differentiation, invasion and cancer progression (Volmer et al. 2005; Paulau M.P. et al. 2010). In order to identify possible predictive markers linked to Notch1 expression and tumour aggressiveness, the secretome of HepG2 and Snu449 cell lines were analysed, with and without knocking down Notch1. Mass spectrometric analysis of conditioned media from silenced cells compared to control cells detects 1103 proteins of which 88 and 53 differentially expressed in HepG2 and Snu449 respectively. The proteins turned out from the Anova analysis as statistically significatives in expression as consequence of Notch1 signalling were annotated using DAVID software (Huang DW et al. 2009; Huang DW et al. 2009_a). A protein enrichment and functional annotation analysis based on cellular function and subcellular localization of these differentially expressed proteins let to identify pathway and physiological functions linked to expression of Nocth1. (Fig.20) Secreted proteins resulted to be downregulated after Notch1 knock down were 51 in HepG2 and 26 in Snu449 cell line. In addition, proteins upregulated after Notch1 knock down were 27 in Snu449 and 37 in Hepg2. HepG2 showed a wider range of secreted proteins dependent on Notch1 signal but the homogeneity of functions and localization of Snu449 differential secretome was confirmed also in upregulated protein class. (Fig. 20 A). A cluster organization based on the functional annotation analysis let to have a general sense of mechanisms and functions involved in Notch1 dependent secretome. Functions regulated by Notch1 seems to be linked to cellular trafficking, signalling and metabolisms, but also to cellular adhesion, motility and cytoskeletal homeostasis. Between these last proteins that are demonstrated or predicted to be involved in functional mechanism linked to invasion and interaction with the extracellular matrix, seven were selected to be validated through western blot analysis. The choice was based on 1) functional relevance with tumour progression, 2) presence in both cell lines 3) score and 4) association with cellular pathways known to be relevant for HCC 5) previous evidence of regulation by Notch signalling (at least one).

Regulators of metalloprotease activity and known to be antiangiogenic factors, Thrompospondin-1 and Thrombospondin-2 (THBS 1-2) were detected in WB and they result to be expressed by both cell lines. Thrombospondin-1 is increased in Snu449 cells after Notch1 depletion, but it did not show changes in expression between Notch1 knock down and controls in HepG2 cell line in western blot, whereas spectrometry analysis have reported a downregulation also in Notch1 KD HepG2 cells (Fig. 20A). Notably, in Snu449 cells both isoforms of 168 and 198 kDa result to be expressed, whereas in HepG2 only the higher molecular weight form was detectable. Thrombospondin-2 secretion instead was increased in Snu449 cells knocked down for Notch1 but diminished in HepG2 silenced cells, suggesting the crosstalk with some other factors in one of two cell lines considered. The meaning of the different regulation after the silencing of the same Notch1 receptor remain to be defined, but it could be referred to a different cellular contest giving reason of an opposite effect and it could not depend on a different specific protein expression, but just by a different secretion rate in the two cell lines. Moreover, depending on complex and contradictory role of Thrombspondins in cancer, it may reflect different stage of tumour progression for the cell lines used as models (Kazerounian S. et al., 2008).

Another factor involved in cellular adhesion revealed by mass spectrometry was Intercellular adhesion molecule-5 (ICAM-5). Even if it is an ICAM's family member with a more tissue specificity for neurons, it was detected in western blot in both cell lines, and its secretion result to be increased after Notch1 silencing. Nevertheless, no significative increase was observed in total protein expression, suggesting Notch dependent variation in protein localization or secretion. Despite ICAMs high conservation and its homology around 50% with ICAM-1, the most intercellular adhesion molecule expressed by epithelia (Mizuno T., 1997), an antibody against a specific domain of human ICAM-5 let us to avoid cross-reaction with others ICAMs and a false positive result.

In the group of protease inhibitor, an interesting molecule emerged from spectrometric analysis was the serine/threonine protein kinase mammalian target of Rapamycin (mTOR), as it is one of the most important factor driving HCC progression in cell growth as regulator of protein synthesis and effector of the high activated PI3K/akt pathway (Tanaka S. et al. 2002). This kinase resulted present in the extracellular compartment at different level between cells expressing or not Notch1. The loss of the receptor lead to a drastical reduction in mTOR levels of the protein in the extracellular compartment of both HepG2 and Snu449 cells knocked down for Notch1, suggesting a role in decrease of cell growth and cell motility (Zhou H and Huang S., 2010) .It was already known a Notch1 signalling dependent activation

of mTOR that plays a crucial role downstream Notch1 activation in mediating its pro-survival activity .(Mungamuri S. K. et al. 2006) It is not surprisingly that Notch1 silencing in HCC cells shows a downregulation of mTOR, even if it was never described before a regulation on total protein levels, neither the presence of the kinase in the extracellular compartment. The total protein expression seems to be downregulated only in SNU449 cells, instead extracellular level of serine-protease are downregulated in both cell lines after Notch1 silencing. Considering these evidence and the crucial role in cancer progression that is achieved by mTOR pathway activation, the presence of the protease in extracellular compartment is a new interesting aspect for diagnosis, prognosis and to understand cancer progression mechanisms.

As fith protein selected for validation, Protein C inhibitor SERPINA5 was investigated as member of the plasma serine protease inhibitor that is mainly expressed in the liver and released in plasma. (Suzuki K at al. 1989) Levels of the protein in Notch1 knocked down cells resulted strongly upregulated in HepG2 cell line and also augmented in Snu449 cells, suggesting a possible role as tumour suppressor for HCC, as observed in ovarian serum borderline tumour (SBT) (Sieben L.G.L et al. 2005).

Also the matrix interacting protein Cingulin was analysed for expression with western blotting in supernatant of Notch shRNA and control HCC cells, but despite of the detection through mass spectrometry and annotation analysis, no expression was found in western blot.

Finally, the expression of E-Cadherin 82kDa shed form was revealed in secretion of HepG2 cell line, with a higher expression level in control cells, confirming the reduced amount of E-Cadherin after Notch1 knock down and the change towards a less aggressive phenotype of these cells. Indeed, shed E- Cadherin mediates cancer invasion and metastasis spreading (Noe V. et al 2001; Ryniers F. et al. 2002), so its reduction after Notch1 knockdown further sustain Notch1 pro – invasive role in HCC.

Serum presence of Notch1 silencing dependent secreted factors involve the signalling in prediction of patient outcome and cancer progression.

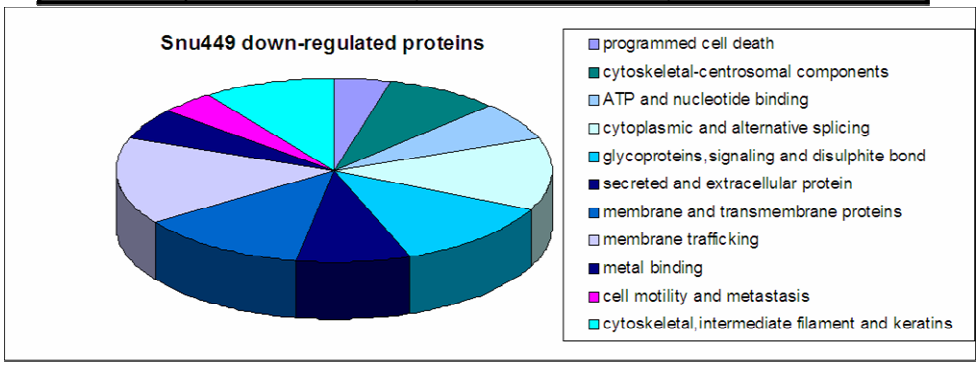
Proteins validated by western blot and obtained as significative changed in Notch1 knock down cells were analysed for their serum presence and expression in serum obtained from patients followed up for cirrhosis and HCC insurgence and progression. As control of normal serum level a pool of serum proteins obtained by healthy donor blood was used. Western blot signals were quantified with optical densitometry and normalized against total protein

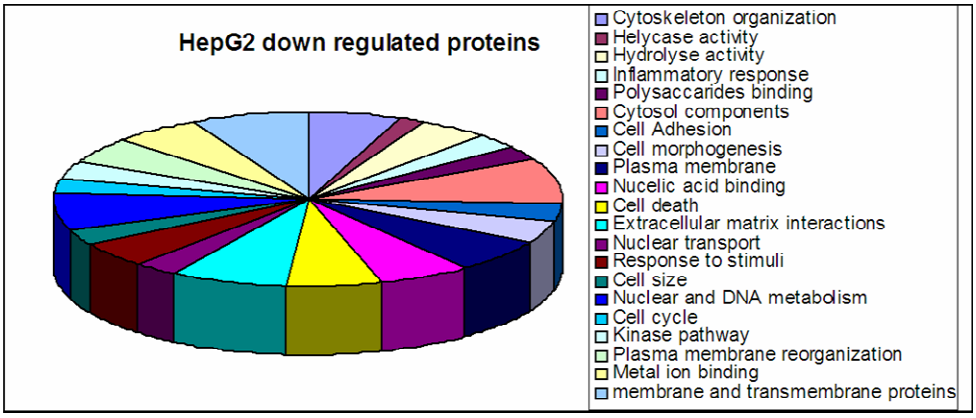
quantity evaluated with ponceau staining of the membranes. Variations were evaluated considering 4 groups: healthy, cirrhotic, early HCC and advanced HCC.(Fig.20B) Thrombospondin-1 (THSB-1), an antiangiogenic factor regulated positively by p53 and negatively by some oncogene (Kazerounian S. et al. 2008) showed statistical significative variations from healthy and sicks, with a drastic reduction, but between the pathological conditions a significative difference was observed between cirrhotic and HCC, whereas two HCC classes were not so different. Neverthless, the same functional direction of a protective role of this protein was suggested, as it was already evident by the upregulation observed in Notch1 silenced cells. Thrombospondin- 2 (THSB-2) is higly similar in sequence and function with Trombospondin-1, bringing the TSR domain characteristic of the subgroup A and mediating anti angiogenic, pro- apoptotic functions, moreover interacting with receptors and affecting signal transduction. (Carlson et.) Plasma levels of Thrombospondin-2 showed reduction, starting from healthy samples and passing through all groups, with significative variations between cirrhotic and early ($p < 0,01$) and between early and advanced samples ($p< 0,05$). This variation is significative of the protective role in cancer as THSB-2 is responsible to inhibit metastasis, growth and angiogenesis. (Kazerounian S. et al. 2008). ICAM-5 is a member of the intercellular adhesion molecule family, composed by 5 cell surface glycoprotein involved in cell adhesion and signalling, playing important roles in development. Despite of its physiological function, it has identified as both pro-invasive and anti-metastatic in a wide range of cancers. For the first time ICAM-5 serum levels were identified associated with HCC, as previously identified in serum only as marker of brain injury (Guo H et al. 2000). Serum ICAM-5 was detected in healthy donor, with values hightly similar to cirrhotic patients. Conversely, early HCC patients showed higher level compared to cirrhotics ($p < 0,01$), whereas HCC advanced cancer showed lowest level of ICAM-5 serum, compared both to early ones and cirrhotics ($p < 0,001$ and $p < 0,01$). Significance of these variations has to be established, but the high statistical significative decrease in advanced HCC seems to have a relevant meaning and give to ICAM-5 a potential value as HCC marker. The serine protease mTOR, effector of the PI3K pathway, has been identified not only in secreted compartment of HCC cells, but also in serum of patients. Both these localization were no reported before. Despite the total absence of detection in health donors, mTOR was detected in serum of cirrhotic, early and advanced HCC patients but no significative variations between classes was detected. Nevertheless, the appearance of detectable levels of mTOR in serum further confirms data about mTOR secretion observed *in vitro* and suggests a possible link between inflammation and cancer physiology in liver. Conversely, SERPINA-5 (PAI-3) a serin

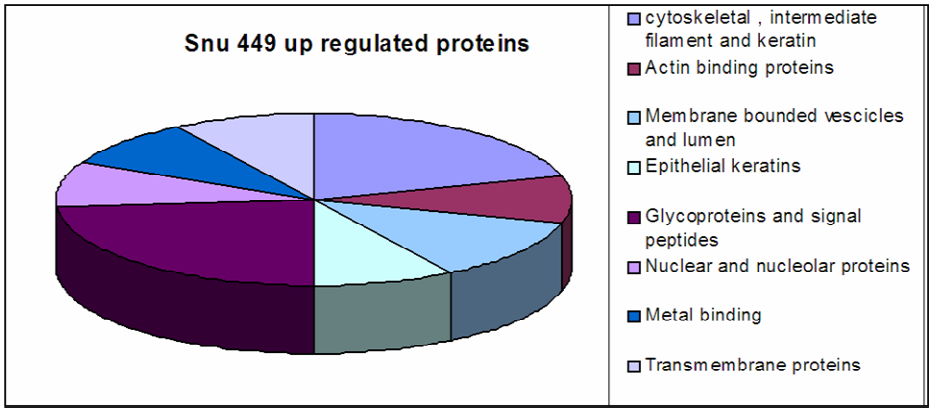
protease inhibitor involved in coagulation is a secreted protein normally released in the blood circle. Serum levels detected in patients were lower than healthy donors ones in all groups, but differences between groups were not statistical significative. Only a significative change in expression between cirrhotic and advanced HCC was observed ($p< 0,05$) with an increase in HCC. Due to its main role is in inhibition of the extracellular matrix degradation and to the inhibitory effect of Notch1 on its expression, it is possible to hypothesize a function in serum patient's level compared to HCC progression. (Sieben L.G.N. et al, 2005)

More significative is the detection in serum of E- Cadherin shed form of 80 kDa that is an accepted marker of cancer metastatization and its detection in serum has been associated with cancer progression and tumour grade (De Wever O. et al. 2007). An increase of soluble E-Cadherin was observed in all sick groups compared to healthy. It is interesting that E-Cadherin soluble form is released also as consequence of inflammation (Nold C. et al. 2012). Nevertheless, between cirrhotic and cancer and in progression of HCC, higher levels were observed, even if the difference between early and advanced HCC patients was not statistical significative.

Cell line	Diff.expressed	Down	Up
Snu449	53/1103	26	27
HepG2	88/1103	51	37







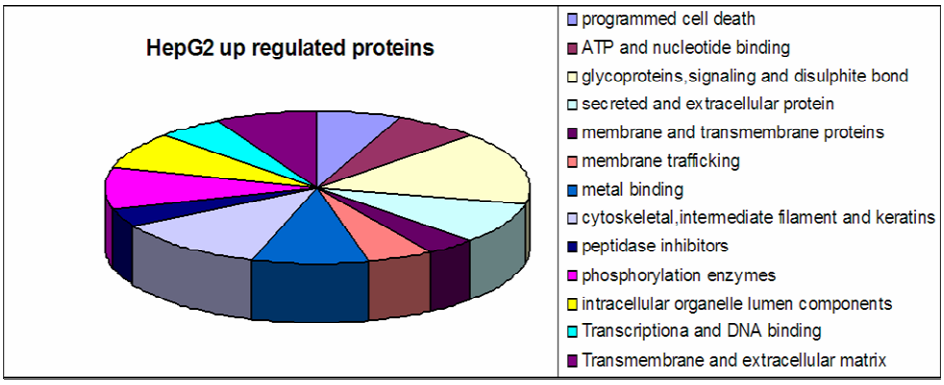


Figure 20 : Schematic representation of annotational clustering of Notch1 regulated protein.

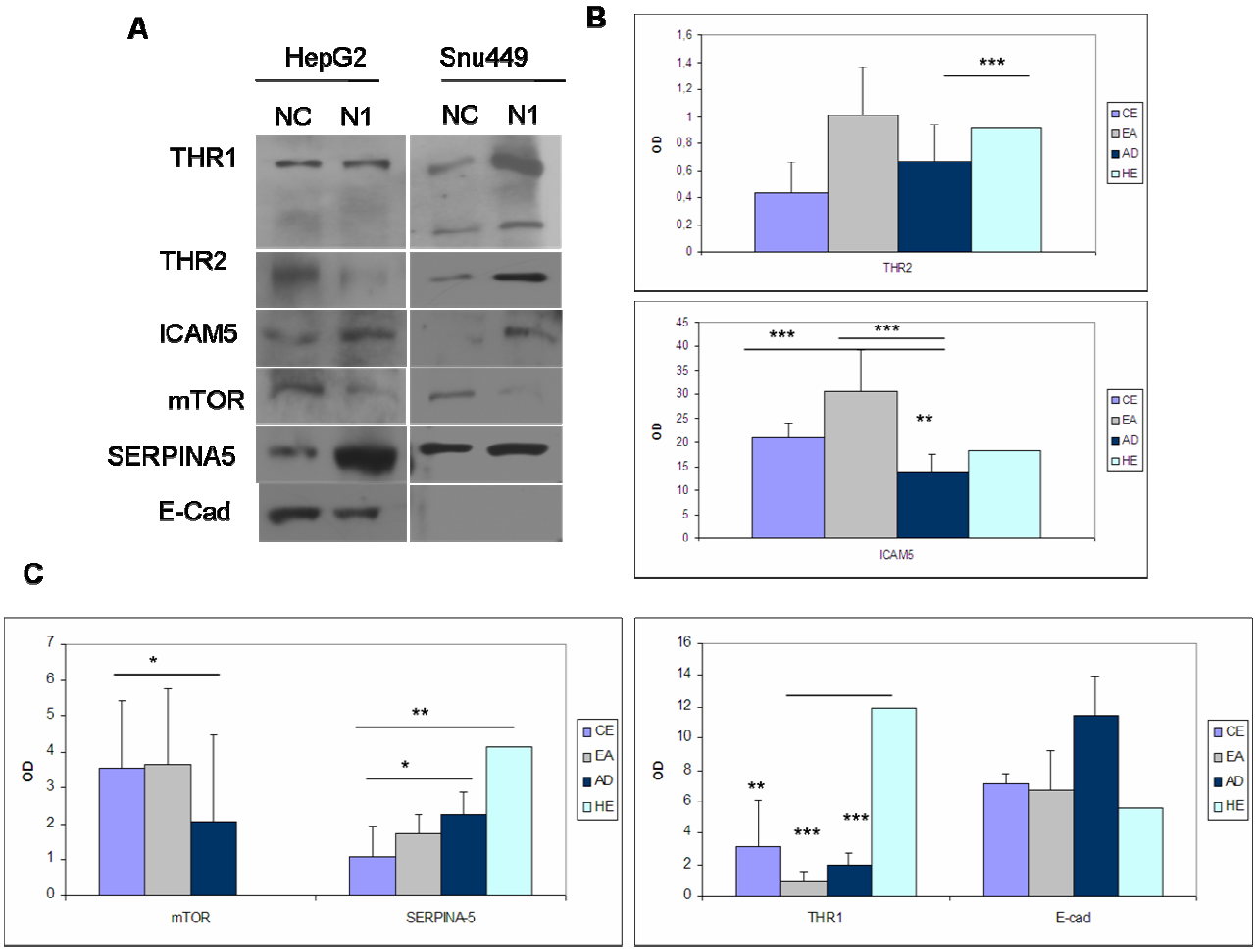


Figure 21. Secretome analyisis shows changes dependent by Notch1 expression and protein release in serum.A) Western blot analysis for protein detected in proteomic analysis in supernatant of Notch1 silenced, in HepG2 and Snu 449 cells. THR1: Thrombospondin-1; THR2: Thrombospondin-2;ICAM-5; mTOR; SERPINA-5 and E- Cadherin. **B-C)** Western blot analysis on serum of the same proteins. Protein were extracted by patients serum and from a pool of healthy donors. CE:cirrhotics; EA: early HCC; AD:advanced HCC; HE: healthy donors pool. Western blot bands were qunatitated and OD normalized using a standard sample in each experiment. Columns: meaning between 8 patients serum; Bars: standard deviation. Statistica significance was evaluated with t test, *:p<0,05;**:p<0,01; ***: p<0,001.

