UNIVERSITÀ DEGLI STUDI DI TRIESTE

Sede Amministrativa del Dottorato di Ricerca
Laboratorio Nazionale Centro Interuniversitario per le Biotecnologie

XX CICLO DEL
DOTTORATO DI RICERCA IN
MEDICINA MOLECOLARE

HUMAN NOTCH1 AND PIN1 UNVEIL A MOLECULAR CIRCUITRY INVOLVED IN TUMORIGENESIS

Settore scientifico-disciplinare **BIO/13**

DOTTORANDO

LUCA TIBERI

COORDINATORE DEL COLLEGIO DEI DOCENTI

CHIAR.MO PROF. ***GIANNINO DEL SAL***
Università degli Studi di Trieste

SUPERVISORE E RELATORE

CHIAR.MO PROF. ***GIANNINO DEL SAL***
Università degli Studi di Trieste

CORRELATORE

DOTT.SSA ***ALESSANDRA RUSTIGHI***
Università degli Studi di Trieste

INDEX

1. INTRODUCTION

1.1 Notch receptors and ligands

In 1917, Thomas Hunt Morgan and colleagues described a strain of *Drosophila* with notches at the end of their wings (Morgan et al., 1917). This curious trait was attributed to a partial loss of function of what would later be identified as the Notch gene. The Notch gene, which was cloned in the mid-1980s by groups of Artavanis-Tsakonas (Wharton et al., 1985) and Young (Kidd et al., 1986), encodes a type I transmembrane receptor. In *Drosophila* only a single Notch protein and two ligands (Delta and Serrate) are present , while mammals, such as mice and humans, possess four Notch proteins Notch1–4 (Weinmaster et al., 1992, Lardelli et al., 1994, Uyttendaele et al., 1996), and five ligands, named Delta-like-1,-3 and -4 (DLL1,DLL3 and DLL4) (Radtke et al., 2003) and Jagged1, Jagged2 (JAG1 and JAG2) which are Ser-like ligands (Figures 1a,1b) (Radtke et al.,2003).

In mammals, the Notch receptor is produced as precursor that is modified in the secretory pathway. It is cleaved in two parts by Furin protease in the trans-Golgi and is exposed to the cell surface as heterodimeric receptor (Blaumueller et al., 1997, Loegat et al., 1996). The Notch heterodimer consists of noncovalently associated extracellular and transmembrane domains. The extracellular domain contains the signal peptide and 36 tandemly repeated copies of an epidermal growth factor-like motif (EGF-like). The transmembrane subunit contains a short extracellular domain, the membrane-spanning region, and an intracellular domain (termed ICD) containing several conserved motifs. Stable association of the two Notch subunits is dependent on a newly described heterodimerization domain comprising the carboxy-terminal end of the extracellular subunit and the extracellular amino-terminal end of the transmembrane subunit (Figure 1c) (Sanchez-Iriazzy et al., 2004).

The amino-terminal EGF-like repeats participate in ligand binding, whereas the LN repeats (present in the extracellular domain) prevent signalling in the absence of ligand. The cytoplasmic extension of Notch contains a RAM domain, six ankyrin (also known as CDC10) repeats, two nuclear-localization signals, a transactivation domain (TAD) and a PEST sequence (Figure 1c). Although the structures of the four Notch receptors are very similar, they show differences in the extracellular and cytoplasmic parts. The Notch1 and Notch2 receptors contain 36 EGF repeats in their extracellular domain, whereas Notch3 harbours 34 and Notch4 only 29 repeats (Bray et al., 2006). Additional differences are found within the

cytoplasmic domain; specifically, Notch1 contains a strong TAD, and Notch2 a weak TAD and no TAD is present in Notch3 and Notch4, however all the four receptors have transactivation activity. The main structural differences between the ligand family members are the number and spacing of EGF-like repeats in the extracellular domain and the presence of a cysteine-rich domain (LN domain, which is located downstream ofthe EGF-like repeats) in Ser, JAG1 and JAG2 (Figure 1c).

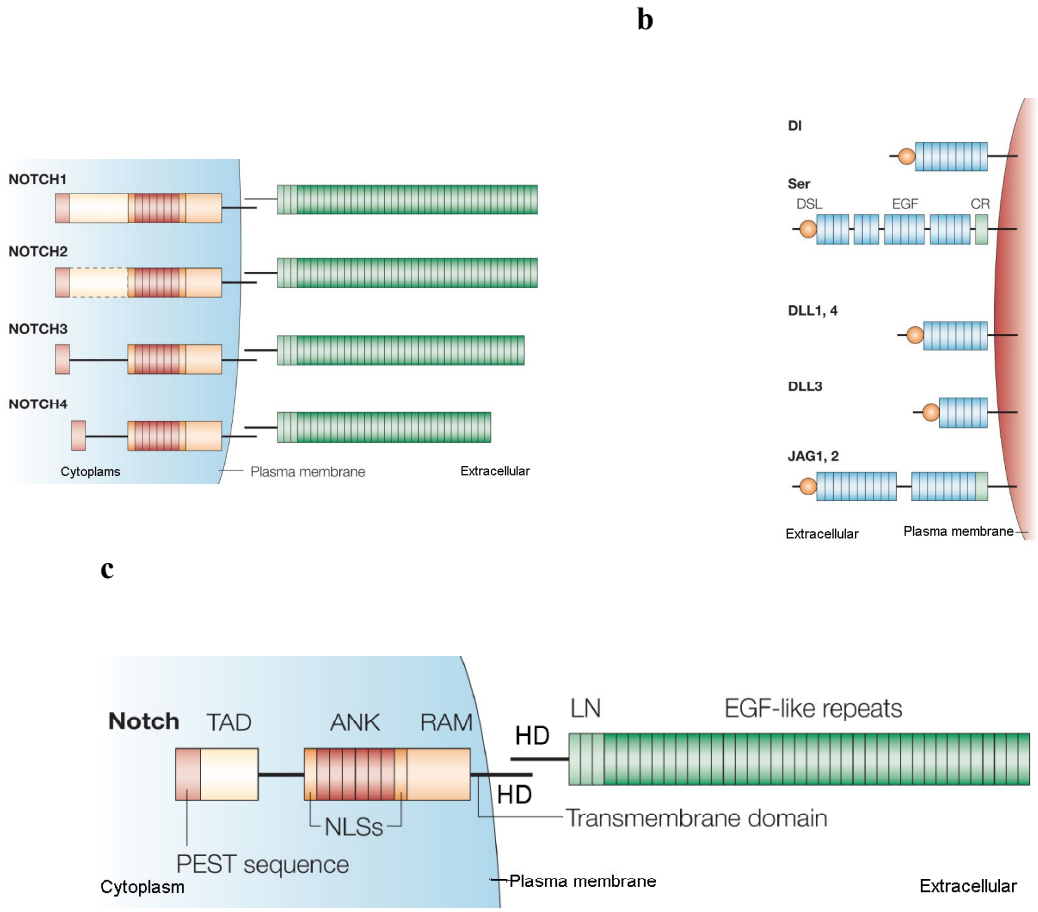

Figure 1 Structure of Notch proteins and their ligands

a) Scheme of Notch receotors. Notch receptors (Notch1-4) are presented on the cell surface as heterodimers. **b)** Scheme of Notch ligands. Two transmembrane-bound ligands for Notch have been identified in Drosophila, named Delta (Dl) and Serrate (Ser). The vertebrates possess three Delta homologues, called Delta-like (DLL)-1, -3 and -4, and two Serrate homologues, Jagged 1 (JAG1) and Jagged 2 (JAG2). Serrate, Jagged1 and Jagged2 harbour a cysteine-rich domain (CR) following the EGF-like repeats. **c)** Domain structure of the eterodimer Notch receptors. The ectodomain of Notch receptors contains EGF-like repeats and a cysteine-rich Notch/Lin12 domain (LN); this is followed by a transmembrane domain, the RAM domain and six ankyrin repeats (ANK), two nuclear-localization signals (NLSs), followed by the transactivation domain (TAD) and a PEST sequence. Adapted from Radtke et al., 2003.

1.1.1 The Notch pathway

Notch signaling is initiated by a receptor-ligand interaction between two neighbouring cells,

notably the ligand is expressed in the signaling sending cell while the receptor in the signaling receiving cell. This cell–cell contact is an important prerequisite to trigger the signalling event leading to two successive proteolytic cleavages, that liberate the cytoplasmic portion of Notch (NICD) from the membrane in the receiving cell.

The first cleavage, caused by an ADAM disintegrin and metalloprotease occurs on the transmembrane subunit close to the transmembrane domain outside the cell (Bray et al., 2006). The second occurs within the transmembrane domain of Notch and is induced by a multisubunit protease called gamma secretase that contains presenilin, nicastrin, PEN2 and APH1 (Figure 2) (Fortini et al., 2002, De Strooper et al., 1999). The second cleavage releases the Notch intracellular domain (NICD), which then translocates to the nucleus and cooperates with the DNA-binding protein CSL (named after CBF1, Su(H) and LAG-1) and its co-activator Mastermind (Mam) to promote transcription. In the absence of Notch signalling, CSL binds to the promoters of its target genes and recruits corepressors and histone deacetylases, which inhibit transcription (Kao et al., 1998). When NICD is present, it competes with the inhibitory proteins for CSL binding. It then recruits co-activators including Mastermind and histone acetyltransferases, which convert CSL from a transcriptional repressor to a transcriptional activator (Bray et al., 2006, Fryer et al., 2002) (Figure 2).

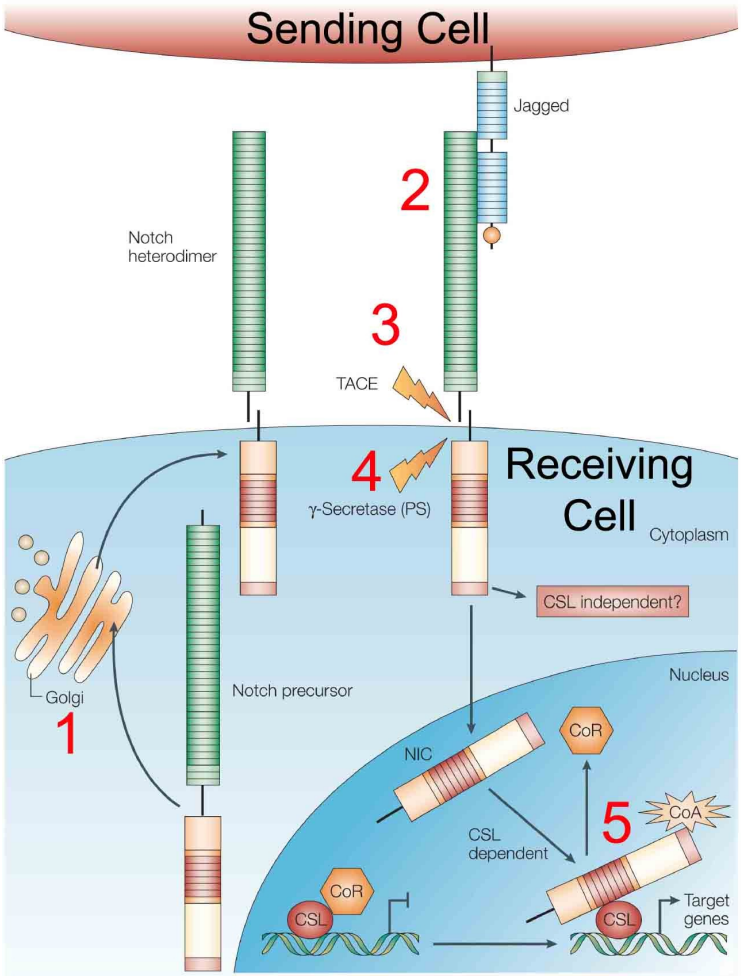

Figure 2. The Notch pathway

Notch proteins are synthesized as precursor proteins that are processed by a furin-like convertase in the Golgi (1) before being transported to the cell surface, where they reside as heterodimers. **2)** Interaction of Notch receptors with their ligands, such as Delta-like or Jagged (2), leads to a cascade of proteolytic cleavages. The first cleavage is mediated by TACE (3), followed by a second cleavage mediated by the gamma-secretase activity of presenilins (PS,4), which liberates the cytoplasmic domain —Notch intracellular domain (NICD). The liberated NICD enters the nucleus and binds to the transcription factor CSL, which displaces co-repressors (CoR) and recruits co-activators (CoA), leading to transcriptional activation of downstream target genes (5). Adapted from Radtke et al., 2003.

1.1.2 Regulation of Notch-ligand activity

Expression of Notch ligands during development is quite dynamic and contributes significantly to differential activity of the pathway. In some developmental contexts, the ligand is produced by a distinct population of cells. However, under many circumstances, differential ligand transcription is not sufficient to explain why certain cells become the signal-sending cells. Indeed not only post-transcriptional modification of the receptors, but also of the ligands were found to be important in Notch pathway. The most important mechanism seems to rely on ubiquitination. Indeed two E3 ubiquitin ligase were identified, Neuralized (Neur) and Mind bomb (Mib), that interact directly with Notch ligands and are required for ligand activation (Figure 3) (Le Borgne et al., 2005).

Loss of Neur in *Drosophila melanogaster* or *Xenopus leavis* and of Mib1 in *Danio rerio* results in neurogenic phenotypes (Le Borgne et al., 2005), typical for loss of Notch function. In normal cells, the extensive trafficking of Notch ligands is compromised in the absence of Neur or Mib, as ligands accumulate at the cell surface but are inactive (Le Borgne et al., 2005b).

This observation indicates that regulation of ligand activity by Neur and Mib is intimately associated with endocytosis and it requires the ubiquitin binding protein Epsin (Wang et al., 2005). Different models have been proposed to explain the link between ubiquitylation, endocytosis and ligand activity (Le Borgne et al., 2005). For example, ligand endocytosis could generate a 'pulling force' on a bound receptor that causes a conformational change in the Notch heterodimerization region (Parks et al., 2000). Another possibility is that ubiquitylation promotes ligand clustering. Indeed, Notch activation is more effective if ligands are clustered through fusion to an Fc moiety or through immobilization on plastic (Varnum-Finney et al., 2000). A third possibility is that ubiquitylation permits trafficking into an endocytic compartment, which enables ligand modification or results in re-insertion of the ligand into specific membrane domains. Two observations support this model. Segregation of RAB11, a component of the recycling endosome or mutations in an exocyst component SEC15, influence signalling in the *Drosophila* sensory organ precursors (SOP), a well establish system to study Notch pathway (Emery et al., 2005). Whatever the mechanism for ligand activation, regulation of E3 ligases represents a crucial step for controlling the activity of the Notch pathway.

The localization of ligands within the cell is important for effective signalling and might be influenced by other proteins. For example, Echinoid, an immunoglobulin C2-type cell-adhesion molecule, colocalizes with Notch and Delta at adherens junctions in *Drosophila*.

Genetic interactions indicate that Echinoid functions as a positive regulator to promote Notch signalling (Escuder et al., 2003). Echinoid colocalizes with Delta in endocytic vesicles, and Echinoid-mediated adhesion could favour Notch–Delta interactions. Consistent with this notion, it has been shown that altered cyto-architecture of cells can affect their signalling potential.

Furthermore, the intracellular domains of some Notch ligands contain protein–protein interaction motifs (for example, PDZ-binding motifs) that can bind to intracellular scaffolding proteins (Wrigth et al., 2004).

Structurally, the ligands share many characteristics with Notch itself and are prone to similar modifications including proteolytic processing (Mishra-Gorur et al., 2002). However, the purpose of ligand cleavage remains unclear. One hypothesis is that proteolytic processing of the ligand contributes to ligand downregulation (Mishra-Gorur et al., 2002). For example, loss of the metalloprotease Kuzbanian-like, which has been shown to cleave Delta, results in ectopic Notch signalling in certain locations. Another hypothesis is that cleaved or secreted ligands antagonize Notch signalling, because, under most circumstances, soluble ligand fragments inhibit receptor signalling (Le Borgne et al., 2005). It is also possible that cleavage of transmembrane ligands could transmit an intracellular signal through activities that are associated with the ligand's intracellular domains. Further investigations are needed to identify all of the functional consequences of ligand proteolysis on Notch signalling in vivo.

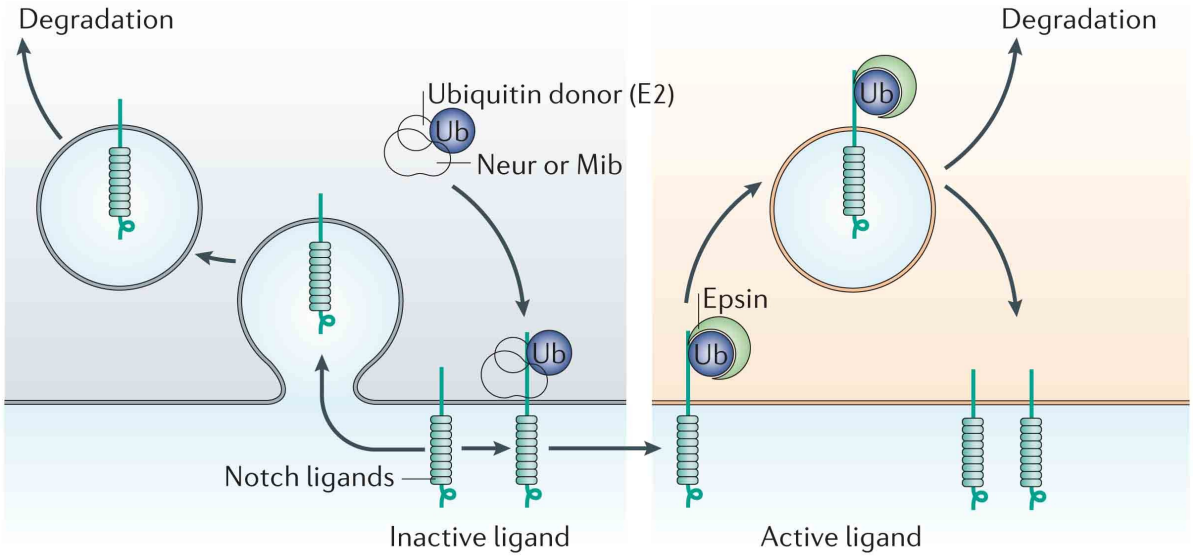

Figure 3. Ligand activation entails ubiquitylation

The E3 ubiquitin ligases Neuralized (Neur) and Mind bomb (Mib) interact directly with Notch ligands. LEFT, inactive ligand: Prior to modification by Neur or Mib, ligands are inactive, and can be endocytosed and degraded. Neur- or Mib-mediated ubiquitylation (Ub) of Notch ligands is required for Epsin-mediated endocytosis. RIGHT, active ligand: Ligands are then competent to signal either because endocytosis is directly associated with receptor activation or because it allows entry into a specific compartment or membrane domain that renders ligands active. They can also be targeted for degradation. E2, ubiquitin-conjugating enzyme. Adapted from Bray et al., 2006.

1.1.3 Notch-receptor maturation

Notch receptors have broad expression patterns in many tissues, but analyses of where cleavage occurs or where target genes are expressed reveal a well-regulated profile of activation. These observations indicate that the activity of the receptor must also be regulated through post-transcriptional mechanisms.

Notch proteins have a large extracellular domain that consists of multiple EGF-like repeats, which are sites for glycosylation (Haines et al., 2003). The enzyme O-fucosyl transferase (O-Fut) adds the first fucose and is essential for the generation of a functional receptor (Okajima et al., 2005). Indeed depletion of O-Fut in *Drosophila* and mice results in phenotypes that resemble those associated with lack-of-Notch signalling. 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 (Okajima et al., 2005). Multiple EGF repeats in Notch have the potential to be modified and, therefore, a large repertoire of

differentially modified receptors could be generated. It has been shown that these glycosyl-modifications alter the capability of ligands to activate Notch. For example, in dorsal cells of the *Drosophila* wing, the glycosil transferase Fringe potentiates activation by Delta and renders Notch resistant to activation by Serrate. For example, Serrate binds with higher affinity to Notch fragments that have been fucosylated and with lower affinity to fragments that have been further modified by Fringe (Okajima et al., 2003).

Mutation of the glycosylation site in EGF-repeat 12, a crucial repeat for ligand binding, allows activation of Notch by Serrate even in the presence of Fringe, which indicates that this is a key site for modification (Yang et al., 2005). Furthermore, Lunatic Fringe, a mammalian homologue of Fringe, potentiates Delta binding in in vitro studies and promotes Notch activity. These observations indicate that glycosylation patterns might do more than producing an all-or-none effect on different ligands.

1.1.4 Proteolytic cleavage of Notch

Notch signaling is initiated by a receptor-ligand interaction that triggers the signalling event leading to two successive proteolytic cleavages. The first cleavage, caused by an ADAM disintegrin and metalloprotease occurs on the transmembrane subunit close to the transmembrane domain on the outside of the cell (Bray et al., 2006). Two metalloproteases have been implicated in the S2 cleavage, ADAM10 (also known as Kuzbanian; Kuz) and tumour-necrosis factor-alfa (TNFalfa)-converting enzyme TACE, also known as ADAM17, that have partially redundant roles (Fortini et al., 2002, Brou et al., 2000). The S2 protease cleavage remains an important aspect for investigation, particularly because studies of metalloproteases reveal the potential for regulation by external factors, membrane environment and intracellular signalling pathways.

The S3 cleavage occurs within the transmembrane domain of Notch and is induced by gamma-secretase, an enzymatic complex that contains presenilin, nicastrin, PEN2 and that liberate the cytoplasmic portion of Notch (NICD) from the membrane in the receiving cell. Presenilins (PS1 and PS2) are required for intramembrane cleavage of an increasing number of type I membrane proteins, including the amyloid precursor protein of Alzheimer's disease (Xia et al., 2003). Moreover, regulated intramembrane proteolysis is a novel mechanism involving proteases that hydrolyze their substrates in a hydrophobic environment. Genetic and biochemical studies have implicated the proteolytic processing events that lead to amyloid-beta-peptide generation in the pathogenesis of Alzheimer's disease. Mutations in APP and the presenilins (PS1 and PS2) cause familial early-onset Alzheimer's disease, and presenilin

mutations lead to increased processing of APP by gamma-secretase in vitro and in vivo(Lendon et al., 1997).

PS1-deficient mice show developmental abnormalities consistent with altered Notch signalling (Wong et al., 1997, Shen et al., 1997) and genetic interactions between the notch homologues glp-1 and lin-12 and the presenilin homologues sel-12 and hop-1 in C. elegans provide indirect evidence for the involvement of the presenilins in the Notch signalling pathway (Levitan et al., 1995, Levitan et al., 1998). Furthermore, PS1-deficient mice and PS-1/PS-2 double knockout mice had a marked decrease in NICD generation. (De Strooper et al., 1999).

Mutagenesis, affinity labeling, biochemical isolation, and reconstitution in cells reveal that PS, in complex with co-factors nicastrin, APH-1 and PEN-2, apparently contains the active site of gamma-secretase, a membrane aspartyl protease (Figure 5).

PS contains YD and LGXGD motifs in two transmembrane domains that is postulated to constitute an aspartyl protease active site. PS1 and PS2 are 467 and 448-residue polypeptides, respectively, and share ~60% sequence similarity. Full-length PS undergoes endoproteolysis to form stable N-terminal (NTF) and C-terminal (CTF) fragments, which remain associated and establish the active form of the enzyme (Xia et al., 2003). Investigation of the PS-containing high-molecular-weight (HMW) complex indicates that additional cofactors intimately associate with PS to form the active gamma-secretase complex. In common with anti-PS1 antibodies, anti-nicastrin antibodies can precipitate the functional gamma-secretase complex (Elser et al., 2002) and although glycosylation of nicastrin is not absolutely required for gamma-secretase activity, mainly the mature form of nicastrin (glycosilated) was identified in the HMW gamma-secretase complex, and levels of nicastrin in cells closely correlate with PS levels (Xia et al., 2003). Reduction of PS levels leads to a concomitant reduction in nicastrin levels, and downregulation of nicastrin expression decreases the levels of stabilized PS molecules.

Interestingly, one of the emerging approaches for blocking Notch signaling is suppression of cleavage by gamma-secretase to prevent generation of the oncogenic NICD and suppress the Notch activity.

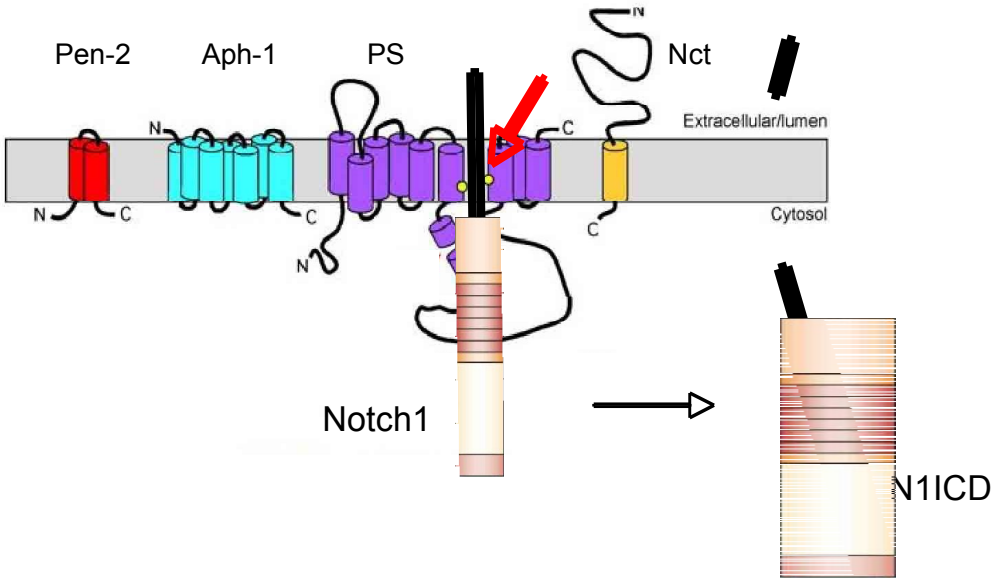

Figure 5. Schematic representation of the components of the gamma-secretase complex and Notch1 cleavage

Schematic rappresentation of the transmembrane domains of Presenilin-1 (PS). In yellow the two aspartyl residues, present in NFT and CTF. Nicastrin (Nct), APH1 and PEN2 are shown in yellow, baby blue and red respectivly. Gamma seretase site of cleavage is indicated by the red arrow.

1.1.5 Notch endocytosis and trafficking

Endocytosis is used by eukaryotic cells to regulate nutrient internalization, signal transduction, and the composition of the plasma membrane. However, a more complex picture is emerging, in which endocytic pathways integrate various signals, thereby contributing to a higher level of cellular and organismal organization. In this way, endocytosis and cell signaling are intertwined in many biological processes, such as cell motility and cell fate determination (Polo et a., 2006). Notch is a cell-surface receptor, so its expected location is the plasma membrane. However, a substantial amount of Notch is targeted for degradation and a large fraction of Notch is detected in the cytoplasm in compartments of the endocytic pathway. Studies in *Drosophila* have shown that Notch colocalizes with the RAB GTPases RAB5 and RAB7, which are both markers of the endocytic pathway. Moreover, Notch accumulates in intracellular structures when the endocytic progression is perturbed (Wilkin et

al., 2004).

Entry into the endosomal and multivesicular-body-sorting pathway is thought to be intimately linked with ubiquitylation of transmembrane proteins. Several E3 ligases that target Notch have been identified. The Itch/Su(dx) family of HECT domain E3 ligases are predominantly negative regulators of Notch signalling, by targeting Notch for its degradation. A second E3 ligase that binds to Notch, within the ankyrin repeats, is the RING finger protein Deltex (Le Borgne et al., 2005). Intriguingly, in several mammalian cells, including lymphoid cells and neurons, Deltex antagonizes Notch. Perhaps the precise balance of different E3-ligase activities dictates the outcome on Notch localization and activity. These ubiquitin modifications could potentially influence the duration of the receptor is located on the surface, its accessibility to ligands, or its capability to interact with gamma-secretase. It is evident that Notch is subject to different types of post-transcriptional regulation.

The activity of Numb, a well characterized Notch inhibitor, also involves endocytosis. Numb is asymmetrically segregated into one of two daughter cells in several lineages, and a search for mutants giving Numb-related phenotypes identified α-adaptin, a component of the adaptor protein-2 (AP2) complex that links cargoes to clathrin coats of transport vesicles. Numb interacts with α-adaptin and with Notch, so it could directly recruit Notch into endocytic vesicles. Furthermore, mammalian Numb promotes Notch ubiquitylation (McGill et al., 2003). However, partial rescue of Numb phenotype is observed with Numb proteins that lack the α-adapt ininteraction domain, which is indicative of alternative mechanisms of Numb-mediated antagonism.

Recently it was demostrated, that a monoubiquitination event takes place on the NdeltaE molecule, a constitutively active form of the Notch receptor that mimics the intermediate TACE-processing product generated after ligand binding(Gupta-Rossi et al., 2004). This modification is a prerequisite for gamma-secretase cleavage of NdeltaE. The major site of monoubiquitination was localized to a conserved lysine residue K1749 in mouse Notch1. It was proposed that this ubiquitination step and endocytosis are required in the context of the full-length receptor for its gamma secretase-dependent cleavage.

Glycosylation and proteolytic processing steps have a crucial influence on receptor activity, and are potentially important steps for drug intervention. Ubiquitylation and endocytic trafficking can modulate the amount of receptor that is available for signalling and could therefore provide powerful mechanisms to tune the activity of the pathway.

