Prolyl-isomerase Pin1 controls normal and cancer stem cells of the breast by counteracting the Fbxw7-oncosuppressive barrier on the Notch signalling pathway

Alessandro Zannini
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Settore scientifico-disciplinare BIO/13

DOTTORANDO
Alessandro Zannini

COORDINATORE DEL CORSO DI DOTTORATO
Prof. Guidalberto Manfioetti

SUPERVISORE DELLA TESTI e RELATORE
Prof. Gianpiero Del Sal

CO-SUPERVISORE
Dott.ssa Alessandra Rustighi

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“Non troverai mai arcobaleni se guardi in basso”

- C. Chaplin

Dedico questa tesi ai miei genitori,
a mia mamma per essere il mio pilastro,
a mio papà per essere la mia roccia.
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2. ABSTRACT

Cancer stem cells (CSCs) are proposed to be responsible for breast cancer heterogeneity, chemotherapeutic treatment failure, metastatic spread and disease recurrence. The precise identification of the molecular bases that govern the induction and maintenance of CSCs and their aggressive phenotypes is of utmost importance, since it may provide the rational to develop effective therapeutic strategies. In particular there is a considerable effort in finding common pathways, mutations or histological features that might be targeted for therapy, overcoming breast cancer heterogeneity.

Here we now demonstrate that CSC self-renewal, chemoresistance, tumour growth and metastases formation capabilities’ are under direct control of Pin1’s enzymatic activity on the Notch signalling pathway. In particular Pin1 protects the nuclear activated forms of Notch1 and Notch4 (N1/4-ICD) from their E3-ubiquitin-ligase Fbxw7α, thereby boosting their protein levels and transcriptional activity. Fbxw7α acts as a potent inhibitor of CSCs maintenance by promoting protein degradation of N1- and N4-ICD, and, as a consequence, this ubiquitin-ligase strongly decreased tumour growth and metastases dissemination in vivo. Interestingly, concomitant over-expression of Pin1 almost completely recovered all these aggressive breast cancer traits.

In tissues from breast cancer patients, we observed Notch signalling over-activation despite presence of the negative regulator Fbxw7α, which relied on high Pin1 protein levels. Notably, activation of the Notch-Pin1 axis correlated with poor prognosis in these patients.

As a consequence of our findings, suppression of Pin1 holds promise in reverting aggressive phenotypes in breast cancer through shrinkage of CSCs number and a concomitant gain in chemosensitivity, carrying important implications for breast cancers therapy.
Breast cancer is the most common cause of cancer death among women (522,000 deaths in 2012) and the most frequently diagnosed tumour type in 140 out of 184 countries worldwide (Bray et al., 2013). Despite death rates have dropped by 34% from 1990 to 2013 in the United States due to an earlier diagnosis, better histopathological classifications and more specific pharmacological treatments (Desantis et al., 2014; Weigelt et al., 2005), a significant percentage of these patients still die. This is partly due to lifestyle changes that cause increased incidence, but also because of treatment failures and the inability to hinder dissemination and growth of metastases (Polyak, 2011; Steeg and Theodorescu, 2008). From a clinical point of view, the main goal of treatment is eradication of the tumour and prevention of metastatic growth. Even though therapeutic strategies have evolved, the three main pillars on which clinicians still rely are represented by surgery, radio- and chemotherapy. Unfortunately, despite increasing advances, when a tumour enters the metastatic phase, the probability of a cure is extremely reduced (Longo et al., 2011). The main cause of the mild improvement of the clinical management of malignant breast cancers resides in their intrinsic inter-tumoural and intra-tumoural heterogeneity. Breast cancer is not a single disease but a collection of breast diseases, with different genetic variations, gene expression profiles, histopathological features, pharmacological response and clinical outcomes (Kreso and Dick, 2014; Stingl and , 2007; Vargo-Gogola and Rosen, 2007).

The possibility to associate mutations or cancer properties to a specific cell type or stage of differentiation could contribute to better classify similar tumours, and to propose tumour type specific interventions. Indeed, breast cancer heterogeneity suggests the hypothesis that breast cancers can initiate in different cell types, either breast epithelial stem cells or their progeny. However, the cellular hierarchy of the mammary gland and the cell of origin of different classes of breast tumours are still open questions that await elucidation.

3.1.1 Classification of breast cancer subtypes: advances and pitfalls

Gene expression profiles and histological staging offered the opportunity to classify human breast cancers in 5 different major subtypes: normal-like, luminal A, luminal B, HER2+, basal-
like and claudin-low (Eroles et al., 2012; Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003; Sotiriou et al., 2003). This type of classification is useful not only for assessment of prognosis but also for the best fitting therapeutic regime.

The luminal A breast cancer is the most common subtype, detected in almost 50% of breast cancer patients. They are characterized by expression of estrogen receptor (ER), its target genes and progesterone receptor (PR) in the luminal epithelium, low rate of proliferation, low histological grade, good prognosis and they respond to ER modulator drugs.