DISCUSSION 2

Notch signalling is implicated in a wide range of function in cell behaviour, both during development and in adult tissues. It is important in differentiation, proliferation and survival and its deregulation has been associated to a different cancers, both solid tumours and leukemias. (Artavanis-Tsakonas S. et al. 1999; Ranganathan P. et al. 2011; Lobry C. et al. 2011). Neverthless, it has a highly cell-context dependent role, activating different downstream pathway, with specific context dependence (Radtke and Raj, 2003) and receptor specific activities (Borggrefe T. and Oswald F. 2009). Notch1 is the most studied between four mammalian receptors and an extensive literature is present. It was associated to oncogenesis and cancer progression as it could maintain the transformed phenotype of Ras transformed cancer or it can act as tumorigenic factor, inducing cancer development (Weijzen S. et al 2002; Allenespach E.J. 2002; Pears W.S. et al. 1996; Aster JC et al. 1997). A role in HCC was already supposed by ours previous studies as Notch1 was found overexpressed in tumor cells but not in the sourrounding tissue and implicated in cell proliferation (Giovannini C., et al. 2009). The role of Notch1 in inducing Epithelial to Mesenchymal transition was supported by the strong involvement of the pathway during this mechanism in development, but also by direct evidences in pancreatic cancer cells, osteosarcoma and kidney cancers (Thiery J.P. et al. 2009; Bao B. et al. 2011; Zhang P. et al. 2010; Saad S. et al. 2010). In HCC it has not yet defined an involvement in EMT, but starting from evidences of its role in other cancer and its role in changing in adhesion during crest neural formation, somitogenesis and angiogenesis, we hypothesize a control of this cellular event even in liver and in hepatocellular carcinoma. (Grego-Bessa J. et al. 2004, Thiery J.P. et al. 2009) In liver was described a role of Notch1 in controls of angiogenesis and vasculature development, showing altered structure and endothelial overgrowth followed to Notch1 activation, together with a spontaneous development of angiosarcoma, suggesting a tumour suppressive role of Notch1 in endothelium (Dill M.T. et al 2012, Dou G.R. et al. 2008). Moreover, Jagged1 mediated activation of Notch1 has been shown to induce EMT in breast cancer, so due to high Notch1 and Jagged1 expression in HCC, an additional evidence supports our hypothesis of Notch1 involvolvement in HCC invasion (Leong K.G. et al. 2007; Giovannini C. et al. 2006; Giovannini C. et al. 2009). Evidence of reduced proliferation after Notch1 downregulation in HCC was reported, in agreement with our present findings (Ning L. et al. 2009). Conversely, a report about the opposite effect on HCC proliferation and apoptosis has been published, despite different cell lines model were used (Qi R. et al. 2003). Neverthless, the role of

Notch1 in invasion and metastatization of HCC is not yet well defined. Notch1 in hepatocellular carcinoma cells seems to be involved in cytoskeletal rearrangements and in differentiation, as was observed after Notch1 silencing in three cell line used to analyse Notch1 function in this cancer. Due to its pivotal function as regulator of EMT and changes in morphology observed cells were subjected to functional characterization, to asses EMT induction and modified invasion properties dependent on Notch1. EMT is induced by injury and wound healing assays let to test the acquisition of the metastable state that give the capability to migrate maintaining contacts and Notch1 confers this property to HCC cells (Thiery J.P. et al. 2009). Characterization of Notch1 silenced cells showed an involvement in cell motility and cell invasive capability in presence of Matrigel, together with an effect on MMP-9 activity that was not active without Notch1 expression. Changes in invasion capability *in vitro* might be dependent on MMP-9, an extracellular gelatynase that mediated matrix degradation and it is active in cells that underwent invasion. (Liabbak N. B. et al. 1996). MMP-9 results reduced in both total expression and activation of the proenzymatic form, in total protein extracts and supernatant of Notch1 knocked down cells. We confirmed previous evidences of Notch1 regulation on MMP-9 expression and activity in pancreatic cancer and in in prostate cancer cells, in which a direct Notch1 transcriptional regulation was reported. (Wang Z. et al 2006, Bin Hafeez B. et al. 2009). The reduced invasion and MMP-9 activity in Notch1 depleted cells suggests to investigate the activation of Epithelial to Mesenchymal transition (EMT) driven by Notch1, because the switch from a polarized and epithelial phenotype to a mesenchymal one occurs during cancer cell invasion, with the acquisition of the matrix degradation capability and induction of proteolitic enzymes (Steeg PS, 2006; Yao D. et al., 2011; Hanahan D. and Weinberg R.A., 2011). Carcinoma metastatization is a multistep process that consists in 1) detachment and migration from the primary tumour site, 2) invasion of neighbouring tissue and penetration through basement membrane, 3) entrance in blood or lymphatic vessel 4) survive to anoikis in circulation 5) exit the circle and be attached to an other tissue 6) form micrometastatic nodule 7) adapt the surrounding stroma and proliferate to form a macrometastasis (Steeg PS, 2006). EMT program activation gives ability to invade, to resist apoptosis and to disseminate to cancer cells and it is a multifaceted process that is activate at different degree and with stable or transient features during invasion and it constitute a real program behind cancer invasion (Thiery et al. 2009; Hannahan D. and Weinberg R.A., 2011). Notch signalling and TGF-β are the main pathway that control EMT in development and cancer and evidences of regulated Snail expression by Notch1 in heart development, neural crest and differentiation were

reported (Grego-Bessa J. et al. 2004; Timmermann L.A. et al. 2004; Cornell and Eisen, 2005). Some of the pleiotropic transcription factors that drives EMT are Snail, Twist and Slug and after Notch1 silencing Snail and Twist result downregulated. Slug was not evaluated because in hepatocellular carcinoma it was demonstrated to have not relation with EMT induction and E- Cadherin expression (Yang M.- H. et al. 2009). They are transcriptional repressor of *E-Cadherin*, Snail act directly binding an E-box at the promoter of *E-Cadherin*, wheareas Twist is an indirect repressor (Thiery J .P . et al 2009). Their first function inhibiting *E –Cadherin* gene expression is to reduce cell to cell contact and cell to matrix adhesion, and we observed a strong upregulation of E -Cadherin mRNA in Notch1 KD cells concomitantly with Snail and Twist reduction. Indeed, the ability to detach from the matrix and to loose cell contact is considered a marker of EMT activation, as E-Cadherin is the main component of cellular junction in epithelia leading to maintain their shape and it is loss in function in cells that acquire the capability to invade. (Hannahan D. and Weinberg R.A., 2011; Cavallaro U. and Christofori G., 2004). Conversely, N- Cadherin, normally expressed in neurons and in mesenchymal cells during organogenesis results to be expressed in all cells with reduction in Notch1 KD cells. De novo expression of N-Cadherin (Neuronal Cadherin), normally expressed by stromal and fibroblast cells, promote cell motility and migration, with an opposite effect than E- Cadherin. All our HCC cell lines showed expression of N-Cadherin suggesting a“cadherin switch” that occurs in development and let to change morphogenesis and interacting features. Also in cancer progression N- Cadherin expression consequent to cadherin switch change interacting features of these cells that may interact with stromal and endothelial cells, promoting invasive capability (Cavallaro U. and Christofori G., 2004). Interestingly, N-Cadherin expression can overcome E-Cadherin mediated adhesion, being sufficient to give a malignant and invasive phenothype, even in presence of E-Cadherin. (Hazan R.B.et al.,2000; Li G.et al. 2001; Nieman M. T. et al.,1999). In our cell models, the strong upregulation of E-Cadherin in mRNA it is not translate in protein expression, Indeed only HepG2 cells expresses E-Cadherin and it results also downregulated in Notch1 silenced cells. This evidence is opposite to all the other features of Notch1 silenced cells that bring in direction of a less aggressive phenotype, consequent to a reversion of EMT program activated by Notch1 expression. Indeed, Snail and Twist are not only repressor of E- Cadherin but they are transcriptional regulators that activate EMT program that is elicited by acquisition of mesenchymal function and repression of epithelial genes (Thiery J.P. et al. 2009). Snail is the pivotal factor that determs the onset of the transition, regulating both E- Cadherin and N-Cadherin expression, wheareas Twist maintains the migratory phenotype (Peinado et al, 2007;

Kang Y. and Massague J.,2004). Moreover, E-Cadherin downregulation alone is not sufficient for EMT and its re-expression in Twist overexpressing cells could not restore the epithelial phenotype (Thiery J.P. et al. 2002; Kang Y. and Massagué J.,2004; Yang J. et al. 2004). Moreover, Twist is responsible of spreading during development and it confers specific intravasation capability to cancer cells, giving specific metastatic advantage and no advantage to primary tumour, also conferring metastatic features to non metastatic HCC cells (Mikulits W., 2010). Notch1 silenced cells show reduced expression of two EMT program supporter Twist and Snail, moreover the mesenchymal markers N-Cadherin, smooth muscle actin (alpha-SMA) and Vimentin are downregulated. These components of the cytoskeleton are specific of mesenchymal cells and their expression in epithelial cell is associated with EMT and cytoskeleton reorganization, events that occurs during cell motility. Alpha- SMA is a marker of the hepatocyte conversion toward fibroblastoid cells that can intervene also in fibrogenesis (Dooley S. et al. 2008) and its regulation has been associated with Notch1 and Snail expression.(Saad S. et al. 2010) Silencing of Notch pathway with DAPT induces α-SMA repression, whereas Vimentin results diminished at transcriptional and post transcriptional level and it marks the intermediate filament switch from cytokeratin, expressed by epithelia, to the mesenchymal cell intermediate component, suggesting a rearrangement of the cells and loss of cell polarity (Savagner P. 2010). Moreover, a direct induction of alpha-SMA expression has beeen reported after NICD1 transfection in kidney cells, suggesting that its regulation of EMT pass also through alpha-SMA induced expression (Nyhan C. K. et al. 2010). In addition, cytokeratins markers of epithelium as CK18 and CK8 result respectively not changed or upregulated after Notch1 downregulation, supporting Notch1 induction of EMT program (Ding W. et al. 2010). These cytokeratins are expressed in cytosolic filament and also at the surface of hepatocytes and they are used as specific HCC markers for diagnostic purpose. (Wells M. J. et al 1997; Stroescu C. et al. 2006) The exclusive expression of CK18 by HepG2 and Snu449 cell line suggests a higher level of dedifferentiation and reprogramming of Snu398 cell line. Conversely, CK8 is expressed by all cell lines and it is upregulated concomitantly with Notch1 silencing, supporting the reversion toward a hepatocyte characteristic phenotype (Van Zijil et al. 2009). Moreover, the reduced expression of the liver progenitor marker CK19 in HepG2 cells silenced for Notch1 indicate the same effect of Notch1 expression in activation of EMT, suggesting transdifferentiation to progenitors hepatocytes and stemness like property of HCC cells, with association to tumour metastasis (Kim H. et al 2011; Mikulits W. 2010; Andersen JB et al. 2010; Ding S.J. et al.2004).

To confirm the induction of EMT by Notch1 in hepatocellular carcinoma cells, well established markers of aggressivity were evaluated, alpha-fetoprotein and c-Met. Only HepG2 cell line expresses alpha-fetoprotein even if the 70% of human HCCs samples re-express the liver specific fetal protein which correlates with metastatic potential and cancer progression. (Peng S. Y.et al. 2004). This protein elicits cancer cell to escape lymphocytes immune surveillance and it is assumed as prognostic factor to predict poor survival and faster HCC progression (Iida H. et al. 2005; Singhal A. et al. 2011). Hepatocyte Growth factor (HGF) acts on the tyrosine kinase receptor c-Met (Hepatocyte Growth factor Receptor, HGFR) and it activates a signalling that controls survival, cytoskeleton rearrangements, cell dissociation, motility and angiogenesis and regeneration in liver.(Pediaditakis P. et al. 2001; Huh CG et al.2004) Interestingly, HGF is produced by mesenchymal, whereas the receptor is expressed on the membrane of epithelial cells even if only during development and in progenitors. The re-expression of c-Met by HCC cells is associated to poor prognosis in HCC and with EMT and metastatic potential (Xie Q. et al. 2001; Son G. et.al. 2006). HGF function in inducing invasion has been well documented, such as the induction of HCC migration through activation of c-Met with phosphorylation.(Stolz DB et al. 1994; Nakanishi et al. 1999). HGF is re-expressed by HCC cells that drives EMT with an autocrine and paracrine feed- forward mechanism that drives the progression of EMT and cancer cells growth and invasion *in vivo* (Ding W. et al. 2010). The reduction in expression of total HGF receptor in Notch1 silenced cells strongly confirms a contrast effect of Notch1 depletion on EMT activation and it suggests a crosstalk between Notch1 and c-Met. Notch1 seems to activate HGF/c-Met pathway regulating c-Met amount at post-transcriptional level, maybe downregulating level of microRNAs like mir199a-3p and mir199a* that targets c-Met (Fornari F. et al 2010; Kim.S. et al. 2008 Mir199). In addition, since HGF is able to induce EMT in vitro, Notch1 may interact with this pivotal pathway to regulated EMT induction. (Stolz D.B. et al, 1994). Despite the way by which Notch1 regulates c-Met, this is a strong evidence of regulation of metastatic potential by Notch1, as c-Met has been demonstrated to be linked with metastatic capability and stem cells like property *in vivo* and *in vitro* in HCC, together with evidence of associations of inthahepatic metastatizationa and reduced survival. (You H. et al, 2011; Singhal A. et al 2011). The reduction of alphafeto-protein, c-Met and MMP-9 in Notch1 KD suggests a reversion of the EMT driven by Notch1. Taken together, these data suggest that Notch1 activity is upstream of Snail, Twist, c-Met pathway and MMP-9 activation and it regulates directly or indirectly cytoskeleton remodelling (α-SMA, Vimentin, Cytokeratins), paracrine communication (alpha-feto protein, c-Met) and cell adhesion (N- Cadherin),

sustaining EMT program and invasion of HCC cells. Maybe, regulation of E- Cadherin, α-SMA and MMP-9 expression could depend by Snail (Saas S. et al., 2010) but the evidence of a wider control exerts by Notch1 is given by the regulation at multiple levels of different pathways that undergo EMT and invasion.

Despite of these results and the observed upregulation of E- Cadherin mRNA in Notch1 knock-down cells, the E- cadherin protein levels results downregulated and the protein expressed only by HepG2 cells. The absence of expression in Snu398 and Snu449 together with the expression of Vimentin and N-Cadherin and the absence of cytokertin 8 expression in Snu398 cells and of cytokeratin 8 and 18 in both cell lines suggest a mesenchymal phenotype of Snu cells opposite to a more epithelial one for HepG2 cells, that lacks the expression of some mesenchymal markers and maintain citokertins expression. Nevethless, independent from the basal phenotype of the cells the role of Notch1 is crucial in inducing EMT and conferring invasive capabilities. In Snu cell lines Notch1 regulates the expression of E-Cadherin at transcriptional levels, despite a post-transcriptional regulation leads to completely abrogate protein expression. In a similar way HepG2 cells, despite of E-Cadherin protein expression, showed a post transcriptional regulation driven by Notch1. Nevertheless, this regulation could not impede protein formation. Indeed, also HepG2 cells that loose Notch1 expression have higher level of mRNA but they expressed lower level of full length 120kDa and the 80kDa forms respect to negative control after western blot analysis. E-Cadherin mediates membrane anchoring, adhesive recognition, cytoskeletal interactions and receptors modulation and its downregulation in tumours is associated with tumour grade, invasion, metastasis and survival. (Vleminckx K. et al, 1991; Birchmeier W. and Behrens J, 1994, Hirohasci S.,1998). Dependent on its function in cell adhesion this function in cancer is lost by mutation, transcriptional repressor, promoter hypermethylation or proteolitic degradation (Cavallaro U. and Christofori G., 2004). This last inactivating mechanism involves Metalloproteases (MMPs - 3,7,9 and 14) that cleave the transmembrane protein producing a soluble 80 KDa form of the protein correspondent to the extracellular domain in a mechanism called ectodomain shedding (Davies G et al. 2001, Noe V. et al. 2001). This cleavage alters the adhesive capability of the protein and this shed form promote cancer invasion by interfering with normal E- Cadherin interactions (Noe V. et al.,2001; Ryniers F. et al. 2002; Symowicz J. et al. 2007). In HepG2 silenced for Notch1 despite the induced rescue of EMT and invasion dependent by Notch1 depletion, E-Cadherin proteins levels resulted downregulated, but the functional localization at the plasma membrane is correct, as shown by IHC. Neverthless, the reduction in total amount evident also in IHC and the

concomitant reduction of 80KDa form of the protein suggests a variation in total expression linked to Notch1 more than altered function through MMP-9 cleavage. Moreover, the reduced 80kDa form release in Notch1 silenced cells probably depend by the inactivation of MMP-9 observed and this sustains a less invasive phenotype for these cells, as MMP-9 mediated E-Cadherin inactivation has been identified as useful strategy in ovarian carcinoma (Cowden Dahl K.D. et al. 2006). Moreover, in ovarian carcinoma MMP-9 mediates not only E-Cadherin cell junction disruption, but also ectodomain shedding, that is associated with metastasis dissemination (Symowicz J. et al. 2007). Soluble E- Cadherin release consequent to ectodomain shedding, is present not only in cancer but also in physiological context and it could be mediated by ADAM metalloproteases either than MMP-9 (DavieS G: et al. 2001). The cleaved fragment might be an autocrine/paracrine competitor for endogenous E-Cadherin function or serve as anchor for migration when bound to extracellualar matrix (De Wever O. et al. 2007). It is well established a role for shed E- Cadherin in cancer, such as to stimulate cancer scattering, invasion and promotion of cell junction disruption. So the decreased presence of E-Cadherin shedding observed in Notch1 silenced cells supports Nocth1 role in inducing cancer invasion trough altering E- Cadherin adhesion and shed E- Cadherin release in HCC.

Starting from E-Cadherin full protein form opposite effect compared to all other markers for invasion and aggressiveness regulated by Notch1, we hypothesize that E-Cadherin expression may favour invasion capability in HCC cells. To elucidate this hypothesis, E- Cadherin was silenced in HepG2 cells and overexpressed in Snu398 and Snu449 cells. The silencing of E-Cadherin in cells that have maintained its expression is traduced in reduced invasion and expression of aggressive markers. On the other side, the forced expression of E-Cadherin in HCC cells causes augmented invasion and higher aggressive markers expression. Despite a lot of papers in which E-Cadherin expression is associated to reduced invasion and EMT also in HCC (You et al. 2011; Liu L. et al. 2010; Cavallaro U. and Christofori G. et al. 2004), together with an inverse association between E- Cadherin surface expression and metastatic HCC (Fransvea E. et al. 2008), our data sustains an opposite function *in vitro* cell culture, in particular when Notch1 drives the modified phenotype. Indeed, E-Cadherin expression in cells silenced for Notch1, is sufficient to restore the basal phenotype of Notch1 only expressing cells, suggesting that Notch1 control in EMT and invasive properties might be dependent by E- Cadherin. But, an additive effect of concomitant expression of Notch1 and E- Cadherin lead to a more aggressive phenotype in Snu398 cells that have lost E- Cadherin expression compared to control. The pivotal role of Notch1 in invasion of HCC is evident by

the almost complete abrogation of invasion capability in Snu398 cells silenced for Nocth1. The E- Cadherin additional expression in these cells almost restore the basal phenotype of Notch1 only expressing cells, suggesting a role of Notch1 in control of invasion that is partially independent but also partially mediated by E- Cadherin expression. (Fig.18 D)The concomitant upregulation of aggressive markers analysed with E- Cadherin re-expression in Snu398 cells together with the reduction of these markers in HepG2 silenced for E- Cadherin expression, confirm the positive role in inducing a more aggressive phenotype enrolled by E-Cadherin in HCC cells *in vitro*. In addition, evidence of HCCs that retain E- cadherin expression and that make intrahepatic metastasis enforce our results and suggests a transient loss of E-cadherin (Osada et al. 1996).