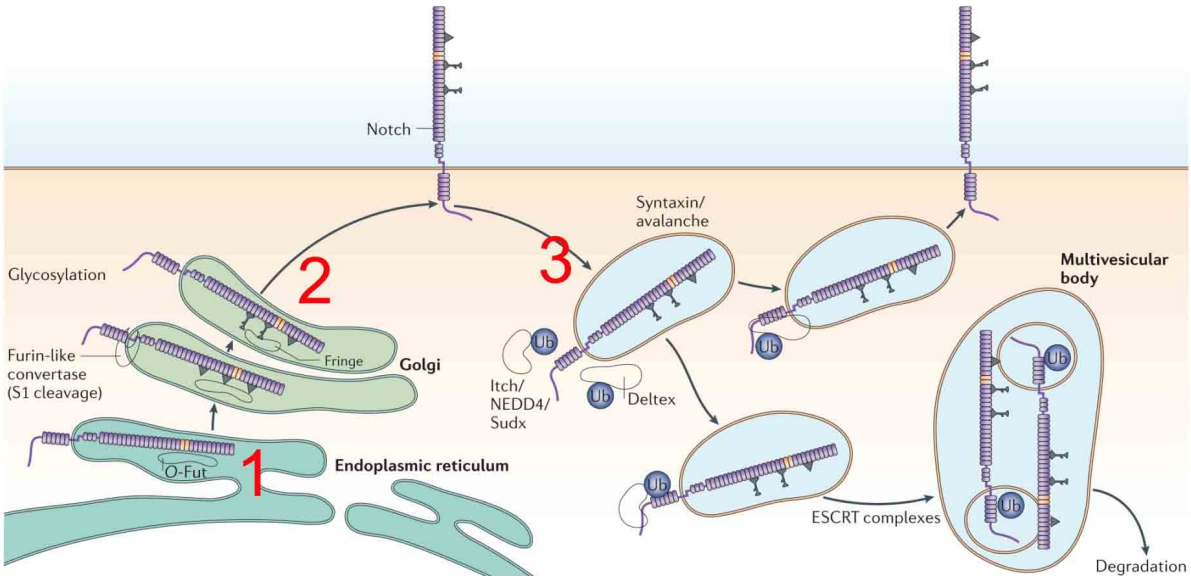

Figure 4 . Processing and trafficking regulate Notch-receptor activity

Notch maturation. Notch (purple) is produced in the endoplasmic reticulum where it interacts with the O-fucosyl transferase (O-Fut; green, 1) and is transported to the Golgi. In the Golgi, it is processed by Furin-like convertase (grey, S1 cleavage, 2) and glycosylated (shown as dark grey protrusion from Notch) by O-Fut and other glycosyltransferases (for example, Fringe) before export to the cell surface. Notch that is endocytosed from the cell surface can be recycled or degraded through the multivesicular-body pathway (3). Actions of the ubiquitin ligases Deltex and Itch/NEDD4/Su(dx) regulate trafficking, although their precise roles are not yet clear. Other proteins (syntaxin, ESCRT complexes) that affect trafficking are indicated, but their sites of action are hypothetical and remain to be fully clarified. Ub, ubiquitin.

Adapted from Bray et al., 2006.

1.1.6 Notch nuclear activity

Following Notch activation, N1ICD enters the nucleus and directly regulates the expression of target genes. Among the Notch targets, the best characterized are the bHLH (basic-helix–loop–helix) genes of the E(spl)/HES class and recently several new N1ICD targets have been identified: cyclin D1, NF-kB, PPAR, myc, p21Waf1/Cip1 (Bray et al., 2006) .

N1ICD enters the nucleus and binds the transriptional factor CSL. This DNA binding protein is the essential effector of the Notch transcriptional activity and has been highly conserved throughout evolution (84% identity between human and *Drosophila* proteins).

In the absence of Notch activity, CSL proteins recruit co-repressors. In mammalian cells, CSL co-repressors include SMRT and SHARP (Kao et al., 1998), which in turn recruit CtBP or other global co-repressors. Two other CSL-interacting proteins, SKIP and CIR (CBF1-interacting co-repressor), are also part of the repression complex. Homologues of these mammalian proteins exist in *Drosophila* and have been linked with CSL or Notch signalling. In mice and *Drosophila*, the phenotypes that are produced by depleting the single CSL are similar but not identical to loss-of-Notch function. Initially, these differences led to the speculation about CSL-independent Notch signalling.

The precise mechanisms that are involved in Notch-dependent transcription are not known yet, although studies in mammalian cells have revealed a number of recruited cofactors. N1ICD forms a trimeric complex with CSL and the co-activator Mastermind (Mam), which is essential for N1ICD-dependent transcription *in vitro* and *in vivo* (Wilson et al., 2006) (Figure 6). Although Mam binds with high affinity to the CSL/NICD complex in an interaction that requires the ANK domain of NICD, neither NICD nor CSL bind to Mam separately (Nam et al., 2006). The structure of the complex, resolved by X-ray crystallography, explains why both CSL and the ANK domain of NICD are required for Mam binding. The interface between the two proteins forms an extended groove made up of the ANK domain of NICD on one side and the C-terminal domain (CTD) of CSL on the other. Mam is nestled within this groove as a long helix, making extensive contacts with both proteins. Mam in turn recruits the histone acetylase p300, which promotes assembly of initiation and elongation complexes (Wallberg et al., 2002).

Immediately C terminal to the ANK of N1ICD there is a stretch of ~100 amino acids that has been implicated in functional interaction with cytokine-signaling pathways (Biga set al., 1998), which is followed by a second functional nuclear localization sequence. Amino acids 2155 to 2374 encompass a transcriptional activation domain (TAD) followed by an OPA sequence. The C-terminal TAD of N1ICD has been shown to associate with the

transcriptional coactivators PCAF and GCN5 (Kurooka et al., 2000), but this domain alone is not sufficent for the N1ICD transcriptional activity.

The emerging picture is that Notch signalling requires recruitment of histone acetyltransferase complexes and exchange of histone variants to activate transcription. In addition, BRE1, a homologue of the yeast histone 2B ubiquitin ligase, is crucial for Notch function in vivo and stimulates Notch-dependent transcription in a transient transfection assay (Bray et al., 2005). Together the data show that Notch activity is highly sensitive to chromatin modifications and histone re-arrangements that could contribute to target-gene specificity.

Furthermore, overexpression of two Polycomb group epigenetic silencers enhances Notch-induced hyperproliferation and also causes hypermethylation of the tumour suppressor gene Rb, indicating further mechanisms that could constrain the accessibility of enhancers and cooperate with Notch to confer different programmes of gene expression (Ferres-Marco et al., 2006).

Therefore, binding of tissue-specific activators contributes to robust target-gene expression, and can explain the specificity of Notch responses in different cell types.

The assembly of the co-activator complex not only promotes transcription, but also results in turnover of N1ICD. This is achieved by recruitment of factors such as cyclin-dependent kinase-8 (CDK8), which phosphorylates N1ICD, rendering it into a substrate for the nuclear ubiquitin ligase FBW7 (Fryer et al., 2004, Thompson et al., 2007). In mammalian cells, FBW7 preferentially interacts with a phosphorylated form of N1ICD and the expression of a dominant negative FBW7 leads to increased expression of Notch targets (Thompson et al., 2007, Wu et al., 2001). This interaction requires the C-terminal PEST region, consistent with observations that Notch with C-terminal truncations are very stable, and in humans contribute to oncogenicity (Thompson et al., 2007). Degradation of N1ICD result in the dissociation of Mam and other co-activators, but it is unclear whether CSL proteins would also be affected or whether they remain intact on the DNA.

Recently another kinase has been found to modulate Notch1 turn-over. Genetic studies have shown that the *Drosophila* homolog of glycogen synthase kinase-3beta (GSK3-beta), Shaggy, may act as a positive modulator of the Notch signaling (Ruel et al., 1993, Ramain et al., 2001). GSK3-beta is a serine/threonine kinase and is a component of the Wnt/wingless signaling cascade (Kim et al., 2000). It was observed that GSK3-beta was able to bind and phosphorylate N1ICD in vitro, and attenuation of GSK3-beta activity reduced phosphorylation of N1ICD in vivo (Foltz et al., 2002). Functionally, ligand-activated signaling through the endogenous Notch1 receptor was reduced in GSK3-beta null fibroblasts,

implying a positive role for GSK3-beta mammalian Notch signaling. These studies reveal that GSK3 modulates Notch1 signaling, possibly through direct phosphorylation of the intracellular domain of Notch, and that the activity of GSK3-beta protects the intracellular domain from proteasome degradation.

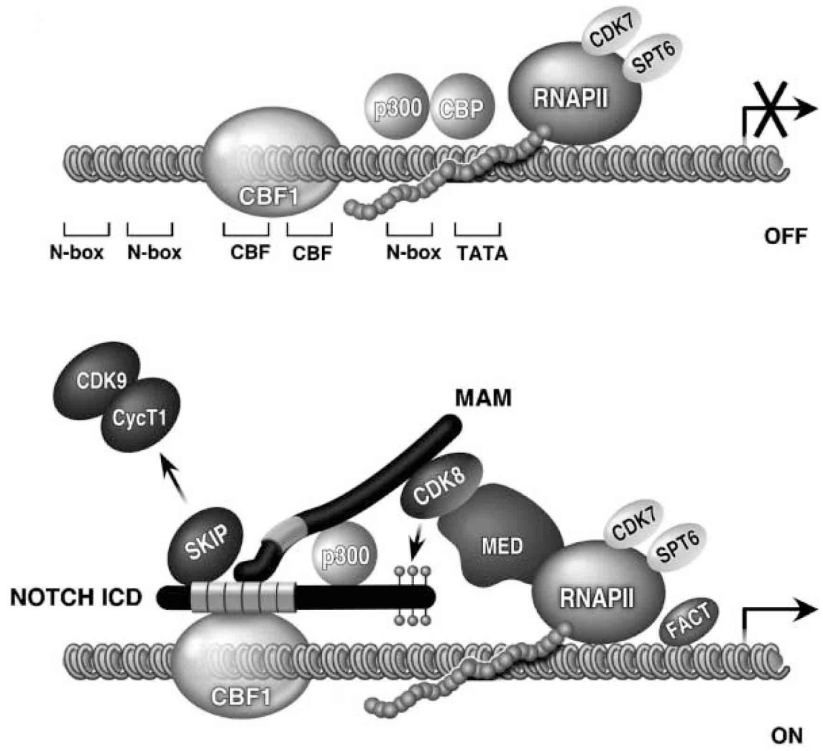

Figure 6. Model for Notch-activation of the HES1 gene.

The proteins bound to the promoter prior to signaling are indicated in light colors (CBF1, RNAPII, CBP/p300, CDK7, and Spt6), whereas proteins that are recruited together with the NICD are shown in darker colors (MAM, SKIP, Med220, CDK8, CDK9/P-TEFb, FACT). Binding of MAM to p300 and CycC:CDK8 can promote phosphorylation of p300, hyperphosphorylation of the NICD PEST domain (open circles), and facilitate Fbw7-mediated ubiquitination of the NICD to disassemble the Notch enhancer complex. Adapted from Fryer et al., 2004.

1.2 Notch functions

The Notch pathway functions during diverse developmental and physiological processes, can broadly be subdivided into three categories:
Bynary cell-fate decision, Differentiation and Stem-cell maintenance (Figure 7).

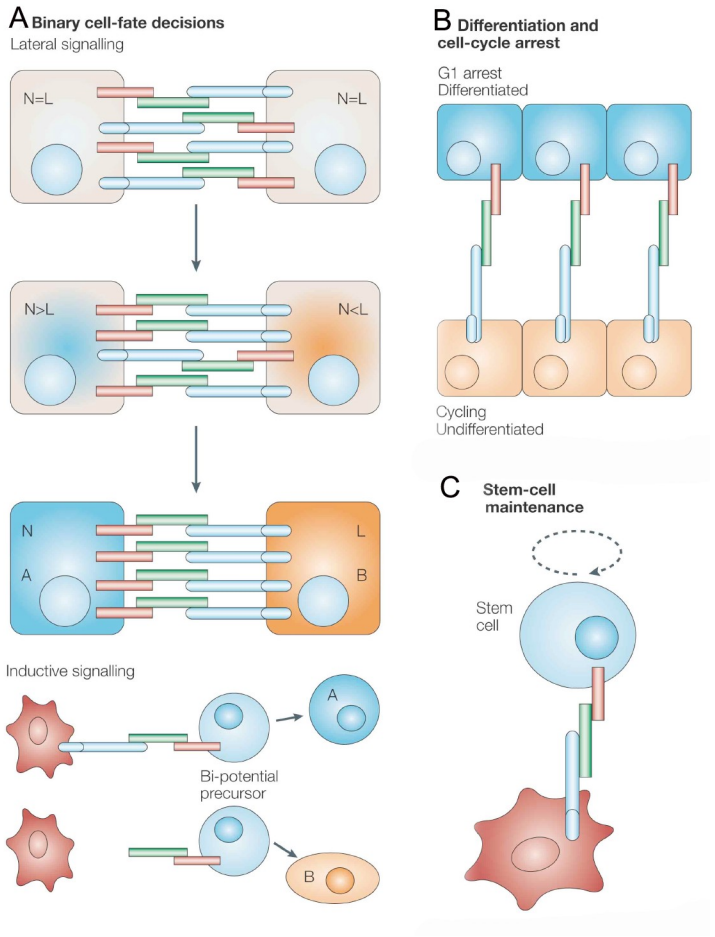

Figure 7. Notch signalling has pleiotropic effects in many different organs.

Three main effects are represented schematically. **a)** Notch signalling influences binary cell-fate decisions via lateral or inductive signalling. In lateral signalling, two equipotent cells that initially express equal amounts of ligand (L) and Notch (N) gradually express either the ligand or Notch. The Notch-expressing cell receives activation signals from the ligand-presenting neighbouring cell, resulting in these two cells adopting very different fates. Inductive signalling occurs between two different cell types. A bi-potential precursor cell is instructed to adopt a particular cell fate (for example, cell fate A) after interacting with a distinct neighbouring cell expressing Notch ligands. In the absence of this Notch signal, the precursor cell would adopt another fate (for example, cell fate B) by default. **b)** A Second property of Notch is its ability to influence differentiation and cell-cycle progression. Notch signalling between two developmentally related cells can initiate terminal differentiation processes and induce cells to exit the cell cycle. **c)** Notch signalling can maintain stem cells or precursor populations in an undifferentiated state. (Adapted from Radtke et al., 2003).

1.2.1 Bynary cell-fate decision

Bynary cell-fate decision during neurogenesis in flies and vertebrates was the first function of Notch to be well characterized (Artavanis-Tsakonas et al., 1999). This is most apparent during the development of neuronal-precursor cells of the sensory organs (SOP, *Drosophila*), which originate from a group of equipotent cells that have the capacity to develop into either neuronal-precursor cells or epidermal cells. Initially, the precursor cells express Notch and its ligand, but the concentrations of these proteins start to differ between neighbouring cells. Small differences in receptor and/or ligand concentrations are amplified over time, leading to cells that exclusively express either Notch or its ligand. The cell that receives Notch signals is inhibited from pursuing the path that leads to neuronal development and adopts an epidermal-cell fate, whereas cells that exclusively express ligands are driven into the neuronal-cell fate, a process called Latheral Inhibition (Kimble et al., 1997).

Another protein involved in SOP fate is Numb. The *Numb* loss-of-function phenotype is, in many respects, opposite to that associated with the loss of Notch. At each asymmetric division, there is a differential level of Notch signaling between the two daughter cells that is opposite to the Numb levels. This different segregation leads to the different fates of the two cells. Experiments in which double mutants of *Notch* and *numb* were generated place *numb* genetically upstream of *Notch*. The contrasting phenotypes of *Notch* and *numb*, together with the discovery that the proteins can bind to each other directly, implied that Numb dictates the fate of SOP progeny by negatively regulating Notch (Guo et al., 1996).

Notch signalling can also occur between two deveopmentally distinct cells referred to as inductive cell-fate determination (Artavanis-Tsakonas et al., 1999). In this case, Notch and its ligands are expressed exclusively on two different cell types. The cell expressing the receptor, and therefore the recipient of the Notch signal, is induced to differentiate into a particular cell lineage. For example, a bipotential mouse neural-crest stem cell can be induced by Notch to adopt a glial-cell fate as opposed to a neuronal one by Notch ligands expressed on neuroblasts (Morrison et al., 2000).

Mouse thymic epithelial cells expressing ligands for Notch1 induce early lymphocyte precursors to adopt the T-cell fate as soon as they enter the thymus, whereas in the absence of Notch1 signalling these precursors adopt the B-cell fate as the default pathway (Inductive cell-fate determination) (Osborne et al., 2007).

1.2.2 Differentiation

Besides influencing the choice between two possible cell fates, Notch signalling can induce or enhance terminal differentiation. Notch signalling in epidermal stem cells thus differs from other progenitor cell populations in promoting, rather than suppressing, differentiation .In human skin, Notch signalling initiates a terminal differentiation programme (Lowell et al., 2000). In the adult mouse skin and keratinocytes this differentiation programme is triggered by Jagged-mediated Notch signalling, which induces early differentiation markers and cell-cycle arrest by upregulating p21WAF1/Cip1 (Rangarajan et al., 2001). Interestingly in others tissues Notch signaling inhibits differentiation such as during maintainance of stem cells in instestine and brain.

1.2.3 Role of Notch in Embryonic Development

Mutations have been introduced in mice for each of the four *Notch* genes (*Notch1–Notch4*) and four Notch ligand genes (*Delta-like1*, *Delta-like4*, *Jagged1*, and *Jagged2*). Mice, homozygously disrupted with either *Notch1* (Molofsky et al., 2004, Swiatek et al., 1994) or *Notch2* (Conlon et al., 1995, Hamada et al., 1999) are fatal at approximately embryonic day 11 . *Notch3*-null (Krebs et al., 2003) and *Notch4*-null mice (Krebs et al., 2000) survive without any apparent phenotypic abnormalities. Homozygous inactivation of *Delta-like1*, *Delta-like4*, or *Jagged1* causes embryonic lethality during E9.5–E12.5 (73), and *Jagged2*-null mice die perinatally (Hrabe et al., 1997). These findings indicate that most of the individual *Notch* and ligand genes have non redundant roles in mouse embryogenesis. Somitogenesis, abnormal vasculature formation, increased cellular apoptosis, excessive neuronal differentiation, etc., are observed in these mutant mice. There are, however, both similar and dissimilar phenotypes in these mice, and the causes of fatality in early to midgestation stages are not likely to be uniform in the knockout mice for each gene. On the other hand, successful progression to midgestation stages implies that Notch signaling is unnecessary for the very early stage of embryogenesis, including the fertilized egg stage (Jiang et al., 1998). This is consistent with the fact that activation of Notch signaling in Embryonic Stem Cells (ESCs), which are derived from the inner cell mass, does not block differentiation of ESCs (Shi et al., 2005).

However a predominat role of Notch pathway activity has been observed in regulating stem cell of mammary gland, central nervous system, vessels, hematopoietic system, intestine and muscle.

1.2.4 Notch Role in the Central Nervous System During Embryogenesis

Among undifferentiated neuroectodermal cells with the same potential during *Drosophila* embryogenesis, some cells will eventually express Delta at higher levels, which sends a signal to surrounding cells that uniformly express the Notch receptor. Cells receiving the signal are blocked from differentiating to neuroglioblasts (NGBs) and eventually assume another differentiation fate. On the other hand, cells that express Delta differentiate to NGBs and subsequently to neurons and glial cells. Accordingly, insufficient levels of Notch signals result in the "neurogenic phenotype," in which all cells with neuronal potential differentiate into neurons.

A conditional Notch1 knockout study (Scmitt et al., 2004) provides further support that Notch signaling inhibits the premature onset of neurogenesis. These studies, as well as those of CSL knockout mice (Lutolf et al., 2002, Hitoshi et al., 2002), suggest that, in addition to the differentiation blockade, this pathway is required for the maintenance and expansion of the neural stem/progenitor cell pool. Inactivation of HES1 and HES5 in mice variably induces precocious neuronal differentiation accompanied by a decrease in neural progenitors. Together, these studies indicate that HES genes are the conserved targets of Notch-CSL signaling for regulating the expansion and differentiation of neural progenitors.

Importantly, Notch signaling either promotes or, by default, facilitates glial cell fate, perhaps as a consequence of inhibiting neuronal cell fate (Morrison et al., 2000). Therefore, Notch signaling might act on neural stem cells in two steps: (a) initially inhibiting neuronal fate while allowing for glial cell fate; and (b) then promoting differentiation to astrocytes while inhibiting differentiation to both neurons and oligodendrocytes.

1.2.5 Notch role in Vasculature Formation

Mice with disrupted Notch receptors display various abnormalities in blood vessel formation, such as proliferation and migration of endothelial cells, smooth muscle differentiation, vascular remodeling processes, and arterial-venous identification. These studies describe the involvement of Notch1 (Krebs et al., 2000), Notch3, and Notch4 receptors and Delta-like4 (Krebs et al., 2004) and Jagged1 ligands in vasculature formation.

1.2.6 Stem cell functions

In mammals, a wide variety of cells use the Notch signaling system for embryonic development and, in adults, maintenance of stem cells. Together with other signaling pathways, such as Wnt and hedgehog, Notch signaling pathway regulates stem cell self-reneawel and is involved in various stem and early progenitor cell systems in both the developmental and adult phases (Mofofsky et al., 2004). Adult stem cells are considered to maintain homeostasis of cells and tissues throughout life. The adult stem cells maintain the number of stem cells, as well as terminally differentiated cells, during normal turnover and repair damage after injury.

Involvement of Notch signaling occurs during both normal status and injury in various stem cell systems:

- Recently, osteoblasts have been identified as one of the bone marrow Hematopoietic Stem Cell (HSC) niches and Notch signaling is actively involved in HSC maintenance and growth in these niches (Zhang et al., 2003). Notch ligands such as Jagged1 and signal transmission to the Notch receptor-expressing HSCs might be one of the molecular mechanisms underlying the regulation of HSC in the bone marrow.
- Involvement of Notch signaling in postdevelopmental stem cell systems is best understood in the skin, particularly in the hair follicles. Notch1, Notch2, and Notch3 are expressed and differentially localized to various layers of the hair follicle (Pan et al., 2004).
- Intestinal epithelial stem/progenitor cells are localized in the basal area of the crypts and continuously supply multiple types of mature cells. These cells express Notch receptors and molecules necessary for Notch signaling. The administration of gamma-secretase inhibitors induces gross histologic changes in the intestinal epithelial layer of mice, such as an increased number of Goblet cells, endocrine cells, and abnormal crypts (Searfoss et al., 2003, Wong et al., 2004, van Es et al., 2005).
- Satellite cells are stem cells of skeletal muscle fibers. Notch signaling is insufficient for the regeneration of injured muscle in aged mice. Notch inhibition impairs regeneration in young mouse muscle, and forced Notch activation restores the regenerative potential to aged mouse muscle (Conboy et al., 2005). Thus, Notch signaling is a key determinant of the muscle regenerative potential that declines with age.

1.2.7 Roles of Notch Signaling In Adult Stem Cells: Mammary gland

The mammary gland in humans and in other mammals is a dynamic organ that undergoes significant developmental changes during pregnancy, lactation, and involution. It is likely that the cellular repertoire of the human mammary gland is generated by a stem cell component. These stem cells have a unique capacity for self-renewal as well as for generating the three lineages that comprise the lobulo-alveolar structure of the adult gland: myoepithelial cells forming the basal layer of ducts and alveoli, ductal epithelial cells lining the lumen of ducts, and alveolar epithelial cells synthesizing milk proteins (Rudland et al., 1997, Hennighausen et al., 2001). Under the regulation of systemic hormones, as well as local stromal epithelial interactions, these cells proliferate extensively, differentiate during each pregnancy and lactation, and undergo apoptosis during mammary involution.

A unique property of stem cells is their ability to undergo self-renewal divisions. In normal organogenesis this process is tightly regulated, while deregulation of self-renewal might be one of the key events involved in carcinogenesis (Pardal et al., 2003).

Indeed, cell signaling pathways and transcription factors involved in the self-renewal of normal stem cells have all been implicated in carcinogenesis. These pathways include Hedgehog, Notch and Wnt, as well as the transcription factor B lymphoma Mo-MLV insertion region 1 (Bmi-1). The existence of self-renewing multipotent mammary stem cells has been clearly demonstrated by transplantation studies in mice and rats (DeOME et al., 1996, Smith et al., 2001, Kim et al., 2000). Fragments of mammary epithelium marked with mouse mammary tumor virus were able to regenerate a new gland after transplantation into a mammary fat pad cleared of its epithelial components (Kordon et al., 1998).

Serial transplantation of the clonally derived outgrowth recapitulated the entire functional repertoire of the gland, demonstrating the existence of self-renewing and multipotent mammary stem cells. A recent study in mice combining long-term labeling in vivo using bromodeoxyuridine with immunosorting and transplantation showed that mammary stem cell antigen-1 (SCA-1)-positive population is enriched in progenitor cells able to regenerate the gland in vivo (Welm et al., 2002). The cultivation of normal mammary stem and progenitor cells has been limited by the lack of suitable systems that permit the propagation of these cells in an undifferentiated state. When primary cultures of mammary epithelium from rodents or humans are cultured on solid substrate, they undergo limited replication and differentiate in a process that is regulated by hormonal factors, extracellular matrix, and cell–cell interactions (Muschler et al., 1999, Romanov et al., 2001, Reynolds et al., 1996). A major advance in neural stem cell research was achieved when it was found that an undifferentiated multipotent population of neural cells can be grown in suspension as neurospheres (Weiss et al., 1996).

On the basis of the hypothesis that stem cells might be able to grow in anchorage-independent conditions, was developed a novel culture system for human mammary epithelial stem and progenitor cells. In culture system in vitro, mammospheres are grown in suspension and are enriched in mammary stem/progenitor cells capable of self-renewal and multi-lineage differentiation. It was also shown that mammospheres contain cells capable of clonally generating complex functional ductal alveolar structures in reconstituted three-dimensional culture systems in Matrigel, and when combined with human mammary fibroblasts they are able to reconstitute the mammary tree in the cleared mammary fat pad of NOD/SCID mice (Liu et al., 2006). The use of this culture system has enabled to begin to elucidate the pathways that regulate the self-renewal and differentiation of normal mammary stem and progenitor cells.

Recently the mammosphere system described above was used to study the role of Notch1 signaling in mammary cell fate determination. As shown in Figure 8 Notch signaling can increase mammospheres formation as observed by *in-vitro* experiments (Krause et al., 2002). These findings suggested that Notch1 signaling is active in several distinct developmental stages of the mammary gland and that Notch acts as a regulator of asymmetric cell fate decisions also in mammary gland. Notch1 activation promoted the self-renewal of stem cells, whereas in later stages of development it biased cell fate decisions in mammary progenitor cells toward the adoption of a myoepithelial cell fate versus an epithelial cell fate (Dontu et al., 2003).

The Notch pathway was shown to be involved also in the normal development of the mammary gland. In vitro, overexpression of the constitutively active form of Notch4 inhibits the differentiation of normal breast epithelial cells. Smith and colleagues also demonstrated that, in vivo, Notch4 has an important role both in normal mammary development and in carcinogenesis.

Recently Notch1 was shown to be a key regulator of asymmetric cell division in human breast epithelial stem cells (Clarke et al., 2005, Clarke et al., 2004). These findings about the role of Notch in promoting the self-renewal of mammary stem cells, in addition to previous observations that it can function as a proto-oncogene (Uyttendaele et al., 1998, Soriano et al., 2000), suggest that abnormal Notch signaling might be involved in carcinogenesis, through the deregulation of normal mammary stem cell self-renewal.

The current knowledge of the Notch signaling pathway in various types of stem and early progenitor cells. Ex vivo stem cell expansion is fundamental to the success of stem cell-based regeneration medicine, and it is likely that Notch signaling has a role in stem cell expansion.

The effects of Notch signaling on progenitor cell survival have been demonstrated, and tumorigenic aspects must be considered.