The first possible explanation of this unusual role of E- Cadherin in invasion and adhesion of HCC might be a "collective invasion" as the subtype of cancer invasion that could involves HCC, as other epithelial cancers (Friedl and Wolf, 2008, 2010). Even if HCC is a high metastatic tumour, the more frequent site of metastatization is the liver. The high frequence of intrahepatic metastatization suggests that epithelial cancer cells have to go through an EMT process, but if they maintain the capability to migrate remaining adherent each other, they result more apt to colonize another site in the liver. This phenotype is physiologically linked to EMT, as cells that activate the program may acquire a "metastable" state which allows them to move while maintaining loose contact than migrating as individual cells (Thiery J.P. et al. 2009). Moreover, a crucial feature of metastatization process has to be considered that is the plasticity of the invasive-growth program of cancer cells. The idea that cancer cells routinely pass through a complete EMT program seems to be semplicistic and the metastatic process shows a higher complexity which has to be considered in interpreting results and suggesting therapies (Hanahan D. and Weinberg R.A.; 2011). Indeed, the activation of EMT may occur only partially and cells may enter the EMT program only acquiring new mesenchymal traits while continuing to express residual epithelial traits (Thiery J.P. et al. 2009). This might be the case of HCC cells. Indeed more than one type of cell migration exists and this process is the result of a complex intregration more elements, such as extracellular determinants, cell-cell and cell-matrix adhesion and protease functions. Depending of cellular and extracellular features, ameboyd, mesenchymal or collective invasion occurs. About ephytelial cells, as hepatocytes, multicellular streaming or collective invasion are the most probably type of migration that can activate, together with individual mesenchymal invasion if they dedifferentiate acquiring a fibroblast like phenotype (Friedl P. and Wolf K., 2009). Belong to the model of cell streaming and collective migration, in which

cells maintain cell to cell contact, the maintenance of E-Cadherin expression led to local invasion, such us intrahepatic one. Moreover, mesenchymal migration may be possible if we consider the process as a dynamic events. Also in chain migration cells can loose and re-acquire cell to cell contact, in an evolving mechanism that is driven by the cellular contest, the extracellular matrix stimuli, while both individual and collective kind of migration, cells activate protease degradation and integrin interaction to proceed in the movement. (Friedl P. and Wolf K., 2008; 2009). In a study about intrahepatic metastatic capability of HCC cell, it has been demonstrated that E-Cadherin expression favour metastatization of cells when inoculated both in intrasplenic and intrahepatic injection. LiNM, the cell line that expresses vimentin but that have lost E-cadherin has undergone EMT, but is unable to form liver metastasis, whereas LiHM that has retained E-cadherin expression produce multiple liver metastasis. The forced expression of E-Cadherin in LiNM give them the ability to form intrahepatic metastatis in both site of injection. These cells have undergone EMT and express both Vimentin and E-Cadherin and they are more metastatic, like our Notch1 expressing cells compared to the counterparts that loose the EMT activator, Notch1, and E-Cadherin expression (Snu398, Fig17A) (Osada T. et al. 1996). Despite to the epithelial phenotype shown by HepG2 cells (Fuchs B. C. et al. 2008), also in these cells that retains E-cadherin and express Notch1 the loss of E-Cadherin reduces their invasive capability. In addition, in an in vivo model of colorectal cancer metastatization in nude mice, the injected human cells that expressed E-Cadherin showed more spreading to lymph node and peritoneums compared to control or Snail overexpressig cells that could not invade the peritoneum. In peritoneal metastasis analysis the authors found increased E- Cadherin protelysis and nuclear translocation, whereas in local and lymph nodes tumours, E- Cadherin was overexpressed but not nuclear (Cespedes MV. Et al. 2010). Interestingly, these data suggest that also the overexpression of E- Cadherin without proteolytic disruption and nuclear translocation is associated to tumorigenesis. In our in vitro model was detected only in membrane, without detection in cytoplasm and nuclei. (Fig17B) Moreover, in prostate cancer the aberrant as the decreased expression of E-Cadherin have been associated with cancer metastatization. In a great expression study conduced on 1220 prostate cancer patients was observed a significative correlation of high E- Cadherin expression and grater tumour size, together with the detection of high E-Cadherin expression in metastatic cancer (Rubin M.A.). These data sustain the possibility that also in epithelial cancers higher expression of E-Cadherin could favour cancer progression. Toghether with these considerations, another aspect of the metastatic colonization has to be considered. The second hypothesis to explain these results about E-

Cadherin role in HCC might be its role in MET, that is mesenchymal to epithelial transition that occurs in the site of colonization and let cells to re-gain hystopathological traits of the primary tumour. It has well established that in MET, epithelial cells that underwent EMT, re-express E-cadherin to adhere and form a new mass in the new tissue site (Chao Y. et al. 2010;Yao D. et al. 2011).

In order to confirm and to better interpret *in vitro* results, an *ex vivo* analysis was performed for Notch1 and E- Cadherin expression and their link with metastatic capability. Data confirmed a positive correlation between Notch and E-Cadherin expression in agreement with *in vitro* results, but only in patients with multifocal cancers, that are tumours that underwent to intrahepatic metastasis. This evidence supports a causative role in metastatization for Notch1 and E-Cadherin expression. Interestingly, Notch1 and E-Cadherin higher expression correlate with time to recurrence. Even if an *in vivo* approach could be resolutive in confirming E-Cadherin negative role in HCC together with Notch1, these *ex vivo* observations strongly supports *in vitro* results.

In order to hypothesize a translational application for these evidences in a prognostic approach, analysis of the secreted protein associated with Notch1 expression were performed, using cell lines showing an epithelial and mesenchymal phenotype, HepG2 and Snu449 respectively.

Secretion of plasma proteins is a characteristic function of hepatocytes. (Crane L.J. 1977) Neverthless, the secretome is composed by actively secreted and shed proteins of the transmembrane and extracellular compartment of the cells. The cancer secretome study is a promising and powerful tool to detect key components in regulatory pathways governing functional acquisition of tumour growth and invasion. (Pavlou M. P: et al 2010).In Notch1 dependent secretome of HCC cells, the presence of a lot of molecules involved in cell-cell adhesion and cell-matrix interactions supports our evidences about Notch1 role in regulating metastatization of HCC and it evidences how its regulation could involve factors and pathways, not only through transcriptional regulation as predictable, but also at post transcriptional level. Variations observed in rate release of secreted proteins or transmembrane molecules subject to shedding suggest that Notch1 is involved in mechanisms of interaction and of cell to cell communication, regulating secretion pathways and mechanisms as metastatization in cancer at a higher level than simply the cellular one.

The high interest for Notch1 secretion control is supported by the evidence of the crucial role of the microenvironment in cancer development, in particular in liver, where interactions between tissue and blood circle is so close. Proteins that acts normally in the extracellular

compartment are so interesting, as thrombospondins, because their possible modification of cancer microenvironment in tissue and also in the whole organism. These matricellular glycoproteins are secreted and presents specific binding motif to interact with extracellular factors and exert their numerous functions (Meng H. et al. 2009).

Thrombospondins are a family of secreted proteins involved in tissue remodelling in development, wound healing and neoplasia. Thrombospondin-1 and 2 are characterized by TSR domain that mediate antiangiogenic activity, inhibition of migration and induction of apoptosis, whereas the TSP domain retains ability to interact with cell surface proteins. Thrombospondin-1 is a strong anti-angiogenic factor, crucial modulator of extracellular proteases and it was linked to tumour invasion and cancer growth in a wide range of cellular model *in vivo* and *in vitro* (Kazerounian S. et al 2008). Thrombospondin-1 results to be differentially regulated after Notch1 silencing in our cell lines, with significative modification in only Snu449 cell line. Complex and contradictory role of thrombospondin-1 was already evident from when in 1987 Tuszynski GP and colleagues demonstrate its function of promoting cell-substratum adhesion in a variety of cells, included epithelial and endothelial cells (Tuszynsky GP et al. 1987 Science) and its role in potentiate metastatization through supporting cancer cell seeding. (Tuszynsky GP et al. 1987 Cancer Res). Moreover, evidences of tumour suppressive effect of its expression and inverse correlation with malignancy in lung, breast and bladder cancer was reported (Zajchowsky D.A. et al.; Campbell S.C. et al, 1998). An evidence of tumour suppression role was demonstrated by in vivo studies in which Thrombospondin-1 expressing cells show reduced cancer cell growth and an antiagiogenic effect of this protein in the early stage of the tumour, inhibiting neovascularization and metastatization, hampering tumour growth through the presence of high circulating THSB-1. (Lawler J. et al. 2001; Naumov G.N. et al. 2006) In this way, the strong upregulation observed in Snu449 Notch1 KD cell may justify the reduced aggressive phenotype and suggests an inhibited vascularization and possible augmented cell adhesion in our model, as its main function seems to be the anti-angiogenic activity. Indeed, on one side it may activate matrix degradation activating plasmin formation, but on the other side it could also induce cell to matrix adhesion and cell to cell contact through its integrins interacting domain. In these contradictory roles the relative level of protein expression result to be crucial, with promoting invasion at high rate of expression and inducing adhesion if it is low expressed. (Roberts D. D. et al. 1996; Albo D. et al 1997; AlboD et al. 1999) From these observations and many papers about both inducing and contrasting metastatatization capability mediated by THBS-1, it is difficult to interpret the significance of our results *in vitro*. (Roberts D. D. et al. 1996;

Firley V. et al 2011; Nucera C. et al. 2010; Moon Y. et al. 2005; Kazerounian S. et al. 2008). For that, a silencing approach against THSB1 will be developed in the future to define its role in our cell model and in HCC. It is almost known that the outcome of Thrombospondin-1 expression depends from a lot of aspect: type of tissue and tumour, expressing cell type, tumour phase and multiple interactions of this protein with other cellular or extracellular components, as cellular receptors and ECM molecules.(Roberts D. D. et al. 1996; Kazerounian S. et al. 2008) Indeed if in tumour cell expression is frequently linked to a tumour suppressor effect, the high expression of the protein in the tumour microenvironment can led to an opposite effect, trough complex range of interaction, as evident with no variations *in vitro* experiments and strong reduced tumorigenesis and metastasis *in vivo* (Zabrenetzky V. et al. 1994 For that, neither tissue specificity can explain the dynamic and complex role of Thrombospondin-1 function in cancer. The clinical significance of THSB-1 expression in HCC seems to be related to invasion and poor survival, but concomitantly high level of the pro-angiogenic factor VEGF were reported, leading to a non direct dependent effect of the mostly anti-angiogenic factor. (Poon RT. Et al, 2004) Moreover, a study on the phenotype of high aggressive alpha-feto protein HCC shows a reduced mRNA and protein expression of THSB-1 in tumour samples, compared to non cancerous surrounding tissue (Iida H. et al. 2005). Interestingly, THSB-1 results to be strongly inhibited in expression by some oncogenes and many are associated with HCC progression as Osteopontin, Ras and Myc, whereas p53 tumour suppressor induces its expression, suggesting and confirming a tumour suppressor function (Limei Z. et al. 2010; Kazerounian S. et al. 2008). In addition, from the present work is evident for the first time that Notch1 positively regulates Thrombospondin-1, with a prevalent Notch1 oncogenic role in HCC. Supporting evidence of a positive outcome of the TSHB-1 upregulation observed in Notch1 KD cells comes from patients' serum analysis. Indeed, considering a high expression level in health serum, statistical significative reduction of serum level of the protein were observed in cirrhotic and cancer affected patients, neverthless the minimum value was observed in early HCC respect to advanced stage. These data could be indicative of the multiple roles of thrombospondin-1 in biology of cancer, inhibiting angiogenesis in the early stage of tumour and affecting cellular adhesion in the advanced one. (Kazerounian S. et al. 2008). This last function may be significative for advanced HCC because relative low levels could give increased cell adhesion, even if too high expression could activate plasmin and invasion. (Kazerounian S. et al. 2008; Lawer J. and Detmar M.,2004). Interestingly, considering variations of TSHB-1 levels between cirrhotic patients and hcc samples, a significative reduction of THSB-1 is

evident, suggesting a possible role in inhibition of angiogenesis and cancer growth in these first stages of the cancer progression. The relative effect of the expression of Thrombospondin-1 in each stage of sample is not defined and so the effective function have to be investigated and confirmed. Moreover, an absolute quantitation might be necessary to could appreciate variations during cancer development and to have a reference to analyse patients' serum levels. Neverthless, these variations of plasma levels might be indicative of cancer progression and a useful prognostic tool.

Concerning Thrombospondin-2, this protein shows high sequence homology with the first one, maintaining key functional TSRs domains, which mediate the antiangiogenic activity. The high level of sequence identity suggests that they have similar activity. In spite of this similarity, the unique THSB-1 RFK domain activates TGF-beta, whereas the WSXW of thrombospondin-2 can antagonize this function. In this way, they could be considered modulators of TFG-beta signalling activation, with a unique inhibiting role of THSB-2 (Roberts D.D. 1996). The anti-angiogenic activity consists in migratory inhibition and apoptosis induction of endothelial cells. Anti angiogenenic effect is mediated by TSR domain of both TSHB1 and TSHB2, even if TSHB2 shows a stronger effect on vessel size and endothelial cell apoptosis. The role of inhibitor of tumour growth, cell metastasis and angiogenesis has been demonstrated also for this protein, together with its downregulation in tumour cells. (Kazerounian S. et al 2008). The expression of THSB-2 antagonizes growth in lung carcinoma and in squamos cell carcinoma only with the presence of its N- terminal domain in the extracellular space (Streit M. at al 2002; Noh T. H. et al.2003). Interestingly, a different function of THSB-2 respect to THSB-1 has been documented in pancreatic cancer, in which the crosstalk of thrombospondin-2 with others factors of the extracellular space let to inhibit cancer invasion, through downregulation of MMP-9 and uPA activity mediated by THSB-2 expression and reduced invasion. (Nakamura M. 2008) These data may be important also in HCC, considering the pivotal role of MMP-9 and uPA in metastatization of this cancer.

In Nothc1 KD HCC in vitro model, levels of THSB-2 results upregulated in Snu449 cells that lack Notch1, but on the contrary HepG2 cell line show its downregulation. Starting from characterization of protein function, it may possible to have a tumour suppressor effect in Snu449 and the opposite effect in HepG2, but also the contrary or the same outcome in both cell line, as defined by the dual nature of these proteins, like thrombospondin-1. Physiological function of Thrombospondin-1 and 2 are similar but not identical, as evident from the respective knock out mice phenotype, that show differential spatial and temporal alteration in

gene expression, but the same effect in suppression of lymph node metastasis and angiogenesis (Hawighorst T. et al. 2002). THSB2 has been less studied compared to the THSB1 form but the effect in HCC was analyzed only by Lin W. in 2011 and associated with migration in HepG2 in vitro model of HDAC inhibition and consequent TSHB-2 expression. Silencing of THSB-2 in this cell line is traduced in a reversion of the migratory phenotype observed. (Lin W.H. et al. 2011). These results suggest a role in inducing invasion in HepG2 cell line *in vitro* and they are in agreement with the strong reduction in extracellular amount of THBS-2 after Notch1 silencing observed in only HepG2 cells, together with a reversion of the aggressive features of the cells. Maybe, the contradictory upregulation of THSB-2 in Snu449 shNotch1 cells might reflects a different stage of tumour progression of the original tumour or an interactions with others factors that modify the amounts of detected protein, despite of the same less aggressive phenotype observed. In serum analysis of free THSB-2 in HCC patients the amount of THSB-2 shows alternate variations. Reduction is observed in cirrhosis compared to healthy and in advanced cancer compared to early lesions, suggesting a tumour suppressive role that has to be overlapped for cancer progression. Despite of that, in early HCC patients, levels of the protein are higher than in cirrhosis, suggesting a similar behaviour of HepG2 cell line. Moreover, the higher expression of early patients also comparing them with advanced cases, suggests a complex and maybe interacting action of this protein with other extracellular factors in determining the outcome on cancer physiology. An example of a higher grade of interaction of this thrombospondin compared to the 1 type with other cellular component is done by the demonstration of Thrombospondin-2 activation of Notch3 signalling, that is not given by Thrombospondin-1.The regulation of NICD3 trancriptional activity is subjected to the presence of the complete Notch3 receptor and is activated by direct interaction between proteins. (Meng H. et al.). This specific THSB-2 regulation is an example of thrombospondin specificity and pathways crosstalk. The inverse regulation of Notch signalling was not reported before and the possibility of Notch3 regulatory loop on Thrombospondin-2 exists may be interesting to be investigated.

A member of the immonoglobulin superfamily ICAM-5 is a transmembrane glycoprotein involved in intercellular adhesion and immune response, interacting with integrins and modifing the mediated integrins activation of T- cell, with an anti-inflammatory role (Tian L. et al. 2008). Impairment of cellular adhesion is one of the first step that lead to metastatic invasion and changes in expression of ICAMs play a critical role in this process (Johnson J. P., 1991; Hannahan D. and Weinber R. A., 2000). In Notch1 silenced cells of HCC, ICAM-5 results increased in secrete with no significative change in total protein expression (data not

shown), suggesting augmented adhesion with the extracellular matrix and sustaining the role of Notch1 in induce acquisition of necessary capabilities for invade. Maybe, the regulation on ICAM-5 expression at the membrane surface is controlled by Notch1 expression through an indirect mechanism that do not involve change in expression. No association was previously observed for Notch1 regulation on ICAM-5, even if the interaction between the pathway and the well conserved locus of ICAMs was already known. Recently, the role of Notch1 in inducing invasion and migration of breast cancer cells was associated to activation of ICAM-1, protein with high homology with ICAM-5, and previously identified at genetic and molecular level as causative of breast cancer invasion (Wang J. et al. 2011; Rosette C. at al, 2005; Chen H. et al 2006). Neverthless, in other cancers ICAM-1 have showed to have anti-metastatic and anti-proliferative functions (Chen H. et al 2006); conversely in liver cancer a pro-invasive role has been identified (Sun J.J. et al. 1998). Despite of contradictory reports on ICAM-1, ICAM-5 function in cancer is not yet understood. Two genetic studies on SNPs in this locus associates the presence of the SNPs with prostate cancer or exclude this association. (Kammerer S. et al, 2004; Hankui C. et al 2006). Even if it is specifically expressed in brain, transcriptome studies report expression in other tissue, comprised liver and HepG2 cell line (Gene Card, Weizman Institute). Our data for the first time associates ICAM-5 with Notch1 expression and HCC progression. Data also suggest that Notch knock down might increase cell to cell adhesion trough a higher membrane localization of ICAM-5, maybe interfering on intercellular turn-over, that leads to a higher release in the extracellular compartment and in plasma. Seric ICAM-5 shows similar level in healthy donors and cirrhotic patients, higher level in early HCC cases and a drastic reduction in advanced HCC, compared to all others group, with high statistical significance. These *in vivo* data with Notch1 induction of ICAM-5 secretion in our cell model support the involvement of ICAM-5 in adhesion and a tumour suppressive role in HCC progression even if molecular details have to be investigated, for example to interpret the increased in early HCC compared to cirrhotics. ICAM-5 serum high levels induced by Notch1 expression might become a significative predictive marker of Notch1 expressing cancer and of its metastatic potential in HCC.

The pivotal role of mTOR activity in HCC tumorigenesis and cancer progression is evident from a lot of works that identify the PI3K/Akt/mTOR pathway as one of proliferation pathways that drives liver carcinogenesis (Tanaka S. 1997, He X. et al. 2008; Avila et al 2006). Considering the wide range of cytokines like insulin and interleukin family proteins that activate PI3K pathway, the role of the downstream kinase mTOR is crucial in a microenvironment of cronic inflammation like the cirrhotic liver that frequently generates

HCC. The interaction of IGF and EGF to their receptors, that are overexpressed in HCC and activates PI3K induces the serine/threonine kinase AKT that phosphorylates mTOR, increasing cellular proliferation, cell survival and inhibiting apoptosis. (Whittaker S. et al. 2010; Roberts LR and Gores GJ 2005). Activity of mTOR has been observed in 45% of HCC in its downstream target p70 S6K phosphorylation and the use of Rapamycin *in vitro*, a inhibitor of mTOR, have reduced cell proliferation of HCC cell lines. (Roberts LR and Gores GJ 2005; Sahin F. et al.2004). Moreover, in HCC sample analysis the activation of mTOR pathway was observed and the effects of blockade of this pathway in xenograft model have showed reduced tumour growth and increased survival (Villanueva et al. 2008). In addition, mTOR role in cancer has been widespread to other functions, as cell motility and tumour metastasis, through interaction with actin cytoskeletal proteins (Berven et al. 2004; Zhou and Huang, 2010). MTOR has been identified to act in two molecular complex in the cytoplasm: mTORC1and mTORC2 that contain respectively mTOR associated with raptor and rictor regulatory proteins. These two complex phosphorylates different targets, as 4E-BP is phosphorylated by mTORC1 and Akt by mTORC2, regulating respectively protein synthesis, cell growth and survival in one hand and actin cytoskeleton dynamic and motility on the other. The reorganization of actin cytoskeleton is the primary mechanism of cell motility and it is essential for cell migration (Zhou and Huang, 2010). The role in inducing cell motility and metastatization is evident by tumour model treatment with rapamycin, that shows reduced metastasis and angiogenesis in mouse models (Guba et al. 2002).This role in cell migration and angiogenesis, through reduction in transcription of VEGF, underline the importance of mTOR in cancer progression and not only in tumorigenesis, together with the relevance of its extracellular detection in secretome and serum, for the first time. Despite of no significative variation in HCC advanced patients mTOR serum levels compared to early and cirrhotic cases, the complete absence of detection in serum of healthy donors underline a relevant role for mTOR serum activity in tumorigenesis. The reduction in its amounts in Notch1 knock down cell is not surprising, as Notch1 is known to be positive regulator of mTOR activity (Mungamuri S.K. et al. 2006; Zhao N. et al. 2010; Muellener M.K. et al.2011; Gutierrez A. et al. 2007).

Clinical trials for HCC theraphy using mTor kinase inhibitors are under investigations because the pathway is considered one of the two involved in oncogenic addiction of HCC. (Tanaka S. et al 2002; Tanaka S. et al. 2010).