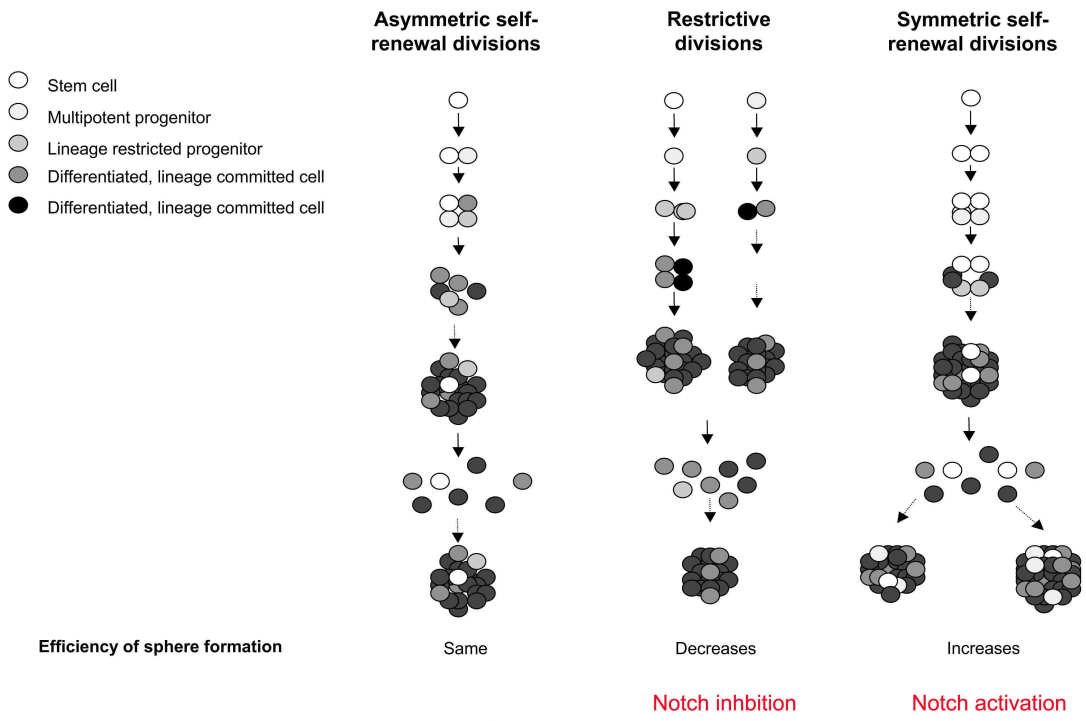

Figure 8

Figure 8. Effect of stem cell self-renewal

a) Asymmetric stem cell self-renewal divisions result in constant mammosphere numbers in serial passages. **b)** Proliferation of stem cells or progenitor cells through restrictive divisions results in a decreasing number of spheres in serial passages (Notch inhibition) **(c)** Symmetric self-renewal divisions of stem cells result in an increasing number of mammospheres in serial passages (Notch activation). Adapted from Li uet al., 2005.

1.2.8 Notch1 and epithelial-mesenchymal transition

As occurs during development, Notch cooperates with other signaling pathways in the transformation process. Notch has recently been shown to promote epithelial-to-mesenchymal transition (EMT) during cardiac valve formation, via E-cadherin downregulation (Greco-Bessa et al., 2004). One implication of this work is that Notch, acting through a similar mechanism, may also be involved in the EMT process that occurs during tumor progression and converts polarized epithelial cells into motile, invasive cells (Timmerman et al., 2004).

1.2.9 Notch1 and angiogenesis

The realization that tumours require new blood vessels (angiogenesis) to provide oxygen and nutrients led to many efforts to develop anticancer therapeutics that block tumour angiogenesis. The best validated of these approaches involves the blockade of the vascular endothelial growth factor (VEGF) signalling pathway, either by blocking VEGF itself or by blocking the primary VEGF receptor, VEGFR2. One such signalling pathway is the Notch system, and recent studies have focused attention on one particular ligand for the Notch receptors, Delta-like ligand 4 (DLL4), which is normally induced by VEGF as a negative-feedback regulator of vascular growth (Thurstone et al., 2007). Angiogenesis during tumor growth is also promoted by hypoxic condition and interestingly in a recent paper it was shown that hypoxia-induced HIF-1alfa potentiates Notch activation (Pear et al., 2005).

In summary, the work of Gustafsson et al. has shown a link between hypoxic signaling and Notch. As both of these pathways are associated with multiple aspects of neoplasia, it will be important to determine the precise contexts in which these pathways influence the transformed state. Hopefully, these results will guide the development of therapeutics based on the intersection of Notch and hypoxic signaling.

1.3 Notch and Cancer

1.3.1 Notch as an oncogene

The oncogenic role of Notch was first identified in human T-cell neoplasia. Some of these cancer cells possess a specific chromosomal translocation involving the Notch1 locus on chr.7 and the T-cell-receptor-(TCR)beta locus on cr.9. The fusion of these two loci — t(7;9) (q34;q34.3) — was identified in a T-cell acute lymphoblastic leukaemia (T-ALL) (Reynolds et al., 1987).

Importantly, the t(7;9) translocation does not juxtapose the entire human NOTCH1 gene to the TCRβ locus, but just the carboxy-terminal region from within the EGF-repeat 34 of NOTCH1. This leads to expression of a truncated NOTCH1 protein that corresponds to the cytoplasmic portion. As all T-ALLs with t(7;9) translocations show this feature, it was proposed that deregulated expression of the cytoplasmic portion of the NOTCH1 protein causes T-ALL in humans (Figure 9). This interpretation is supported by the generation of mouse models for T-ALL. Mice reconstituted with haematopoietic progenitor cells expressing the human N1ICD proteins develop T-cell leukaemia. During this process, they generate immature double-positive (DP) T cells in the bone marrow (Pear et al., 1996) with simultaneous inhibition of B-cell development, indicating that NOTCH1 signalling drives haematopoietic progenitor cells into the T-cell lineage (Pui et al., 1999).

Indeed, loss-of-function experiments in which Notch1 in bone-marrow progenitors was inactivated, show that Notch1 is essential for normal T-cell lineage commitment. Aberrant N1ICD expression in bone-marrow progenitors leads to immature DP T cells that are developmentally blocked at this stage. These DP T cells were initially of polyclonal origin and not cycling (Osborne et al., 2006). With increasing time, however, these mice develop highly aggressive monoclonal T-cell tumours, which indicates that additional mutations cooperate with N1ICD to transform non-cycling cells into aggressive, rapidly cycling tumour cells. Experimentally, Notch1 can collaborate with c-Myc (Girard et al., 1996). Overexpression of N1ICD in haematopoietic cells gives rise exclusively to T-cell neoplasia. No tumours of myeloid origin have been described so far, indicating that N1ICD cooperates with T-cell-specific signals to exert its oncogenic potential.. Proviral integration of the Moloney murine leukaemia virus (MuLV) or feline leukaemia virus into Notch1 or Notch2, respectively, causes T-cell leukaemia (Girard et al., 1996). In both cases, the cytoplasmic domain of the Notch proteins is expressed under the influence of the viral promoter. Consistent with these findings, forced expression of the Notch ligand Dll4 also results in the

development of T-cell leukaemia (Yan et al., 2001) and T-cell lymphoproliferative disease in another (Dorsh et al., 2002). Finally, Notch activity participates not only in the initiation of cancers, but also in their maintenance, as shown by Weng et al. for Notch-transformed T-ALL cells (Weng et al., 2003).

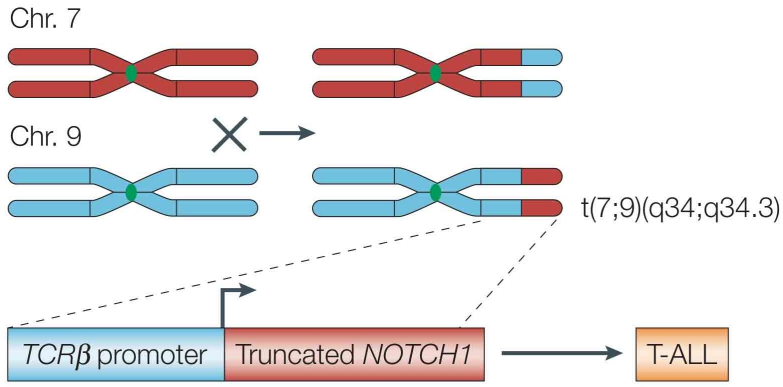

Figure 9. Notch1 in T-ALL

Schematic rappresentation of translocation occuring in T-ALL between cr.7 and 9. This leads to the expression of truncated Notch1 transcripts (N1ICD) from the TCRbeta promoter, causing T-ALL. From Radtke et al., 2003.

1.3.2 Other tumors

Based on the known role of Notch signaling in regulating epithelial differentiation in developing mouse pancreas recently it has been found that the Notch pathway activation is a direct consequence of EGF receptor signaling in exocrine pancreas. Moreover, it was demonstrated that Notch is a requisite downstream mediator of TGF-alfa induced changes in epithelial differentiation (Myamoto et al., 2007).

These studies define a novel role for Notch in regulating metaplastic conversion between epithelial cell types and suggest an important link between EGF receptor signaling and Notch pathway activation in the context of pancreas tumorigenesis. Deregulated expression of Notch receptors, ligands, and targets is also observed in other solid tumors, including cervical, head and neck, endometrial, renal, lung, ovarian, and prostate carcinomas, osteosarcoma, mesothelioma, gliomas, and medulloblastomas (Zagouras et al., 1995, Dang et al., 2000, Rae et al., 2000, Santagata et al., 2004, Hopfer et al., 2005, Fan et al., 2004, Pupow et al., 2005, Weijzen et al., 2002, Bocchetta et al., 2003, Hendrix et al., 2002). High-level expression of Notch-1 and Jagged-1 is associated with metastasis in prostate cancer (Santagata et al., 2004). Hodgkin's lymphomas, anaplastic large-cell non-Hodgkin's lymphomas, some acute myeloid leukemias, B-cell chronic lymphoid leukemias, and multiple myeloma also show deregulated expression of Notch receptors or ligands .

1.3.3 Breast Cancer

Several studies highlight the potential importance of the Notch1 signaling pathway in human breast cancer and underscore the necessity for future studies to better understand how this gene contributes to mammary epithelial cell transformation. The first evidence that aberrant Notch1 signaling might play a role in mammary tumorigenesis came from the identification of MMTV insertions in the *Notch1* locus in MMTV-*Neu* mammary tumors (Dievart et al., 1999). To identify genes that collaborate with *Neu*/*erbB2* in mammary tumorigenesis, MMTV-*Neu* mice were infected with the MMTV virus and tumors arising in these mice were analysed to identify the insertion sites of the MMTV provirus. Surprisingly, in MMTV-*Neu* tumors, *Wnt-1* was the only affected locus (4/24 tumors) among the genes previously known as affected by MMTV insertion (Callahan et al., 1996). However, in 2/24 tumors the MMTV provirus had inserted into the *Notch1* locus. These insertions caused rearrangements in the *Notch1* gene leading to expression of a truncated Notch1 protein. Both of the MMTV insertions into the *Notch1* locus occurred upstream of the Notch1 transmembrane domain, in a manner similar to that observed in Notch4(*int-3)* mammary gland tumors (Robbins et al.,

1992), leading to expression of the constitutively active intracellular domain of Notch1. The absence of MMTV insertions into the majority of loci previously identified as targets for provirus insertion indicates that, in the context of an *erbB2*/*Neu* transformed mammary gland, disruption of specific loci may be selected for and contribute to the tumorigenic process.

Thus, Wnt-1 and Notch1 may contribute to Neu-induced tumorigenesis, instead other MMTV integrations do not. Alternatively, analysis of a greater number of MMTV-infected/MMTV-*Neu* transgenic mice may reveal additional MMTV integration sites involved in breast cancer tumorigenesis.

A recent study has implicated Notch1 in human mammary tumorigenesis and also provides insight into the possible mechanism by which Notch1 exerts its tumorigenic effect (Weijzen et al., 2002). An assessment of human breast cancer cases demonstrated that of seven human breast tumors analyzed, Notch1 was highly expressed in all of the cases and the highest levels of Notch1 expression were observed in Ras-positive tumors (Weijzen et al., 2002). In the Ras-transformed fibroblasts, downregulation of Notch1 expression using antisense technology led to a significant decrease in the proliferation rate of these cells. Moreover, inhibition of Ras signaling blocked upregulation of N1ICD indicating that Ras acts upstream of Notch1. In addition, expression of oncogenic Ras led to higher levels of Delta-like1 and Presenilin1 proteins, which may increase Notch1 processing thus accounting for the high levels of the Notch1 intracellular cleavage product found in these cells. Importantly, hTERT/SV40 T antigen-expressing human fibroblasts expressing N1ICD proliferate more rapidly than control cells and form colonies in soft agar, highlighting the possibility that N1ICD may mediate, at least in part, the tumorigenic effects of oncogenic Ras. Furthermore, cleavage of Notch1 is induced by the MAPK/SAPK pathway upon oncogenic Ras activation. These studies indicate that, crosstalk between Ras and Notch1 may play a role in the tumorigenic process.

To investigate the consequences of modulating Notch activity in the murine mammary epithelium, mouse lines were generated carrying MMTV LTR-driven transgenes encoding a constitutively activated form of the human Notch1 receptor (hN1ICD) (Kiaris et al., 2004). In the case of the MMTV-hN1ICD animals, they develop lactation-dependent tumors that, however, regress at weaning, because the neoplastic cells apparently retain the ability to respond to apoptotic cues encountered during the process of mammary involution. Eventually, these regressing neoplasms apparently evolve into nonregressing adenocarcinomas. Moreover, by exploring the phenotypic consequences of antagonizing the endogenous Notch activity in the mammary epithelium through the transgenic expression of Deltex, cyclin D1 was found to be an in vivo target of Notch1 signals. Consistent with this observation, it was

shown that Ras-induced tumorigenesis, which depends on the presence of cyclin D1 activity, can be suppressed by expressing Deltex (Kiaris et al., 2004).

The demonstration that the expression of Deltex interferes with Ras-driven oncogenesis in the mammary gland is also an indication, that the mammary tissue is sensitive to Notch signals and consequently suggests the existence of a crosstalk between Ras and Notch signals in this tissue via cyclin D1. Indeed, Cyclin D1 overexpression is now established as a common feature of many human tumors (Hulit et al., 2004,). Thus, cyclin D1 transcription is up-regulated in Notch1-induced murine mammary tumors that arise during lactation (Kiars et al., 2004). In rat kidney epithelial cells containing an estrogen-inducible activated Notch1 (RKE-ER-N1ICD), cyclin D1 mRNA levels increase when the N1ICD is activated and the cells are transformed (Ronchini et al., 2000). Thus, cyclin D1 transcription might have been up-regulated in RKE-ER-N1ICD or by a mitogen-activated signal transduction pathway (Albanese et al., 1995).

Recent data showed that the expected high level of N1ICD transgene expression in tumors resulted in up-regulation of known Notch1 transcriptional targets, such as Hes1 and Hey1, and was paralleled by high Myc transcript levels. Indeed Myc is a direct Notch1 target. These results unveil a Notch1/Myc relationship by using this mouse model that documented a strong association between high levels of Notch1 and Myc expression in human breast cancers.

Interestingly, recent evidence suggests that Wnt or Notch signaling may also be deregulated in human breast cancer (Ayyanan et al., 2006). Indeed, increased Wnt signaling is sufficient to cause transformation of primary HMECs (human mammary epithelial cells), with early activation of the DDR followed by a cascade of events resulting in the tumorigenic phenotype. Expression of Notch ligands Dll1, Dll3, and Dll4 is increased, and Notch activation is required for Wnt-induced transformation both in vitro and in vivo. Finally, analysis of a substantial number of human breast carcinomas indicates that these findings are likely to be relevant to the clinical situation.

Therefore, both Wnt and Notch signaling pathways are important in maintaining and amplifying progenitor cells in different tissues (Koriken et al., 1998), including the breast (Li et al., 2005).

Thus, in concomitance with the biochemical events described above, a further factor to be considered is the existence of subpopulations of HMECs with different susceptibility to malignant transformation that may be selectively amplified by activation of Wnt and/or Notch signaling pathways.

The crosstalk between these pathways may impinge especially on early steps of breast carcinogenesis, with potential impact on novel treatments and/or prevention of breast cancer.

1.3.4 Clinical DATA

Aberrant activation of Notch receptors has been shown to cause mammary tumors in mice and recently Notch1 and the Notch ligand Jagged1 overexpression are also associated with poor clinical outcomes in human breast cancer (Reedijk et al., 2005).

Recently in situ hybridization was used to analyze expression of Notch ligands and receptors in human breast cancer. High levels of JAG1 and Notch1 were noted in a subset of tumors with poor prognosis pathologic features. Patients with tumors expressing high levels of JAG1 or Notch1 had a significantly poorer overall survival compared with patients expressing low levels of these genes. These data (a) identify novel prognostic markers for breast cancer, (b) suggest a mechanism whereby Notch is activated in aggressive breast tumors, and (c) may identify a signaling pathway activated in poor prognosis breast cancer which can be therapeutically targeted.

Recently, same results have been obtained through immunohistochemical analysis of the same breast cancer cases (N=127) that patients with tumors expressing high levels of JAG1 protein had a worse outcome than those with tumors expressing low levels (Dikson et al., 2007). It was also describe that when tumors were classified as either high or low for JAG1 mRNA or protein expression, there was only 65% agreement between the two methods of expression analysis. When JAG1 mRNA and protein data were combined, patients with tumors expressing low levels of both had a 10-year survival of 53% and median survival of 131 months. In comparison, patients with tumors expressing either high levels of JAG1 protein, mRNA or both had reduced 10-year survival and median survival (31%, 19%, 11% and 77, 43, 23 months respectively; $P<0.0001$).

These data show that quantification of JAG1 mRNA and protein levels in breast cancer can be used to identify patients who have a significant survival disadvantage and who may benefit from therapies (such as gamma-secretase inhibitors) that target signaling through the Notch pathway.

A much clearer indication has come from two surveys examining Numb expression (Pece et al., 2004), a negative regulator of Notch pathway, and the mRNA levels of Notch receptors and their ligands (Reedijk et al., 2005) in breast carcinoma samples. Numb was downregulated in about 50% of tumors due to ubiquitination and proteosomal degradation, and its levels were inversely correlated with grade and proliferation rate (Pece et al., 2004)

(Table1). Furthermore, the authors showed that colony formation was reduced by reintroducing Numb into epithelial cells derived from Numb-negative tumors.

		NUMB			
	N	class 1 (%)	class 2 (%)	class 3 (%)	p-value
All subjects	321	38.3	16.8	44.9	
Grade1	70	11.4	17.1	71.4	
Grade2	124	44.4	17.7	37.9	
Grade3	127	47.2	15.7	37.0	<.0001
Ki-67 < 22%	158	26.6	15.8	57.6	
Ki-67 ≥ 22%	163	49.7	17.8	32.5	<.0001
LN - Negative	231	39.4	16.9	43.7	
LN - Positive	90	35.6	16.7	47.8	0.492

Table1. Correlation between Numb status and clinical-pathological features.

Class 1-3 define three types of tumors with different Numb immunoreactivity.

Class-1 (38.3% of the cases) tumors showed Numb staining in <10% of the neoplastic cells, whereas class-2 and -3 tumors (16.8% and 44.9% of investigated cases, respectively) showed Numb immunoreactivity in 10–50% and >50% of the tumor cells, respectively. Thus, more than one half of all breast tumors (classes 1 and 2 combined) had reduced levels of Numb. Remarkably, a strong inverse correlation was found between Numb expression levels and tumor grade (P = 0.001) and Ki67 labeling index (P = 0.001), which are known indicators of aggressive disease. N, number of tumors analyzed; Ki67, proliferative index; LN, lymph nodes. P value was obtained using the Mantel-Haenszel Chi square statistics.

Adapted from Pece et al., 2004

1.3.5 Notch and p53

A mechamism by which Notch1 modulates cellular transformation is the negative role of Notch signaling on p53 transactivation function (Kim et al., 2007). The N-terminal fragment of N1ICD, which can interact with p53, inhibits p53 phosphorylation and represses p53 transactivation. In addition, Notch signaling downregulated p53-dependent apoptosis induced by UV irradiation.

Others studies confirm the crosstalk between Notch1 and p53 pathways. It was shown that cells expressing N1ICD are chemoresistant in a wild-type p53-dependent manner (Mungamuri et al., 2006).

Additionally, a new function for human Numb has been described as a regulator of tumor protein p53 (Colaluca et al., 2008). Numb enters in a tricomplex with p53 and the E3 ubiquitin ligase HDM2 (also known as MDM2), thereby preventing ubiquitination and degradation of p53. This results in increased p53 protein levels and activity, and in regulation of p53-dependent phenotypes. Biologically, this results in an aggressive tumour phenotype, as witnessed by findings that Numb-defective breast tumours display poor prognosis.

As said above, aberrant Notch signaling contributes to more than half of all human T-cell leukemias, and accumulating evidence indicates Notch involvement in other human neoplasms. Recently it was shown that Notch suppresses p53 in lymphomagenesis through repression of the ARF-mdm2-p53 tumor surveillance network. This shows that continued Notch activity is required to maintain the disease state. Furthermore, it was proposed that suppression of p53 by Notch is a key mechanism underlying the initiation of T-cell lymphoma.

1.3.6 Notch Inhibitors as Cancer Therapeutics

Numerous studies have proposed inhibition of Notch signaling as a strategy for cancer treatment (Nickoloff et al., 2003). Selective strategies include antisense, monoclonal antibodies, and RNA interference. Nonselective strategies include gamma-secretase inhibitors, and Ras signaling inhibitors. gamma-Secretase inhibitors (GSI) have the most immediate therapeutic potential. GSI cbz-IL-CHO has Notch-1 – dependent antineoplastic activity in Ras-transformed fibroblasts (Weijzen et al., 2002).

Like other small-molecule agents, GSIs have multiple effects. Gamma-Secretase cleaves all Notch receptors and some ligands but also ErbB4, syndecan, CD44, and other proteins (Nickoloff et al., 2003, Kopan et al., 2004). Therapeutically, this may actually be advantageous because many cancers coexpress two or three different Notch homologues. Some GSIs may also affect proteases other than gamma-secretase. Pharmacologic studies

with GSIs need to carefully address target specificity, and a complementary transcriptional silencing approach is necessary in each model. Accumulating preclinical evidence has led to the opening of phase 1 trials of a GSI in T-ALL and breast cancer (Miele et al., 2006).

The pathways affected by Notch inhibition are likely to be context dependent, and rational combinations of GSIs with other antineoplastic drugs will require mechanistic studies in individual models (Miele et al., 2006). Looking beyond GSIs, ADAM inhibitors may find clinical uses to inhibit the first ligand-induced Notch cleavage. In light of recent evidence, blocking specific E3 ligases responsible for ubiquitination of Notch ligands or monoubiquitination of Notch receptors may be an alternate approach. Small molecules that interfere with Notch coactivator binding may selectively inhibit CSL signaling but not non-canonical signaling. Depending on the relative importance of Notch downstream pathways in individual cancers and normal tissues, this may be an advantage or a disadvantage compared with drugs that block receptor activation. An important question is whether selective inhibition of one of the four Notch homologues may be therapeutically desirable. Finally, it will be important to establish whether cancer stem cell – targeted agents are optimally active alone or in rational combinations (e.g., a Notch inhibitor with a Hedgehog inhibitor).

1.3.7 Notch1 as tumour suppressor

Evidence that Notch signalling is not exclusively oncogenic but is also tumour suppressive comes from studies on skin. The epithelium of the skin comprises several layers of keratinocytes that are at various stages of terminal differentiation. In normal tissue, proliferating keratinocytes are present mainly in the basal layer of the epithelium. Basal cells proliferate for a limited number of times before initiating terminal differentiation. This event is marked by their migration to the next layer (the spinous layer) where the cells cease proliferation and become committed to terminal differentiation.

Notch1 and Notch2, as well as Jagged1 and Jagged2, are exclusively expressed in the suprabasal layers of murine epidermis (Rangarajan et al., 2001).

Notch signaling also promotes commitment of keratinocytes to differentiation and suppresses tumorigenesis. p63, a p53 family member, has been implicated in establishment of the keratinocyte cell fate and/or maintenance of epithelial self-renewal. It was shown that p63 expression is suppressed by Notch1 activation in both mouse and human keratinocytes (Nguyen et al., 2006). In turn, elevated p63 expression counteracts the ability of Notch1 to restrict growth and promote differentiation. p63 functions as a selective modulator of Notch1-dependent transcription and function, with the Hes-1 gene as one of its direct negative targets.

Thus, a complex cross-talk between Notch and p63 is involved in the balance between keratinocyte self-renewal and differentiation.

1.4 The Prolyl-isomerase Pin1

1.4.1 Phosphorylation of proteins on Ser/Thr-Pro motifs

Post-translational modifications of proteins, such as phosphorylation, are important signalling mechanisms involved in many cellular pathways, such as those controlling cell cycle, transcription, DNA repair, cell differentiation and proliferation. The phosphorylation of proteins on serine or threonine residues that immediately precede a proline (pSer/Thr-Pro) is a central signaling mechanism that has been shown to play an important role in regulating different biological processes and pathological conditions (Lu et al., 2002). Ser/Thr-Pro motifs are exclusive phosphorylation sites for a large number of Pro-directed protein kinases that play essential roles in signal trasduction and cell cycle progression. These include all cyclin-dependent kinases (CDKs), which control cell cycle transition, as well as most of the mitogen-activated protein kinases (MAPKs), and glycogen synthase kinase 3β (GSK-3β).

Many oncogenes and tumor suppressors themselves are directly regulated by Pro-directed phosphorylation and/or trigger signalling pathways involving Pro-directed phosphorylation. The peptidyl-prolyl *cis/trans* isomerase Pin1 has been identified and characterized, which specifically regulates the conformation of specific Pro-directed phosphorylation sites in certain proteins (Ranganathan et al., 1997). Pin1 is a peptidyl–prolyl isomerase that binds specifically to those motifs upon phosphorylation thereby switching the intervening peptide bond from *cis* to *trans* conformation and *vice versa*, providing an additional level of regulation (Yaffe et al., 1997) (Fig.10).

1.4.2 The prolyl-isomerase Pin1

The human Pin1 isomerase gene maps to the chromosome 19p13 and Pin1 protein is a member of the evolutionarly conserved peptidyl-prolyl isomerase (PPIase) family of proteins. Prolyl isomerases comprise three structurally distinct subfamilies; the cyclophilins, FK506-binding proteins (FKBP) and the parvulins. Pin1 is a parvulin of approximately 18 kDa protein composed of two functional domains, an aminoterminal WW domain (amino acids 1–39)(Yeh et al., 2007) involved in protein–protein interaction, and a COOH-terminal PPIase domain (amino acids 45–163) that functions as isomerase (Figure 10). These two domains are separated by a short flexible linker region. Pin1 is unique from the other parvulin family members because it recognizes Ser/Thr–Pro motifs only following phosphorylation, working in concert with protein kinases that phosphorylate these motifs as well as protein phosphatases that dephosphorylate them to control the activity and/or stability of their common targets.

PIN1 was discovered through a yeast two-hybrid screen designed to identify proteins that interact with never in mitosis gene A (NIMA), an essential mitotic kinase in Aspergillus nidulans (Lu et al., 1996). The overexpression of NIMA in yeast is lethal because it induces premature chromosome condensation followed by cell death. The overexpression of Pin1 prevented this, therefore it was suggested that Pin1 might function as a regulator of mitosis.

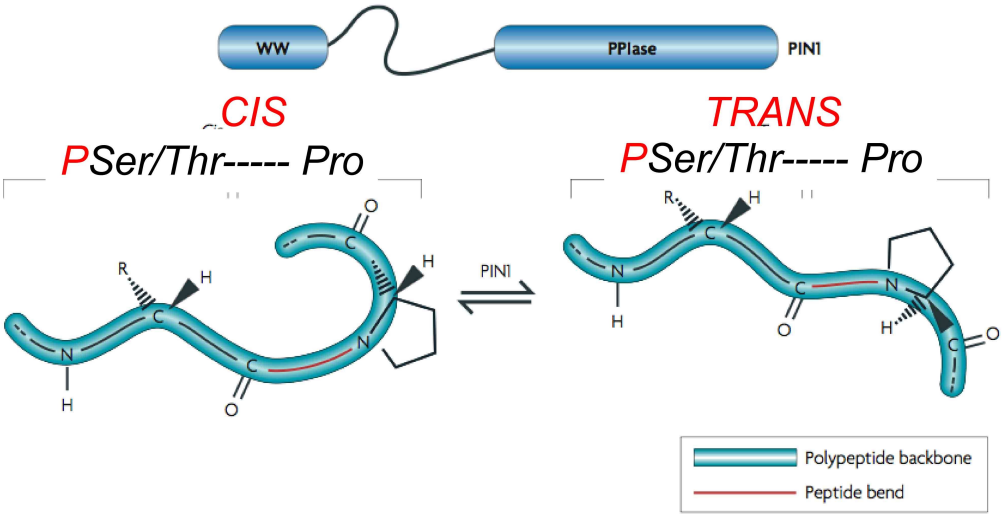

Figure 10. Pin1 structure and function

The protein Pin1 is shown in the upper part of the figure. Pin1 is an 18 kDa protein consisting of a WW binding domain and a PPiase catalytic domain. The lower part of the figure shows convertion of the conformation of the target protein from *cis* to *trans* conformation and *vice versa*. Adapted from Yeh et al., 2007.

1.4.3 Pin1 is involved in several cellular processes

Given numerous phosphorylated substrates of Pin1 it is involved in several aspects of cellular processes:

- Evidences was recently provided that Pin1 has an important function in chromosome condensation during mitosis. It was demonstrated that the interaction of Pin1 with chromatin is greatly elevated in G2/M phase and that this correlates with the presence on chromosomes of several mitotic phosphoproteins, especially topoisomerase(Topo)IIα(Xu et al., 2007). Indeed, purified Pin1 and cdc2/cyclin B kinase were by themselves sufficient to induce condensation. This reflects the ability of Pin1 to increase TopoIIα phosphorylation by cdc2/cyclinB in vitro, which in turn dramatically increased formation of a TopoIIα/Pin1/DNA complex.

- Interestingly the peptidyl prolyl isomerase Pin1 modulates also RNAP II function during the cell cycle (Xu et al., 2007b). Several assays indicate that the inhibition largely reflects Pin1 activity during transcription initiation and not elongation, suggesting that Pin1 modulates RNAP II activity, during an early stage of the transcription cycle.

- Another physiological function of Pin1 is regulating fibroblast proliferation and extracellular matrix deposition. Eosinophilic inflammation is due to chronic asthma that often culminates in subepithelial fibrosis with variable airway obstruction. Pulmonary eosinophils (Eos) are a predominant source of TGF-β1, which drives fibroblast proliferation and extracellular matrix deposition. Pharmacologic blockade of Pin1 in a rat asthma model selectively reduced eosinophilic pulmonary inflammation, TGF-β1 and collagen expression, and airway remodeling. These data suggest that pharmacologic suppression of Pin1 may be a novel therapeutic option to prevent airway fibrosis in individuals with chronic asthma (Shen et al., 2008).

- Another function of Pin1 is to regulate B cells. Antigen-specific B cells are selected in germinal centers, the structure in which these cells proliferate while accomplishing genome-remodeling processes such as class-switch recombination and somatic hypermutation. These events are associated with considerable genotoxic stress, which cells tolerate through suppression of DNA-damage responses by Bcl-6, a transcription factor required for the formation of germinal centers. Interestingly the expression of Bcl-6 is regulated by DNA damage through a signaling pathway that promotes Bcl-6 degradation. After DNA damage has accumulated, the kinase ATM promotes Bcl-6 phosphorylation, leading to its interaction with the isomerase Pin1 and its degradation by the ubiquitin-proteasome system (Phan et al., 2007). Because Bcl-6 is required for the maintenance of germinal centers, these findings suggest that the extent of genotoxic stress controls the fate of germinal center B cells by

means of Bcl-6.

1.4.4 Pin1 mouse models

Mice knock-out for *Pin1* gene in a mixed background (129SvJae/C57BL/6) develop tissue defects as they age. Such defects include retinal atrophy, decreased body weight, testicular atrophy and lack of breast epithelial expansion during pregnancy (Liou et al., 2002).

Most of these phenotypes are remarkably similar to those of cyclin D1-deficient mouse phenotypes. Of several phenotypes ob served in Pin1-/- mice, the alterations in retina and mammary gland seemed to be most drastic. Pin1-/- mice show dramatic impairments in cell survival or proliferation in the retina, especially at old age. Moreover, it is very clear and striking that in pregnant Pin1-/- female, mammary epithelia cells fail to undergo massive proliferation in the development of alveolar structures and ductal side branching. Pin1 is highly expressed in retina and mammary gland compared with other tissues, and the depletion of Pin1 causes a dramatic retinal atrophy and mam mary gland impairment (Liou et al., 2002). In another study both male and female Pin1-null mice on imbred C57BL/6 showed a reduced number of germ cells owing to an impairment in primordial germ cell (PGC) expansion, and led to a profound decrease in fertility (Atchinson et al., 2003). Although Pin1 depletion was previously reported to lead to apoptosis or mitotic arrest, neither of these defects occurred in the PGCs. Instead, the PGCs had a marked increase in duration of the cell cycle owing to a prolonged G1–S transition. These studies indicated that Pin1 is important in controlling the G1–S phase transition (Atchinson et al., 2003).

1.4.5 Pin1 and cell-cycle checkpoints

- Analyses of Pin1-null mouse embryo fibroblasts (MEFs) from mice of a mixed genetic background (129SvJae/C57BL/6) provide additional support for a role of PIN1 in proliferation that is unrelated to its function in mitosis(Fujimori et al., 1999). Fujimori et al. found that Pin1-null MEFs showed slower growth than wild-type MEFs. Moreover, Pin1-/- MEFs rendered quiescent owing to serum deprivation were markedly delayed in cell cycle re-entry in response to stimulation with serum. Additional studies by an independent group confirmed these growth defects by showing that serum-arrested Pin1-/- MEFs were resistant to cell cycle re-entry in response to insulin-like growth factor 1 (IGF1)(You et al., 20002). Moreover, following this delayed return to asynchronous growth, Pin1-/- MEFs had slower

doubling times than wild-type MEFs. These results are supported by a study in A. nidulans showing that reduced expression levels of PINA (the gene that encodes Pin1) result in an increase in the time required to complete a nuclear division cycle, which was primarily due to the lengthening of the G1–S transition(Joseph et al., 2004). Finally, it has been reported that the expression of Pin1 is positively regulated by E2F such that Pin1 expression is increased as cells progress from G0 into the S phase of the cell cycle(Ryo et al., 2002). These results, together with those from both mixed and C57BL/6 Pin1-null mice, indicate that PIN1 has a role in in G0–G1–S progression in addition to its originally proposed role in mitotic progression.

Recently has been developed a screening method to isolate Pin1 inhibitors (Uchida et al., 2003). The chemical library screen identified PiB as the most potent inhibitors with IC50s of 1.5 μM. For comparison, juglone, the only previously reported Pin1 inhibitor, which is irreversible and rather nonspecific has an IC50 of 5 μM. PiB has antiproliferative activity against a variety of cancer cell lines. Pin1 expression levels differed between the various cancer cell lines and cells with a low level Pin1 were less sensitive to inhibitor treatment than cells expressing high levels of Pin1. The effects of PiB on the growth of wild-type and Pin1-/- MEFs were also examined. PiB inhibited proliferation of wild-type MEF whereas the same concentration did not inhibit the growth of Pin1-/- MEFs.

The results suggest that one of the targets of PiB is Pin1 and that inhibition of Pin1 is apparently important for the antiproliferative activity of PiB. However, this does not rule out the possibility that PiB may have other targets in addition to Pin1 (Uchida et al., 2003).

- During the G0/G1-S phase transition, the timely synthesis and degradation of key regulatory proteins is required for normal cell cycle progression. Two of these proteins, c-Myc and cyclin E, are recognized by the FBW7alfa E3 ligase of the Skp1/Cul1/Rbx1 (SCF) complex. FBW7 binds to a similar phosphodegron sequence in c-Myc and cyclin E proteins resulting in ubiquitylation and degradation of both proteins via the 26 S proteosome (van Drogen et al., 2006).

SCF(FBW7alpha) binds a complex containing cyclin E, Cdk2, and the prolyl cis/trans isomerase Pin1 and promotes the activity of Pin1 without directly ubiquitylating cyclin E (van Drogen et al., 2006). However, due to the action of this FBW7alfa-Pin1 complex, cyclin E becomes an efficient ubiquitylation substrate of FBW7gamma.

It was also shown that Pin1 also regulates the turnover of cyclin E in mouse embryo fibroblasts (Yeh et al., 2006). The absence of Pin1 results in an increased steady-state level of cyclin E and stalling of the cells in the G1/S phase of the cell cycle. The cellular changes that

result from the loss of Pin1 predispose Pin1 null mouse embryo fibroblasts to undergo more rapid genomic instability when immortalized by conditional inactivation of p53 and sensitizes these cells to more aggressive Ras-dependent transformation and tumorigenesis.

The stability of c-Myc is regulated by multiple Ras effector pathways. It was shown that Ser 62 is dephosphorylated by protein phosphatase 2A (PP2A) before ubiquitination of c-Myc, and that PP2A activity is regulated by the Pin1 prolyl isomerase. Furthermore, the absence of Pin1 or inhibition of PP2A stabilizes c-MycThus, Ras-dependent signalling cascades ensure transient and self-limiting accumulation of c-Myc, disruption of which contributes to human cell oncogenesis.

- There is emerging evidence that Pin1 regulates the function of the p53 family of tumour suppressors in response to genotoxic insults. The p53 family members p53 and p73 both undergo proline-directed phosphorylation after DNA damage or stress exposure. Pin1 can directly bind to, and presumably isomerize, multiple phosphorylated Ser/Thr-Pro motifs in p53 (Ser 33, Ser 46, Thr 81 and Ser 315) and p73 (Ser 412, Thr 442 and Thr 482) (Zacchi et al., 2002, Mantovani et al., 2004, Wulf et al., 2002, Zheng et al., 2002). As a result, Pin1 increases the protein half-life of p53 by inhibiting its binding to the Mdm2 ubiquitin ligase, which regulates the degradation of p53. Furthermore, recently it was found that Pin1 is required for efficient loading of p53 on target promoters upon stress. Accordingly, tumor-associated mutations at Pin1-binding residues within the p53 proline-rich domain hamper acetylation of p53 by p300. After phosphorylation of p53 at Ser46 triggered by cytotoxic stimuli, Pin1 also mediates p53's dissociation from the apoptosis inhibitor iASPP, promoting cell death. In tumors bearing wild-type p53, expression of Pin1 and iASPP are inversely correlated, supporting the clinical relevance of these interactions (Mantovani et al., 2007). Pin1 also increases the stability of p73 by promoting its binding to and acetylation by p300. At the same time, Pin1 enhances the DNA-binding activity and transcriptional activity of p73 towards its target genes. Functionally, Pin1 seems to be required for maintaining DNA damage checkpoints, protecting against DNA-damage-induced apoptosis, and also to accelerate p53-dependent apoptosis by enhancing pro-apoptotic genes. However, these studies have been restricted to cell lines or embryonic fibroblasts, and it remains unclear to what extent Pin1-dependent regulation of p53 or p73 contributes to the DNA damage response in vivo, or even to tumour suppression. To investigate the physiological relationship between Pin1 and p53 and Notch1, recently Pin1-/-p53-/- mice were created (Takahashi et al., 2007). P53-deficient mice spontaneously developed lymphomas, mainly of thymic origin, as well as generalized lymphoma infiltration into other organs, including the liver, kidneys and lungs. Ablation of Pin1, in addition to p53, accelerated

the thymic hyperplasia, but the thymocytes in these Pin1-/-p53-/- mice did not infiltrate other organs. The thymocytes in Pin1-/-p53-/- mice had significantly higher levels of N1ICD than the thymocytes of p53-/- or wild-type mice. These results suggest that Pin1 control the proliferation and differentiation by regulating the N1ICD level, but these data have been obtained in the absence of p53 and in thymocytes.

1.4.6 Pin1 and cancer

A connection between Pin1 and cancer was first suggested when Pin1 was observed to be overexpressed in human cancer tissues (Wulf et al., 2001, Ryo et al., 2001). More extensive studies revealed that (with very few exceptions, such as neurons) in normal tissues Pin1 expression is associated with cell proliferation, and that it is overexpressed prevalently in human cancers, including cancer of the breast, prostate, lung and colon (Bao et al., 2004). Furthermore, increased Pin1 levels are highly predictive of cancer recurrence after prostatectomy (Ayala et al., 2003). Analysis of molecular markers for cancer revealed a close correlation between Pin1 and cyclin D1 (Wulf et al., 2001). Because cyclin D1 is an important cell-cycle regulator that is known to have a key role in the development of many cancers, this connection has led to a series of in vitro and in vivo studies that demonstrate an essential role of Pin1 in regulating cyclin D1 expression and turnover through multiple mechanisms)Wulf et al., 2001, Ryo et al., 2001, Liou et al., 2002) (Fig. 1). Phosphorylation of cyclin D1 by glycogen synthase kinase-3beta (GSK-3beta) on the pThr 286-Pro motif promotes its nuclear export and subsequent ubiquitin-mediated degradation. However, Pin1 can directly bind to and presumably isomerize the pThr 286-Pro motif of cyclin D1 and thereby prevent its nuclear export and ubiquitin-mediated degradation, resulting in cyclin D1 stabilization (Lou et al., 2002). Furthermore, Pin1 can also regulate cyclin D1 gene expression by at least three different mechanisms.

First, following the activation of c-Jun N-terminal kinases (JNKs) in response to growth-stimulating conditions, Pin1 can target to the pSer 63/73-Pro motifs in c-Jun and increase c-Jun transcriptional activity towards its target genes such as cyclin D1 (Wulf et al., 2001)). Because c-Jun has been shown to be subjected to ubiquitin-mediated degradation in a JNK-dependent manner, it is possible that Pin1 might increase protein stability of c-Jun, although it has not been examined.

Second, Pin1 can target to the pThr 246-Pro motif in beta-catenin and prevent beta-catenin from interacting with the tumour suppressor APC (adenomatous polyposis coli gene product). This enhances transcriptional activity of beta-catenin towards its target genes, including

cyclin D1, as do many genetic mutations in beta-catenin or APC that are found in human cancer (Moon et al., 2002). Consistent with this notion, Pin1 overexpression and beta-catenin gene mutations have recently been shown to be distinct oncogenic events in human hepatocellular carcinoma (Pang et al., 2004).

Third, after cytokine treatment, Pin1 can target to the pThr 254-Pro motif in the p65/RelA subunit of NF-kappaB and prevent NF-kappaB from binding to its inhibitor IkappaB. This prevents p65/RelA nuclear export and its subsequent degradation by a ubiquitin-mediated pathway (Ryo et al., 2003). This leads to enhanced nuclear accumulation, protein stability and transcriptional activity of NF-kappaB towards its target genes such as cyclin D1 and IkappaB. This may provide an explanation for the puzzling phenomenon that NF-kappaB activity is upregulated in the presence of elevated IkappaB in some cancer samples.

With the same net result of enhancing pro-proliferative signalling, Pin1 also inhibits negative feedback of MAPK signalling: in response to growth-stimulating conditions, Raf kinase is activated by Ras and then triggers a protein kinase cascade, leading to the activation of MAPKs. MAPKs can phosphorylate and inactivate Raf in a negative feedback mechanism (Dougherty et al., 2005). Pin1 prevents Raf kinase from being turned off after growth stimulation by promoting dephosphorylation of Raf (which is inhibited by phosphorylation) probably as a result of the conformational specificity of the phosphatases involved.

In addition, Pin1 is transcriptionally regulated by the E2F family of transcription factors in response to growth factors and other stimulating conditions such as activation of Her2/Neu or Ras (Ryo et al., 2002). This suggests the existence of a positive feedback loop, in which Her2/ Neu or Ras activation leads to an increase in Pin1 via E2F-mediated transcription, and Pin1 in turn positively regulates cyclin D1 function at the transcriptional level and also through post-translational stabilization.

Despite the multitude of molecules that are affected by Pin1-mediated prolyl isomerization, and despite the diverse mechanisms it modulates, the common denominator for Pin1 activity in cancer cells seems to be the amplification of pro-proliferative signalling at the level of proline-directed phosphorylation. A recent study has provided the first genetic data on the role of Pin1 in cancer development in vivo (Wulf et al., 2004). Pin1-deficienct mice are largely protected from breast cancer induced by oncogenic c-Neu or v-Ha-Ras, but not by c-Myc (Wulf et al., 2004), as is the case for cyclin-D1-null mice (Yu et al., 2004), demonstrating an essential role for Pin1 in tumorigenesis that is induced by certain oncogenes (Figure 11).

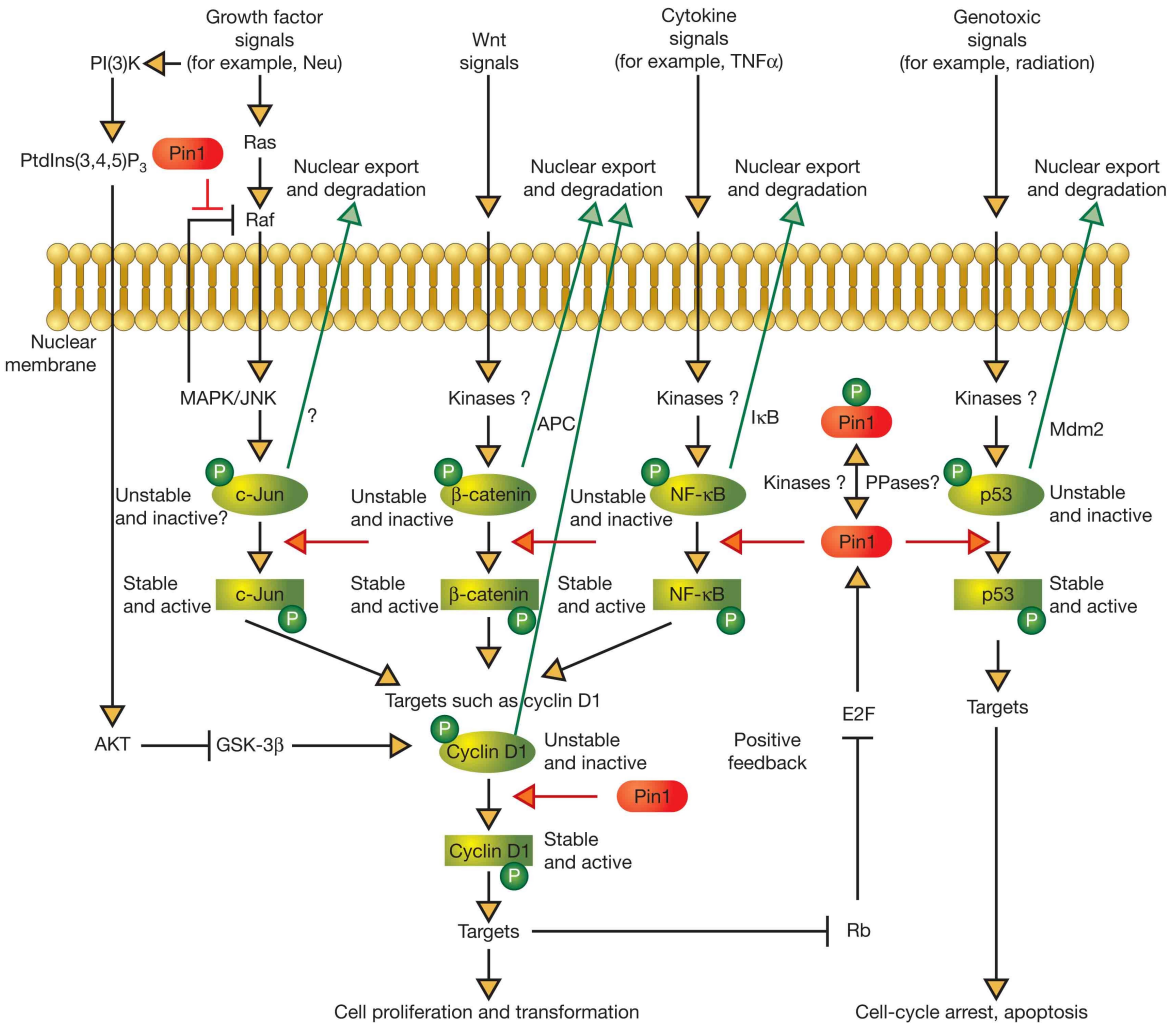

Figure 11. Pin1 regulates phosphorylation signalling following phosphorylation

The left part of the figure shows that Pin1 regulates c-Jun stability upon MAPK/JNK phosphorylation. In the middle; Pin1 regulates beta-catenin, cyclin D1 and NF-kB. Pin1 is an E2F target gene, This suggests the existence of a positive feedback loop, in which Her2/Neu or Ras activation leads to an increase in Pin1 via E2F-mediated transcription, and Pin1 in turn positively regulates cyclin D1 function.

The rigth part of the figure shows that Pin1 can increase the nuclear localization and protein half-life of p53, thereby involving cell-cycle arrest and apoptosis. Adapted from Wulf et al.,2005.

1.4.7 Pin1 regulation

Pin1 function is tightly regulated by multiple mechanisms under normal growth conditions

and during the cell cycle. One such mechanism is inhibitory phosphorylation on sites including Ser 16 at the centre of the pSer/Thr-Pro-binding pocket of the WW domain (Lu et al., 2002). This phosphorylation abolishes the ability of Pin1 to interact with its substrates. Interestingly, Pin1 seems to be hypophosphorylated in breast cancer, suggesting that Pin1 is overexpressed and also activated during oncogenesis. Although protein kinase A can phosphorylate Pin1 in vitro, it remains unclear as to which are the physiological kinases and phosphatases that regulate Pin1 phosphorylation in vivo.

Moreover, it is reported that the human Pin1 binds to Plk1 from mitotic cell extracts in vitro(Eckerdt et al., 2005). It was demonstrate that Ser-65 in Pin1 is the major site for Plk1-specific phosphorylation, and the polo-box domain of Plk1 is required for this phosphorylation. Interestingly, the phosphorylation of Pin1 by Plk1 does not affect its isomerase activity but rather is linked to its protein stability. Pin1 is ubiquitinated in HeLa S3 cells, and substitution of Glu for Ser-65 reduces the ubiquitination of Pin1. Furthermore, inhibition of Plk1 activity enhances the ubiquitination of Pin1 and subsequently reduces the amount of Pin1 in human cancer cells. Since previous reports suggested that Plk1 is a substrate of Pin1, our work adds a new dimension to this interaction of two important mitotic regulators.

2. AIM

Increasing evidences provided by several groups have shown that deregulated Notch1 is involved in human breast cancer. For example down-regulation of Numb, that negatively regulates Notch1, has been observed in about 50% of breast carcinomas (Pece et al., 2004). Furthermore, Notch1 cross-talks with several pathways that are subverted in Breast Cancer such as Ras, Wnt, c-Myc, suggesting that Notch could be a target for drug-based therapy of breast cancer (Miele et al ., 2006). Expression of Notch1 and Ras correlates in breast carcinomas and Notch1 signaling is induced by the MAPK/SAPK pathway upon oncogenic Ras activation (Weijzen et al., 2002).

Phosphorylation-specific prolyl-isomerizzation catalyzed by the prolyl-isomerase Pin1 has recently emerged as a potent signal transduction mechanism of several pathways. Pin1 has been shown to be involved in several human malignancies and in breast cancer through modulation of a multiplicity of pathways. Alteration of Pin1 have been implicated in the amplification of oncogenic signals (Yeh et al., 2007) and cooperates with the Neu/Ras pathway in mammary tumorigenesis (Wulf et al., 2004).

Based on these data the aim of my thesis was to unveil a possible role of Pin1 in regulation the Notch1 pathway and in particular in breast cancer.

3. RESULTS

3.1 Pin1 interacts with human Notch1

Since Pin1 binds phosphoSerine/Threonine-Proline sites, first of all we tested if human Notch1 (NP_060087) contains these motifs. Indeed, the cytoplasmic region of Notch1 harbors many Ser/Thr-Pro motifs, whose phosphorylation might generate binding sites for Pin1. By using SkBr-3, MDA-MB-468 and MCF-7 human breast cell lines Notch1 was immunoprecipitated and probed with MPM-2 antibody that recognizes this kind of sites. This experiment showed that endogenous Notch1 is phophorylated in Ser/Thr-Pro motifs (Figure 1a). Notably in this experiment we used an antibody that recognizes the transmembrane subunit of Notch1 protein (N1TM). This subunit of the Notch heterodimer contains a short extracellular domain, the membrane-spanning region, and an intracellular domain termed N1ICD. The extracellular subunit has not been considered.

To verify if Pin1 binds endogenous Notch1 protein same lysates (as in immunoprecipitation assay) were subjected to GST or GST-Pin1 pull-down followed by anti-Notch1 Western blot. Consistently, endogenous Notch1 present in these cells bound to GST-Pin1 but not to GST (Figure 1b). This interaction required a functional Pin1 binding domain because GST-Pin1 mutans impaired for binding GST-Y23A, GST-PPiase) did not bind endogenous Notch1 protein in GST-pull down assay (Figure 1c).

Furthermore this interaction relied entirely on phosphorylation, as demonstrated by the lack of binding upon treatment of cell lysates with phosphatase (Figure 1d). Moreover, the interaction between Notch1 and Pin1 occurs *in vivo* among endogenous proteins as shown by Western blot analysis of anti-Notch1 or anti-Pin1 co-immunoprecipitates from SKBr-3 cells (Figure 1e). Finally to evaluate if the binding between these two proteins is direct we performed a Far-western assays. To this end, we utilized a membrane-tethered Notch1 derivative (N1DE) that is constitutively processed by gamma-secretase since it lacks most of the extracellular domain (Schroeter et al., 1998). To performe the experiment with this membrane tethered N1DE we used the gamma secretase inhibitor (GSI) DAPT during the whole experiment. Control or anti-Flag antibody immunoprecipitates from pcDNA3-N1DeltaE-FLAG transfected SKBr-3 cells (GSI-treated), were subjected to Far Western using purified GST-Pin1 as a probe. Western Blot analysis with an anti-Pin1 antibody shown that the binding between endogenous Notch1 and Pin1 is direct (Figure1f).

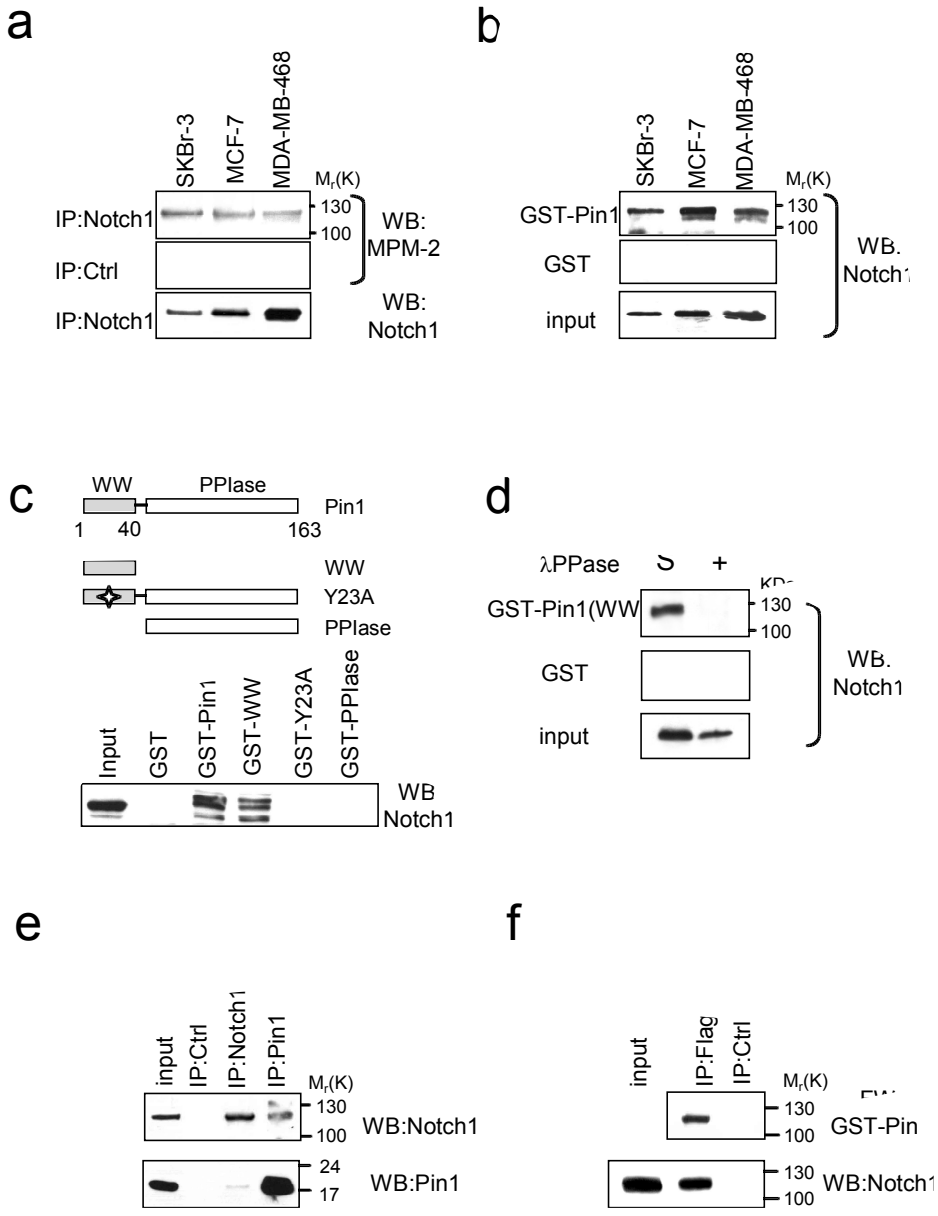

Figure 1. Pin1 binds to human Notch1

a) Endogenous Notch1 is phophorylated in S/T-P sites. Control antibody- (Ctrl) or anti-Notch1 immunoprecipitates (IP) from indicated cells were probed with anti-MPM-2 in Western blot (WB) followed by stripping and anti-Notch1 Western analysis to show immunoprecipitated protein. **b)** Notch1 binds to Pin1. Same lysates as in (a) were subjected to GST or GST-Pin1 pull-down followed by anti-Notch1 Western blot. Input lane shows 5% of total lysate. **c)** Schematic of full length GST-Pin1 and its truncation or point mutants GST pull-down of lysate from SKBr-3 cells was performed incubating with GST, GST-Pin1 or the indicated GST-Pin1 mutants, followed by anti-Notch1 Western blot. **d)** GST-Pin1 or GST pull-down experiments were performed using mock- or lambda phosphatase treated lysates from SKBr-3 cells, followed by anti-Notch1 immunoblot. **e)** Notch1 and Pin1 interaction occurs *in vivo*. Western blot analysis of control antibody, anti-Notch1 or anti-Pin1 co-immunoprecipitates from SKBr-3 cells. **f)** Notch1 and Pin1 interaction is direct. Control or anti-Flag antibody immunoprecipitates from GSI-treated, pcDNA3-N1ΔE-FLAG transfected SKBr-3 cells, were subjected to Far Western using purified GST-Pin1 as a probe, followed by anti-Pin1 immunoblot. Anti-Notch1 Western analysis of the upper panel after

stripping is shown. a)-d) Molecular weight standards are indicated in KDa.

3.2 Pin1 positively modulates Notch1 transcriptional activity

After the binding evidence we asked wheter Pin1 was able to modulate Notch signaling pathway, by analyzing the transcriptional activity of Notch1 in luciferase reporter assays.