Between serine protease enzymes regulated by Notch1 in the extracellular space and in membrane associated contest emerged from high throughput proteomic analysis, SERPINA5

resulted to be interesting not only for its higher liver specific expression (Suzuki. K. 1989), but also because it is physiologically present in plasma, so bringing itself a high intrinsic predictive potential if it would result relevant for HCC progression (Jackson TP. et al. 1997). Interestingly, SERPINA5 results to be upregulated in cellular supernatants when Notch1 signalling was swiched off, supporting a role of the pathway in tumour progression and suggesting one of the mechanism through which it may assert this function. Indeed, SERPINA5 is an inhibitor of urokinase plasminogen activator (uPA), the effector member of the MAPK pathway that inhibits plasminogen processing and consequent ECM degradation through MMP-9 (Sieben L.G.N. et al, 2005). Increased uPA activity has been considered the most sensitive factor affecting HCC invasion and to be associated with reduced survival and recurrence of HCC (Itoh T. et al., 2000; Zheng Q. at al. 2000). For this involvement in matrix remodelling and its physiological extracellular localization, SERPINA5 could be considered an effector of Notch1 metastatic potential in HCC and a useful predictive factor of a more aggressive phenotype of this cancer, even if its association in HCC pathogenesis was not report before. In prostate cancer instead SERPINA5 has been associated to metastatization and tumour progression, with the loss of SERPINA5 expression in high-grade prostate tumours (Cao Y. at al.2003; Glasscock L.N. et al. 2005). In addition, a role in breast cancer metastatization and cell growth has been demonstrated, together with an antiangiogenic function independent from its protease inhibitor activity (Asanuma K, 2007). In liver tissue a role for SERPINA 5 was identified in regulating regeneration, through interaction with HGFA, the main activator protease of HGF. In addition, the main source of plasma SERPINA5 is the liver, leading to measure and correlate SERPINA5 directly to liver function and maybe to hepatocellular carcinoma progression. Indeed, SERPINA5 has been described as strong regulator of liver regeneration through inhibition of HGFA, the main activator protease of HGF (Miyazawa K. et al. 2010). The reduced HGF activation dependent on SERPINA5 causes a delay in liver regeneration (Hamada T. et al. 2008, Suzuki K. 2010), but we also suppose it could reduce tumour growth rate, abrogating the proliferation and pro-invasive activity of hepatocyte specific cytokine HGF. As supporting evidence of its predictive value, our results about serum value in patients suggest a correlation between SERPINA5 plasma levels and cancer progression, even if the significance is reached only for reduction in cancer compared to healthy patients. Neverthless, in a recent study of proteomics biomarkers of Transcatheter Arterial Embolization (TACE), they have identified SERPINA5 as marker to monitor the efficiency of the therapy. This analysis gives reason of a role of SERPINA5 in the progression of HCC (Li CY et al. 2011).

Finally, shed E- Cadherin form detection in supernatants and in the serum of patients, constitute a significative aspect in Notch1 characterization in HCC and a marker for mechanisms that underline HCC progression. Indeed, shed- E-Cadherin has been associated with inhibition of adhesion and cellular aggregation, together with the ability to induce invasion in vitro (Ryniers et al. 2002). Many reports demonstrate *in vitro* and *in vivo* the role of soluble E-Cadherin form in inducing invasion and metastatization of cancer. (De Wever O. et al. 2007) The mechanism underline its formation are proteolitical cleavage by extracellular proteases and the shed form in the extracellular space acts as modulator of E- Cadherin full form adhesion, moreover acts as a anchor for metastatic spread cells during invasion. Interestingly, shed E- Cadherin is found in human fluids indicating loss of diffusion barrier and its plasma levels may reflect tumour volume and the extent of the barrier defect (De Wever O. et al. 2007). The shed E-Cadherin form has been identified in serum of patients affected by different cancers and correlated with tumour grade and cancer progression. For example, in colorectal cancer it is associated with tumour grade and presence of liver metastasis, (Wilmanns C. et al. 2004) whereas in gastric cancer it is accepted and used as prognostic marker of disease recurrence and metastasis development. (Velikova G. et al 1997; Gofuku J. et al. 1998; Chan AO-O et al. 2003 and 2005; Juhasz M. et al. 2003) Also for HCC a study demonstrate that serum soluble E-cad levels were elevated in patients with HCC, and high serum soluble E-cadherin (> or = 8,000 ng/ml) was associated with early recurrence or extrahepatic metastasis, suggesting serum soluble E-cad as potential prognostic marker for HCC (Soyama A. et al. 2008). In our patients variations of serum levels of soluble E-Cadherin were observed, with a statistical significative increase between early HCC and advanced one, whereas similar values were observed between cirrhotic and early HCC, suggesting the dependence of E-Cadherin shedding by inflammation (Pittard AJ. Et al. 1996), but also its higher levels could be associated with advanced stages and cancer progression, confirming the prognostic potential of this marker. Moreover, due to its association with Notch1 expression, this and the other soluble protein that shows significative variation depedent by Notch1 expression, might be indicator of a defined phenotype of high expressing Notch1 cancer, and constitute markers to predict cancer progression or define stategies for therapy.

Taken together, present results evidence that Notch1 is involved in inducing EMT and invasion capability of HCC cells, being a strog negative prognostic marker to predict HCC progression in clinic practice. Moreover, the associated secreted protein modulation and their possible detection in serum of patients, makes this predictive tool more powerful. The use of

Notch1 expression as predictor of recurrence after surgical hepatectomy results a new tool, together with the use of serum markers identified, it will help to predict HCC recurrence..

Recently, some published papers report evidences of Notch1 role in development and progression of HCC, with a specific involvement in invasion. Lim S.O and colleagues document Notch1 involvement in HCC oncogenesis from a in vivo analysis, confirming previous observations of our group (Giovannini C. et al. 2009). They indicate a necessary link with p53 status in the outcome of Notch1 expression, indeed Notch1 and Snail expression induce invasiveness in presence of p53 wild type, whereas it decreased invasiveness in absence of wild type p53.(Lim S. et al. 2011). They describe the behaviour of a subset of HCC advanced tumours in which the coexpression of Notch1, Snail and p53 wild type protein expression, explain their high grade and invasiveness. They suppose the necessary coexpression of Snail with Notch1 to induce invasion. These data are in agreement with our findings, also considering that the cellular models we used carried wild type or functional p53 proteins.(Biology of Snu)

Another work shows involvement of Notch1 in HCC metastasis to the lung, reporting Notch1 and Snail expression only in metastatic cell lines and showing a reduction in metastatic capability when Notch1 is silenced. Moreover they report a downregulation of E-Cadherin level that they refer to Notch- Snail control of EMT, but also in cells that did not express Notch1 and which they consider non metastatic, the same E- Cadherin low or absent level were observed, together with no variation in N-Cadherin. Even if this evidence shows a link for Notch1 in inducing liver metastasis, the molecular mechanism underline this phenotypic effect is not clarified. Neither, the relation between Notch1- Snail and E-Cadherin expression in patients samples results not convincing. (Wang X.Q. et al. 2011). In agreement with our findings, they observed that Notch1 activate Snail expression, inducing invasion and EMT. Conversely, Lim SO. in 2011 reports a direct interaction between Snail and Notch1, suggesting that NICD1 binds and induces degradation of Snail, acting as inhibitor of Snail dependent invasion (Lim SO. et al. 2011). These controversial evidences make necessaries other study to further define the molecular mechanisms underline Notch1 activation, in order to establish the effective pathway in HCC. Neverthless, evidences from *ex vivo* samples let us to confirm the *in vitro* findings and to conlcude that Notch1 and E- Cadherin expression correlate with higher metastatization of HCC.

The main aspect emerging from this study, taking together results at molecular level, *in vitro* and *ex vivo* data, together with proteomic profiling is the broad spectrum of cellular events

that Notch1 regulates directly and indirectly in HCC cells. Indeed, not only it is involved in cellular process undergoing cancer progression, but a wide spectrum of crosstalk and interaction might render its function more complex and efficient. For example, Raghu H. and colleagues in 2011 published evidence of a reciprocal regulation of Notch1 and uPA activity, together with the involvement of mTOR activity in glioblastoma. They demonstrate the existence of a feedback loop by wich uPA inhibits Notch1 expression at mRNA level when in the meantime Notch1 activates uPA expression and activity. This Notch1 activator effect on uPA is traduced in activation of mTOR, Akt and ERK as they result downregulated after both Notch1 and uPA silencing. These results are in agreement with our hypothesis of a complex and interchanged regulation of Notch1 in invasion and metastasis. Indeed, also in our Notch1 KD cells mTOR activity seems to be reduced and the uPA pathway activation might be regulated by Notch1 through its negative regulators SERPINA5 and Thrombospondin-2 (Nakamura M. 2008). Moreover, the regulation of ICAM seems to have a broad spectrum of biological functions, more than cell adhesion, such as migration, signal transduction and also regulation of gene expression (Juliano RL 2002; Cavallaro U. and Christofori G., 2004), suggesting that others mechanism may be involved in this new ICAM protein associated with Notch1 regulation. In addition, the apparent regulation on uPA activity underline multiple level of interference of Notch1 in invasion, that is uPA activity may be the most sensitive factor affecting HCC invasion in the plasminogen activation system and is a strong predictor for the recurrence and prognosis of HCC (Singhal A. et al. 2011). Interestingly, a crosstalk between Notch1 and TGF-β has been reported in kidney epithelial cells, together with the evidence of an interconnected crosstalk in regulating alpha-SMA, E- Cadherin and THSB-1. Authors showed a direct regulation of Notch1 on alpha-SMA, whereas E- cadherin seem to be only partially regulated by Notch1. THBS-1 instead was evaluated only after TGF-β treatment and after promoter in silico analysis for Notch1 binding sites the authors excluded a direct regulation, even if they observe an induction after Notch1 inhibition (Nyhan et al. 2010). These results are partially in agreement with our data, supporting the high conservation and the pivoltal role of Notch in invasion and cytokine response, either as required pathway for TGF-β induced response.

Notch1 may be considered a powerful pathway in driving invasion and progression of HCC. Notch1 seems to activate a cascade mechanism involving 1) EMT activators (Snail, Twist) 2) cytoskeletal rearrangement and consequent motility of the cells, both at intracellular and extracellular level (N- Cadherin, Vimentin, alpha-SMA, mTOR) 3) cell to cell and cell to matrix adhesion (E-cadherin, cMet, ICAM5) 4) activate ECM degradation, regulating

directly and indirectly MMPs expression and activation (MMP-9, THBS-1; THBS-2, mTOR; SERPINA5) 5) induce angiogenesis (THBS-1; THBS-2, SERPINA5, uPA). The spectrum of factors regulated by Notch1 expression suggests a pivotal role in regulating all steps of invasion and metastatization of HCC and it define Notch1 as oncogenic pathway on progression of HCC.

RESULTS 3

Notch3 regulates p53 levels in HCC cell lines trough transcriptional and post transcriptional mechanisms, without affecting p53 transcriptional activity

Notch3 knockdown in HepG2 and Snu398 HCC cell lines have shown a regulation on p53, indeed after Notch3 stable knockdown, p53 protein level resulted upregulated. (Giovannini C. et al., 2009) (Fig 22A). Given this observation, we asked if Notch3 regulates p53 expression transcriptionally or through post-transcriptional mechanisms. As the activation of Notch pathway is mediated by NICD3 induced transcriptional activation of target genes by NICD3 we firstly asked if Notch3 regulates TP53 through a transcriptional regulation. P53 mRNA levels showed an increase in both cell respectively 1.5 and 2.7 fold in HepG2 and Snu398 cells, after Notch3 silencing (Fig.1A). In order to confirm the effect observed after Notch3 shRNA, an expression plasmid containing the the active form of Notch3 (NICD3) was transiently transfected in HepG2 cells carrying wild type TP53 gene. Due to low transfection efficiency for large DNA constructs, the plasmid was cross-linked with a fluorophore and 24h post transfection, cells were sorted in order to divide overexpressing NICD3 from others. In agreement with the upregulatory effect observed in Notch3 knockdown cells, in NICD3 overespressing cells a strong downregulation of p53 protein levels was observed, whereas a less dramatic change of mRNA were observed in semiquantitative PCR. (Fig 1B, lower part).Moreover, to exclude the endogenous effect of Notch3 expressed by the cell and confirm this result, the same transfection was performed in HepG2 Notch3KD cells, in order to have a rescue effect. After NICD3 expression, p53 protein demonstrate a strong downregulation, whereas p53 mRNA did not show significant variation.(Fig.22) Given to these results and the observations that p53 mRNA variation in Notch3 silenced cells does not reflect the protein up-regulation that it is 5 fold higher in silenced cells compared to controls, we hypothesize prevalence of Notch3 post-transcriptional regulation on p53. In physiological conditions p53 activity is required immediately after cell stimuli and it coordinates a fine-tuned network of cellular responses. For that, p53 is mainly subjected to post transcriptional and post translational mechanisms of regulation and stabilization, than to transcriptional ones (Kruse J-P and Gu W.). So, we hypothesize that the increase in p53 protein levels was dependent on these altered regulatory mechanisms. The main post-transcriptional regulator of p53 is Mdm2, an ubiquitin ligase that mediate p53 degradation and changes in activity, through poliubiquitination or monoubiquitination.(Haupt Y. et al. 1997, HondaR. Et al. 1997, Brooks et al. 2006; Li M. et al. 2003; Lee JT and Gu W. 2010) Polyubiquitination mediates

proteasome dependent degradation of the protein, that in the natural setting of the cells has a short half-life. A higher stabilization of the protein may depend on p53 post transcriptional modifications able to alter Mdm2-p53 interaction and give reason of higher p53 level in Notch3 KD cells. Phosphorylation at Ser-20 and Ser-15 of p53 may alter the interaction with Mdm2 and prevent p53 target for degradation (Chehab et al. 1999; Unger et al. 1999). Neverthless, in Notch3 KD cells no significative variation was observed for Ser-20 phosphorylation comparing to controls (Giovannini C. et al. 2009). This residue is localized in the interaction domain of p53 that binds Mdm2 and its faible variation in phosphorylation status could not justify the high upregulation in p53 protein level. (Fig1C). The other p53 residue that might alter the interaction with Mdm2 when phosphorylated is Ser-15, modified after DNA damage, its phosphorylation activates p53 (Shien et al. 1997; Tibbets et al. 1999, Unger et al. 1999, Chehab et al. 2000). No significative variation was observed in phosphorylation status at these residues after Notch3 silencing, in both HepG2 and Snu398 cells. (Fig.1C) This evidence suggests that the higher Notch3 silenced cell response to doxorubicin (Giovannini C. et al. 2009), might depend on the increased p53 total level that bring to a grater apoptotic response, than to the presence of an already activated p53 protein after Notch3 silencing. Moreover, the luciferase assay with the reporter controlled by p53 consensus site in HepG2 demonstrates that p53 in Notch3 silenced cells was not more active in transcription than the control's one, but the stronger response to doxorubicin observed has to be referred to higher total p53 protein levels. (Fig1D) Taken together, these results suggest that the increase in p53 protein levels after Notch3 silencing is not dependent by altered p53 phosphorylation that are able to impair Mdm2 binding.

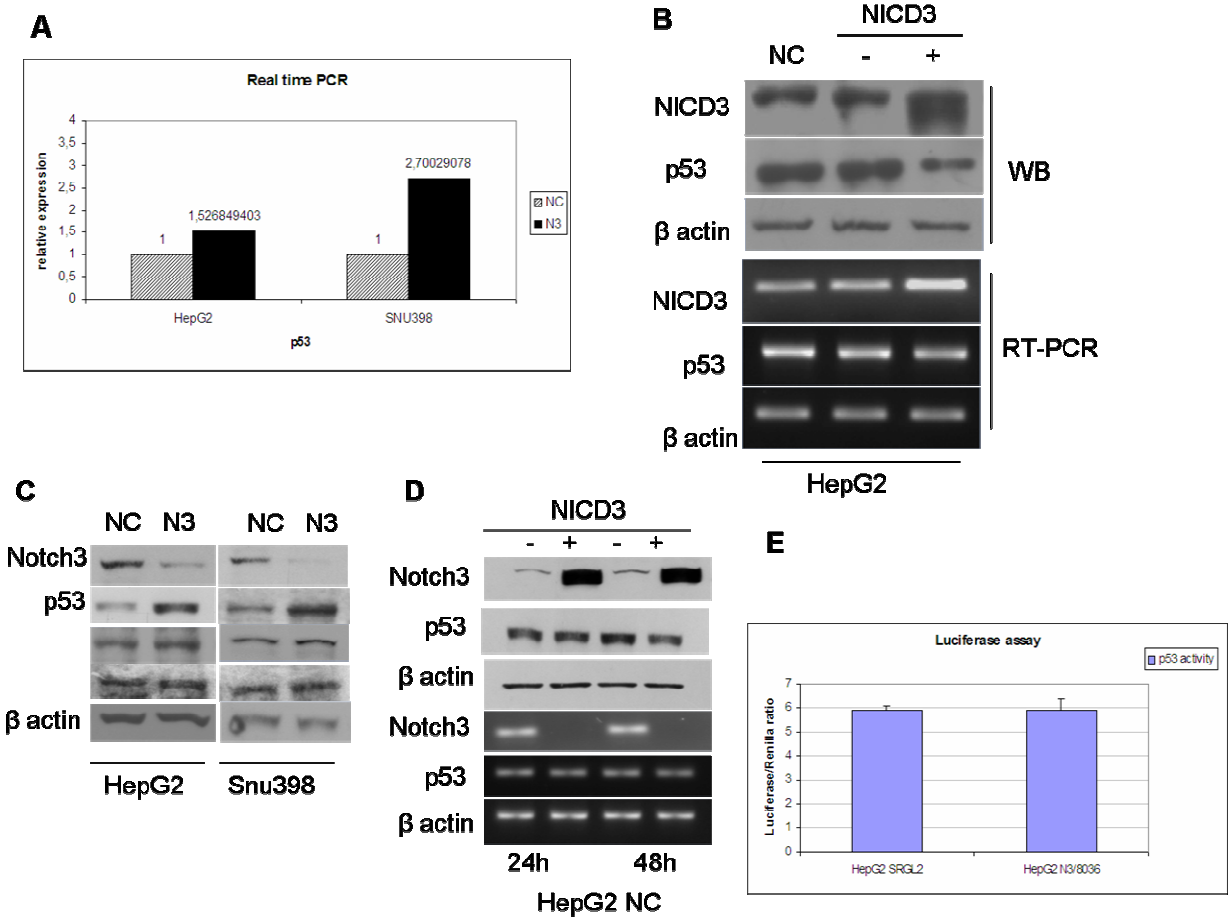


Figure 1: Notch3 regulates p53 through transcriptional and post-transcriptional mechanisms. A) Real Time PCR for p53 in HepG2 and Snu398 cell lines steadily silenced for Notch3. Results represents fold increase in relative expression comparing Notch3 silenced cells with negative controls. Expression was calculated on the basis of 2(δδ Ct) of each sample normalized for beta actin and putting negative control expression value as 1.Values of fold increase in expression are indicated on the top of the columns. NC: Negative control of shRNA; N1: Notch3 shRNA. **B)** Transient transfection of NICD3 expression vector in HepG2 cells. Analyses were performed 48h after transfection. Upper: Western blot for Notch and p53 expression, β actin was used as houseekeeping loading control. Lower: RT- PCR for Notch3, p53 and β actin on cDNA obtained by the same cells. NC: negative control, cells transfected with HSG empty vector.; NICD3 -: cells transfected with the NICD3 vector but sorted as negative to the fluorescence, not effectively transfected; NICD3+ : cells transfected with the NICD3 vector sorted as positive to the fluorescence, effectively transfected with NICD3 plasmid. **C)** Western blot analysis of HepG2 and Snu398 Notch3 silenced cells. Notch3, p53, p-p53 phsphorylated at Ser20; p-p53 phosphorylated at Ser-15 and β actin as houseekeeping loading control. **D)** Transient transfection of NICD3 expression vector in HepG2 shRNA Notch3 cells. Analyses were performed24 and 48h after transfection. Upper: Western blot for Notch and p53 expression, β actin was used as houseekeeping loading control. Lower: RT- PCR for Notch3, p53 and β actin on cDNA obtained by the same cells. NC: negative control, cells transfected with HSG empty vector.; NICD3 -: cells transfected with the NICD3 vector but sorted as negative to the fluorescence, not effectively transfected; NICD3+ : cells transfected with the NICD3 vector sorted as positive to the fluorescence, effectively transfected with NICD3 plasmid. **E)** Luciferase assay in HepG2 NC and N3

silenced cells. Cells were transfected with p53 responsive plasmid (pp53TA-luc) or the empty vector (pTA-luc) in negative controls and Notch3 Knocked down cells. Luciferase activity was evaluated 24h after transfection and measured as ratio on Renilla activity. Results were reported as ratio between pp53TA-luc on pTA-luc transfected cells for NC and N3.