SKBr-3 were transfected with the pcDNA3-N1DeltaE-FLAG plasmid to express N1DE. Since N1DE is constitutively processed, it releases active intracellular domain (N1ICD) that translocates to the nucleus where it acts as a transcriptional transactivator (Table 1). Luciferase reporter assays were performed with the Notch1 target promoter HES-1 (Jarriault et al., 1995) and with a TK-renilla plasmid as control for the transfection efficiency. Co-transfections experiments in SKBr-3 cells showed that over expression of wild-type Pin1, but not a catalytically inactive Pin1 mutant Pin1-S67E (Zhou et al., 2000), could enhance the transcriptional activity of N1DE (Figure 2a). Notably, overexpression of wild-type Pin1 and the catalitic inactive Pin1 mutant does not increase the luciferase activity of HES-1/Luc indicating that the effect of Pin1 is Notch1 dependent (Figure 2a). Importantly, GSI treatment confirmed a specific function of Pin1 on Notch1 pathway.On the other hand, either ablation of Pin1 expression by RNA interference (RNAi) or inhibition of its activity by the small molecule inhibitor PiB (Uchida et al., 2003), significantly reduced the transcriptional activity of N1DE on experiments performed as above (Figures 2b, 2c). Together the binding and luciferase assays indicate that Pin1 interacts with human Notch1 and regulates its function through its prolyl-isomerase activity.

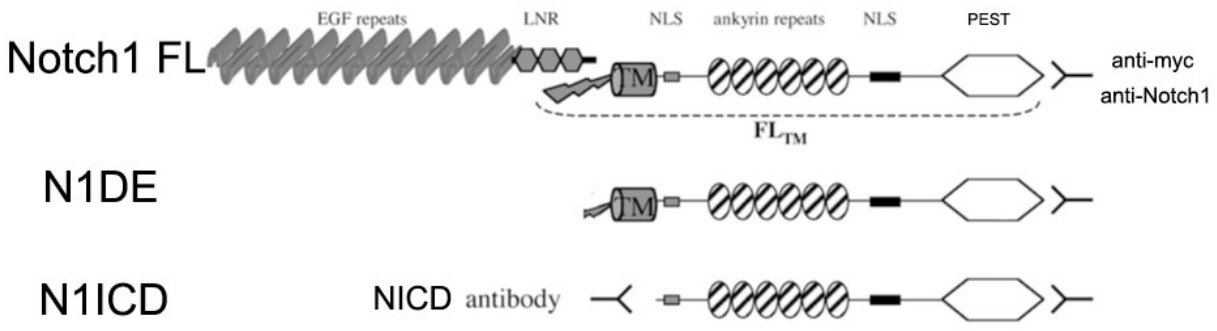

Table 1. Constructs used

Notch1 derivates N1DE, N1ICD forms and the epitope recognized by the antibody used. Note that NICD antibody recognizes the gamma-secretase product N1ICD, whereas the anti-myc and anti-Notch1 antibodies recognize all the forms.

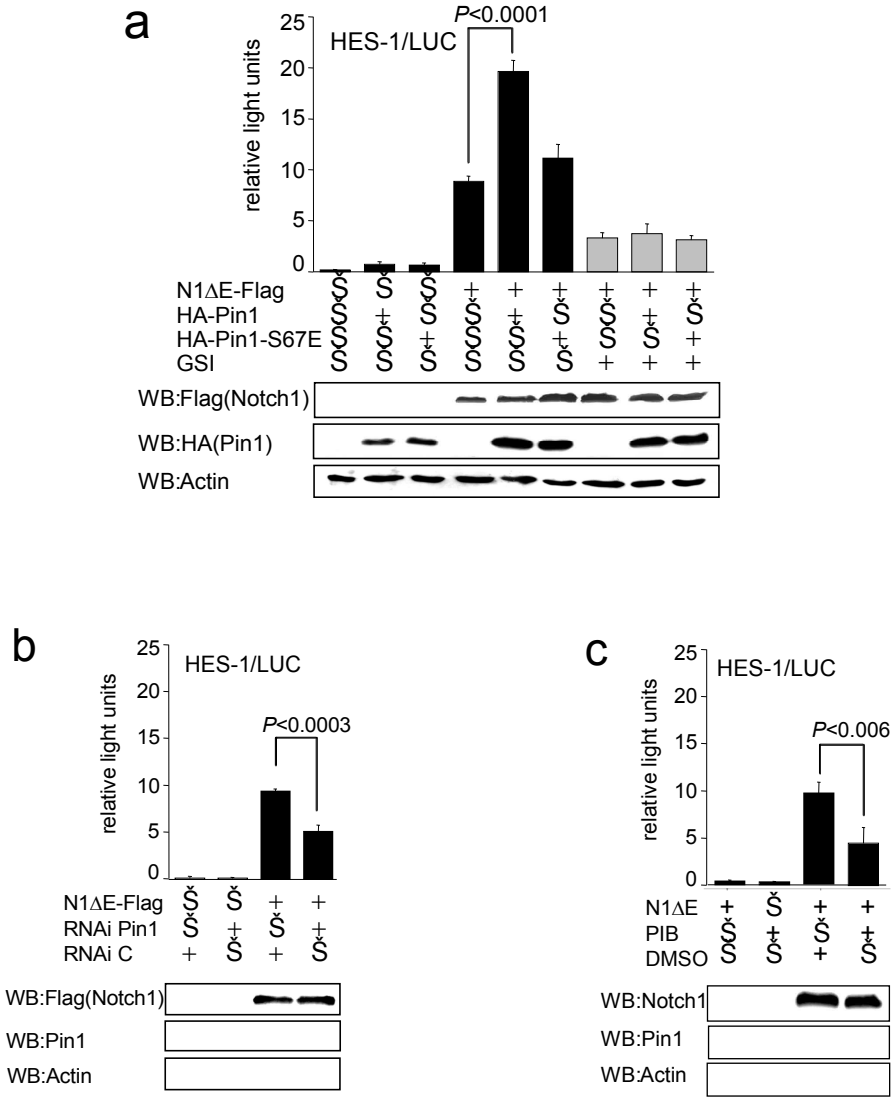

Figure 2. Pin1 affects Notch1 activity

a) Pin1 affetcs Notch1 transcriptional activity.Luciferase assay in SKBr-3 cells co-transfected with pGL2HES-1/LUC and N1DE expression vector (pcDNA3-N1DE-Flag). Expression vectors for either wild-type (HA-Pin1) or catalytically inactive (HA-S67E) Pin1 were co-transfected, with gamma-secretase inhibitor (GSI), where indicated (grey bars). Histograms represent means and s.d. of at least three independent experiments; **b)** Specific knock down of endogenous Pin1 affects Notch1 transcriptional activity. Transcriptional activity of N1DE was tested on HES-1/LUC in SKBr-3 cells in presence of either a Pin1 specific (RNAi Pin1) or control siRNA (RNAi C). **c)** Pin1 inhibitor (PIB) reduces N1ΔE transcriptional activity. Luciferase assay of SKBr-3 cells co-transfected with HES-1/LUC and pcDNA3-N1ΔE-Flag and treated with PIB or vehicle. Histograms represent means and s.d. of at least three independent experiments. a)-c) *P* values are indicated for the observed differences and cell lysates were analyzed by Western blot.

3.3 Pin1 co-localizes with N1DeltaE in the membrane compartment

We next performed a confocal immunofluorescence analysis of Pin1 and N1DE in SKBr-3 to evaluate the subcellular localization of both proteins. Cells were transfected with pcDNA3-N1DeltaE-myc and treated with GSI to keep N1DE uncleaved in the membranes. As shown in Figure 3 NDE and Pin1 co-localize at the cell membrane compartment before gamma-secretase cleavage either with overexpressed Pin1 (oe Pin1) or endogenous Pin1 (end.Pin1).

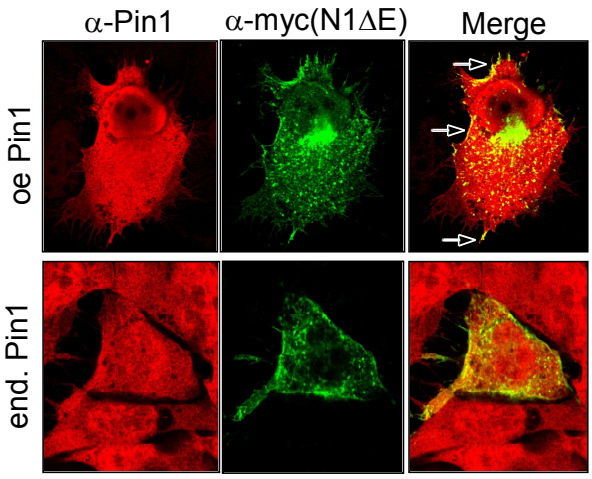

Figure3. Pin1 co-localizes with N1DE

Confocal immunofluorescence analysis was performed on SKBr-3 cells overexpressing myc-tagged N1DE (green) and endogenous (end.) or overexpressed (oe) Pin1 (red) in presence of GSI. The white arrows indicate the co-localization between NDE and Pin1

3.4 Pin1 affects Notch1 processing by gamma-secretase

To understand the role of Pin1 in modulating Notch signaling we tested its role in perturb Notch1 processing. To this end we transfected SKBr-3 cells with pcDNA3N1DE-FLAG and with two different siRNAs for Pin1. As shown in Figure 4a and 4b knock-down of Pin1 with siRNA, in N1DE-transfected cells, resulted in decreased levels of processed N1ICD .

We next analyzed the effects of Pin1 over-expression on the processing of endogenous Notch1, by measuring the amount of NICD produced upon a short treatment with EDTA in culture (Figure 4c). This treatment has been shown to trigger Notch1 cleavage by gamma secretase (Rand et al., 2000) and it is blocked by GSI. Ablation of Pin1 expression by RNA interference (RNAi), reduced Notch1 cleavage after EDTA treatment in SKBr-3 (Figure 4c).

This effect was not dependent on changes in active presenilin levels upon Pin1 overexpression, as judged by Western blot against N- and C-terminal fragments of presenilin-1 (NTF and CTF) that consist the core of the gamma-secretase enzyme (Figure 4c).

To confirm these data we over-expressed HA-Pin1, by retroviral infection, in human MCF-10A. After the infection we selected a stably expressing NDE population of MCF-10A cells (polyclonal) and we performed the same experiment as above. As shown in Figure 4d in these cells the efficiency of endogenous Notch1 processing triggered by EDTA clearly increased compared with empty vector infected cells.

Finally, we measured the EDTA-induced processing of endogenous Notch1 in $Pin1^{+/+}$ and $Pin1^{-/-}$ primary Mouse Embryo Fibroblasts (MEFs) (Fujimori et al., 1999). Lower levels of NICD were detected in $Pin1^{-/-}$ compared to $Pin1^{+/+}$ cells (Figure 4e). Importantly, Notch1 processing could be completely rescued in $Pin1^{-/-}$ MEFs upon re-introduction of Pin1 expression (Figure 4e), suggesting that the observed effect was dependent on Pin1 expression.

We next analyzed wheter the reduced cleavage of Notch1 was due to a diminished binding of Notch1 to presenilin. Performing Co-IP experiments in MEFs $Pin1^{+/+}$ and $Pin1^{-/-}$ we have found that there are no binding differences between these two proteins (data not shown).

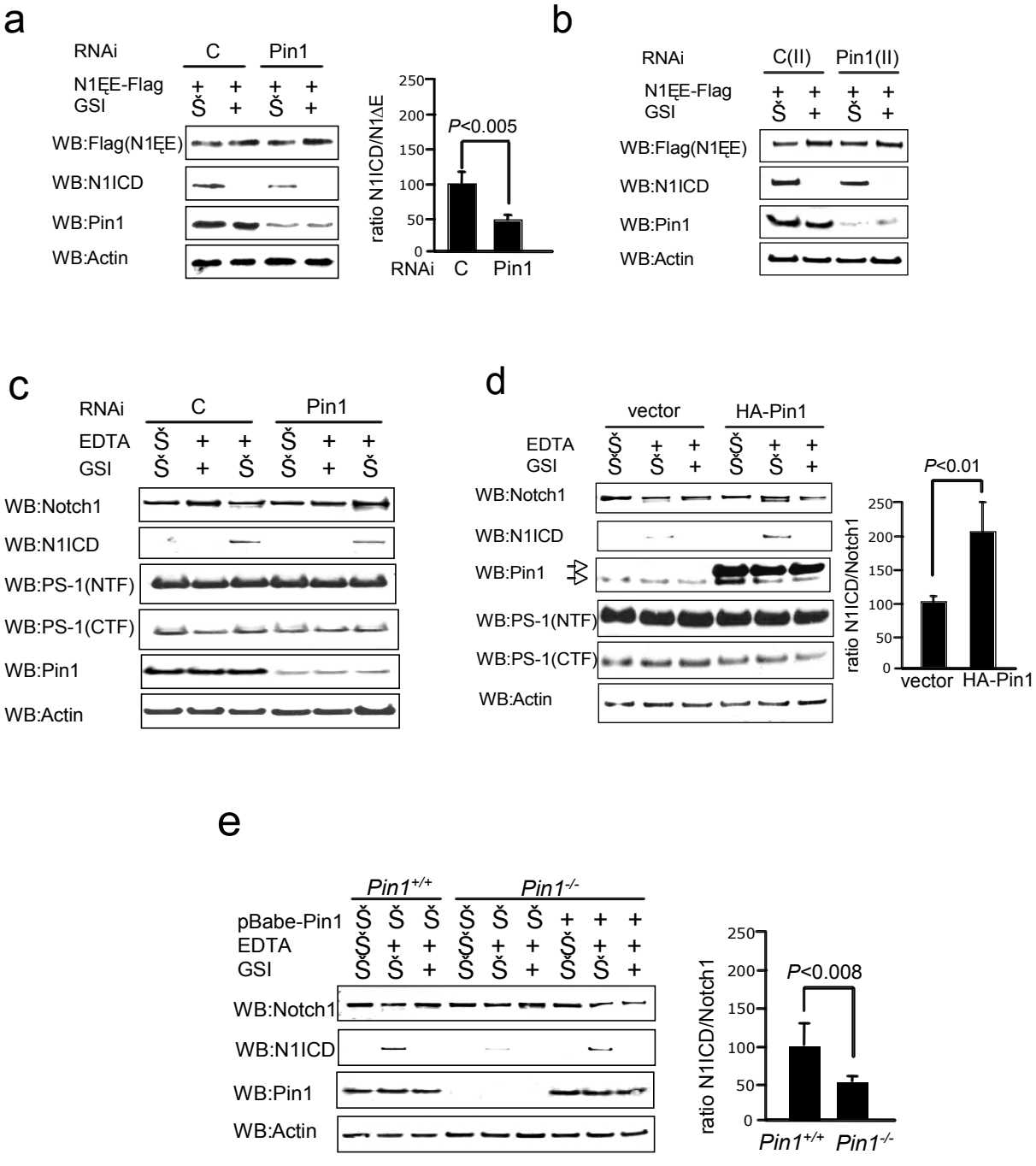
a
RNAi
C
Pin1
N1ĘE-Flag
GSI
WB:Flag(N1ĘE)
WB:N1ICD
WB:Pin1
WB:Actin
ratio N1ICD/N1ΔE
P<0.005
RNAi C Pin1
b
RNAi
C(II)
Pin1(II)
N1ĘE-Flag
GSI
WB:Flag(N1ĘE)
WB:N1ICD
WB:Pin1
WB:Actin
c
RNAi
C
Pin1
EDTA
GSI
WB:Notch1
WB:N1ICD
WB:PS-1(NTF)
WB:PS-1(CTF)
WB:Pin1
WB:Actin
d
vector
HA-Pin1
EDTA
GSI
WB:Notch1
WB:N1ICD
WB:Pin1
WB:PS-1(NTF)
WB:PS-1(CTF)
WB:Actin
P<0.01
ratio N1ICD/Notch1
vector HA-Pin1
e
Pin1+/+
Pin1-/-
pBabe-Pin1
EDTA
GSI
WB:Notch1
WB:N1ICD
WB:Pin1
WB:Actin
P<0.008
ratio N1ICD/Notch1
Pin1+/+ Pin1-/-

Figure 4

Figure 4. Pin1 affects human Notch1 processing by gamma-secretase

a-b) Ablation of Pin1 impairs constitutive processing of N1DE. Western blot analysis of N1DE-Flag processing in SKBr-3 cells transfected with control (RNAi C) or Pin1 specific siRNA (RNAi Pin1) and treated with vehicle or GSI. **c)** Ablation of Pin1 impairs Notch1 cleavage, by gamma-secretase SKBr-3 cells were left untreated (–) or activated with EDTA, or EDTA and GSI as control (Rand et al., 2000). **d)** Pin1 overexpression enhances Notch1 cleavage by gamma-secretase. Western blot analysis of endogenous Notch1 processing from MCF-10A cells infected with pLPC (vector) or pLPC-HA-Pin1 and left untreated (–) or activated with EDTA, or EDTA and GSI as control (Rand et al., 2000). Black arrows show migrations of endogenous Pin1 (lower arrow) and overexpressed HA-Pin1 (upper arrow). **e)** Notch1 processing decreases in *Pin1*$^{-/-}$ MEFs and is rescued by reintroduction of Pin1**.** *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ MEFs were mock treated or treated with EDTA as above. In parallel, the same experiment was performed with *Pin1*$^{-/-}$ MEFs infected with a Pin1 retroviral expression vector (pBabe-Pin1 a)-d) Molecular weight standards are indicated in KDa.

a)-e) Densitometric recording of cleaved (N1ICD) and uncleaved (N1DE or Notch1 untreated) protein levels was performed by Image J software and the ratio between N1ICD and the corresponding uncleaved form, was calculated. The mean values of the ratios (percentage) from three independent experiments were compared, setting control ratio average to 100% ± standard deviations, and are shown in the graphs, and *P* values for the observed differences are indicated.

3.5 Pin1 enhances Notch1 processing by binding to the STR region

To start dissecting the mechanism by which Pin1 promotes Notch1 processing, we then mapped the Pin1-interacting domains on Notch1.

To this end, several N1DE deletion mutants were generated (Figure 5a), over-expressed in HEK 293T cells in the presence of GSI, and tested for binding to GST-Pin1(WW) *in vitro*. As shown in Figure 5b, C-terminal truncation of N1DE deleting the PEST domain (dP) led to a decreased binding to Pin1, that was further reduced upon deleting also the TAD domain (d2171). The interaction was abolished in d2120 upon further removal of the Serine-Threonine-rich region STR (Ingles-Esteve et al., 2001). Lack of STR alone also reduced the interaction of Pin1 to both, N1DE and N1DE-dPest (dSTR, dP-dSTR).

These experiments reveal that Pin1 binds the Notch1 protein in three domain, the STR, OPA and the PEST domain.

To identify which of the Pin1-interacting regions were important for Notch1 cleavage, we tested the processing efficiency of all deletion mutants upon over-expression in SKBr-3 cells. We could observe that only those mutants lacking the STR region (d2120, dSTR, dSTR-dP) showed a reduced cleavage as compared to N1DE or to mutant d2171, which contain the STR region (Figure 5c-d).

The above results might suggest that Pin1, upon binding to the STR region, could produce a conformational change leading to proficient cleavage of Notch1 by gamma-secretase.

The STR region contains four Ser/Thr-Pro motifs, which are putative MAPK targets. Indeed we observed that the binding of d2171 to GST-Pin1(WW) was reduced by treatment of intact cells with inhibitors of MEK/Erk or JNK (Figure 5e). Thus, we mutated these putative Pin1-binding sites by Ser/Thr to Ala substitutions in construct d2171 (d2171-4M), and observed a reduction of binding to Pin1 (Figure 5f). Importantly, mutation of these Pin1 target sites reduced the efficiency of d2171 processing by gamma-secretase as shown in Figure 5g.

Notably, these mutations rendered the cleavage insensitive to Pin1 over-expression (Figure 5h). All these data suggest that conformational changes of the STR region upon Pin1 binding are necessary for an efficient cleavage by gamma-secretase.

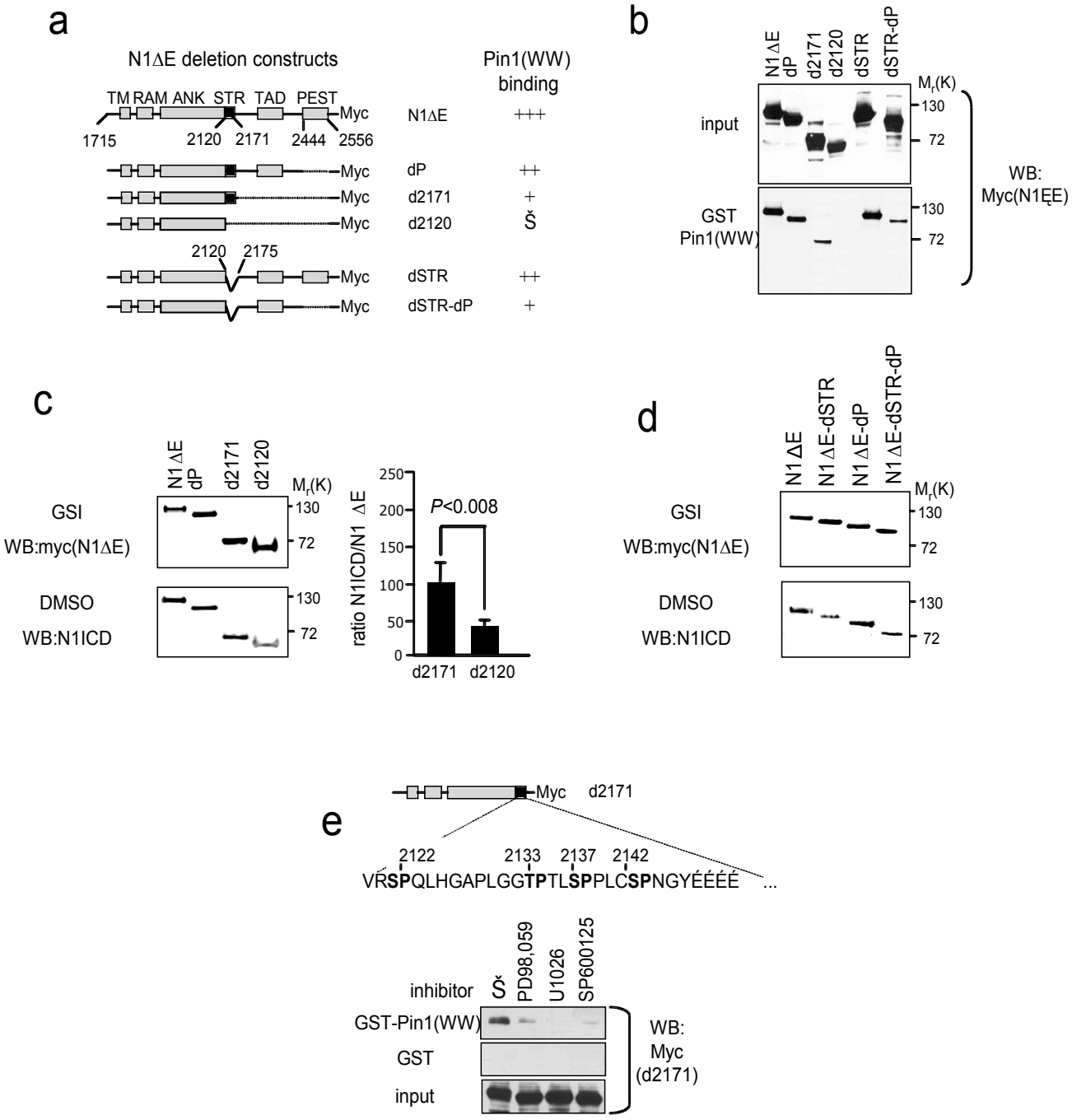
a
N1ΔE deletion constructs
Pin1(WW) binding
TM RAM ANK STR TAD PEST
Myc
1715
2120
2171
2444
2556
N1ΔE
+++
dP
++
d2171
+
d2120
Š
2120
2175
dSTR
++
dSTR-dP
+
b
N1ΔE
dP
d2171
d2120
dSTR
dSTR-dP
Mr(K)
input
GST
Pin1(WW)
130
72
WB:
Myc(N1ĘE)
c
GSI
WB:myc(N1ΔE)
DMSO
WB:N1ICD
ratio N1ICD/N1 ΔE
P<0.008
d2171
d2120
d
N1ΔE
N1ΔE-dSTR
N1ΔE-dP
N1ΔE-dSTR-dP
e
Myc
d2171
2122
2133
2137
2142
VRSPQLHGAPLGGTPTLSPPLCSPNGYÉÉÉÉ ...
inhibitor
Š
PD98,059
U1026
SP600125
GST-Pin1(WW)
GST
input
WB:
Myc
(d2171)

Figure 5

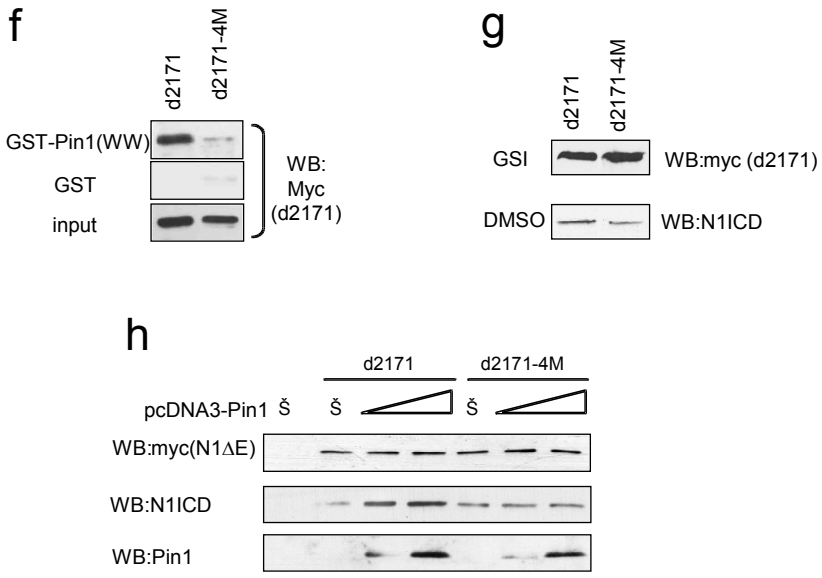

Figure 5. Pin1 improves cleavage of human Notch1 by binding to the STR region

a) Schematic of pBabe-N1DE-myc deletion constructs used in experiments in (b). TM: transmembrane, RAM: CSL interacting, ANK: ankyrin, STR: Ser/Thr rich, TAD: transactivation, PEST: Pest domain. Numbering refers to Swissprot entry P46531. Interactions with Pin1 (b) are indicated next to the constructs; +++ strong, ++ intermediate, + weak, – no binding. **b)** Mapping of Pin1 binding domains of Notch1. Constructs shown in (a) were over-expressed in HEK 293T cells, treated with GSI and lysates subjected to GST-Pin1(WW) or GST (not shown) pull down assay, followed by Western blot with anti-myc antibody. Molecular weight standards are indicated in KDa. **c-d)** N1DE proteins lacking STR are impaired in processing. Cleavage of N1DE deletion mutants was analyzed by Western blot upon over-expression in SKBr-3 treated with either GSI or DMSO. Upper panels show uncleaved inputs (GSI treated), while in the lower panels the cleavage product recognized by the Val 1744 antibody (N1ICD) is shown. N1ICD and N1DE protein levels were recorded as in Figure 2 and the ratio (percentage) between N1ICD (cleaved) and the corresponding uncleaved form is shown in the diagram. The ratio of N1DE-d2171 and N1DE FL were set to 100% ± standard deviations. The mean values of the ratios from at least three independent experiments were compared, and *P* value for the observed difference is indicated. **d)** Interaction of Pin1 with the STR region is impaired by MAP/SAPK inhibitors. Schematic of STR domain showing S/T-P motifs (bold). pBabe-d2171-myc was tested for Pin1 binding as in (b) either in absence (-) or presence of indicated kinase inhibitors. **e)** Mutation of S/T-P motifs in STR (d2171-4M) impairs binding to Pin1. Constructs pcDNA3-d2171-4M-myc and pcDNA3-d2171-myc were

tested for Pin1 binding as in (b). **f)** Mutation of S/T-P motifs in STR (d2171-4M) impairs processing by gamma-secretase. pcDNA3-d2171-myc or pcDNA3-d2171-4M-myc were overexpressed in SKBr-3 cells and their processing analyzed as in (c). **g)** Absence of S/T-P sites in the STR region of Notch1 hampers Pin1-dependent increase of processing by gamma-secretase. Comparable amounts of d2171 or d2171-4M were over-expressed in *Pin1*$^{-/-}$ fibroblasts along with increasing amounts of Pin1 and processing was then analyzed as in (c).

3.6 Pin1 potentiates Notch1 transforming activity

The role of Pin1 in potentiating Notch1 signaling prompted us to investigate the impact of Pin1 on Notch1 transforming activity. To this aim we decided to perform a soft agar assay in the immortalized normal mammary cell line MCF-10A. N1DE was over-expressed in these cells either alone or together with HA-Pin1, and cells were then tested for the ability to grow in soft agar. Consistent with previous reports, (Stylianou et al., 2006) activated Notch1 was able to sustain anchorage-independent growth of these cells (Figure 6a), while ectopic expression of Pin1 alone could not induce the formation of colonies in soft agar, it significantly increased the colony-forming efficiency of N1DE, when over-expressed together (Figure 6a). This was not observed in the presence of GSI, thus confirming that the effect of Pin1 required Notch1 activity. Soft-agar assays were also performed in MCF-10A cells stably infected with a vector expressing a Pin1-specific short harpin RNA(shRNA) to down-regulate Pin1. Importantly, ablation of Pin1 by shRNA could block soft agar growth induced by N1DE, suggesting that Pin1 is required for Notch1-induced cell transformation (Figure 6a).
Western blot analysis of N1DE expressing clones used in these experiments revealed that Pin1-specific shRNA led to a decrease in the levels of both cleaved Notch1 and of the Notch1 target Hes-1 (Figure 6b, compare lanes 5 and 4). On the other hand, Pin1 over-expression in N1DE infected MCF-10A clones enhanced the levels of both cleaved Notch1 and Hes-1 (Figure 6b, compare lanes 5 and 6). Confirming our previous data, that Pin1 modulates Notch1 pathway activation by increasing N1ICD production and endogenous protein levels of Hes-1.

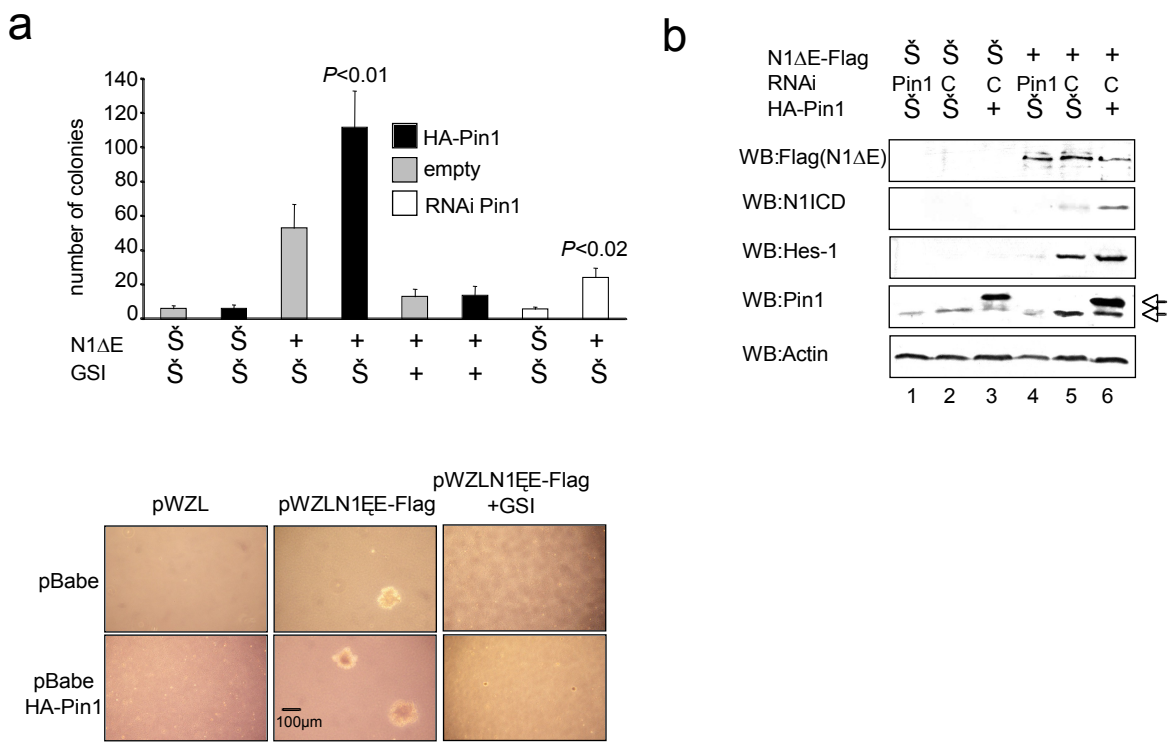

Figure 6. Pin1 impacts on human Notch1 transforming activity

a) Notch1 transforming activity is modulated by Pin1. Normal MCF-10A breast epithelial cells were transduced with retroviruses encoding N1DE-Flag along with empty (grey bars) or HA-Pin1 (black bars) expressing vectors and grown in soft agar with or without GSI treatment. In parallel, downregulation of Pin1 was assayed by co-infection of N1DE-Flag expressing cells with Pin1 specific (Pin1)(white bars) or control (C) shRNA encoding vectors. After 3 weeks colonies were counted. Histograms represent means and s.d. of three independent experiments, with *P* values for the observed difference between N1DE and N1DE with either HA-Pin1 or RNAi Pin1, are indicated. Representative phase contrast images of soft agar colonies of the indicated clones are shown in the lower panel. **b)** Protein lysates were analyzed in Western blot (except from GSI treated colonies), arrows indicate endogenous (lower) and over-expressed (upper) HA-Pin1, respectively. c) and d) middle panels show Western analysis of protein lysates and lower panels show representative phase contrast images of the above colonies.

3.7 Pin1 is a novel Notch1 target gene

In the above experiments, we consistently observed higher levels of endogenous Pin1 expression in cells stably expressing N1DE as compared to vector-transfected cells (Figure 6b, compare lanes 2 and 5, and Figure 6c, compare lanes 1 and 3). Notably, these increased Pin1 levels could be reversed by treatment with GSI, similarly to the Notch1-target gene HES-1 (Figure 6c, compare lanes 3 and 6). This evidence prompted us to investigate if Notch1 could perturb endogenous Pin1 expression.

To test whether Notch1 induces Pin1 at the transcriptional level, we performed semi-quantitative RT-PCR from MCF-7 cells over-expressing N1ICD and we observed an increased Pin1 mRNA level compared to the vector-transfected cells (Figure 7a). As control we checked the mRNA levels of the Notch1 target Hes-1 (Figure 7a).

Moreover H1299 cells expressing N1ICD-ER fusion protein (Ronchini and Capobianco, 2000) demonstrated that, upon induction of N1ICD nuclear translocation by 4-OHT, Pin1 mRNA levels raised, similarly to those of Notch targets Hes-1 and Hey-1 (Maier and Gessler, 2000) (Figure 7b). These results are consistent with the presence of three putative CSL-binding sites within the human Pin1 promoter, that are partially conserved also in the mouse gene (Figure 7c). We therefore cloned the human Pin1 promoter upstream of the luciferase gene, and demonstrated that its activity could be specifically induced by over-expression of N1DE (Figure 7c).

Finally to establish if N1ICD associates with the human Pin1 promoter we performed a chromatin immunoprecipitation (ChiP) in HEK 293T cells. After cromatin immunoprecipitation with an anti-Notch1 antibody we amplified the DNA with two pairs of primers, covering the Pin1 promoter region containing the two distal CSL-binding sites (BS1) and the proximal one (BS2) respectively.

This analysis demonstrated that only the two distal BS1 on the endogenous Pin1 promoter were specifically bound by N1ICD (Figure 7d), while no chromatin association was detected upon GSI treatment.

These data confirm that Pin1 is a new direct target of Notch1

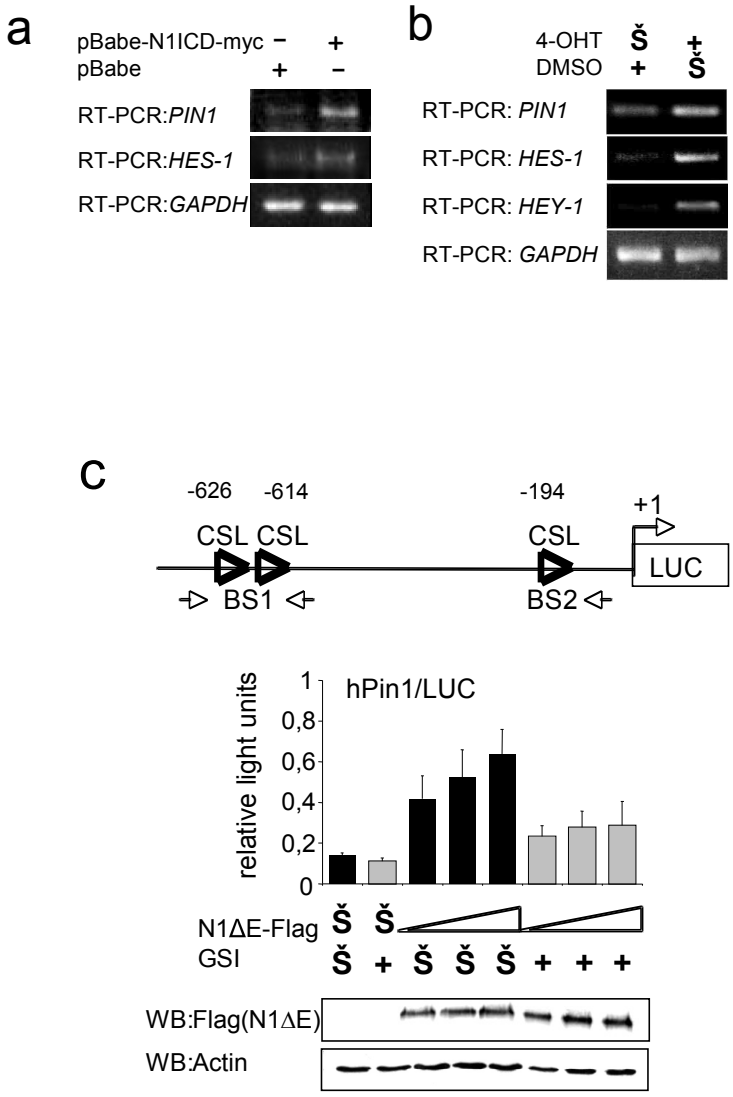
a
pBabe-N1ICD-myc − +
pBabe + −
RT-PCR:PIN1
RT-PCR:HES-1
RT-PCR:GAPDH
b
4-OHT Š +
DMSO + Š
RT-PCR: PIN1
RT-PCR: HES-1
RT-PCR: HEY-1
RT-PCR: GAPDH
c
-626 -614
-194
+1
CSL CSL
CSL
LUC
BS1
BS2
hPin1/LUC
relative light units
1
0,8
0,6
0,4
0,2
0
N1ΔE-Flag Š Š
GSI Š + Š Š Š + + +
WB:Flag(N1ΔE)
WB:Actin
d
DMSO
GSI
ChIP
αNotch1
αHA
Chromatin inputs
Primers
C+ C- BS1 BS2 C+ C- BS1 BS2

Figure 7

Figure 7. Pin1 is a novel transcriptional target of human Notch1

a) N1ICD enhances Pin1 mRNA levels. RT-PCR analysis of the indicated genes from MCF-7 cells stably transfected with an empty or N1ICD expressing vector. **b)** N1ICD activation enhances Pin1 mRNA levels. RT-PCR analysis of *Pin1*, *HES-1* and *Hey-1* in H1299 cells stably expressing ER-N1ICD after 24 hours of mock (EtOH) or 4-OH-tamoxifen (OHT) induction. a) and b) Human *GAPDH* has been included for normalization. **c)** Notch1 activates the human Pin1 promoter. (upper part) Schematic of human Pin1 promoter (genomic sequence from -713 to +53 with respect to the ATG) depicting CSL binding sites and indicating the primer sets used for ChIP analysis in (d). The depicted genomic region of the human Pin1 promoter was PCR amplified and cloned into pGL3/LUC. (lower part) Reporter assays of pGL3hPin1/LUC upon N1ΔE-Flag expression in SKBr-3 cells in presence (grey columns) or absence (black columns) of GSI. Histograms represent means and s.d. of at least three independent experiments. Cell lysates were analyzed by Western blot. **d)** Notch1 associates with the human Pin1 promoter. Binding of endogenous N1ICD to the human Pin1 promoter, analyzed by Chromatin Immunoprecipitation analysis in HEK 293T cells, treated either with DMSO or GSI, is shown. Semi-quantitative PCR analysis of Pin1 promoter (sequences BS1 and BS2) or HES-1 promoter as positive control (C+) or HES-1 intron (Fryer et al., 2004) as negative control (C-) were performed on α-Notch1 or unrelated (α-HA) chromatin immunoprecipitates.

3.8 Expression of Pin1 and activated Notch1 (N1ICD) correlates in human breast cancer

Next we analyzed the levels of the Notch1 targets Pin1 and Hes-1 in several breast tumor cell lines of lobular (SkBr-3, MCF-7) and ductal (MDA-MB-468, MDA-MB-435, MDA-MB-231) origin, known to have deregulated Notch activity (Stylianou et al., 2006).

All these cell lines showed high levels of both HES-1 and Pin1 as compared to the normal human mammary MCF-10A cell line (Figure 8a).

These results suggested a cross-talk between Pin1 and Notch1 in breast carcinogenesis. We sought for evidence, in primary breast cancers, that could corroborate this possibility. To this end, we performed immunohistochemical analysis of Pin1 and N1ICD expression on serial sections from the same breast tumor tissue microarrays (in collaboration with Pier Paolo di Fiore, Salvatore Pece and Paolo Nuciforo, IFOM Milan). This analysis highlighted a significant direct correlation between Pin1 expression levels and accumulation of N1ICD in these tumors (Figures 8b, 8c): those tumors exhibiting high levels of nuclear N1ICD (59 out of 147) showed also high Pin1 protein levels in the majority of the cases (65%), while low levels of N1ICD (88 out of 147) were paralleled by low levels of Pin1 (85%).

Similar results were obtained when the expression levels of the downstream Notch target gene HES-1 were analyzed by immunohistochemistry on the same tissue microarrays and compared to Pin1 expression (Figure 8b, 8c).

This correlation between N1ICD and Pin1 protein levels in human breast cancer samples supports our hypothesis of a possible positive loop between these two proteins that could be relevant for human carcinogenesis.

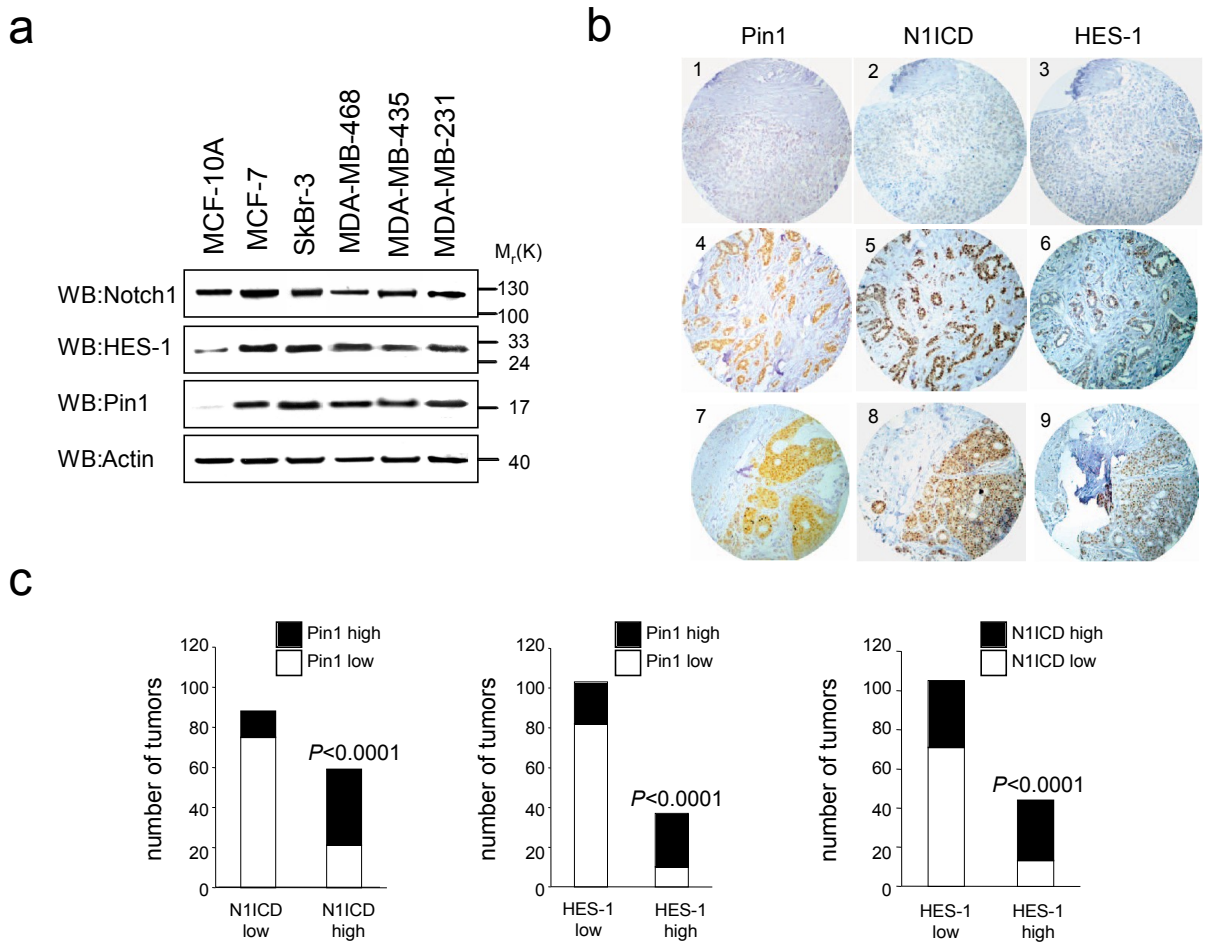

Figure 8. Expression of Pin1, Notch1 and HES-1 in breast cancer cell lines and in serial sections of breast carcinoma samples

a) Western Blot analysis of a human immortalized breast cell line (MCF-10A) and five breast cancer cell lines (MCF-7,Sk-Br3, MDA-MB-468, MDA-MB-435, MDA-MB-231) is shown for Notch1, Pin1 and HES-1, normalized by actin levels. **b)** Representative samples showing immunohistochemical analysis of breast tissue microarrays performed with the indicated antibodies on adjacent sections of the same array. (1-3) An example of breast carcinoma negative for all three proteins; (4-9) examples of moderate to strong expression for N1ICD, Pin1 and HES-1 on two different breast cancer samples **c)** Graphs showing correlation between Pin1, HES-1 and N1ICD expression levels in breast cancer. *P* values of observed differences, according to pearson chi-square, are indicated.

4. DISCUSSION

Cancers arise as somatic cells mutate and escape the control of cell cycle, survival or death. These processes are normally regulated by several signalling pathways and recently several studies highlight the potential importance of the Notch1 signaling pathway in human cancer. Notch activation has been involved in several mouse models of mammary carcinogenesis and recently also in human breast cancer. Importantly loss of Numb, a negative regulator of Notch1, has been observed in about 50% of breast carcinomas (Pece et al., 2004).

Expression of Notch1 and Ras correlates in breast carcinomas and expression of the Notch1 pathway ligand Jagged1 is a potential markers for breast cancer progression (Reedijk et al., 2005). Furthermore, the Notch1 interplay with pathways such as Wnt, c-Myc, is subverted in breast cancer, thus suggesting that Notch could be a target for drug-based therapy of breast cancer . Intriguingly also the prolyl-isomerase Pin1 is crucial in breast cancer and in several human malignancies through modulation of these pathways. Alterations of Pin1 have been implicated in the amplification of oncogenic signals, as demostarted by its cooperation with the Neu/Ras pathway in mammary tumorigenesis (Wulf et al., 2001) .

The aim of this work was to unveil a possible role of Pin1 in the regulation of Notch1 pathway in breast cancer. Pin1 directly interacts with phosphorylated Notch1, and increases Notch1 cleavage by gamma-secretase. Accordingly, Pin1 contributes to Notch1 transforming properties in human breast cells. Notch1 in turn up-regulates Pin1, thus establishing a feed-forward loop that amplifies Notch1 signalling. Importantly human breast cancers bearing elevated levels of Pin1 have also deregulated expression of activated Notch1 and Hes-1 and these data underscore the relevance of our observations for human carcinogenesis.

The increasing evidence that Notch signalling can drive the growth of a wide range of tumors, from leukaemia to breast cancer, has recently promoted intense studies of the mechanisms that lead to alteration of this pathway during carcinogenesis . Several crucial aspects however remain obscure, in particular concerning the regulation of Notch1 activation and how its downstream effects contribute to the neoplastic phenotype. One critical point in Notch activation entails proteolytic cleavage by gamma-secretase, but several mechanisms that impinge on this step are still elusive .

- The results presented in this thesis have shown that, in addition to the extracellular domain of the receptor, also the intracellular serine/threonine rich (STR) region of Notch1 may be required for efficient processing. This region is conserved between Notch1 and Notch2 and

has been shown to be phosphorylated in response to cytokine-induced differentiation of 32D cells .

These results, obtained by mimicking Notch1 activation by EDTA treatment or ectopic expression of constitutively cleaved N1DE, allow us to hypothesize that Pin1 binds to critical Ser/Thr-Pro sites within STR upon phosphorylation, producing a conformational change of Notch1, that favors processing by gamma-secretase. Indeed, we have shown that deletion of this region, or mutation of the critical phosphorylation and Pin1 binding sites, dramatically impairs the constitutive processing of N1DE. Notably, the cleavage of this Notch1 mutant is insensitive to Pin1 over-expression.

These effects were not dependent on changes in active presenilin levels upon Pin1 overexpression, as judged by Western blot against N- and C-terminal fragments of presenilin1.

All these data support the hypothesis that conformational changes of the STR region upon Pin1 binding are necessary for an efficient cleavage by gamma-secretase. All these data suggest that Notch1 processing was shown to be strongly increased in Ras-transformed cells . The observation that Pin1 binding to Notch1 involves phosphorylation events induced by MAPK/SAPKs, coupled with the evidence that Pin1 expression is induced by Ras indicates a role of Pin1 in the activation of Notch1 by oncogenic stress.

Recently it was demostrated, that a monoubiquitination event takes place on the NDE molecule, which is constitutively cleaved form of Notch receptor (Gupta-Rossi et al., 2004).

This modification that occurs during endocytosis seems to be a prerequisite for gamma-secretase cleavage of NDE, since not monoubiquitinated NDE is less cleaved by gamma-secretase, but still interact with PS when overexpressed. Therefore it is temping to predict that as it does with activate Notch at the membrane, the complex being then ubiquinated and internalized to reach the endocytic compartment where the gamma-secretase is active. These data suggest that ubiquitination could be required for an efficent Notch1 cleavage. We can speculate that Pin1 could also modulate Notch1 processing by influencing the mono-ubiquitination of Notch1 receptor.

- N1ICD transcription factor activates specific subsets of downstream targets. The mechanistic basis for such selectivity is not easily understood because N1ICD binds to promoters via an interaction with the same protein, CSL. Recent analyses have demonstrated that the potency of the four vertebrate NICD in a transient transfection assay depends on the promoter, the amino acid composition of the individual Notch proteins, and to a lesser degree on a cellular context. Collectively, these data suggest that a matrix of parameters that include NICD concentration, CBS (CSL binding site) orientation, and promoter context will determine which promoters respond to a particular Notch protein at a given level of ligand binding. Notch signaling is mediated through CBS that can bind to either a low (RTGRGAR) or high affinity (YGTGRGAA) CSL. The best characterized Notch target promoter such as HES1 in mouse contains a highly conserved "SPS" (Su(H) paired sites (Ong et al., 2006), that is composed of two inverted CBS sites (CBS-A and CBS-B) separated by 15-22 nucleotides. As reported previously, it was found that intact CBS A and B are necessary for activation of the HES1 promoter. In fact, a minimal promoter containing only the paired site and a TATA sequence is sufficient to elicit the 60% luciferase activity compared to the HES1 promoter, confirming that the SPS is both necessary and sufficient for Notch-dependent gene expression in vertebrate cells. The HES5 promoter does not contain paired CBS, instead, it has two high affinity CBS located 134 bases apart and three low affinity sites further upstream. Mutating the two most proximal high affinity sites abolished Notch-mediated activation. Moreover, also N3ICD transactivates Hes5 and its activation depends critically on a putative zinc finger transcription factor-binding site (ZFBS; GCCGCCATT) and a binding site for CCAAT/enhancer-binding protein alfa (C/EBPalfa) undifferentiated protein (CUP) (CCGCCG). All three sites occupied 27 bases within the HES5 promoter. These observations suggest that the zinc fingers TF and possibly CUP are recruited to their putative binding site where they act synergistically with the C-terminal region of Notch3. Interestingly, also the human Pin1 promoter contains three CBS, two of these sites are located ~700 bp upstream of the ATG (CBS-A,CBS-B). These two sites are also conserved in mouse Pin1 promoter. These CBS have a low and an high affinity for CSL and are separeted by only 4 nucleotides. Moreover CBS-A/B are required for the NICD transactivation of the Pin1 promoter and are bound by N1ICD, while the third seems not to be bound by N1ICD. Importantly Pin1 promoter has not inverted sites as Hes-1. It remains to be seen if it could be modulated by the

binding with other transcription factors as the Hes5 promoter does. This aspect is very important to unveil new transcription factors that synergistically act with the Notch pathway to regulate Pin1 expression.

- Interestingly Notch1 is also important in several physiological processes and in controlling cell fate in normal conditions. In mammals, a wide variety of cells use the Notch signaling system for embryonic development and, in adults, maintenance of homeostasis. The Notch signaling pathway regulates intestine stem cell self-renewal, together with other signaling pathways, such as Wnt and hedgehog. Notch signals are involved in various stem and early progenitor cell systems in both the developmental and adult phases.

Adult stem cells are considered to maintain homeostasis of cells and tissues throughout life. The adult stem cells maintain the number of stem cells, as well as terminally differentiated cells, during normal turnover and repair damage after injury. Involvement of Notch signaling occurs during both normal status and injury in various stem cell systems.

The mammary gland in humans and in other mammals is a dynamic organ that undergoes significant developmental changes during pregnancy, lactation, and involution. It is likely that the cellular repertoire of the human mammary gland is generated by a stem cell component.

Activation of the Notch pathway results in changes in cell fate, including self-renewal of stem cells or differentiation along a particular lineage. The Notch pathway was shown to be involved in the normal development of the mammary gland. Interestingly mice *Pin1*$^{-/-}$ show failure in massime proliferation of the mammary gland during pregnancy. This evidence suggest a possible role of Pin1 in mammary stem cell regulation by modulating Notch1 pathway, or by other cellular pathways. Moreover Pin1 could be involved in the stem cell mainteinace of several tissues that required the activation of the Notch1 pathway.

For exanple in hematopoietic stem cell and in Intestinal epithelial stem/progenitor cells self renewal. Indeed, the administration of gamma-secretase inhibitors induces histologic changes in the intestinal epithelial layer of mice, and conditional inactivation of CSL in the epithelium of the intestine and colon induces conversion of proliferative crypt cells to postmitotic Goblet cells (Searfoss et al., 2003, Wong et al., 2004, van Es et al., 2005). These findings suggest that Notch signaling in adults functions to maintain intestinal epithelial stem/progenitor cells. Moreover Notch1 pathway cross-talks with Wnt pathway in controlling self-renewal of intestinal stem cell, another important cellular signaling involved in tumorigenesis and modulated by Pin1. Furthermore Pin1 could regulates stem cell self renewal and

tumorigenesis also in intestine.

4.1 Conclusion

During my thesis we have demonstrated a role for Pin1 in enhancing human Notch1 signalling via its prolyl-isomerase activity. Pin1 directly interacts with phosphorylated Notch1, and increases Notch1 cleavage by gamma-secretase. Accordingly, Pin1 contributes to Notch1 transforming properties in human breast cells. Notch1 in turn up-regulates Pin1, thus establishing a feed-forward loop that amplifies Notch1 signalling. Importantly, our finding, that human breast cancers bearing elevated levels of Pin1 have also deregulated expression of activated Notch1 and HES-1, underscores the relevance of our observations for human carcinogenesis.

By up-regulating Pin1, Notch1 improves its own processing and activation. In addition, Pin1 might impinge on N1ICD activity and it could also directly affect some Notch1 transcriptional targets, such as cyclin D1 and NF-kappaB , further amplifying the Notch1 signal.

The relevance of this molecular circuitry in human carcinogenesis could have important implications for therapeutic intervention. Targeting gamma-secretase to block Notch1 signaling is considered a promising strategy for therapy of human cancers, particularly breast tumors . However GSIs, although effective *in vitro*, exhibit a clear toxic effect due to their action on normal stem cells. Here we have shown that Pin1 down-regulation by either RNAi or pharmacological means dramatically reverts Notch1 dependent cell growth, with an effect comparable to that of gamma-secretase inhibitors (Figure 6). It is therefore tempting to predict that in tumors with deregulated expression of both Notch1 and Pin1, a lower dose of GSI could provide a better response if administered in combination with Pin1 inhibitors.