Notch3 controls Mdm2 phosphorylation status

After having excluded a p53 modification impairing the interaction with its main negative regulator Mdm2, we asked if Mdm2 expression was changed after Notch3 silencing. Analysis of Mdm2 mRNA in semiquantitive PCR showed no significative increased in Mdm2 expression.(fig 23A) Whereas in HepG2 and Snu398 cell line a reduction in Mdm2 total protein level was observed after Notch3 silencing, suggesting the existence of a post-transcriptional regulation that could be causative of less Mdm2 total levels. This may be translated in higher p53 protein levels. At the same time, we performed Notch3 knock down in Hep3B p53 null cells, in order to have a model lacking p53 effect, as Mdm2 is a target gene of p53 (WuX. Et al. 1993). In Hep3B Notch3 knockdown cells (KD), Mdm2 mRNA shows no changes, neither the down-regulation in protein levels observed in HepG2 and Snu398 Notch3 silenced cells (Fig.23A).This evidence suggests that the small increase in Mdm2 mRNA could be p53 dependent. So, the regulation of Mdm2 seems to be both p53 dependent and independent. To explain the discrepancy between Mdm2 mRNA and protein levels in HepG2 and Snu398 cells (Fig 23B) after Nothc3 silencing we analyze miR221 expression in RT-PCR. (Fig 23B) asked if the different pre and post transcriptional regulation occurred by Notch3 could be dependent on microRNAs. As miR221 was recently demonstrated to target Mdm2 mRNA (Dongkyun K. et al, 2010) and it is resulted to be upregulated and involved in oncogenic mechanisms of HCC (Fornari F.et al. 2008), we analysed miR221 levels after Nocth3 KD in real time PCR (Fig2B). The reduction in Mdm2 total protein level could be dependent by post transcriptional regulation of miR-221. Indeed, miR221 level results upregulated in HepG2 and Snu398 Nocth3 KD cells, but it results strongly downregulated in Hep3B cells silenced for Notch3. (Fig 23B) This effect excludes, at least in part, the regulation of miR221 in downregulating Mdm2 protein levels in our model. Indeed, miR-221 increases in HepG2 and Snu398 Notch3 KD cells toghether with Mdm2 protein reduction, but not changes in Mdm2 protein levels was detected in Hep3B Notch3 KD loss miR-221 expression compared to control. Probably, another post transcriptional regulator intervenes in Notch3 silenced cells to induce Mdm2 protein level reduction. The hypothesis of a double transcriptional and post transcriptional control of p53 on Mdm2 levels is consistent with physiological strategy of multiple level of regulation in cell physiology. Post transcriptional changes in regulatory modification of Mdm2 was analysed, looking to Ser166 and Thr216

phosphorylation, that respectively regulate reduction in Mdm2 E3-ubiquitin ligase activity and p53 affinity. (Meek 2004; Mayo and Donner, 2002; (Kruse J-P and Gu W., 2009). In Notch3 knocked down cells a reduced activity associated to Thr216 of Mdm2 was observed in all cell lines, whereas for Ser166 in HepG2 and Snu398 cells. (Fig2C) This suggests a Notch3 control of p53 homeostasis through Mdm2. Phosphorylation in Ser166 results in increased nuclear localization and ubiquitinase activity, instead Thr216 phosphorylation weakens Mdm2 activity, indicated by a reduced binding capability of SMP14 antibody. (De Toledo 2000).

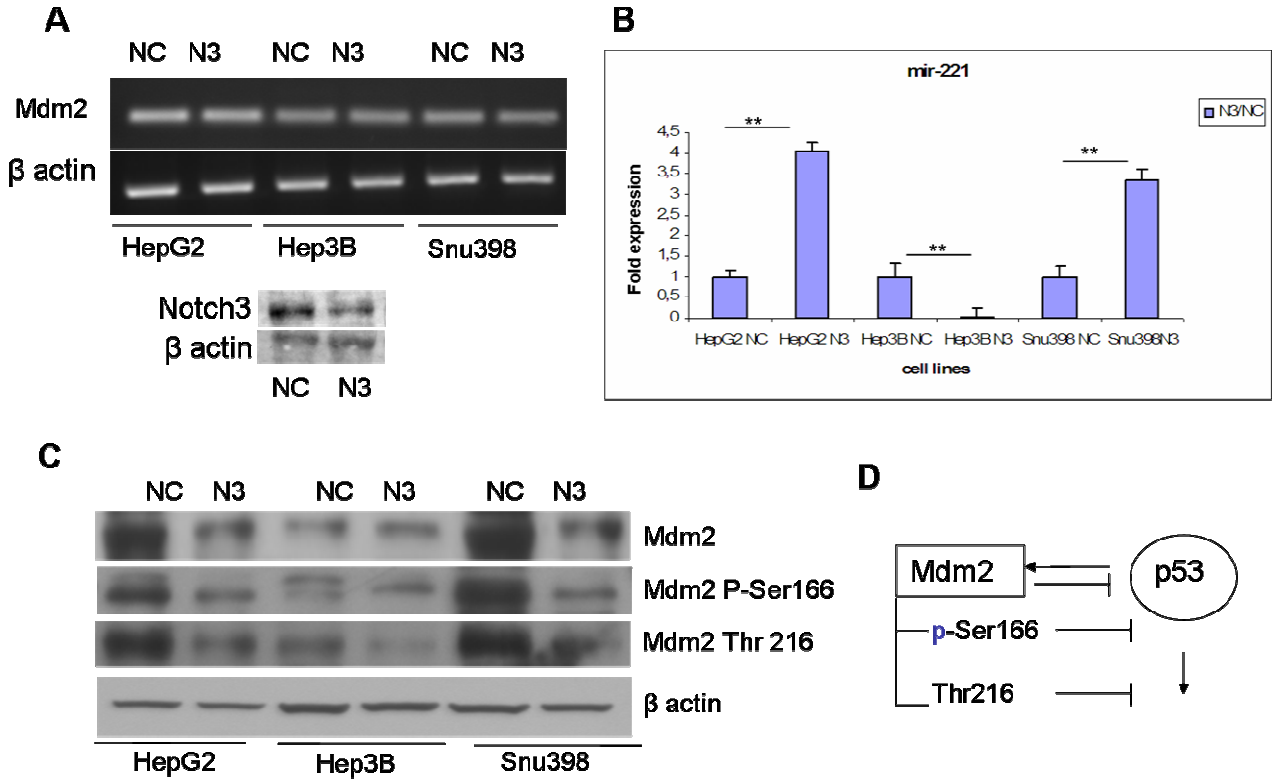


Figure 23: Notch3 regulation on Mdm2 . A) Upper: RT-PCR for Mdm2 on HepG2, Hep3B and Snu398 cells silenced for Notch3. Lower: Western blot for Notch3 in Hep3B cells to verify the effective knock down. **B)** Real time PCR of miR-221 in HepG2, Hep3B and Snu398 cells silenced for Notch3. U6RNA was used as housekeeping. 2(δδCt) of each sample was normalized for the housekeeping and the ratio value N1/NC was displayed. Y axis shows the fold expression in Notch3 silenced cells on negative control. Samples were analyzed in triplicate. Meaning value: columns, standard deviation: bars. Statistical significance obtained by t test is displayed in the graphic. (** P< 0,01). **C)** Western blot for Mdm2, Mdm2 phosphorylated at Ser-166, Mdm2 posphorylation at Thr216 and beta actin used as housekeeping in HepG2, Hep3B and Snu398 cells silenced for Notch3. Mdm2-ser166 antibody is specific for the phosphorylated residue; Mdm2-Thr216 specific antibody (SMP14) recognize only the deposhorylated residue, that is the activated form. NC: Negative control of shRNA; N1: Notch3 shRNA. **D)** Cartoon representing relatives regulation of Mdm2 and p53 with post translational Mdm2 modification and the effect in p53 stability.

Nocth3 regulates p53 and Mdm2 activity through CyclinG1

As the main regulation of the Mdm2 activity and p53 interaction is mediated by phosphorylation (Henning W. et al. 1997; Blattener C. et al. 2002; Meek D.W. and Knippschild U.), we hypothesize that Notch3 may alter the functionality of Mdm2 regulatory kinases or phosphatases, as the phosphorylation status is the result of an equilibrium between them. These two residues Ser166 and Thr216 are known to be both regulated by PP2A serine/threonin phosphatase, which activity is regulated by Cyclin G1, that binds the enzyme and mediates its interaction with Mdm2 (Okamoto K. et al. 2002). Moreover, Cyclin G1 is a specific regulator of p53 turnover and it exerts its function modulating Mdm2 activity acting as scaffold protein for PP2A that could targets many sites in Mdm2 regulatory domains (Meek D. W. and Knippshild U., 2003). So, we asked if Cyclin G1 could be responsible of p53 stabilization together with the modified status of Mdm2 residues analyzed, despite an opposite effect of the phosphorylation status was known for Ser166 and Thr216 residues, respectively activating and inhibiting Mdm2 activity. In Notch3 KD cells, Cyclin G1 protein level results to be downregulated (Fig 24A) partially in agreement with the reduced activity of Mdm2, as evident by an increased phosporylation at Thr216 residue. This last condition is traduced in a higher affinity for the SMP14 antibody that is demonstrated to loss Mdm2 binding capability when the residue Thr216 became phosporylated (Zhang T. and Prives C. 2001). Conversely, the reduced Ser166 phosphorylation of Mdm2 in Notch3 silenced cells was not dependent by CyclinG1 effect (Fig23C-24A). Both post transcriptional modifications of Mdm2 analyzed in Notch3 silenced cells, sustain a reduction of Mdm2 inhibitory effect on p53, together with a reduced CyclinG1, that in the accepted model, directly and/or indirectly exerts an inhibitory effect on p53, despite Ser166 is not dependent by Cyclin G1 (Kimura SH. et al. 2002). Interestingly, Cyclin G1 mRNA level results strongly up-regulated after Notch3 knockdown in HepG2 and Snu398 cells, suggesting a concomitant post-transcriptional mechanism that regulates protein translation. Conversely, in Notch3 Hep3B silenced cells, a parallel reduction in messenger and protein level was detected. A known regulator of Cyclin G1 in HCC is miR-122, that targets Cyclin G1mRNA and it is overexpressed in HCC (Fornari F. et al. 2009). We hypothesize that Notch3 mediated variation of its expression may explain the reduction in CyclinG1 translation despite the higher mRNA levels. Neverthless, real time PCR for miR-122 in Notch3 knock down cells reveals reduction in miR-122 after Notch3

silencing cells compared to controls in HepG2 and Hep3B cells, suggesting that it could not be responsible of the reduced protein expression. (Fig24B)Moreover, despite a similar behaviour in CyclinG1 setting, Snu398 did not express miR-221, suggesting that it is not involved in GyclinG1 negative regulation after Notch3 silencing(Fig.24B). Then, as Cyclin G1 is one of the first identified p53 target gene (Okamoto K. and Beach D., 1994), maybe the higher mRNA level could depend on transcription mediated by increased amount of p53. Moreover, CyclinG1 mRNA resulted upregulated in Notch3 knock down cells except for Hep3B p53 null cells, suggesting that the increase in mRNA was p53 dependent. Indeed, the transient silencing of p53 in HepG2 cells reduces cyclinG1 and Mdm2 mRNA levels, suggesting that p53 in this contest regulates transcriptionally its negative regulators in a feed-back loop. (Fig. 24C) Probably, the effect observed after Notch3 knock down is due to the higher amount of p53 available for target gene transcription, than to changes responsible of transcriptional p53 increased activity.

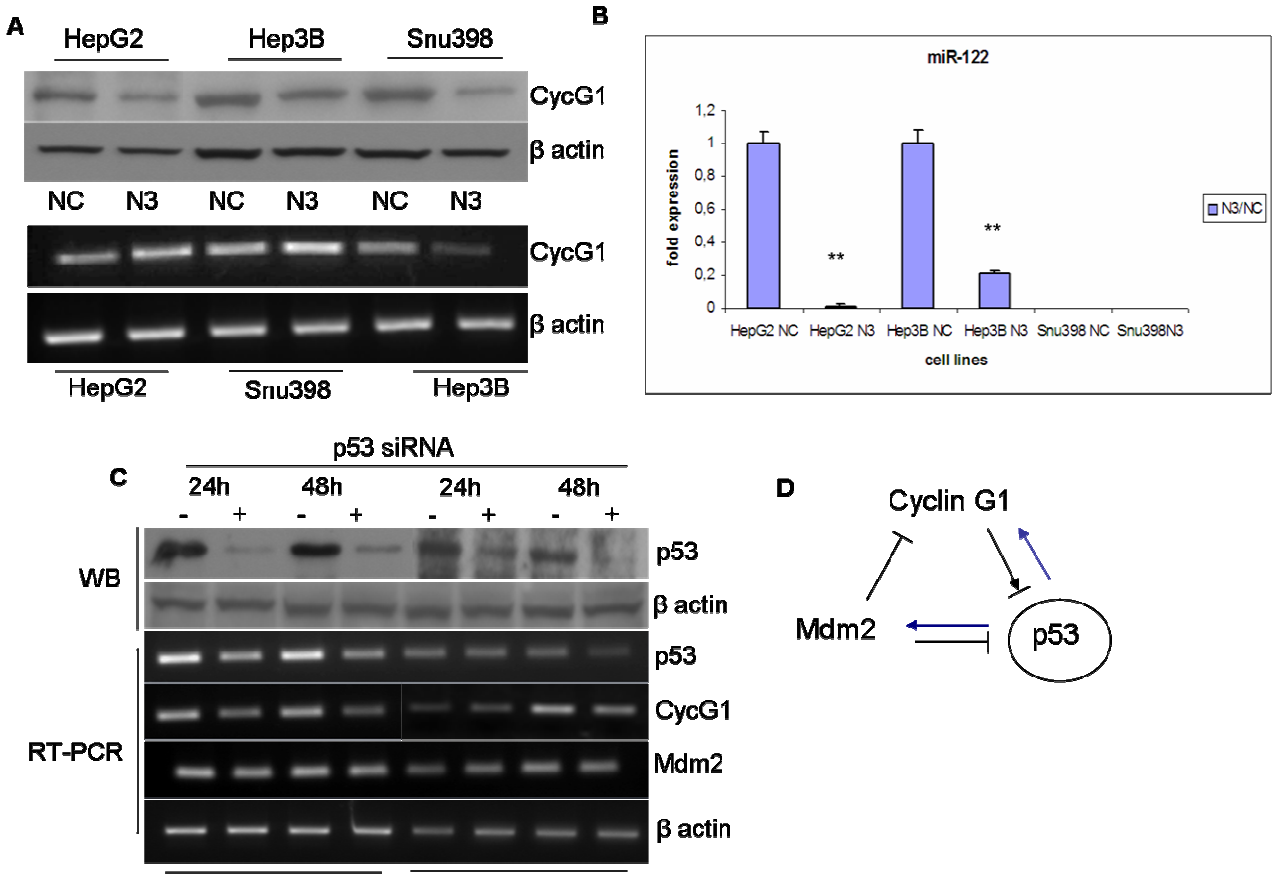


Figure 24: Notch3 regulates CyclinG1 protein, toghether with a feed back loop mediated by p53. A) Western blot analysis for CyclinG1 in HepG2, Hep3B and Snu398 Notch3 silenced cells. CyclinG1 protein results reduced after Notch3 depletion, respectively of 44%

in HepG2, 8% in Hep3B and 52% in Snu398 cells. NC: Negative control of shRNA; N1: Notch3 shRNA. **B)** Real time PCR of miR-122 in HepG2, Hep3B and Snu398 cells silenced for Notch3. U6RNA was used as housekeeping. 2(ΔΔCt) of each sample was normalized for the housekeeping and the ratio value N1/NC was displayed. Y axis shows the fold expression in Notch3 silenced cells on negative control. Samples were analyzed in triplicate. Snu398 cells did not express the miR-122 Meaning value: columns, standard deviation: bars. Statistical significance obtained by t test is displayed in the graphic. (** P< 0,01).**C)** p53 transient silencing in HepG2 cells and Snu398. Cells were analysed 24h and 48h after transfection. Western blot analysis for HepG2 and Snu398 Notch3 silenced cells to asses p53 silencing. RT-PCR for p53, CyclinG1 and Mdm2 after p53 transient knockdown. Beta actin was used as housekeeping for western blot and in RT-PCR. CycG1: Cyclin G1. **D)** Cartoon representing known reciprocal regulation of p53, Mdm2 and CyclinG1

To further define the role of CyclinG1 reduction in Notch3 knock down cells, a transient silencing of Cyclin G1 was performed in HepG2 and Snu398 cells and mRNA silencing was verified with RT-PCR (Fig. 25A). Five hours post transfection, the effect on p53 stability was already evident, with a strong upregulation of protein levels, that are maintained until 48 h after transfection, suggesting that the absence of Cyclin G1 in the regulatory p53 complex, (Otsuka T. et al. 2003; Zhao L. et al. 2003) constitutes a crucial event in inducing p53 stability. Conversely, p53 mRNA levels did not change, confirming the expected post transcriptional regulation. The main interesting effect is the regulation on Mdm2, that results to be more phosphorylated at Thr216 residue (lower SMP14 binding) quickly after Cyclin G1 silencing (5h), whereas it returns in a less phosphorylated condition than the controls at 11h, 18h, 24h and 48h. The restoration of a more dephosporylated status of Thr216 after cyclin G1 silencing could be a compensatory effect given by the activity of the kinase responsible of the Thr216 phosphorylation, probably independent by the presence of CyclinG1. Neverthless, it also might be a feed back loop consequent to the upregulation of p53 that occurs 8h after transfection. Indeed, the absence of the dephosphorylating effect due to lost Cyclin G1 mediated interaction between Mdm2 and PP2A is immediately evident, whereas after 11h Mdm2 phosphorylation at Thr216 rescue to levels higher than basal ones, (Fig25B). Despite the p53 mediated feed back loop evident in cells that loose Cyclin G1 expression in direction of a restoration of low level of phosphorylation in Thr216, Notch3 HCC silenced cells showed a maintenance of the modified status. This maybe dependent by the presence of post transcriptional regulator on CyclinG1 homeostasis, that might contrast the feedback loop effect, probably consequent to high p53 cellular levels. Futhermore, this maintenance maybe depends by the nature of the effect that is constant despite the temporal effect of transient silencing.

The phosphorylation at Ser166 residue of Mdm2 is however always upregulated after Cyclin G1 silencing, suggesting an effective regulation through a loss of the dephosphorylating responsible factor, PP2A. (Fig25B) This data confirms that in Notch3 silenced cells this inactivating variation on Ser166, that is less phosphorylated, it is not mediated by CyclinG1, suggesting that another factor may be involved in this control mechanism. Moreover, the increase in Ser166 suggests also a reduced Mdm2 autoubiquitinatory activity but total Mdm2 levels did not show variations. (Fig25B) Despite variations in Mdm2 activity suggested by these post transcriptional regulation, the strong effect on p53 stability consequent of cyclin G1 silencing, suggests 1) the presence of CyclinG1 in the complex of regulation like a condition *per se* for a correct turnover of the protein or 2) that Cyclin G1 may regulate other functional Mdm2 modifications in order to determ p53 stability. 3) Probably, also a direct interaction of p53 and CyclinG1 occurred independently by Mdm2 (Zhao L. et la. 2003). 4) This evidence also suggests that maybe only Thr216 increased phosphorylation could mediate p53 stabilization of p53.