5. METHODS

Oligonucleotides. All oligonucleotides were purchased from MWG Biotech.

for cloning the human Pin1 promoter:
Forward primer 5'-AGGACGTGGAAGGCCTT-3' (713 bp upstream from ATG)
Reverse primer 5'-GCTGCGGCTCATGCGCT-3' (53 bp downstream from ATG)
for cloning pBabe-N1DE-myc:
Forward primer 5'GAGCGGATCC**ATG**CCGCCGCTCCTGGCGCCCCT-3' (BamHI site underlined and ATG initiation codon in bold print)
Reverse primer 5'-CATCCTTCTCGAG**CTT**GAAGGCCTCCGGAATG-3' (XhoI site underlined, no STOP codon, only last aa = E2555 of human Notch1 in bold print).
for construction of pBabe-N1DE-myc deletion plasmids (dP, d2171, d2120, dSTR, dSTR-dP):
At the 5' end the same forward primer as for pBabe- N1DE-myc was used.
At the 3' end the following reverse primers were used:
for dP and dSTR-dP 5'-TGGCTCGAGGTCTGCCTGGCTCGGCTCTCCACTCAGGA-3',
for d2171 5'-CTTCTCGAGCTTGCTTCCACAGGCCA-3',
for d2120 5'-CGGCTCGAGCACCAGGTTGTACTCGTCCA-3'
for internal truncations in constructs dSTR and dSTR-dP:
reverse primer on aa 2120 (5'-CGGGCTGTCGACCAGGTTGTACTCGT-3')
forward primer on aa 2175 (5'-GAGGCCGTCGACCTCAAGGCACGGA-3')
for construction of d2171-4M:
S2122A forward 5'-AGTACAACCTGGTGCGCGCCCCGCAGCTGCA-3'
S2122A reverse 5'-GCGCACCAGGTTGTACTCGTCCAGCA-3';
T2133A forward 5'-GAGCCCCGCTGGGGGGCGCGCCCACCCTGT-3'
T2133A reverse 5'-GCCCCCCAGCGGGGCTCCGTGCAGCT-3';
T2133A/S2137A forward 5'-GAGCCCCGCTGGGGGGCGCGCCCACCCTGGCGCCCCCGCTCT-3'
T2133A/S2137A reverse 5'- GCCCCCCAGCGGGGCTCCGTGCAGCT-3'
T2133A/S2137A/S2142A forward 5'-TGGCGCCCCCGCTCTGCGCGCCCAACGGCT-3'
T2133A/S2137A/S2142A reverse 5'-GCAGAGCGGGGGCGCCAGGGTGGGCGC-3'.
for generation of pcDNA3-HA-Pin1-S67E by site-directed mutagenesis:
Forward primer 5'- CTGGCTGTGCTTCACCAGCAGGTGCGAGCAG-3'
Reverse primer 5'-AGGGCCGCCGTTCCTGGCTGTGCTTCACCAGCA-3'
for RT-PCR:
HES-1 forward: 5'-GAGAAAAGACGAAGAGCA-3'
HES-1 reverse: 5'-TGTGCTCAGCGCAGCCGT-3'
HEY-1 forward: 5'-GAGGTGGAGAAGGAGAGTGC-3'
HEY-1 reverse: 5'-CTCCGATAGTCCATAGCAA-3'
PIN1 forward: 5'-CATCACTAACGCCAGCCAGT-3'
PIN1 reverse: 5'-TCAAATGGCTTCTGCATCTG-3'
GAPDH forward: 5'-GCCAGTGGACTCCACGAC-3'
GAPDH reverse: 5'-CAACTACATGGTTTACATGTTC-3'
for silencing:

siRNA Pin1(I) 5'-GCCAUUUGAAGACGCCUCG-3'
siRNA Pin1(II) 5'-CGGGAGAGGAGGACUUUGA-3' (upgrade 7 from smart pool, Dharmacon)
siC(I) 5'-CCUUUUUUUUUGGGGAAAA-3'
siC(II) 5'-GUGACCAGCGAAUACCUGU-3' (siRNA for LacZ)
shPin1 5'-GCCATTTGAAGACGCCTCG-3' (from siRNA Pin1(I))
shC (II) 5'-GTGACCAGCGAATACCTGT-3' (from siRNA LacZ))
for ChIP analysis:
BS1 forward 5'-AAAGTTGAGCCCTGCAAAAA-3' (677 bp upstream from ATG)
BS1 reverse 5'-AGGCGGGATAGAGCTTATGG-3'; (364 bp upstream from ATG)
BS2 forward 5'-AGAAGGGGTCGGGAGTTTT-3' (336 bp upstream from ATG)
BS2 reverse 5'-GCTGCCTATTGGCTAGACG-3' (69 bp upstream from ATG)

Plasmids. N1DE proteins were expressed using pcDNA3-N1DE-Flag and a series of derived constructs. It was obtained from human full length Notch1 (pBabe/HA-hNotch1) that was cloned into EcoRI site of pcDNA3 in frame with a Flag-tag at the C-terminus, then to obtain pcDNA3-N1DE a PCR-mediated in-frame deletion of aa 24-1714 was performed. An HA-tag was then inserted at aa 1731. pBabe(Puromycin)-N1DE-myc was generated starting from pcDNA3-N1DE-Flag by PCR and subcloning into BamHI and XhoI digested pBabe and pcDNA3 (Invitrogen) vectors bearing in frame a myc tag followed by two STOP codons 3' to the XhoI restriction site. pBabe-dP, -d2171, -d2120, -dSTR, -dSTR-dP were gereated by PCR starting from pBabe-N1DE-myc. For internal truncations in constructs dSTR and dSTR-dP a SalI restriction site at aa 2175 was exploited to design one reverse annealing primer on sequence corresponding to aa 2120 and one forward primer starting from that of aa 2175 (both containing a SalI restriction site) that were used in combination with the forward and reverse primers of pBabe-N1DE-myc, respectively. After SalI restriction the PCR products (corresponding to N-terminal to aa 2120 and of aa 2175 to the C-terminal end) were united by ligation-mediated PCR using again forward and reverse oligonucleotides of pBabe-N1DE-myc. pcDNA3-d2171-myc was generated by subcloning from pBabe-d2171-myc. Construction of pcDNA3-d2171-4M-myc started from pcDNA3-d2171-myc by introducing sequentially Serine (S) or Threonine (T) to Alanine (A) substitution one after the other by site-directed mutagenesis (OligoEngine). pWZL(Hygromycin)-NdE-Flag was obtained from pcDNA3-N1dE-Flag by PCR and subcloning. Retroviral vector pLPC(Puromycin)-HA-Pin1 was constructed by subloning from pcDNA3-HA-Pin1. pcDNA3-HA-Pin1-S67E was obtained by site-directed mutagenesis from pcDNA3-HA-Pin1 . pGL3-hPin1/LUC was constructed by subcloning a PCR product containing the human Pin1 promoter from HeLa genomic DNA into pGL3 (Promega). All constructs were checked by sequencing.

Plasmid and retroviral vectors. pcDNA3-N1DE-Flag, pBabe-N1DE-myc and deletion constructs for mapping studies, pcDNA3-d2171-myc and pcDNA3-d2171-4M as well as pWZL-N1DE-Flag, pLPC-HA-Pin1 retroviral expression vectors were generated by standard procedures and details appear in Supplemental data. pBabe-Pin1, pGL2-HES-1/LUC and pBabe-ER/N1ICD-myc have been described . For RNA interference, sequences of Pin1 specific or control siRNA and oligonucleotides for shDNA cloned into pRetro.Super (OligoEngine).

Cell lines and treatments. SKBr-3, MDA-MB-468, MDA-MB-435, MDA-MB-231 and MCF-7 are human breast carcinoma cells (MCF-7 were purchased from IZS Brescia, Italy), MCF-10A are human normal immortalized epithelial breast cells, HEK 293T is a human embryonic kidney cell line with SV40 large T, H1299 is a human lung carcinoma cell line. Immortalized *Pin1*$^{-/-}$ fibroblasts have been obtained by spontaneous immortalization from Murine Embryo Fibroblasts of C57BL6/129Sv mixed background . All cells were cultured in DMEM (BioWhittaker^{TM}) supplemented with 10% Fetal Bovine Serum (Gibco) and Penicillin/Streptomycin, except MCF-10A that were maintained in DMEM:F12 HAM'S (1:2) (Sigma) supplemented with 5% Horse Serum (Gibco), 10μg/ml Insulin (Sigma), 0.5 μg/ml Hydrocortisone, 20 ng/ml EGF (Peprotech). Transient transfections and luciferase assays were performed by standard procedures, as described . For creation of stable clones a selection corresponding to the expressed vectors was applied for 2 weeks to transfected or infected cells at the concentrations of 50 μgr/ml for Hygromycin, 2 μgr/ml for Puromycin and 0,5mg/ml for Neomycin, respectively. Gamma-secretase Inhibitor DAPT (Sigma), Pin1 inhibitor PiB (Calbiochem) were dissolved in DMSO and used at a final concentration of 20 and 0.5 μM respectively. Cycloheximide 50 μM (Sigma). For EDTA treatment, cells were washed twice in PBS, then PBS alone or with 5mM EDTA or 5mM EDTA plus GSI was added for 15 min, followed by a 15 min chase in culture medium, washed and collected, as described. For processing experiments with N1DE deletion constructs, transfected cells were split in two parts and treated with either GSI or DMSO. Kinase inhibitors U0126, PD98,059 (Sigma) and SP600125 (Biosource) were solubilized in DMSO and used at a 10 μM final concentration for 30 min. Functioning of the inhibitors was tested in Western blot analyses using endogenous phosphorylated targets of these kinases as markers (anti-pErk1/2, Cell Signaling, anti-pATF-2, Santa Cruz) (not shown).

Antibodies for Western blot and Immunoprecipitation. The following antibodies were used: mouse monoclonal anti-MPM-2 (Upstate), rabbit and goat polyclonal anti-Notch1 (SantaCruz), rabbit polyclonal anti-N1ICD Val1744 (Cell Signaling), mouse monoclonal anti-myc clones 9E10 and 9B11 (Cell Signaling), mouse monoclonal anti–Flag and anti-HA (Sigma), rabbit polyclonal and mouse monoclonal (SantaCruz) anti-Pin1, rabbit polyclonal anti-HES-1 .

In vitro binding, immunoprecipitation, Western blot and Far Western. Pin1 in vitro binding assays and Co-IP, as well as phosphatase treatment were performed by standard procedures, as described . Briefly, for GST pull-down analysis using cell lysates the lysis buffer was supplemented with inhibitors of phosphatase (1 mM sodium orthovanadate, 5 mM NaF) and protease (phenylmethylsulfonylfluoride (PMSF) 1 mM and chymostatin, leupeptin, antipain, pepstatin 10 μg ml^{-1} each). In case of phosphatase treatment inhibitors for phosphatase were left out and 400 U ml^{-1} of lambda phosphatase was added to cell extracts and reaction was continued for 2 h at 30°C following the manufacturers' instructions (New England Biolabs) prior to GST pull-down.
For Pin1 immunoprecipitation assays, cells were collected in PBS pH 8.3 buffer (PBS pH 8.3, 0.1% Tween-20) and lysed by passing through a 26 G needle. For Notch1 immunoprecipitations cells were collected in GST pull-down buffer (150 mM NaCl, 50 mM Tris/HCl pH 7.5, 10% glycerol, 0.1% Nonidet P-40). Cell lysates were cleared with proteinA Sepharose by rocking for 30 min, then Protein A/G sepharose (GE Healthcare) cross-linked antibodies, precleared with 10 mg/ml BSA, were added. Binding reactions were left for a minimum of 4 hours to over night rocking at 4°C. Then beads were washed and bound

proteins were loaded and separated in SDS-Page, followed by Western blotting on Nitrocellulose membranes (Scleicher & Schuell).
Purified GST-Pin1 protein for Far Western analysis was obtained by immobilization, after production in bacteria, on glutathione sepharose 4B beads (GEhealthcare) followed by elutions using reduced GSH as a competitor in Tris/HCl pH8 100mM and NaCl 100mM. The eluted protein was subsequently purified by dialysis.
For Far Western Blot analysis proteins were immunoprecipitated with the indicated antibody, resolved by SDS Page and blotted onto nitrocellulose membrane. Blocking was perfomed for 1h at 4°C in PBS plus not fat dry milk 10%. Blots were then incubated with 1μg/ml GST-Pin1 protein in blocking buffer for 1h. Membranes were washed 4 times in PBS, 0.2% Tween-20. Subsequently recognition by standard Western blot was performed.
Densitometric values of protein levels in Western blot analyses were obtained by Image J software.

Retroviral infection. Ampho/Phoenix packaging cells were transfected with indicated retroviral vectors by a standard calcium phosphate method. After 48 h incubation at 32°C, the supernatants containing viral particles were collected, and infection was performed as described .

Soft-agar analysis. Cells from SKBr3 and MCF-10A stable clones used for soft agar experiments were resuspended in a top layer of the corresponding culture mediums with 0.3% or 0.25% agarose (Gellyphor, Euroclone), respectively, at 10,000 cells per well in triplicate in 6-well plates and plated on a bottom layer of culture medium containing 1% agarose. Every 2 days pharmacological treatments (GSI, PIB, DMSO) were repeated. After three weeks the colonies were counted with a 20X objective on a Olympus CK30 microscope.

RNA extraction and reverse-transcription PCR. Total RNA was extracted with TRIzol (Invitrogen), and 1 μg was reverse-transcribed using SuperscriptIII reverse transcriptase and random primers (Invitrogen) following the manufacturer's instructions. For PCR all primers have been chosen or designed in order to anneal on different exons of the respective cDNA (HES-1 NM_005524; Hey1 (or HERP 2) AF232239; Pin1 NM_006221; GAPDH BC083511).

Chromatin immunoprecipitation (ChIP). Cells were crosslinked with 1% formaldehyde for 10 min, neutralized with 125 mM glycine pH 2.5 and washed in PBS. Nuclei were prepared by hypotonic lysis (5mM Pipes pH 6.8, 85 mM KCl, 0.5% NP40) and centrifugation, and resuspended in RIPA-100 buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.5% deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Sigma), 1 mM PMSF and phosphatase inhibitors (NaF 5 mM, Na_3VO_4 1 mM). Chromatin was sonicated by Bioruptor (Diagenode) to 500-1000 bp average fragment size and cleared by centrifugation. IP was performed overnight at 4°C with either 1 μg of anti-Notch1 antibody (SC-6014R; SantaCruz Biotech.) or as an unrelated control anti-HA-tag antibody (Sigma) at 4 °C. DNA-protein complexes were recovered by protein A/G PLUS-Agarose (SantaCruz Biotech.) and washed sequentially with RIPA-100 and RIPA-250 buffer and LiCl solution (10 mM Tris/HCl pH 8, 1 mM EDTA, 250 mM NaCl, 0.5% Na-deoxycholate, 0.5% NP40), then resuspended in TE, digested with 2U Dnase-free Rnase (Calbiochem) for 30 min at 37°C, and incubated overnight at 68°C with 300 mg/ml Proteinase K (NEB) in 0.5% SDS, 100 mM NaCl to digest proteins and reverse crosslinks. After purification by phenol-chloroform separation and

ethanol precipitation, DNA was resuspended in H_2O and 1/10 volume was used for quantification. 1/10 of input chromatin was amplified as standard for each experiment. PCR products were resolved on 2% agarose gels, visualized by EtBr staining, and quantified with Kodak Digital Science 1d 2.0.2 software. Primer sequences are indicated above.

Tissue Microarray (TMA) construction and Immunohistochemical analysis (IHC). Formalin-fixed and paraffin-embedded tissue specimens for TMA construction were obtained from Istituto Europeo di Oncologia. TMAs were prepared as described previously . Briefly, two representative tumor areas (diameter 0.6 mm) from each sample, identified previously on Hematoxylin and Eosin stained sections, were removed from the donor blocks and deposited on the recipient block using a custom-built precision instrument (Tissue Arrayer, Beecher Instruments). Serial sections (3 μm) of the resulting recipient block were cut, mounted on glass slides and processed for immunohistochemistry with rabbit polyclonal anti-Pin1 (Calbiochem, 1/200), rabbit polyclonal anti-N1ICD (Chemicon, 1/750), and mouse monoclonal anti-HES-1 (MBL, Clone NM1, 1:500) antibodies. A tyramide signal amplification labeling kit (Invitrogen, TSA Kit #21, Cat# T20931) was used for signal enhancement of anti-N1ICD and anti-HES-1 immunohistochemical stains. Arbitrary cut offs for definition of low and high protein expression levels used for statistical analysis were established according to both intensity of staining (from 0 to 3) and percentage of stained cells (from 0 to 100%) in the tumor nuclei. A semiquantitative score from 0 to 300 was obtained. For each gene, cases showing a score below the mean were classified as “low expression” whereas cases above the mean as “high expression”. Statistical significant differences were assessed according to pearson chi-square.

Statistical analyses. For transfection and processing experiments *P*-values were obtained by applying one-tailed, type 2 t-test (assuming equal variances) using Microsoft Excel. For TMA analysis *P*-values were obtained from pearson chi-square test.

6. REFERENCES

Albanese C, Johnson J, Watanabe G, et al. Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J Biol Chem 1995;270:23589–97.

Artavanis-Tsakonas, S., Rand, M. D. & Lake, R. J. Notch signaling: cell fate control and signal integration in development. Science 284, 770–776 (1999).

Atchison, F. W., Capel, B. & Means, A. R. Pin1 regulates the timing of mammalian primordial germ cell proliferation. Development 130, 3579–3586 (2003).

Ayala, G. et al. Pin1 is a novel prognostic marker in prostate cancer. Cancer Res. 63, 6244-6251(2003).

Ayyanan A, Civenni G, Ciarloni L, Morel C, Mueller N, Lefort K, Mandinova A, Raffoul W, Fiche M, Dotto GP, Brisken C. Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism.Proc Natl Acad Sci U S A2006 Mar 7;103(10):3799-804.

Bao, L., Sauter, G., Sowadski, J., Lu, K. P. & Wang, D. Prevalent overexpression of prolyl isomerase Pin1 in human cancers. Am. J. Pathol. 164, 1727-1737 (2004).

Barbareschi M, Pelosio P, Caffo O, et al. Cyclin-D1-gene amplification and expression in breast carcinoma: relation with clinicopathologic characteristics and with retinoblastoma gene product, p53 and p21WAF1 immunohistochemical expression. Int J Cancer 1997;74:171–4

Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J. M., Lukas, C., Ørntoft T, Lukas J, Bartek J. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. (2005) Nature 434, 864–870.

Beverly LJ, Felsher DW, Capobianco AJ. Suppression of p53 by Notch in lymphomagenesis: implications for initiation and regression. Cancer Res. 2005 Aug 15;65(16):7159-68.

Beverly, L. J. & Capobianco, A. J. Perturbation of Ikaros isoform selection by MLV integration is a cooperative event in Notch(IC)-induced T cell leukemogenesis. Cancer Cell 3, 551–564 (2003).

Bigas A, Martin D I, Milner L A. Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. Mol Cell Biol. 1998;18:2324–2333

Blaumueller C.M., Qi H., Zagouras P. and Artavanis-Tsakonas S. Intracellular cleavage of Notchl leads to a heterodimeric receptor on the plasma membrane. Cell 90, 281-291(1997).

Bocchetta M, Miele L, Pass HI, Carbone M. Notch-1 induction, a novel activity of SV40 required for growth of SV40-transformed human mesothelial cells. Oncogene 2003;22:81–9.

Bocchetta, M. et al. Notch-1 induction, a novel activity of SV40 required for growth of SV40-transformed human mesothelial cells. Oncogene 22, 81–89 (2003).

Bray, S. J. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 7, 678-89 (2006).

Bray, S., Musisi, H. & Bienz, M. Bre1 is required for Notch signaling and histone modification. Dev. Cell 8, 279–286 (2005).

Brou C, Logeat F, Gupta N, Bessia C, LeBail O, Doedens JR, Cumano A, Roux P, Black RA,

Israël A.A novel proteolytic cleavage involved in Notch sgnaniling: the role of disintegrin-metalloprotease TACE. Mol Cell 5 207-216(2000).
Callahan R. MMTV-induced mutations in mouse mammary tumors: their potential relevance to human breast cancer. Breast Cancer Res Treatment 1996;39:33–44.
Calvi LM, Adams GB, Weibrecht KW et al. Osteoblastic cells regulate the haematopoieti stem cell niche. Nature 2003;425:841–846
Capobianco AJ, Zagouras P, Blaumueller CM, Artavanis-Tsakonas S, Bishop JM. Neoplastic transformation by truncated alleles of human NOTCH1/TAN1 and NOTCH2. Mol Cell Biol. 1997 Nov;17(11):6265-73.
Cardiff RD, Sinn E, Muller W, Leder P. Transgenic oncogene mice. Tumor phenotype predicts genotype. Am J Pathol 1991;139:495–501.
Chen, Y., Fischer, W. H. & Gill, G. N. Regulation of the ERBB-2 promoter by RBPJkappa and NOTCH. J. Biol. Chem. 272, 14110–14114 (1997).
Clarke RB, Anderson E, Howell A, Potten CS. Regulation of human breast epithelial stem cells. Cell Prolif. 2003;36(Suppl 1):45–58.
Clarke RB, Spence K, Anderson E, Howell A, Okano H, Potten CS. A putative human breast stem cell population is enriched for steroid receptor-positive cells. Dev Biol. 2005;277:443–456.
Colaluca IN, Tosoni D, Nuciforo P, Senic-Matuglia F, Galimberti V, Viale G, Pece S, Di Fiore PP.
NUMB controls p53 tumour suppressor activity. Nature. 2008 Jan 3;451(7174):76-80.
Conboy IM, Conboy MJ, Wagers AJ et al. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 2005;433:760–764
Conlon RA, Reaume AG, Rossant J. Notch1 is required for the coordinate segmentation of somites. Development 1995;121:1533–1545.
Cooper, M. T. et al. Spatially restricted factors cooperate with notch in the regulation of Enhancer of Split genes. Dev. Biol. 221, 390–403 (2000).
Dang TP, Gazdar AF, Virmani AK, et al. Chromosome 19 translocation, overexpression of Notch3, and human lung cancer. J Natl Cancer Inst 2000;92:1355–7
De Strooper B, Annaert W, Cupers P, Saftig P, Craessaerts K, Mumm JS, Schroeter EH, Schrijvers V, Wolfe MS, Ray WJ, Goate A, Kopan R. A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain.Nature.1999 Apr 8; 398(6727):518-22.
De Strooper, B. et al. A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature 398, 518-22(1999).
DeOme KB, Medina D. A new approach to mammary tumorigenesis in rodents. Cancer. 1969;25:1255–1258.
Devgan V, Nguyen BC, Oh H, Dotto GP. p21WAF1/Cip1 suppresses keratinocyte differentiation independently of the cell cycle through transcriptional up-regulation of the IGF-I gene.J Biol Chem. 2006 Oct 13;281(41):30463-70. Epub 2006 Aug 15.
Dickson BC, Mulligan AM, Zhang H, Lockwood G, O'Malley FP, Egan SE, Reedijk M.High-level JAG1 mRNA and protein predict poor outcome in breast cancer. Mod Pathol. 2007 Jun;20(6):685-93.
Dievart A, Beaulieu N, Jolicoeur P. Involvement of Notch1 in the development of mouse mammary tumors. Oncogene 1999;18:5973– 81.
Dontu G, Al-Hajj M, Abdallah WM, Clarke MF, Wicha MS. Stem cells in normal breast development and breast cancer. Cell Prolif. 2003;36(Suppl 1):59–72.
Dorsch, M. et al. Ectopic expression of Delta4 impairs hematopoietic development and leads

to lymphoproliferative disease. Blood 100, 2046–2055 (2002).
Dougherty, M. K. et al. Regulation of Raf-1 by direct feedback phosphorylation. Mol. Cell 17, 215-224 (2005).
Dumont E, Fuchs KP, Bommer G, Christoph B, Kremmer E, Kempkes B. Neoplastic transformation by Notch is independent of transcriptional activation by RBP-J signalling. Oncogene. 2000 Jan 27;19(4):556-61.
Duncan AW, Rattis FM, DiMascio LN et al. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol 2005;6:314–322
Eckerdt F, Yuan J, Saxena K, Martin B, Kappel S, Lindenau C, Kramer A, Naumann S, Daum S, Fischer G, Dikic I, Kaufmann M, Strebhardt K. Polo-like kinase 1-mediated phosphorylation stabilizes Pin1 by inhibiting its ubiquitination in human cells. J Biol Chem. 2005 Nov4;280(44):36575-83
Elenbaas, B., Spirio, L., Koerner, F., Fleming, M. D., Zimonjic, D. B., Donaher, J. L., Popescu, N. C., Hahn, W. C. & Weinberg, R. A. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. (2001) Genes Dev 15, 50–65.
Ellisen, L. W. et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 66, 649–661 (1991).
Emery G., Hutterer A., Berdnik D., Mayer B., Wirtz-Peitz F., Gaitan M.G., Knoblich J.A. Asymmetric Rab11 endosomes regulate Delta recycling and specify cell fate in the Drosophila nervous system. Cell 122, 763-773(2005).
Escuder L.M:, Wei S.Y., Chiu W.H.,Modolell J. and Hsu J.C. Echinoid patterning synergizes with the Notch signaling pathway in Drosophila mesothorax bristle Development 130, 6305-6316(2003).
Esler W.P., Kimberly W.T., Ostaszewski B.L., Ye W., Diehl T.S., Selkoe D.J., complex reveals nicastrin and a gamma substrate.Proc Natl Acad Sci U S A. 2002 Mar 5;99(5):2720-5.
Evan, G. I. & Vousden, K. H. Proliferation, cell cycle and apoptosis in cancer. Nature 411, 342–348 (2001).
Fan X, Mikolaenko I, Elhassan I, et al. Notch1 and notch2 have opposite effects on embryonal brain tumor growth. Cancer Res 2004;64:7787–93.
Ferres-Marco, D. et al. Epigenetic silencers and Notch collaborate to promote malignant tumours by Rb silencing. Nature 439, 430–436 (2006).
Fitzgerald, K., Harrington, A. & Leder, P. Ras pathway signals are required for notch-mediated oncogenesis. Oncogene 19, 4191–4198 (2000).
Foltz DR, Santiago MC, Berechid BE, Nye JS.Glycogen synthase kinase-3beta modulates notch signaling and stability.Curr Biol. 2002 Jun 25;12(12):1006-11.
Fortini, M. E. Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling. Nat Rev Mol Cell Biol 3, 673-84 (2002).
Fryer C.J., Lamar E., Turbachova I., Kintner C., Jones K.A. C.J. Master Notch signaling mediates chromatin-specific transcription and turnover of the Notch enhancer complex. Genes Dev. 16, 1397-1411(2002).
Fryer, C. J., White, J. B. & Jones, K. A. Mastermind recruits CycC–CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol. Cell 16, 509–520 (2004).
Fujimori, F., Takahashi, K., Uchida, C. & Uchida, T. Mice lacking Pin1 develop normally, but are defective in entering cell cycle from G(0) arrest. Biochem. Biophys. Res. Commun. 265, 658–663 (1999).
Gallagher E.,Gao M., Liu Y.C. and Kaeim M. Activation of the E3 ubiquitin ligase Itch

through a phosphorylation-induced conformational change. Proc. Natl. Acad. Sci. USA 103, 1717-1722(2006).
Girard, L. et al. Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. Genes Dev. 10, 1930–1944 (1996).
Girard, L. et al. Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. Genes Dev. 10, 1930–1944 (1996).
Grego-Bessa J, Díez J, Timmerman L, de la Pompa JL. Notch and epithelial-mesenchyme transition in development and tumor progression: another turn of the screw. Cell Cycle. 2004 Jun;3(6):718-721.
Guo M, Jan LY, Jan YN. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron. 1996 Jul;17(1):27-41
Gupta-Rossi N, Six E, LeBail O, Logeat F, Chastagner P, Olry A, Israël A, Brou C Monoubiquitinqtion and endocytosis direct gamma-secretase cleavage of activated Notch receptor. J Cell Biol 166,73-83(2004).
Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, Ruas JL, Poellinger L, Lendahl U, Bondesson M. Hypoxia requires notch signaling to maintain the undifferentiated cell state.Dev Cell. 2005 Nov;9(5):617-28.
Hadland BK, Huppert SS, Kanungo J et al. A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. Blood 2004;104:3097–3105.
Haines N. and Irvine K.D. Glycosilation regulates Notch signaling. Nat Rev Mol Cell Biol 4, 786-797(2003).
Hamada Y, Kadokawa Y, Okabe M et al. Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. Developmen 1999;126:3415–3424.
Hanes, S. D., Shank, P. R. & Bostian, K. A. Sequence and mutational analysis of ESS1, a gene essential for growth in Saccharomyces cerevisiae. Yeast 5, 55–72 (1989).
Hendrix MJ, Seftor RE, Seftor EA, et al. Transendothelial function of human metastatic melanoma cells: role of the microenvironment in cell-fate determination. Cancer Res 2002;62:665–8.[
Hennighausen L, Robinson GW. Signaling pathways in mammary gland development. Dev Cell. 2001;1:467–475. doi: 10.1016/S1534-5807(01)00064-8.
Hitoshi S, Alexson T, Tropepe V et al. Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes Dev 2002;16:846 – 858.
Hopfer O, Zwahlen D, Fey MF, Aebi S. The Notch pathway in ovarian carcinomas and adenomas. Br J Cancer 2005;93:709–18.
Hori K, Fostier M, Ito M, Fuwa TJ, Go MJ, Okano H, Baron M, Matsuno K Drosophila Deltex mediates Suppressor of Hairless indipendent and late-endosomal activation of Notch signaling. Development 131, 5527-5537(2004).
Hrabe de Angelis M, McIntyre J 2nd, Gossler A. Maintenance of somite borders in mice requires the Delta homologue DII1. Nature 1997; 386 717–721.
Hulit J, Wang C, Li Z, et al. Cyclin D1 genetic heterozygosity regulates colonic epithelial cell differentiation and tumor number in ApcMin mice. Mol Cell Biol 2004;24:7598–611
Hurston G, Noguera-Troise I, Yancopoulos GD.The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. Nat Rev Cancer. 2007 May;7(5):327-31.
Imatani, A. & Callahan, R. Identification of a novel NOTCH-4/INT-3 RNA species encoding an activated gene product in certain human tumor cell lines. Oncogene 19, 223–231

(2000).
Izon, D. J. et al. Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1. Immunity 16, 231–243 (2002).
Jeffries, S. & Capobianco, A. J. Neoplastic transformation by Notch requires nuclear localization. Mol. Cell Biol. 20, 3928–3941 (2000).
Jiang R, Lan Y, Chapman HD et al. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. Genes Dev 1998;12:1046 –1057.
Joseph, J. D., Daigle, S. N. & Means, A. R. PINA is essential for growth and positively influences NIMA function in Aspergillus nidulans. J. Biol. Chem. 279, 32373–32384 (2004).
Kadam, S. & Emerson, B. M. Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes. Mol. Cell 11, 377–389 (2003).
Kao, H. Y. et al. A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes Dev. 12, 2269–2277 (1998).
KaoH.Y., Ordentlich P., Koyano-Nakagawa N., Tang Z., Downes M., Kintner C.R., Evans R.M., Kadesch T. A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes Dev. 12, 2269-2277 (1998).
Kidd S., Kelley M.R. and Young M.W. Sequence of the notch locus of Drosophila melanogaster: relationship of the encoded protein to mammalian clotting and growth factors. Mol. Cell. Biol. 6, 3094-3106(1986).
Kim ND, Oberley TD, Yasukawa-Barnes J, Clifton KH. Stem cell characteristics of transplanted rat mammary clonogens. Exp Cell Res. 2000;260:146–159.
Kim SB, Chae GW, Lee J, Park J, Tak H, Chung JH, Park TG, Ahn JK, Joe CO.Activated Notch1 interacts with p53 to inhibit its phosphorylation and transactivation. Cell Death Differ. 2007 May;14(5):982-91. Epub 2006 Dec 22.
Kimble, J. & Simpson, P. The LIN-12/Notch signaling pathway and its regulation. Annu. Rev. Cell Dev. Biol. 13, 333–361 (1997).
Klinakis A, Szabolcs M, Politi K, Kiaris H, Artavanis-Tsakonas S, Efstratiadis A. Myc is a Notch1 transcriptional target and a requisite for Notch1-induced mammary tumorigenesis in mice. 2006 Jun 13;103(24):9262-7.
Kopan R, Ilagan MX. Gamma-secretase: proteasome of the membrane? Nat Rev Mol Cell Biol 2004;5:499–504.
Kordon EC, Smith GH. An entire functional mammary gland may comprise the progeny from a single cell. Development. 1998;125:1921–1930.
Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J. & Clevers, H. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. (1998) Nat. Genet 19, 379–383
Krause DS. Regulation of hematopoietic stem cell fate. Oncogene. 2002;21:3262–3269.
Krebs LT, Shutter JR, Tanigaki K et al. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev 2004;18:2469 – 2473.
Krebs LT, Xue Y, Norton CR et al. Characterization of Notch3-deficient mice: Normal embryonic development and absence of genetic interactions with a Notch1 mutation. Genesis 2003;37:139 –143.
Krebs LT, Xue Y, Norton CR et al. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev 2000;14:1343–1352.
Kumano K, Chiba S, Kunisato A et al. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. Immunity 2003;18:699 –711.

Kunisato A, Chiba S, Nakagami-Yamaguchi E et al. HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo. Blood 2003;101:1777–1783

Kurooka, H. & Honjo, T. Functional interaction between the mouse Notch1 intracellular region and histone acetyltransferases PCAF and GCN5. J. Biol. Chem. 275, 17211–17220 (2000).

L. Kim and A.R. Kimmel, GSK3, a master switch regulating cell-fate specification and tumorigenesis. Curr. Opin. Genet. Dev. 10 (2000), pp. 508–514.

L. Ruel, M. Bourouis, P. Heitzler, V. Pantesco and P. Simpson, Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch. Nature 362 (1993), pp. 557–560.

Lai E.C. Protein degradation: four E3s for the notch pathway. Curr Biol 12, R74-R78(2002).

Lardelli M., Dahistrand J and Lendahl U. The novel Notch homologue mouse Notch3 lacks specific epidermal growth factor-repeats and is expressed in proliferation neuroephitelium. Mech. Dev. 46, 123-136(1994).

Le Borgne R., Bardin A. and Schweisguth F. The roles of receptor and ligand endocytosis in regulating Notch signalin. Development 132, 1751-1762(2005).

Le Borgne R., Remaud S., Hamel S. and Schweisguth F. Two distinct E3 ubiquitin ligase have complementary functions in the regulation of Delta and Serrate signaling in Drosophila. PloS Biol. 3, e96(2005).

Lee MS, Kao SC, Lemere CA, Xia W, Tseng HC, Zhou Y, Neve R, Ahlijanian MK, Tsai LH. APP processing is regulated by cytoplasmic phosphorylation. J Cell Biol. 2003 Oct 13;163(1):83-95.

Lendon C., Ashall F. and Goate A. Exploring the etiology of Alzheimer's disease using molecular genetics. J. Am. Med. Assoc. 277, 825–831 (1997).

Levitan D. and Greenwald I. Facilitation of lin-12-mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene. Nature 377, 351–354 (1995).

Levitan, D. and Greenwald, I. Effects of SEL-12 presenilin on LIN-12 localization and function in Caenorhabditis elegans. Development 125, 3599–3606 (1998).

Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P. & Werb, Z., et al. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. (2003) Proc. Natl. Acad. Sci. USA 100, 15853–15858.

Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, Fujimori F, Uchida T, Hunter T, Lu KP.Free in PMC Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. Proc Natl Acad Sci U S A. 2002 Feb 5;99(3):1335-40. Epub 2002 Jan 22.

Liou YC, Sun A, Ryo A, Zhou XZ, Yu ZX, Huang HK, Uchida T, Bronson R, Bing G, Li X, Hunter T, Lu KP.Role of the prolyl isomerase Pin1 in protecting against age-dependent neurodegeneration.. 2003 Jul 31;424(6948):556-61.

Liou, Y. C. et al. Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. Proc. Natl Acad. Sci. USA 99, 1335-1340 (2002).

Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW, Suri P, Wicha MS. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells.Cancer Res. 2006 Jun 15;66(12):6063-71.

Lman, D. et al. Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. J. Exp. Med. 194, 99–106 (2001).

Logeat F., Bessia C., Brou C., LeBail O., Jarriault S., Seidah N.G. and Israël A. The Notch1

receptor is cleaved constitutively by a furin-like convertase. Proc. Natl. Acad. Sci. USA 95, 8108-8112(1996)
Lowell, S. et al. Stimulation of human epidermal differentiation by delta–notch signalling at the boundaries of stem-cell clusters. Curr. Biol. 10, 491–500 (2000).
Lu, K. P., Hanes, S. D. & Hunter, T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature 380, 544–547 (1996).
Lu, K. P., Liou, Y. C. & Vincent, I. Proline-directed phosphorylation and isomerization in mitotic regulation and in Alzheimer's disease. Bioessays 25, 174-181 (2003).
Lu, K. P., Liou, Y. C. and Zhou, X. Z. (2002b). Pinning down the proline directed phosphorylation signaling. Trends Cell Biol. 12, 164-172
Lu, P. J., Wulf, G., Zhou, X. Z., Davies, P. & Lu, K. P. The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein. Nature 399, 784-788 (1999).
Lu, P. J., Zhou, X. Z., Liou, Y. C., Noel, J. P. & Lu, K. P. Critical role of WW domain phosphorylation in regulating its phosphoserine-binding activity and the Pin1 function. J. Biol. Chem. 277, 2381-2384 (2002).
Lutolf S, Radtke F, Aguet M et al. Notch1 is required for neuronal and glial differentiation in the cerebellum. Development 2002;129:373–385.
Mantovani F, Piazza S, Gostissa M, Strano S, Zacchi P, Mantovani R, Blandino G, Del Sal G. Pin1 links the activities of c-Abl and p300 in regulating p73 function. Mol Cell. 2004 Jun 4;14(5):625-36.
Mantovani F, Tocco F, Girardini J, Smith P, Gasco M, Lu X, Crook T, Del Sal G.The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP. Nat Struct Mol Biol. 2007 Oct;14(10):912-20.
Mason JL, Jones JJ, Taniike M et al. Mature oligodendrocyte apoptosis precedes IGF-1 production and oligodendrocyte progenitor accumulation and differentiation during demyelination/remyelination. J Neurosci Res 2000;61:251–262.
McGill M.A. and McGlade C.J. Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch intracellular domain J. Biol.Chem. 278,23196-23203(2003).
Miele L. Notch signaling. Clin Cancer Res. 2006 Feb 15;12(4):1074-9. Review.
Mishra-Gorur K.,Rand M.D.,Perez -Villamil B. and Artavanis-Tsakonas S. Down-regulation of Delta by proteolytic processing. J. Cell Biol 159, 313-324(2002).
Miyamoto Y, Maitra A, Ghosh B, Zechner U, Argani P, Iacobuzio-Donahue CA, Sriuranpong V, Iso T, Meszoely IM, Wolfe MS, Hruban RH, Ball DW, Schmid RM, Leach SD. Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis.Cancer Cell. 2003 Jun;3(6):565-76.
Molofsky AV, Pardal R, Morrison SJ. Diverse mechanisms regulate stem cell self-renewal. Curr Opin Cell Biol 2004;16:700 –707.
Moon, R. T., Bowerman, B., Boutros, M. & Perrimon, N. The promise and perils of Wnt signaling through beta-catenin. Science 296, 1644-1646 (2002).
Morgan T. H., The theory of the gene. Am. Nat. 51, 513–544 (1917).
Morrison SJ, Perez SE, Qiao Z et al. Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell 2000;101:499 –510.
Morrison SJ, Perez SE, Qiao Z, Verdi JM, Hicks C, Weinmaster G, Anderson DJ. Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell 101, 499–510 (2000).
Mungamuri SK, Yang X, Thor AD, Somasundaram K.Survival signaling by Notch1:

mammalian target of rapamycin (mTOR)-dependent inhibition of p53. Cancer Res. 2006 May 1;66(9):4715-24.

Muschler J, Lochter A, Roskelley CD, Yurchenco P, Bissell MJ. Division of labor among the α6β4 integrin, β1 integrins, and an E3 laminin receptor to signal morphogenesis and β-casein expression in mammary epithelial cells. Mol Biol Cell. 1999;10:2817–2828.

Nagel, A. C. et al. Hairless-mediated repression of Notch target genes requires the combined activity of Groucho and CtBP corepressors. Mol. Cell. Biol. 25, 10433–10441 (2005).

Nam Y, Sliz P, Song L, Aster JC, Blacklow SC. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. Cell. 2006 Mar 10;124(5):973-83.

Nguyen BC, Lefort K, Mandinova A, Antonini D, Devgan V, Della Gatta G, Koster MI, Zhang Z, Wang J, Tommasi di Vignano A, Kitajewski J, Chiorino G, Roop DR, Missero C, Dotto GP. Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation.Genes Dev. 2006 Apr 15;20(8):1028-42.

Nickoloff BJ, Osborne BA, Miele L. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. Oncogene 2003;22:6598–608.

Nickoloff BJ, Qin JZ, Chaturvedi V, Denning MF, Bonish B, Miele L.Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. Cell Death Differ. 2002 Aug;9(8):842-55.

Nicolas M, Wolfer A, Raj K et al. Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 2003;33:416–421

Okajima T., Xu A., Lei L. and Irvine K.D. Chaperone activity of protein O-fucosyltransferase1 promotes notch receptor folding. Science 307, 1599-1603(2005).

Okajima T., Xu A., Lei L. and Irvine K.D. Modulation of Notch-ligand binding by protein O-fucosyltransferase1 and fringe. J. Biol. Chem. 278, 42340-42345(2003).

Okano H, Imai T, Okabe M. Musashi: a translational regulator of cell fate. J Cell Sci. 2002;115:1355–1359.

Osborne BA, Minter LM. Notch signalling during peripheral T-cell activation and differentiation.2007 Jan;7(1):64-75. Epub 2006 Dec 15. Review.

Oswald F, Liptay S, Adler G, Schmid RM. NF-kappaB2 is a putative target gene of activated Notch-1 via RBP-Jkappa. Mol Cell Biol. 1998 Apr;18(4):2077-88.

P. Ramain, K. Khechumian, L. Seugnet, N. Arbogast, C. Ackermann and P. Heitzler, Novel Notch alleles reveal a Deltex-dependent pathway repressing neural fate. Curr. Biol. 11 (2001), pp. 1729–1738.

Pan Y, Lin MH, Tian X et al. Gamma-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. Dev Cell 2004;7:731–743.

Pang, R. et al. PIN1 overexpression and beta-catenin gene mutations are distinct oncogenic events in human hepatocellular carcinoma. Oncogene 23, 4182-4186 (2004).

Pardal R, Clarke MF, Morrison SJ.Applying the principles of stem-cell biology to cancer.Nat Rev Cancer. 2003 Dec;3(12):895-902. Review

Parks A.,Klueg K.M., Stout J.R. and Muskavitch M.A. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development 127, 1373-1385(2000).

Pastorino L, Sun A, Lu PJ, Zhou XZ, Balastik M, Finn G, Wulf G, Lim J, Li SH, Li X, Xia W, Nicholson LK, Lu KP. The prolyl isomerase Pin1 regulates amyloid precursor protein processing and amyloid-beta production.Nature. 2006 Mar 23;440(7083):528-34.

Pear WS, Simon MC. Lasting longer without oxygen: The influence of hypoxia on Notch signaling. Cancer Cell. 2005 Dec;8(6):435-7. Review.

Pear, W. S. et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. J. Exp. Med. 183, 2283–2291 (1996).

Phan RT, Saito M, Kitagawa Y, Means AR, Dalla-Favera R. Genotoxic stress regulates expression of the proto-oncogene Bcl6 in germinal center B cells. Nat Immunol. 2007 Oct;8(10):1132-9. Epub 2007 Sep 9.

Polo S, Di Fiore PP.Endocytosis conducts the cell signaling orchestra.Cell. 2006 Mar 10;124(5):897- 900. Review.

Pui, J. C. et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity 11, 299–308 (1999).

Purow BW, Haque RM, Noel MW, et al. Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. Cancer Res 2005;65:2353–63

Qin JZ, Stennett L, Bacon P, et al. p53-independent NOXA induction overcomes apoptotic resistance of malignant melanomas. Mol Cancer Ther 2004;3:895–902

Radtke F., Raj K. The role of Notch in tumorigenesis: oncogene or tumor suppressor? Nat Rev Cancer 3, 756-767(2003).

Rae FK, Stephenson SA, Nicol DL, Clements JA. Novel association of a diverse range of genes with renal cell carcinoma as identified by differential display. Int J Cancer 2000;88:726–32

Ranganathan, R., Lu, K. P., Hunter, T. & Noel, J. P. Structural and functional analysis of the mitotic peptidyl-prolyl isomerase Pin1 suggests that substrate recognition is phosphorylation dependent. Cell 89, 875–886 (1997).

Rangarajan A, Syal R, Selvarajah S, Chakrabarti O, Sarin A, Krishna S. Activated Notch1 signaling cooperates with papillomavirus oncogenes in transformation and generates resistance to apoptosis on matrix withdrawal through PKB/Akt.. 2001 Jul 20;286(1):23-30.

Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, Aster JC, Krishna S, Metzger D, Chambon P, Miele L, Aguet M, Radtke F, Dotto GP.Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J. 2001 Jul 2;20(13):3427-36.

Rangarajan, A. et al. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J. 20, 3427–3436 (2001).

Reedijk, M., Odorcic, S., Chang, L., Zhang, H., Miller, N., McCready, D. R., Lockwood, G. & Egan, S. E. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. (2005) Cancer Res 65, 8530–8537

Reineke EL, Lam M, Liu Q, Liu Y, Stanya KJ, Chang KS, Means AR, Kao HY. Degradation of the tumor suppressor PML by Pin1 contributes to the cancer phenotype of breast cancer MDA-MB-231 cells. Mol Cell Biol. 2008 Feb;28(3):997-1006

Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol. 1996;175:1–13.

Reynolds, T. C., Smith, S. D. & Sklar, J. Analysis of DNA surrounding the breakpoints of chromosomal translocations involving the beta T cell receptor gene in human lymphoblastic neoplasms. Cell 50, 107–117 (1987).

Robbins J, Blondel BJ, Gallahan D, Callahan R. Mouse mammary tumor gene int-3: a member of the notch gene family transforms mammary epithelial cells. J Virol

1992;66:2594–9. 42.
Robert-Moreno A, Espinosa L, de la Pompa JL et al. RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. Development 2005;132:1117–1126.
Robey, E. et al. An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. Cell 87, 483–492 (1996).
Romanov SR, Kozakiewicz BK, Holst CR, Stampfer MR, Haupt LM, Tlsty TD. Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature. 2001;409:633–637.
Ronchini C, Capobianco AJ. Notch(ic)-ER chimeras display hormone-dependent transformation, nuclear accumulation, phosphorylation and CBF1 activation. Oncogene 2000;19:3914–24
Ronchini, C. & Capobianco, A. J. Notch(ic)-ER chimeras display hormone-dependent transformation, nuclear accumulation, phosphorylation and CBF1 activation. Oncogene 19, 3914–3924 (2000).
Rudland, PS.;Barraclough, R.;Fernig, DG.; Smith, JA. Mammary stem cells in normal development and cancer. In: Potten CS. , editor. Stem Cells. San Diego: Academic Press; 1997. pp. 147–232.
Ryo A, Liou YC, Wulf G, Nakamura M, Lee SW, Lu KP. PIN1 is an E2F target gene essential for Neu/Ras-induced transformation of mammary epithelial cells. Mol Cell Biol. 2002 Aug;22(15):5281-95.
Ryo, A. et al. Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. Mol. Cell 12, 1413-1426 (2003).
Ryo, A., Nakamura, N., Wulf, G., Liou, Y. C. & Lu, K. P. Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. Nature Cell Biol. 3, 793-801 (2001).
Sanchez-Irizarry C., Carpenter, A.C., Weng, A.P., Pear, W.S., Aster, J.C. and Blacklow, S.C., 2004. Mol. Cell. Biol. 24, pp. 9265–9273.
Santagata S, Demichelis F, Riva A, et al. JAGGED1 expression is associated with prostate cancer metastasis and recurrence. Cancer Res 2004;64:6854–7.
Schmitt TM, de Pooter RF, Gronski MA et al. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat Immunol 2004;5:410 – 417.
Scott W. Lowe, Enrique Cepero & Gerard Evan Intrinsic tumour suppression. Nature 432, 307-315 (2004).
Searfoss GH, Jordan WH, Calligaro DO et al. Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor. J Biol Chem 2003;278:46107–46116
Shen ZJ, Esnault S, Rosenthal LA, Szakaly RJ, Sorkness RL, Westmark PR, Sandor M, Malter JS. Pin1 regulates TGF-beta1 production by activated human and murine eosinophils and contributes to allergic lung fibrosis. J Clin Invest. 2008 Feb 1;118(2):479-490.
Shen, J. *et al*. Skeletal and CNS defects in presenilin-1-deficient mice. *Cell* 89, 629–639 (1997).
Shi S, Stahl M, Lu L et al. Canonical Notch signaling is dispensable for early cell fate specifications in mammals. Mol Cell Biol 2005;25:9503–9508.
Smith GH, Chepko G. Mammary epithelial stem cells. Microsc Res Tech. 2001;52:190–203.
Soriano JV, Uyttendaele H, Kitajewski J, Montesano R. Expression of an activated

Notch4(int-3) oncoprotein disrupts morphogenesis and induces an invasive phenotype in mammary epithelial cells in vitro. Int J Cancer. 2000;86:652–659.

Swiatek PJ, Lindsell CE, del Amo FF et al. Notch1 is essential for postimplantation development in mice. Genes Dev 1994;8:707–719.

Takahashi K, Akiyama H, Shimazaki K, Uchida C, Akiyama-Okunuki H, Tomita M, Fukumoto M, Uchida T. Ablation of a peptidyl prolyl isomerase Pin1 from p53-null mice accelerated thymic hyperplasia by increasing the level of the intracellular form of Notch1. Oncogene. 2007 May 31;26(26):3835-45. Epub 2006 Dec 11.

Thompson BJ, Buonamici S, Sulis ML, Palomero T, Vilimas T, Basso G, Ferrando A, Aifantis I.The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia..2007 Aug 6;204(8): 1825-35.

Timmerman LA, Grego-Bessa J, Raya A, Bertrán E, Pérez-Pomares JM, Díez J, Aranda S, Palomo S, McCormick F, Izpisúa-Belmonte JC, de la Pompa JL. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes dev 2004 Jan 1;18(1):99-115.

Ubiquitylation of cyclin E requires the sequential function of SCF complexes containing distinct hCdc4 isoforms. Mol Cell. 2006 Jul 7;23(1):37-48.

Uchida T, Takamiya M, Takahashi M, Miyashita H, Ikeda H, Terada T, Matsuo Y, Shirouzu M, Yokoyama S, Fujimori F, Hunter T.Pin1 and Par14 peptidyl prolyl isomerase inhibitors block cell proliferation.Chem Biol. 2003 Jan;10(1):15-24.

Uyttendaele H, Soriano JV, Montesano R, Kitajewski J. Notch4 and Wnt-1 proteins function to regulate branching morphogenesis of mammary epithelial cells in an opposing fashion. Dev Biol. 1998;196:204–217.

Uyttendaele H.,Marazzi G., Wu G., Yan Q., Sassoon D. and Kitajewski. Notch4/int-3, a mammary proto-oncogene is an endothelial cell-specific mammalian Notch gene. Development 122, 2251-2259(1996).

Van Drogen F, Sangfelt O, Malyukova A, Matskova L, Yeh E, Means AR, Reed SI.Ubiquitylation of cyclin E requires the sequential function of SCF complexes containing distinct hCdc4 isoforms. Mol Cell. 2006 Jul 7;23(1):37-48.

van Es JH, van Gijn ME, Riccio O et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 2005;435:959–963

Varnum-Finney B, Xu L, Brashem-Stein C et al. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med 2000;6:1278–1281

Varnum-Finney B., Wu L., Yu M., Brashem-Stein C., Staats S., Flowers D., Griffin J.D., Bernstein I.D. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. J. Cell Sci. 113,4313-4318(2000).

Wallberg, A. E., Pedersen, K., Lendahl, U. & Roeder, R. G. p300 and PCAF act cooperatively to mediate transcriptional activation from chromatin templates by notch intracellular domains in vitro. Mol. Cell. Biol. 22, 7812–7819 (2002).

Wang W. and Struhl G. Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in Drosophila. Development 132, 2883-2894(2005).

Welcker M, Clurman BE. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation.2008 Feb;8(2):83-93. Review.

Weijzen S, Rizzo P, Braid M, et al. Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nat Med 2002;8:979–86.

Weijzen S, Rizzo P, Braid M, et al. Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nat Med 2002;8:979–86.

Weinmaster G., Roberts V.J. and Lemke G. Notch2: a second mammalian Notch gene. Development 116, 931-941(1992).
Weiss S, Reynolds BA, Vescovi AL, Morshead C, Craig CG, van der Kooy D. Is there a neural stem cell in the mammalian forebrain? Trends Neurosci. 1996;19:387–393.
Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM, Goodell MA. Sca-1pos cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol. 2002;255:42–56.
Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004;306:269–71
Weng, A. P. et al. Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. Mol. Cell Biol. 23, 655–664 (2003).
Wharton K.A., Johansen K.M., Xu T. and Artavanis-Tsakonas S. Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with protein containig EGF-like repeats. Cell 43,567-581(1985).
Wilkin M.B, Carbery AM, Fostier M, Aslam H, Mazaleyrat SL, Higgs J, Myat A, Evans DA, Cornell M, Baron M. Regulation of Notch endosomal sorting and signaling by Drosophila Nedd4 family proteins. Curr Biol 14, 2237-2244(2004).
Wilson, J. J. & Kovall, R. A. Crystal structure of the CSL–Notch–Mastermind ternary complex bound to DNA. Cell 124, 985–996 (2006).
Winkler, K. E., Swenson, K. I., Kornbluth, S. & Means, A. R. Requirement of the prolyl isomerase Pin1 for the replication checkpoint. Science 287, 1644–1647 (2000).
Wolfe M.S. The gamma-secretase complex: membrane-embedded proteolytic ensemble.. Biochemistry 45, 7931-9 (2006).
Wong GT, Manfra D, Poulet FM et al. Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. J Biol Chem 2004;279:12876–12882
Wong, P. C. et al. Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. Nature 387, 288–292 (1997).
Wrigth G.J.,Lesile J.D.,Ariza-McNaughton I. and Lewis J. Delta proteins and MAG1 proteins and interaction of Notch ligands with intracellular scaffolding molecules and its significance for zebrafish development. Development 131, 5659-5669(2004).
Wu, G. et al. SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. Mol. Cell. Biol. 21, 7403–7015 (2001).
Wulf GM, Ryo A, Wulf GG, Lee SW, Niu T, Petkova V, Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. EMBO J. 2001 Jul 2;20(13):3459-72.
Wulf, G. M., Liou, Y. C., Ryo, A., Lee, S. W. & Lu, K. P. Role of Pin1 in the regulation of p53 stability and p21 transactivation, and cell cycle checkpoints in response to DNA damage. J Biol Chem 277, 47976-9 (2002).
Wulf, G., Garg, P., Liou, Y. C., Iglehart, D. & Lu, K. P. Modeling breast cancer in vivo and ex vivo reveals an essential role of Pin1 in tumorigenesis. EMBO J. 23, 3397-3407 (2004).
Wulf G., Finn G., Suizu F., and Lu K.P. Phosphorylation-specific prolyl isomerization: is there an underlying theme? Nat Cell Biol 7;435-441 (2005)
Xia W., Wolfe M.S.Intramembrane proteolysis by presenilin and presenilin-like proteases. 15, 2839-44(2003)
Xu YX, Manley JL. Pin1 modulates RNA polymerase II activity during the transcription cycle.Genes Dev. 2007 Nov 15;21(22):2950-62.

Xu YX, Manley JL.The prolyl isomerase Pin1 functions in mitotic chromosome condensation.Mol Cell. 2007 Apr 27;26(2):287-300.

Yaffe, M. B. et al. Sequence-specific and phosphorylation-dependent proline isomerization: A potential mitotic regulatory mechanism. Science 278, 1957–1960 (1997).

Yan, X. Q. et al. A novel Notch ligand, Dll4, induces T-cell leukemia/lymphoma when overexpressed in mice by retroviral-mediated gene transfer. Blood 98, 3793–3799 (2001).

Yang L.T Nichols JT, Yao C, Manilay JO, Robey EA, Weinmaster G..Fringe glycosyltransferases differentiallly modulate Notch1 proteolysis induced by Delta1 and Jagged1. Mol Biol. Cell. 16,927-942(2005).

Yang X, Klein R, Tian X et al. Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. Dev Biol 2004;269:81–94.

Yeh E, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G, Hahn WC, Stukenberg PT, Shenolikar S, Uchida T, Counter CM, Nevins JR, Means AR, Sears R. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat Cell Biol. 2004 Apr;6(4):308-18.

Yeh ES, Lew BO, Means AR. The loss of PIN1 deregulates cyclin E and sensitizes mouse embryo fibroblasts to genomic instability. J Biol Chem. 2006 Jan 6;281(1):241-51. Epub 2005 Oct 13.

Yeh ES, Means AR. PIN1, the cell cycle and cancer. Nat Rev Cancer. 2007 May;7(5):381-8.

You H, Zheng H, Murray SA, Yu Q, Uchida T, Fan D, Xiao ZX. IGF-1 induces Pin1 expression in promoting cell cycle S-phase entry. J Cell Biochem. 2002;84(2):211-6

Zacchi P, Gostissa M, Uchida T, Salvagno C, Avolio F, Volinia S, Ronai Z, Blandino G, Schneider C, Del Sal G. The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. Nature. 2002 Oct 24;419(6909):853-7.

Zagouras P, Stifani S, Blaumueller CM, Carcangiu ML, Artavanis-Tsakonas S. Alterations in Notch signaling in neoplastic lesions of the human cervix. Proc Natl Acad Sci U S A 1995;92:6414–8.

Zhang J, Niu C, Ye L et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003;425:836–841

Zheng H, You H, Zhou XZ, Murray SA, Uchida T, Wulf G, Gu L, Tang X, Lu KP, Xiao ZX. The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response. Nature. 2002 Oct 24;419(6909):849-53.

Zhu X, Raina AK, Lee HG, Casadesus G, Smith MA, Perry G. Oxidative stress signalling in Alzheimer's disease. Brain Res. 2004 Mar 12;1000(1-2):32-9. Review.

7. Acknowledgements

I would like to thank Giannino Del Sal as supervisor of my Ph.D, Alessandra Rustighi and Alessia Soldano for their fundamental contribution in my thesis.

I thank all my colleagues at the LNCIB for advice and technical help. M. Stebel of the C.S.P.A. of the University of Trieste (Trieste, Italy) for the care of Pin1 mice colonies. M. Donzelli (IFOM-IEO, Milan, Italy) for generation of the pcDNA3-N1DE-Flag construct, to S. Piccolo (University of Padua, Italy) for MCF-10A cells, to T. Sudo (Toray Industries, Inc. Kamakura, Japan) for providing the anti-HES-1 antibody, G. Blandino (Regina Elena Institute, Rome, Italy) for pRSshLacZ, A. Israel (Institute Psteur, Paris, France) for pGL2-HES-1/LUC and T. Uchida (Tohoku University, Sendai, Japan) for providing Pin1 KO mice, and to ICGEB (Trieste, Italy) for access to their facilities. Paolo Nuciforo (IFOM, Milan, Italy) for analysis and evaluation of TMA under the supervision of Salvatore Pece (IFOM, Milan, Italy) and Pier Paolo Di Fiore (IFOM, Milan, Italy); Fred Kaplan and Anthony Capobianco (The Wistar Institute, Philadelphia, U.S.A) for essential reagents.