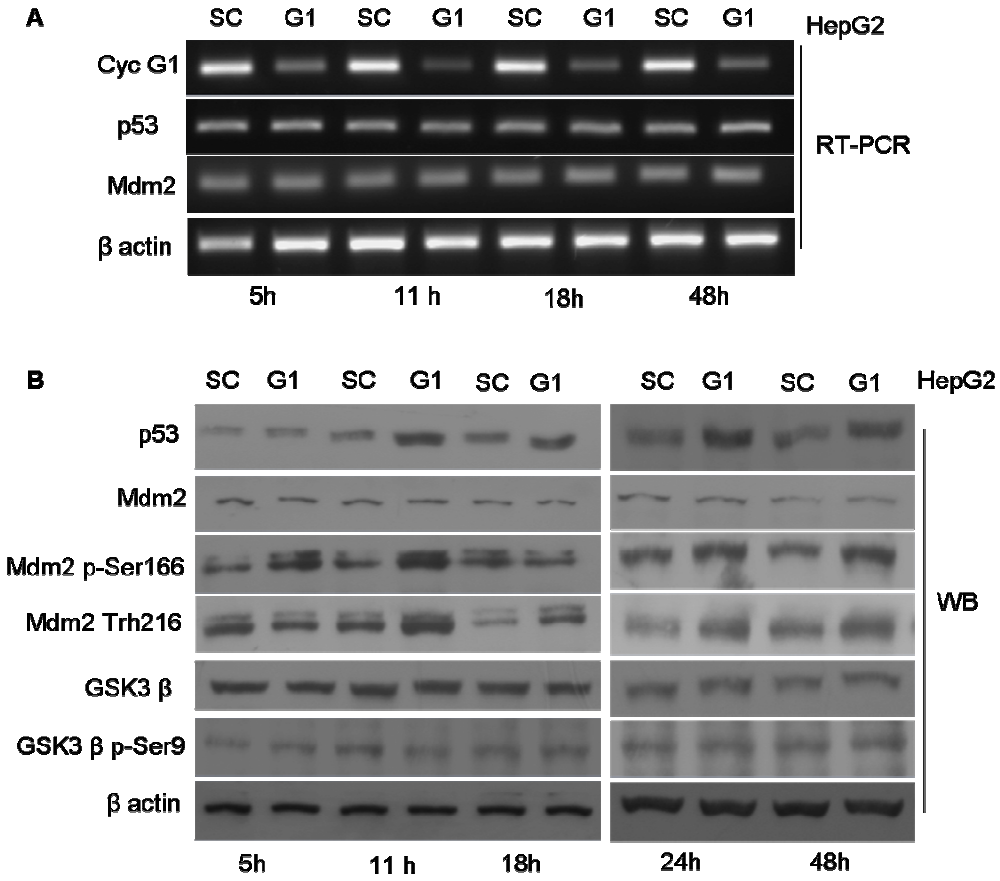


Figure 25: CyclinG1 transient silencing stabilizes p53 and modifyes Mdm2 phophorylations. A) RT-PCR for Cyclin G1 siRNA in HepG2 cells. PCR was conducted for CyclinG1, p53, Mdm2 at 5-11-18-48h after silencing. **B)** Western blot analysis for CyclinG1

siRNA in HepG2 at 5-11-18-24-48h after siRNA tranasfection. Western blot was conducted for p53, Mdm2, Mdm2 phosphorylated at Ser 166, Mdm2 dephosphorylated at Thr 216 (SMP14 antibody); GSK3 β, GSK3 β hosphorylated at the inactivating Ser9 and beta actin as housekeeping. SC: scramble; G1: siRNA against human CyclinG1 RNA messenger.

Mdm2 activity is not regulated by Notch3 through known kinases acivity

Phoshorylation is a fine modulating molecular mechanism that regulates biological processes that is the result of a balance between kinases' and phosphatases' activity and many kinases binds and can phosphorylates Mdm2 at its serines and threonines residues (Meek D. W. and Knippshild U., 2003). So,t o explain Mdm2 post-trascriptional modifications in Ser 166 and Thr216 in Nocth3 silenced cells that may explain the constant higher p53 levels of these cells, we supposed that they could be dependent by Notch3 regulation in activity or expression of kinases. Mdm2 has been demonstrated to be activated by PI3K/Akt pathway, through Ser166 phosphorylation by Akt (Zhou B. P. et al. 2001) and evidence in T-ALL leukaemia of a Notch1 regulatory loop on p53 passing through Akt and Mdm2, further sustain this hypothesis (Palomero T. et al. 2007;Guo W. et al. 200). For that we analysed levels of phoshoAkt (Ser 473), the active form of the kinase, after Notch3 silencing. No variations were observed, suggesting that it is not responsible of altered Ser166 status in these cells. (Fig 26A). Moreover, it has been reported that in hepatocytes, this phosphorylation of Mdm2 is not mediated by AKT but by MEK-ERK pathway (Malmlof M. et al. 2007). Despite this tissue specific regulation, also phospho-ERK (Tyr204) seems not to be changed in activity by Notch3 expression (Fig 26A).

Then, a recent work showed that in HCC the hepato-specific miRNA-122 downregulates GSK3β, we asked if in our Notch3 KD cells in which miRNA-122 is downregulated (FIG3), GSK3β resulted increased in total levels and we hypothesize that it could be responsible of changes in Mdm2 phosphorylation status (Zheng C. et al. 2010). Mdm2 Ser166 neither Thr216 seems to be theorycal GSK3β consensus sites (S/T–XXX-S/P) belonging to NetPhosK analysis (http://www.cbs.dtu.dk/services/NetPhosK/). Neverthless, also residues outsides this consensus site have been demonstrated to be phosphorylated by GSK3β, that however binds Mdm2 and phosphorylates residues in the acidic domain, supporting the model that Mdm2 is a physiological substrate of GSK3β (Dhiel A.J. et al. 1998; Kulikov R. et al. 2005). Despite reduction in miRNA-122 levels, western blot of total GSK3β showed no variations after Notch3 silencing, but despite no differences in activity of AKT, that is the kinase physiologically known to inhibit GSK3β, (Shaw M. et al. 1997) levels of active GSK3β resulted downregulated in Notch3 absence (Fig 26A). Indeed, GSK3β staining

evaluated through a Ser-9 specific antibody that is the inhibitory modification of the kinase increased in Notch3 knockdown cells, suggesting its possible role in regulating the reduced Mdm2 phosphorylation condition.

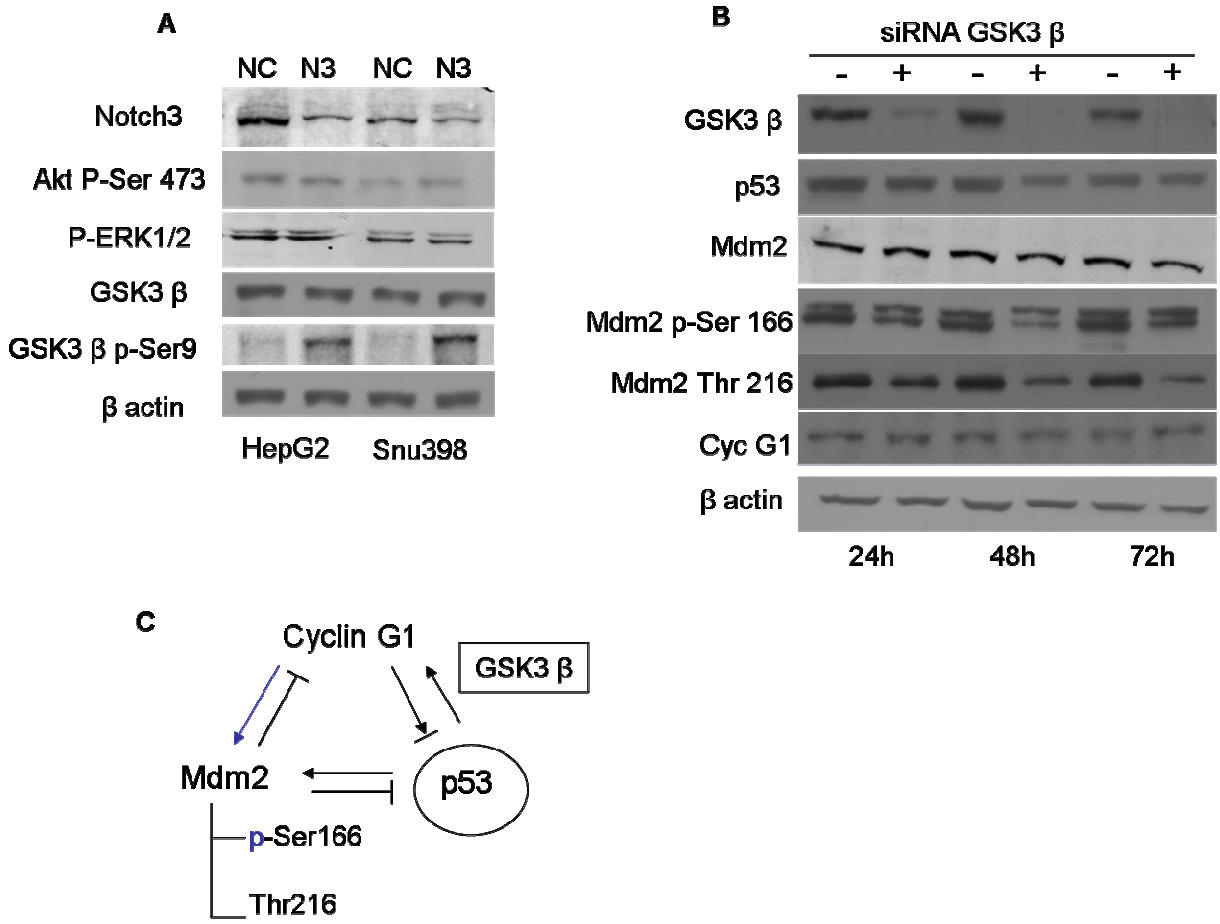


Figure 26: Mdm2 changes in phosphorylation after GSK3 β silencing. A) Western blot analysis for GSK3 β siRNA in HepG2 cells at 24-48-72h after siRNA tranasfection. Western blot was conducted for GSK3 β to asses the interference, p53, Mdm2, Mdm2 phosphorylated at Ser 166, Mdm2 dephosphorylated at Thr 216 (SMP14 antibody), CyclinG1 and beta actin as housekeeping. -: scramble; +: siRNAs pool against GSK3 β human messenger.

GSK3β regulates Mdm2 phosphorylation in Ser166 and Thr216 residues and alters p53 stability

In order to verify if GSK3β activity could modifies Mdm2 phosphorylation at both residues changed in Notch3 silenced cells, we performed a transient silencing of GSK3β in HepG2 cells. Surprinsingly, loss of GSK3β expression determs reduced phosphorylation at Ser166 of Mdm2, whereas the total Mdm2 protein remains unchanged. (Fig26B) This results may suggests a GSK3β activity in directly phosphorylates Ser166. Conversely, Thr216 phosphorylation results unexpectedly increased after GSK3β kinase inhibition, as showed by strong reduction in the SMP14 antibody detection. (Fig 26B). This last modification may

depend by an indirect effect of GSK3β on a molecules involved in Thr216 regulation, such as a phosphatase. Despite the opposite effect in terms of phosphorylation condition, both modifications suggest a less active Mdm2 in p53 binding and degradation as we observed in Notch3 silenced cells. Neverthless, despite changes in potential Mdm2 activity, p53 results strongly downregulated. This evidence is contradictory not only with Mdm2 conditions, but also with experimental evidences reporting the prevented degradation of p53 after GSK3β inhibition (Kulikov R. et al. 2005). The opposite effect of Mdm2 inhibited activity and reduced p53 level after GSK3β silencing on one hand and the increased Mdm2 activity in CyclinG1 siRNA cells, accompanied by p53 increase on the other, could be explained by a collaborative effect. GSK3β and CyclinG1 seems to both act in p53 stabilization and Mdm2 modulation. Moreover, a compensatory effect in Mdm2 modulation consequent to modification in protein silencing could explain the observed conditions, together with an additive element of direct interaction between GSK3β and CyclinG1 with p53 (Otsuka T. et al. 2003).

So, in order to better reproduce the molecular conditions observed after Notch3 depletion, a co-transfection of both CyclinG1 and GSK3β siRNA was performed. (Fig 27) The concomitant loss of functions of two the proteins in p53 regulation could explain prevalent aspects that determ the observed phenotype that is causative of p53 increased levels. Indeed, after effective silencing at 24h Mdm2 resulted less phophorylated at Ser 166, suggesting a reduced activity. Neverthless, after 48h levels Mdm2 seems to have a compensative effect, being higher phosphorylated than the control. Moreover, also Mdm2 Thr216 has an opposite performance in the two timepoints. It results less dephosphorylated (reduced SMP14) at the firs time, so reducing its ubiquitinase activity, and it display a strong compensatory effect in the second time. Neverthless, the combination of the opposite effect on Mdm2 by CyclinG1 and GSK3β as assessed by their respective depletion, seems to be compensated and traduced in a first prevalence of GSK3β regulatory effect and a subsequent CyclinG1's prevalence. Indeed, the effect on the stability of p53 is traduced in a first reduction in p53 levels, followed by strong increased in the second timepoint (48h). This suggests that the effect due to the reduced Mdm2 activity at both phosphorylation sites obtained by double silencing is evident in a second time on the stabilization of p53 protein. GSK3β and CyclinG1 seem to regulate the phosphorylation and the dephosphorylation of Mdm2 at these sites, suggesting that the final outcome of their opposite role is the result of reciprocal contributions. Neverthless, the analysis of CyclinG1 levels after GSK3β silencing and the evaluation of GSK3β total and inactivated level after cyclinG1 silencing was performed. (Fig27)FIG In both cases there were

not variations. Despite a possible effect due to stable altered condition in shNotch3 cells compared to transient effect in double silenced cells, CyclinG1 showing a strong effect on stabilizing p53 seems to be important to regulate protein levels directly. Moreover, GSK3β seems to have a direct effect on stabilization of p53 that seems to be more relevant than Mdm2 regulatory activation. In addition, about GSK3β regulation of p53 it as been defined not only a GSK3β inhibitory effect in stability fully mediated by Mdm2 and the GSK3β mediated phophorylations in Mdm2 acidic domain, but also a direct regulation of GSK3β on p53 that is dependent on GSK3β activity (Kulikov R. et al. 2005; Watcharasit P. et al. 2003).
For that, we hypothesize that despite the lower activity of Mdm2 in GSK3β silenced cells, the different condition between the absence of the protein in GSK3β silenced cells compared to the presence of GSK3β inhibited in activity may explains the effect on p53 stability. Indeed, GSK3β phosphorylated at Ser 9 by Lithium Cloride (LiCl) treatment has been demonstrate to increase GSK3β interaction with p53 and its stabilisation (Watcharasit P. et al. 2003). The condition of GSK3β phosphorylated at Ser 9 and inhibited in activity is correspondent to the situation observed in Notch3 silenced cells and it might reproduce their phenotype. In order to better define GSK3β role in p53 stabilization in our model, treatment with Lithium Cloride 20uM was performed on HepG2 cells, as it was demonstrated to inhibit GSK3β phosphorylating its Ser9 residue (Kirshenboim N. et al. 2004). Cells were treated with LiCl for 2-5-8 h and 24h, in order to analyze p53 changes that are rapids and have a correct kinetic of molecular changes. After GSK3β inhibition, assessed by increase in Ser 9 phosphorylation, p53 resulted stabilized, toghether with a decreased dephosphorylation condition at Thr216, as observed after GSK3β silencig, whereas a reduction in Ser166 phosphorylation was evident only immediately after treatment, followed by no significative changes in this modification (Fig6).
Then, GSK3β inhibition might explain Notch3 silenced cells phenotype only partially in terms of mediating the Mdm2 Thr 216 increased phosphorylation and p53 stabilization that seems to be dependent by a direct effect of GSK3β on p53, independently in part by Mdm2. Indeed, only Mdm2 reduced activity observed after GSK3β siRNA seems not sufficient to induce p53 increased stability. Also Mdm2 reduced ubiquitination capability (SMP14) after LiCl exposure is not sufficient to explain p53 upregulation.

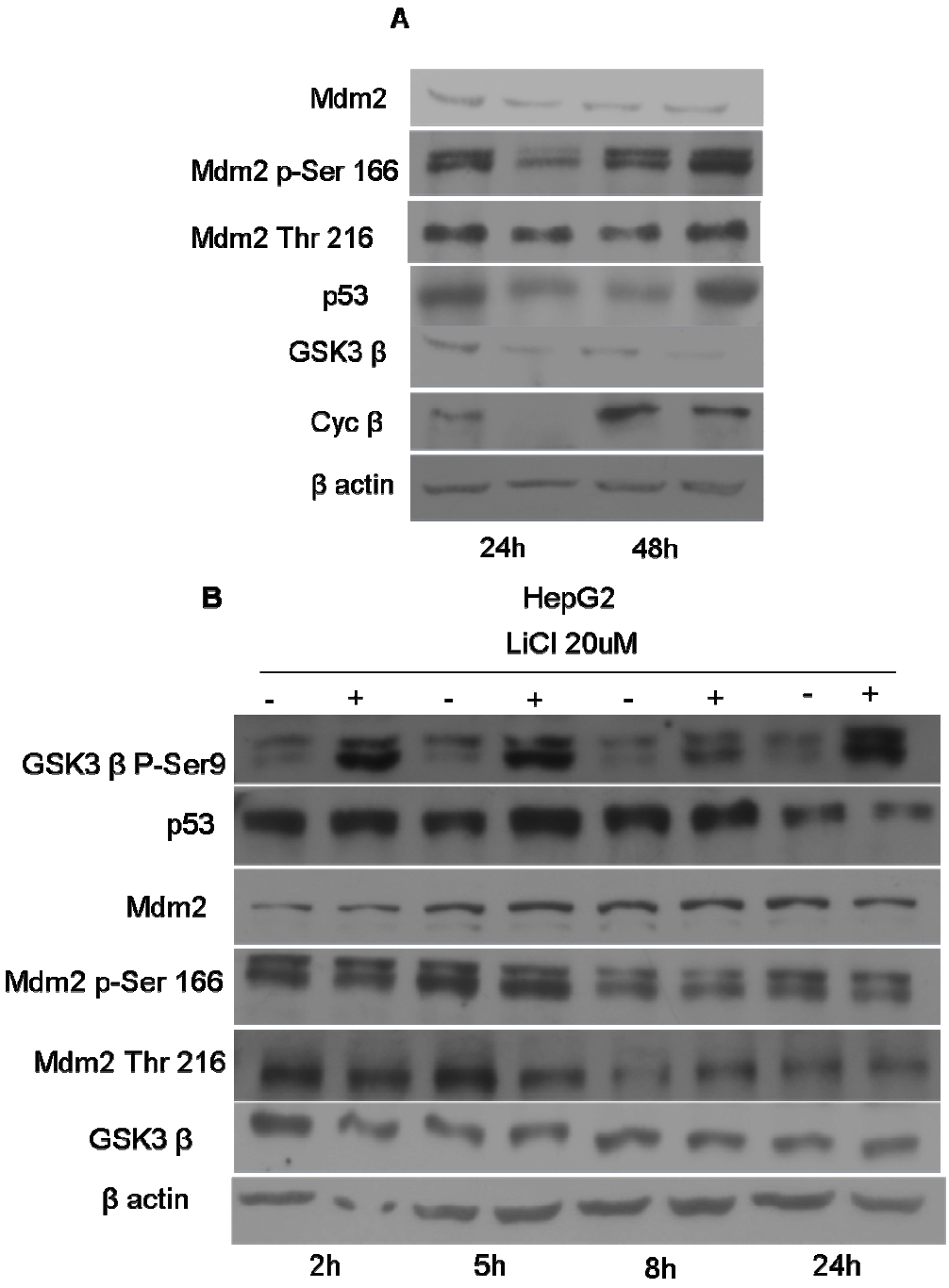


Figure 27: GSK3 β inibition stabilize p53. A) Western blot analysis for siRNA of CyclinG1 and GSK3β in HepG2 cells at 24 and 48h after transfection. Western blot was conducted for GSK3 β and CyclinG1 to asses the effecitve protein inhibition, p53, Mdm2, Mdm2 phosphorylated at Ser 166, Mdm2 dephosphorylated at Thr 216 (SMP14 antibodyand beta actin as housekeeping. **B)** Western blot analysis for Lithium Cloride treatment in HepG2 cells at 2-5-8-24h. Western blot was conducted for GSK3 β P-Ser 9 to asses the inhibition, p53, Mdm2, Mdm2 phosphorylated at Ser 166, Mdm2 dephosphorylated at Thr 216 (SMP14 antibody), GSK3 β and beta actin as housekeeping. -: scramble; +: LiCl 20uM.

Ex vivo analysis : Notch3 correlates with CyclinG1 expression and GSK3β activation

To have further tools to dissect Notch3 and p53 relationship in HCC, an *ex vivo* analysis was performed. It was prevalently directed into establish a correspondence between variations observed *in vitro* after Notch3 silencing, and an effective relation between Notch3 and the elements of p53 regulation *in vivo*. We firstly analysed CyclinG1, due to its attested association in HCC in vivo with cancer progression and invasion capability (Jensen M.R. et al. 2003; Wen W. Et al. 2012), and to the strong evidence on p53 stabilization *in vitro*.

The analysis was conducted in 35 HCC surgical samples by western blot for Notch3 and Cyclin G1 expression. A positive correlation between Notch3 and Cyclin G1 was found.(Fig) In addition, the effective regulation of Notch3 in GSK3β activation has been verified, suggesting that the in vivo setting reflects in vitro data. Indeed, a inverse correlation between Notch3 and GSK3β was assessed in the same patients.(Spermann $\rho=0.666$ $p<0,01$). Neverthless, p53 evaluation, necessary to confirm the effectiveness action of CyclinG1 and GSK3β, is already in progress, as it is conducted only for samples carrying p53 wild type assessed by sequencing. Moreover, Mdm2 will be evaluated after having assessed the prevalent regulatory aspect in term of total expression or post translational modification that affect p53 stability in Notch3 depedent setting.

Secondly, it was conducted a study on an *in vivo* model of HCC, that mimics human carcinogenesis and that could be a useful model to study p53 post translational regulation, as DENA carcinogenesis rarely induce p53 mutation. Carcinogenesis was induced on 12 wistar rats using DENA and cancer tissues were analyzed for protein expression. A positive correlation between Notch3 and CyclinG1 expression was observed, suggesting that also *in vivo* Notch3 could act on CyclinG1. Neverthless, this preliminary evidence requires further analyses on the other supposed actors of p53 regulatory pathway linked to Notch3.

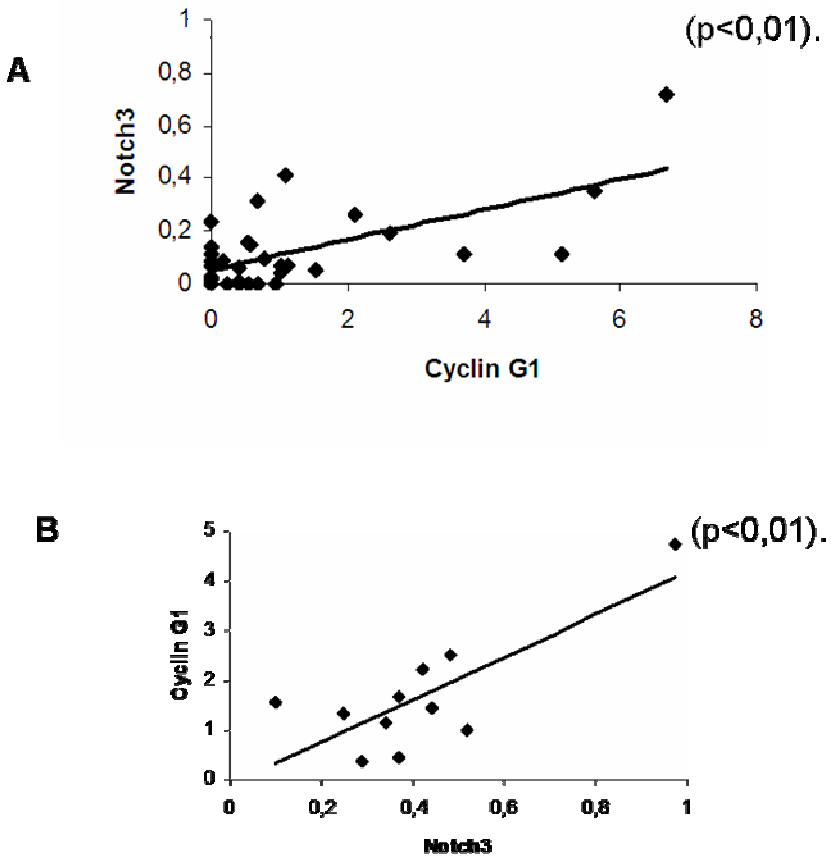


Figure 28: Ex vivo analysis shows correlation between Notch3 and CyclinG1 expression. **A)** Notch3 and Cyclin G1 expression evaluated in western blot in 35 HCC samples. A significative correlation was verified with Pearson's test with (p<0,01).**B)** Notch3 and Cyclin G1 expression evaluated in western blot in 12 samples of liver cancer obtained by wistar rats after induced carcinogenesis. A significative correlation was verified with Pearson's test with (p<0,01).

DISCUSSION 3

Notch signalling has been demonstrate to be involved in a wide range of cellular events, such as apoptosis, cell death, survival and differentiation, showing to be a pathway that could decide the fate of cells (Artavanis-Tsakonas S. et al. 1999). This strong capability in physiological conditions, makes it more important in human pathologies in particular in cancer, in which its deregulation could be translate in different outcomes in terms of oncogenesis or tumour suppressive functions, the possible crosstalk with p53 become crucial. Some evidence of links and reciprocal regulations of Notch1 and p53 were already known, such as Notch1 controls p53 in T-ALL leukemiogenesis and lymphomagenesis, in which Notch1 controls p53 activity through post translational networks, through PI3K/Akt/PTEN and ARF/Mdm2 pathways (Palomero T. et al. 2008; Guo W. et al. 20008; Uren A. G. et al. 2008; Dotto G.P. , 2008; Beverly LJ et al. 2005). Moreover, a direct negative regulation of p53 activation is carried out by NICD1 directly, binding and inhibiting p53 phosphorylation (Kim S. B: et al. 2007). Otherwise some Notch pathway's components were involved in p53 regulation, such as Numb, that increases stability of p53 and acts as tumour suppressor factor (Colaluca N.I. et al. 2007). Neverthless, previously only our work has reported the existence of a regulation between Notch3 and p53 (Giovannini C. et al., 2009). The relevance of this crosstalk is due to the key role of p53. P53 has been called "cellular gatekeeper" or "guardian of the genome" (Levine A.J. 1997; Lane D.P. 1992), due to its role in coordinating the cellular response to a broad range of cellular stresses, acting as node in organizing whether the cell answer with apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism or autophagy (Kruse J-P and Gu W., 2009). P53 is a transcription factor and it controls many effects through transcriptional regulation of different target genes, nevertheless some of these p53 effects are independent from transcription. The main level of p53 regulation is instead characterized by an array of post-translational modifications, both in normal homeostasis and in stress conditions. Despite some post transcriptional regulatory mechanism are known, such as miRNAs (Vilborg A. et al. 2010), this level of regulation is overlapped by post translational modifications, that let to quickly and fine tuning p53 stability and activity. Indeed, more than 36 different amino acids of p53 have been shown to be modified, altering its functionality and its interaction with its regulators (Kruse J-P. and Gu W., 2008 snapshot). Moreover, the six C-terminal lysines of p53 were identified as predominant sites of ubiquitination and regulation by Mdm2, the major E3 ubiquitin-protein that effectively

regulates p53, modifying activity and inducing proteosomal degradation (Haupt Y. et al.1997; Honda R. et al. 1997; Kubbutat et al. 1997). But despite the first idea that p53's phosphorylation sites and lysines constitutes the main molecular strategy for p53 stabilization, many studies indicates that in vivo p53 stabilization involves many different mechanisms, for example finding that Mdm2 is able to degrade p53 also in absence of known ubiquitination sites (Krummel K. A. et al. 2005). For that, the model has to shift to a grater consideration of multiple layers of Mdm2 regulation, and to an understanding of how p53 is stabilized in different tissues and after different stress signals (Kruse J-P and Gu W., 2009).

In this work, we would like to define the molecular events undergoing p53 stabilization after Notch3 depletion, to dissect which is the pathway affected by Notch3 to reduce its activity in HCC.

These regulatory strategies became more important in tumours in which p53 mutation does not occur at the beginning of carcinogenesis, or in which p53 is still wild type during cancer progression. This is the case of HCC, in which p53 mutation frequency vary from 0 to 70% (Osada S. et al. 2004; SoiniY. Et al. 1996) and its mutations seems to occur later in carcinogenesis (Ng I.O. et al. 1995). In this contest, alterations of p53 stability let cancer cells to overcome its tumours suppressive function and in condition of wild type p53 these mechanisms became more important to be understood. (Brooks L.C. and Gu W., 2006).

Due to the nature of the signalling, in dissecting Notch3 regulatory control on p53 we looked if the transcriptional one might be involved. mRNA quantization in real time PCR showed a negative regulation on p53 transcription by Notch3 expression, that might depend by direct or indirect NICD3 activity. Indeed, through the activation of a transcriptional repressor target gene or through RBP-Jk mediated transcription, Notch3 may regulate p53 transcription. Indeed, despite RBP-Jk acts as transcriptional activator, evidence of a competition of RBP-Jk on a regulatory sequence at the promoter of p53, identified a reduced p53 transcription directly dependent by RBP-Jk binding (Boggs K. et al. 2009). Neverthless, in presence of NICD, RBP-Jk might become an activator of transcription, despite the binding sites is recognized and bound by another transcriptional factor, C/EBPβ-2, that activates p53 transcription (Boggs and Reisman, 2006, 2007). In this context, if RBP-Jk could not initiate transcription after Notch binding, it acts as transcriptional repressor as competitor in the binding site. Despite hypothesis on transcriptional regulation, the increased in mRNA is significatively lower than the increase of the protein. The overexpression of a NICD3 activated form of the protein, strongly downregulates p53 protein expression in HepG2, supporting the negative regulation of Notch3 on p53, but at messenger level, small variations

were observed, suggesting that the control is mostly post-transcriptional. Indeed, due to its functional roles, a post-translational regulation makes sense, because p53 activity is required immediately after inducing stimuli and the de novo protein synthesis would delay cellular response (Blattener C., 2008). Neverthless, a promoter study will be performed, in order to find binding sites for RBP-JK and for known transcriptional repressors that are Notch3 target genes and that may mediate Notch inhibitory effect on transcription. A luciferase reporter test will be useful to define repressive or activating function of RBP-JK in this context, together with a Chromatin Immunoprecipitation to demonstrate the binding. Despite different kinetics of stabilization dependent by kind of cellular stressors and mechanisms giving p53 stabilization, they seem all to converge to Mdm2 protein. Indeed, the short p53 half life of less than 20 minutes (Blattener C. et al. 2002) is augmented by changes in p53 post transcriptional modifications that alter its interaction with Mdm2, together with altered Mdm2 functionality through hypophosphorylation or increased degradation due to auto-ubiquitination (Kruse J-P and Wei G. 2009; Blattener C. et al. 2002; Stommel J.M. and Geoeffrey M. W.) .

So, the first step in p53 stabilization was thought to be N-terminal protein phosphorylation in sites Ser-15 (Chehab et al. 2000; Shieh et al. 1997; Tibbetts et al. 1997) and Ser-20, that stabilize the protein preventing its interaction with Mdm2 (Chehab et al. 1999; Unger et al. 1999). We firstly asked if the higher p53 levels consequent to Notch3 silencing was dependent from that. We observed that after Notch3 silencing p53 resulted upregulated, but not changed in Ser-20 and Ser15 phosphorylation, indicating a possible interaction of p53 with its negative regulator. Also luciferase test to asses p53 transcriptional activity, showed that Notch3 silencing does not alter p53 capability to activate target genes transcription, in agreement with our previous data in which the increased in apoptosis observed in Notch3 silenced cells was present only after stress stimuli (Giovannini C. et al. 2009). As p53 showed inaltered capability to bind Mdm2, we thought that Mdm2 could be negatively regulated by Notch3, giving increased p53 level. Mdm2 resulted downregulated after Notch3 silencing in total levels, suggesting that a reduced protein quantity could reduce the negative regulatory effect. This condition is associated with the concomitantly regulation of mir221 by Notch3, whose increase cause a reduction in Mdm2 protein level targeting the higher mRNA levels, probably consequent to p53 mediated transcription, confirmed by p53 transient silencing in HepG2 cells that have a wild type p53 able to induce target gene of its regulators, like Mdm2 and CyclinG1. Nevertheless, the reduction in Mdm2 activity levels might also be dependent by an autoubiquitination process. The balance it between auto ubiquitination and substrate

ubiquitination is done by post-translational modification such as phosphorylation, that is the main layer of regulation of numerous sites and regulates many aspect of Mdm2 activity (Meek D. W. and Knippshild U., 2003). Even if Mdm2 autoubiquitination is a molecular strategy that involve DNA damage kinases and occurs in this stress conditions (Stommel J.M. and Wahl G. M.), evidence of regulation of the autoubiquitinatory activity of Mdm2 by Ser166 status has been reported. The increase in phosphorylation reduces Mdm2 autoubitination capability, suggesting that the reduction in total Mdm2 level after Notch3 silencing could be dependent also by this effect. (Feng J. et al. 2004).

In the contest of Notch3 silencing the main probably strategy of Mdm2 regulation is phosphorylation, as the protein have multiple sites that are regulated by kinases and phophatases and fine modulate Mdm2 activity (Meek D. W. and Knippshild U., 2003). Between them, phosphorylation at Ser166 and Thr216 were respectively associated with increased nuclear entry and E3-ubiquitin ligase activity (Mayo L.D. and Donner D. B. 2001; Meek D.W.2004) and reduced p53 binding affinity and increased ARF binding (Zhang T. and Prives C.,2001). Neverthless, binding of ARF, a physiological negative regulator of Mdm2 that alter it capability to bind p53, is modestly increased after Thr216 phosphorylation (Zhang T. and Prives C.,2001;Lowe and Sherr, 2003). Effectively, Ser166 phosphorylation was reduced, whereas Thr216 increased in its phosphorylation after Notch3 silencing. Based on that, ARF binding increase in Notch3 silenced cells could not be excluded in principle. Neverthless, these two Mdm2 modifications are both regulated by Cyclin G1, a regulator of Mdm2-p53 network that seems to be important in HCC tumorigenesis and cancer behaviour (Jensen M. R. et al. 2003; Fornari F. et al. 2009). CyclinG1 is a transcriptional target of p53 that negatively regulates p53 and it has been associated with proliferative capability of uterine, breast, prostate cancer and hepatocellular carcinoma (Baek W. K. et al. 2003; Reimer C.L. et al. 1999¸ Jensen M. R. et al. 2003). It induced cell cycle progression at G2/M, survival and altered p53 kinetics, through interaction with Mdm2, even if hypotheses of direct binding to p53 were introduced. (Kimura S.H. et al. 2002; Kimura S.H. et al. 2001; Piscopo D.M. and Hinds P.W., 2008; Otsuka T. et al. 2003; Zhao L. et al. 2003). The direct interaction of CyclinG1 and p53, in addition to the binding in a trimeric complex with Mdm2 and P53, is sustained by the strong effect on p53 stabilization independently by Mdm2 activity that we observed after its silencing (Zhao L. et al. 2003; Kimura S. et al. 2002). Indeed, CyclinG1 resulted downregulated after Notch3 silencing and probably responsible of changes in Thr216 phosphorylation status, due to its capability to bind and regulate the serine/threonin phosphatase PP2A, which dephosphorylates both residues in vitro and in vivo (Okamoto K. et

al. 2002; Kimura S. et al. 2002). Only Thr216 in Notch3 silenced cells resulted more phosphorylated and it could be translatable in an effect of CyclinG1 and PP2A phosphatase. Neverthless, only 5h after transient cyclinG1 silencing the same change has been detected, despite the longer effect on p53 stability. For that, our present data do not let us to suppose that Thr216 change in Notch3 knocked down cells is dependent by CyclinG1. Conversely, Ser166, that is also PP2A's target, resulted higher phosphorylated after CyclinG1 siRNA, in agreement with the absence of PP2A, but decreased in Notch3 silenced cells, showing that this regulation in our shRNA model could not be dependent by CyclinG1. In addition, the higher Thr216 phosphorylation reduces Mdm2 activity, instead Ser 166 increased Mdm2 degradating capability on p53. So, it seems that CyclinG1 mediate an opposite and controversial effect on Mdm2 and p53, with an outcome that is not clear and that underline a possible condition-depedent function of CyclinG1 in attenuating or increasing Mdm2 activity (Piscopo and Hinds).

Interestingly, it has demonstrated an increased crucial function of CyclinG1 as scaffold protein in Mdm2-p53 complex, indeed, not only cyclinG1 binds and activate PP2A to let its phosphatase activity on Mdm2, (Okamoto K. et al. 2002) but also it mediated ARF- Mdm2 interaction. In absence of CylcinG1, Mdm2 and ARF interaction is not possible, suggesting that ARF inhibitory activity could not be the explanation for the increase p53 levels in our cellular contest of Notch3 silencing (Kimura S.H. et al. 2002).

The alternative way of phosphorylation on Mdm2 by Notch3 is the regulation through inactivation of kinases. Here we showed that Notch3 activity does not impair AKT neither ERK activity, despite previously literature associates Notch3 signalling to crosstalk with PI3K pathway, in particular tissue or tumour contest (Wang T. et al. 2007; Vo K. et al. 2011; Raimondi L. et al. 2011). Despite Akt is the main regulatory kinase of Ser166 (Mayo L.D. and Donner D.B. 2001 Zhou B. P. et al. 2001) and despite a specific regulation of ERK in Ser166 of Mdm2 demonstrated for the liver (Malmlof M. et al. 2006), we did not found variations consequent Notch3 silencing that could justify Mdm2 changes. Instead, an other member of PI3K pathway resulted regulated in activity by Notch3, GSK3β. Notch3 changes the status of phosphorylation at Ser 9 of GSK3β, the inactivating modification of the kinase. (Sutherland et al. 1996) Indeed, this serine/threonine kinase firstly identified in glycogen synthesis pathway, but also implicated in other cellular signalling pathways (Grimes C.A and Jope R.S. 2001), is constitutively active and regulated through inactivation (Cross A.D. et al. 1995). GSK3β was already identified as Mdm2 regulator of the phosphorylation status of two sites in acidic domain of Mdm2, in which the phosphorylation increased Mdm2 degradation

of p53 (Kulikov R. et al. 2005). We found increased inhibited GSK3β after Notch3 impairment and we hypothesize that it could also regulates other sites, being able to bind and interact with Mdm2-p53 complex. Moreover, despite the identification of a consensus sites, GSK3β have showed to be able to modified residues outside of the consensus motif (Diehl J. et al. 1998; Alt J. et al. 2000). So, we test if GSK3β silencing it could justify reduced Ser166 phosphorylation in Noch3 silenced cells, and we surprisingly found a reduced phosphorylated status for Ser166, together with an increased phosphorylation at Thr16, that is translatable to a reduced SMP14 binding affinity. This result is correspondent to Mdm2 status in Notch3 silenced cells and it identifies Ser166 as possible direct or indirect target of GSK3β. Neverthless, the opposite increased Thr216 phosphorylation, that weakens Mdm2 activity, but that is unaspectable after kinase depletion, may suggest an inhibiting regulation of GSK3β on PP2A, that might regulate this site. In our contest it seems to be possible, but only after specific silencing or inactivation of the phosphatase it would be understandable. In this direction, experiments including PP2A evaluation have to be planned, indeed if the regulation of GSK3β is not detected on CyclinG1, the effect of GSK3β on PP2A has to be investigated. Recently, it has been published that GSK3β could negatively regulate PP2A, both reducing its expression and inactivating it through Tyr307 inhibitory phosphorylation, mediated by tyrosine kinase PTP1B (protein tyrosine phosphatase 1B), directly regulated by GSK3β (Yao X.Q. et al. 2011). This identify a possible double regulation of GSK3β, that seems to have an inhibitory effect on the activating function of PP2A/CyclinG1 on Thr216, on the other hand to have an activating function on Mdm2 through increasing Ser116 phosphorylation. Neverthless, in our conditions, the absence of GSK3β protein causes reduced Ser166 and increased Thr216 phosphorylation, that are both inhibitory modulations of Mdm2 and reflect the Mdm2 condition in Notch3 silenced cells. Despite this functional aspect, levels of p53 resulted strongly downregulated, suggesting that GSK3β have a direct stabilizing function on p53 indipendent by Mdm2, or that it regulates other Mdm2 functional residues. Indeed, it has been demonstrated that GSK3β directly bind and regulates p53, with a stabilization and activation effect on p53 through an Mdm2 indipendent mechanism (Whatcharasit P. et al. 2002 PNAS; Whatcharasit P. et al. 2003). Moreover, considering Notch3 silencing that comprises both loss of CyclinG1 and of GSK3β activity, we tried to combine these conditions, in order to understand if a collaborative or reciprocal regulatory effect could explain Notch3 knock down phenotype. Distinguishing each condition, they seem to suggest that both the two Mdm2 modification are not enough to explain p53 stabilization. The double silencing of CyclinG1 and GSK3β reflect at 24h the effect of GSK3β silencing and at 48h

CyclinG1 silencing effect, both on Mdm2 and p53. So, it does not clarify which effect is prevalent, but suggests opposite and compensatory effects due to p53 regulation. Of course, it is possible that the condition observed in Notch3 silenced cells is dependent by the stable CyclinG1 downregulation and GSK3β inactivation, but also an additional function mediated by the presence of GSK3β protein, despite its inactivation but due to its p53 and Mdm2 binding capability, might be important. For that treatment with LiCl was conducted, as it is a GSK3β inhibitor that acts phosphorylating Ser 9 (Kirshenboim N. et al., 2004), and so mimicking the condition of present but inactive GSK3β. Perfoming a treatment in HepG2 cells, we effectively found an increase in p53 protein levels, together with a reduced Mdm2 activity for Thr216, as we observed in GSK3β, further confirming that this kinase might participate in its regulation. However, no significative changes in Ser166 phosphorylation were observed as was after GSK3β inhibition. This evidence suggests that the solely inhibition of GSK3β may justify p53 increased in Notch3 silenced cells and it indicates a new indirect regulation of GSK3β on Thr216 residue of Mdm2.

The direct interaction of GSK3β and p53 was reported to be present in the nucleus, without changing of GSK3β localization consequent to its inhibition that might explain different results between GSK3β silencing and inactivation (Zimjewski J.W: and Jope R.S. 2004; Gautam N. B. and Jope S.R., 2001). The interaction between GSK3β and p53 has been reported to modify p53 acetylation and phosphorylation at many sites, changing p53 activity (Eom T-Y and Jope R.S., 2009; Beurel E. et al. 2004; Qu L. et al.2004; Pluquet O. et al. 2005). GSK3β effect on p53 could be to increase apoptotic response and regulating transcriptional activity, phosphorylating p53 in Ser 33 and giving a mechanism of p53 activation independent from DNA-damage (Ghosh J.C. and Altieri D.C. 2005 ; Turenne G. and Price B.D. 2001). But all these regulations that causes p53 activation are dependent by GSK3β activity (Watcharasit P. et al. 2002), whereas it has been observed higher p53 stabilization in condition of GSK3β inhibition. Watcharasit P. et al. in 2003 showed that the inhibition of GSK3β does not alter the capability of the kinase in binding p53, whereas the Ser 9 phosphorylation increases the interaction capability of GSK3β with p53 and its stabilization. (Watcharasit P. et al., 2003). To asses if also in our model this could be an explanation, an immunoprecipitation will be performed for p53, to show the capability to bind both GSK3β and Mdm2 proteins, also after Lithium Cloride treatment.

Taken together, these data suggests that the stabilization of p53 in Notch3 silenced cells is mediated not only by Mdm2, but also by CyclinG1 downregulation and GSK3β inhibition, that both could increase p53 stability. Nevethless, further studies are necessary to really

understand and confirm their contribution and also eventual reciprocal regulation. In the future, experiments will be performed in order to combine CyclinG1 and GSK3β inhibition through Lithium Cloride treatment, performing the treatment on cells silenced for CyclinG1 and reproducing the condition observed in Notch3 silenced cells. In addition, silencing of GSK3β in Notch3 silenced cells, will be useful to exclude and confirm a possible stabilizating effect of GSK3β on p53. In order to investigate this aspect, immunoprecipitation in Notch3 silenced cells will be conduced, to test if the increased p53 levels are linked to an effective binding increase between p53 and GSK3β. In order to confirm a role of Mdm2, CyclinG1 and GSK3β in p53 stabilization independently by possible compensatory effects mediated by p53 itself, experiments of Mdm2 and CyclinG1 silencing, will be performed in Hep3B cells, p53 null, but which express both Mdm2 and CyclinG1. In particular, Mdm2 silencing it is necessary to exclude that Mdm2 reduction in total levels after Notch3 silencing could be enough to induce p53 increase.

A further aspect of p53 regulation have to be excluded in order to better define the regulation on p53. Indeed, the reduced activity of Mdm2 could lead to a monoubiquitination targeting on p53, instead of a poliubiquitination that determs its degradation. As monoubiuitination induces p53 nuclear export and citoplasmatic localization, immunofluorescence analyses for p53 are necessary at least in Notch3 silenced cells. (Lohrum et al. 2001; Li et al. 2003; Marckenko et al. 2007). Furthermore, to really detected if p53 is monoubiquitinated an immunoprecipitation followed by ubiquitin specific blot it could be done.

The crosstalk between Notch3 and GSK3β pathway was recently reported (Kashikar ND et al. 2011; Li C: et al. 2011), but Notch3 regulated activation of GSK3β was never described. GSK3β inactivation at Ser 9 in physiological conditions it has been linked not only to Akt/PKB activity, (Shaw M et al. 1997) but also to protein kinase C and protein kinase A (PKC and PKA) activity (Goode N. et al. 1992; Fang X. et al. 2000), which might be negatively regulated by Notch3. This aspect also will be considered in future directions.

Ex vivo analysis showed a correlation between expression level of Notch3 and CyclinG1, suggesting a positive regulation of Notch3 on CyclinG1 also *in vivo*. In addition, the same protein expression analysis was performed on hepatocellular carcinoma obtained by rats in which carcinogenesis has been induced by DENA. This kind of analysis let to compare liver sample of similar aetiology, in an animal model of carcinogenesis that even if caused by DENA, highly reflects the molecular characteristics of human cancer, such as low frequency of p53 mutation and chronic inflammation. This model was useful to confirm the association between Notch3 and CyclinG1 expression, that showed a significative correlation, further

confirming Notch3 regulation on CyclinG1. Neverthless, in animal samples, the characterization of GSK3β status, together with Mdm2 expression in both animal and human samples will be evaluated, in order to better define the regulatory loop also in vivo. It is in progress the analysis of p53 levels in these same samples, in order to really characterize the p53 loop mediated by Notch3. It is however difficult to evaluate p53 in vivo, due to its difficult to detect the wild type protein in immunohystochemestry and western blot, because it has a rapid turnover and it is detectable as an effect of accumulation consequent to p53 mutation (Lee S.N. et al. 2002). So, analyses with ELISA will be performed on samples that showed a wild type TP53 after sequencing.

Finally, in this work it has been shown that Notch3 expression affects p53 stability in HCC mainly through post transcriptional and post-translational mechanisms. P53 resulted stabilized, but not changes in its activity. The stabilization is the result of a multiple control of Mdm2 total protein expression and activity modulation (Ser166, Thr216), CyclinG1 expression and GSK3β inactivation, mediated by Notch3 expression. Reciprocal regulations have to be better investigated. Neverthless, Notch3 silencing led to p53 stabilization and reduction in CyclinG1 expression and GSK3β inactivation, which constitute other then their capability to control p53 protein levels, two powerful pathways in HCC progression.

Despite this, the real mechanism through which Notch3 regulates p53 stability in HCC has to be elucidated. Indeed, despite reduction in Mdm2 activity, changes in the analysed phosphorylations seem to not be really effective on p53 stability. Indeed, after cyclinG1 silencing, they both suggest an increased Mdm2 activity, despite p53 is stabilized. In the same way, after GSK3β silencing, they showed a reduced activity, together with a decrease in p53. This last condition might be explained by a direct additional GSK3β effect on p53 stabilization, but the concomitant difference also in phosphorylation between GSK3β silencing and inactivation on Ser166 status, suggests that another level of regulation exist. Moreover, the expected regulations after kinases and phosphatases loss of function are not confirmed. Instead, an opposite effect in Ser166 increased phosphorylation after CyclinG1 silencing was observed, with an increase in Thr216 phosphorylation status after GSK3β. This strongly supports a reciprocal regulation of these elements and the involvement of other regulators, that are probably regulated directly or indirectly by GSK3β and its effector maybe regulates these sites. A further hypothesis is about CyclinG1: its loss of function does not induces the attended effect on Ser166 and Thr216 target, despite that p53 increases. The effective outcome of these regulations is already controversial, also because these sites seem to have opposing effect on MDM2 and so the biological effect mediated by CyclinG1 is not

entirely clear. (Meek D.W. and Kinppschild U., 2003) In addition, Thr216 phosphorylation has been associated with ATM kinases (De Toledo et al. S.M. et al. 2000) but differents evidence suggest a specie specific regulation, suggesting that the range of regulation is more complex to dissect. (Meek D.W. and Kinppschild U., 2003)

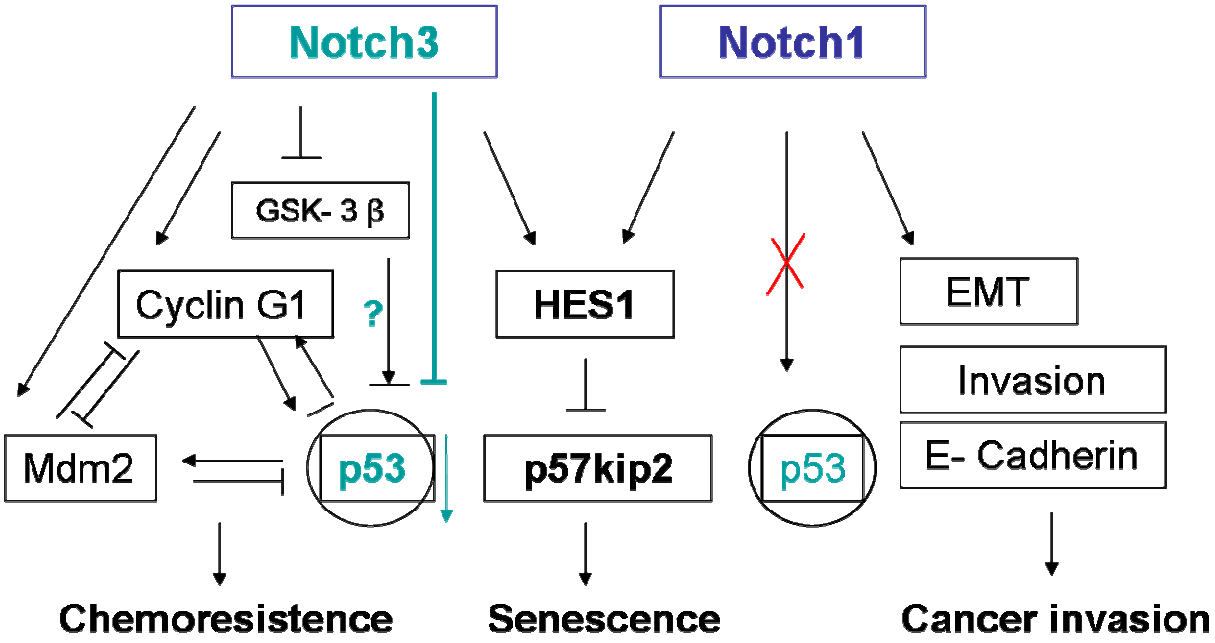


Figure 30: General scheme of Notch1 functional regulation in human hepatocellular carcinoma.

CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis, the involvement of Notch signalling in HCC has been approached, *in vitro* through analysis of Notch3 and Notch1 depletion in human hepatocarcinoma cell lines and *ex vivo* analysing receptors and identified interactors expression, in human HCC and in rats livers after carcinogenesis induction. Notch1 and Notch3 resulted overexpressed in HCC (Giovannini C. et al. 2009), suggesting a pivotal role in this cancer, but due to the high range of cellular processes regulated by Notch signalling in physiological conditions, their deregulation in cancer showed a multifaceted role, being oncogenetic or tumour suppressive, also in the same cancer in particular conditions (Radtke F. and Raj K., 2003). So, the context has a great importance in analysis of Notch signalling pathway outcomes. Moreover, in controls of downstream pathways, Notch1 and Notch3 showed redundant and not redundant functions, having similar but also significative different structural characteristics. (Bellavia D.)

In this work we would like to define some common and uncommon pathway of Notch1 and Notch3, in order to better understand Notch signalling function in HCC.

Firstly, we demonstrated that Notch1 and Notch3 regulate the transcriptional target Hes1, which in turn is able to inhibit CDKN1C/p57 kip2 transcription. The overespression of two receptors, suggests a high Hes1 levels in HCC that could mediate the reduced CDKN1C/p57 kip2 expression and induces cell cycle progression in cancer cells. Dependent by these evidences, it could be favourable to inhibits Notch receptors activation or expression, using specific antagonist or siRNA approach, or silencing Hes1, in order to overcome this pro-proliferative effect mediated by Hes1. However we demonstrated that in HCC cells CDKN1C/p57 kip2 overexpression induces both cell cycle arrest and senescence, both in CDKN1C/p57 kip2 overexpressing cells and also in Hes1 silenced and Notch1 and Notch3 silenced cells. Senescence is considered a tumour suppressive process and in principle, this strategy could be advantageus (Campisi J. 1997; Prieur A. and Peeper S. 2008). Analysing ex vivo expression data with the time to recurrence associated with Hes1 and CDKN1C/P57 kip2we found a surprising protective effect of high HES1 expression, together with an opposite negative effect in recurrence for high CDKN1C/p57 kip2 expression. These data suggests that the high CDKN1C/p57 kip2 expression and consequent senescence induction may constitute a negative factor for HCC recurrence. Indeed, senescence could also be a negative factor *in vivo*, giving chemoresistance and sustaining cell proliferation and/or invasion. (Campisi J.and D'Adda di Fagagna, 2007,Freund A. et al. 2010) So, if the high

CDKN1C/p57 kip2 expression lead to a negative outcome due to senescence induction and Hes1 expression is a positive elements to contrast cancer recurrence, Notch1 and Notch3 silencing for therapeutical aims seems not useful. CDKN1C/p57 kip2 also block the cell cycle progression and it has been attributed a positive function in contrast proliferation, but this effect might also induce increased cancer progression instead of contrasting it, because not proliferating cells could display aggressive feature.

Secondly, we identified some new downstream factors regulated by Notch3 that mediate its control on p53 protein levels. Indeed, p53 resulted upregulated after Notch3 silencing, suggesting that Notch3 activity mediate p53 inactivation in HCC. This gives a great advantage to cancer cells and it constitutes a mechanism of p53 inactivation for cells in which p53 is not mutated. Notch3 seems to regulate CyclinG1 and Mdm2 with post translational mechanisms, increasing CyclinG1 expression and Mdm2 expression and activity. Indeed, Notch3 silencing lead to the downregulation of both and inactivation of Mdm2 together with GSK3β inactivation, which seems to have a function in regulating Mdm2 and directly p53. This specific Notch3 regulatory pathway on p53 opens many applicative strategies.

Thirdly, a Notch1 function in HCC has been identified in inducing EMT and invasion *in vitro,* through activation of EMT inducing factor and E- Cadherin regulation. Notch1 seems to induce this aggressive phenotype activating EMT program, but also through E- Cadherin, which expression increases invasion capability of epithelial and also in mesenchymal HCC cells when exogenously expressed. This evidence is supported by ex vivo data, showing a positive correlation between the Notch1 and E- Cadherin expression. Moreover, patients with higher Notch1 and E- Cadherin expression levels showed a shorter time to recurrence.

Taken together, Notch1 and Notch3 seem to have an oncogenic role in HCC, even if in some particular conditions, they could show partial tumour suppressive effects, such as could be senescence. Conversely, if senescence induced by their depletion will increase the recurrence in HCC patients, others Notch1 and Notch3 oncogenic functions might be inactivated by receptors silencing. Neverthless, we do not know the real outcome of senescence in vivo. Then, a study to evaluate senescence on HCC samples will be useful to establish the effective induction in vivo and to investigate the correlation between Notch receptors and recurrence. Moreover, further investigation will be necessaries to extend evaluation of Hes1 function *in vivo*, because of its protective role in recurrence and because conversely it is one of the main downstream mediators of both receptors, which instead show negative correlation with recurrence ex vivo and oncogenic functions *in vitro*. So, an animal model will be useful to elucidate this apparent contradiction. Moreover, in considering therapeutical strategies that

take account of all these evidences, some considerations have to be done. 1) Senescence was not analysed *in vivo*, so the possibility to asses if it really occurs after Hes1 silencing and CDKN1C/P57 kip2 expression let to confirm the effect on cancer outcome, making possible to delucidate senescence role in HCC progression. This will explain the ex vivo data of recurrence and it will suggest an effective or not desirable therapy based on Notch1 and Notch3 depletion. 2) Despite previous data reported about a different capability to activate Hes1 by Notch1 and Notch3 (Beatus et al. 1999; 2001), in our *in vitro* model they seems to be both able to activate their target Hes1 or both required to have high transcription. In this context, the silencing of only one of two genes will not sufficient to maintain Hes1 higher level or whereas sufficient to have Hes1 critical expression to regulate CDKN1C/P57 kip2.This evidence underline a complex setting in transcriptional activities of NICD, which depend by RBP-Jk expression and other factors, together with reciprocal NICD regulation in context of concomitant activation of different Notch receptors (Shimizu K et a. 2002). Moreover, different levels of activated NICD may exert a regulatory control in a dose dependent manner, being able to activate or repress some targets, with an outcome dependent by the expression and the context (Lathion S. et al. 2003). For that, it will be necessary to do an *in vivo* study with an animal model, in order to establish if it exist a correlation in vivo between Notch1 and Hes1, to foresee the effect of Notch1 silencing in Hes1 expression. Indeed, Notch1 inactivation will be advantageus to contrast progression of HCC. As preliminary investigation, association of ex vivo expression data about Notch1 and HES1 expression will be useful to establish an effective link in expression between them *in vivo*. Moreover, also Notch4 receptor is highly expressed by HCC samples (Gramantieri L. et al. 2007;), and it is not note if it could activate HES1, even some indirect evidence are present in endothelium (Quillard. T et al. 2010). In this case, the contemporary depletion of Notch1 and Notch3 toghether with maintenance of Hes1 expression driven by Notch4, it could be a successfull therapeutical strategy. 3) Notch1 regulation on E-Cadherin expression seems not to be mediated by Hes1, as it does not show repressive effect on this protein. In this context and for our knowledge, Notch1 inhibition and consequent loss of Hes1 expression might be negative only in term of CDKN1C/p57 kip2 loss of inhibition. So, in the perspective to silence Notch1 for therapy and eliminate its pro invasive effect, it will be useful to analyze the concrete possibility of Hes1 to regulate CDKN1C/p57 kip2 expression in vivo, for each patient. Indeed, CDKN1C/p57 kip2 promoter is frequently silenced by epigenetic changes in HCC (Schwienbacher C. et al. 2000). In this condition, Hes1 regulatory effect might be ineffective. Moreover, as previously demonstrated (Fornari F. et al. 2008), miR221 controls

CDKN1C/p57 kip2 translation and it is high expressed in hepatocellular carcinoma also in vivo. So, it will be a useful tool to be associated to Notch1 and/or Notch3 silencing in order to both eliminate Notch signalling mediated functions and have low CDKN1C/p57 kip2 impaired expression, evaluating its expression and performing Notch signalling depletion in patients that have high mir-221 levels.

4) Notch3 has showed a strong regulatory effect on p53 stabilization, that in condition of damage induction (doxorubicin treatment), leads to a higher apoptotic response, if Notch3 depletion is associated with chemotherapeutical stresses (Giovannini C. et al. 2009). So, it will be better to associate Notch3 depletion with chemotherapy, in order to have an apoptotic strong response immediately, reducing the possibility of an induced senescence and consequent resistance.

Indeed, also other effect of Notch1 in regulating invasion, might give an important contribution in preventing recurrence, taking care of previous observations about the possibility to apply this strategy relatively to Hes1 mediated CDKN1C/p57 kip2 regulation.

Considering results form all three analyzed aspects of Notch1 and Notch3 signalling in HCC, it is evident which complex network underwent their activation. Taking together, it is interesting to asses the presence of redundant and not redundant functions, also including other possible overlapping and not yet identified regulations. Moreover, it is important to notice that the oncogenic and tumour suppressive effect of Notch signalling in HCC is linked to the contest, supporting the growing idea that the concept of oncogene or tumour suppressor is too restrictive and too absolute. Indeed, senescence, that is considered a tumour suppressive process in cancer, it is activated by oncogenes, indicating how the boundary between the two classes could be difficult to define (Radtke F. and Raj K., 2003). Moreover, senescence is also considered a deleterious factor for cancer progression, due to secretion of pro-proliferative factors and proteins inducing malignant progression (Campisi J and D'Adda di Fagagna F., 2007). Indeed, the effect of a protein on the cell is determed not just by its biochemical properties, but also by cellular context in which the protein is present. In this perspective, also double functions of Notch signalling in cancer could be determed by the contest, defining the oncogenic or tumour suppressive outcome of Notch signalling activation (Radtke F. and Raj K., 2003).

Considering this observations and results emerged in this work, the context makes the difference in Notch signalling functions. In cancer context but simply in cell dependent context, its inactivation or activation brought to different consequence. Indeed, not only loss of Hes1 expression could induce cell cycle exit that might have negatives outcomes, but also

in cancer context, a protein involved in adhesion, such as E- Cadherin could confers pro invasive features. For that, based on our evidence, Notch inhibitory therapy has to be established after tumours characterization. The use of γ secretase inhibitors () as Notch signalling inhibitors, has to be taken with care for therapy of HCC, because the complete loss of functions of all three Notch expressed in HCC could not bring a positive outcome. Indeed, we have evidence of some opposite effect linked to Hes1 depletion, but the signalling is highly interconnected and controls many cellular processes to make possible others similar situation. Moroever, we only know only in part Notch1 and Notch3 interactions in HCC, whereas nothing is described for Notch4 in this context, despite its high expression in tumour. (Ref giovan) So, it emerges the necessity to characterize Notch signalling in each tissues, cancer or cellular setting, to try to preview and understand its real functions.

For that, the therapy could be based on selective silencing of Notch1 and Notch3 receptors, based on possible Hes1 capability to repress CDKN1C/p57 kip2. If others regulatory factors intervene in its regulation (epigenetic or miRNAs), both receptors could be silenced to reduce invasion and possible recurrence and to sensitize to chemotherapy. Indeed, the association of Notch3 silencing with chemotherapy is the more evident exemple of positive effect in inhibits Notch signalling in HCC.

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