The luminal B subtype is less present than the A type but these tumours are more aggressive, with high proliferation rate, worse prognosis and they do not have a specific treatment regime as not always responding to ER modulators.

The HER2 positive subtype represents at least 15-20% of all breast cancers, and they are characterized by a high expression of the HER2 gene or amplification of HER2 gene, or up-regulation of modulators of its pathway characterizes them. These tumours display high proliferation rate, high histological grade and worse prognosis. From a clinical point of view the discovery of anti-HER2 molecules strongly changed the median survival of these patients, reducing the metastases formation but also curbing primary tumours.

The Basal-like subtype represents 10-20% of all breast carcinomas that expresses genes usually present in normal breast myoepithelial cells. This subtype of cancer is characterized by large tumour size, high histological grade, high frequency of lymph node affection and poor overall survival. They are characterized by lack of expression of the 3 key molecular targets present in the great proportion of breast tumours, namely ER, PR and HER2. For this reason, a large percentage of Basal-like breast tumours are also called Triple Negative breast cancers, or TNBC. Moreover, since there is no specific molecular target to date, the therapeutic regime for these patients still relies on chemotherapy.

The Claudin-low subtype represents 12-15% of breast tumours and it is defined by low expression of genes involved in the regulation of tight junctions, inter-cellular adhesion (claudin-3, -4, -7) and epithelial markers (E-Cadherin). They are similar to basal-like tumours, but they differ by the fact that the claudin-low subtype is strongly infiltrated by immune system cells and moreover they are characterized by overexpressions of genes linked to mesenchymal differentiation and epithelial-to-mesenchymal transition (EMT).

This subtype classification is not only relevant to define the most fitting therapeutic approach, but it is also useful to predict the clinical outcome and to provide, at times, possible explanation
to the failure of the pharmacological regime. In addition, the recent discovery of other breast cancer subtypes (Eroles et al., 2012; Sims et al., 2007), as a consequence of more comprehensive genomic and transcriptomic analyses, unveiled that a definitive classification is still far to come. However, such classifications are inherently problematic, since they are based on molecular analyses on a heterogeneous population, and they don’t take into account that, in a tumour cell population, there is a collection of various statuses of differentiation, cellular commitment, and mutational landscapes (Gerlinger et al., 2012). Moreover, the heterogeneity in breast cancer is even more complex. For example a HER2 breast cancer subtype is classified on the basis of the percentage of cells in the tumour mass display amplification of its gene – or up-regulation of its pathway – that exceeds a particular cut-off. Thus, even if a tumour is classified as HER2, not all tumour cells exhibit high levels of this receptor (Wolff et al., 2013), leading to the existence of genetic and epigenetic intra-tumour variability (Marusyk and Polyak, 2010; Polyak, 2011). Thus, even though personalized medicine using targeted agents is considered a more promising way to target individual tumours than simple chemio- or radiotherapy, intra-tumour heterogeneity represents an important hurdle to the achievement of a complete patients’ response.

Different approaches in cancer research are converging to provide more insights into the underlying mechanisms of tumour heterogeneity and uncovering how these are linked to therapy resistance, tumour progression, and recurrence. In this regard, therapies aimed at targeting “cancer stem cells” have gained much attention (Figure 1) (Kreso and Dick, 2014).

![Figure 1. Stemness as a guiding principle that governs therapeutic response](image)

Three fields in biology—cancer genetics, epigenetics, and microenvironment—are coming together to provide increasing clarity to the processes that determine stemness and in turn influence clinical outcome. These three factors can influence stemness simultaneously, but they can also act independently over time. Through evolutionary time, different forces can impact a cell’s stemness properties and thereby shape tumor progression and therapeutic response (Kreso and Dick, 2014).
Introduction

It is increasingly acknowledged that a subpopulation of cancer cells, termed cancer stem cells (CSCs) play a major role in cancer growth, metastasis formation and chemoresistance in breast cancer (Dean et al., 2005; Stingl & Caldas, 2007; Visvader & Lindeman, 2012). Like their normal counterpart, CSCs are able to self-renew and maintain a reservoir of cancer-initiating cells, which may produce a more differentiated progeny of cells and contribute to intra-tumour heterogeneity (Stingl & Caldas, 2007). Current breast cancer research aims at better classifying breast cancer subtypes and defining the molecular circuitries of subtype-specific CSCs, trying to find common pathways, mutations or histological features that might be targeted for therapy, overcoming breast cancer heterogeneity. Indeed, there is emerging evidence, that despite the multiplicity of molecular signatures, receptors expression, histopathological features and intra-tumour heterogeneity, a common characteristic that could underlie these differences, are the CSCs (Kreso and Dick, 2014; Magee et al., 2012; Pece et al., 2010; Stingl and Caldas, 2007; Visvader and Lindeman, 2012).

3.1.2. Normal stem cells of the mammary gland

The mammary gland is a tubulo-alveolar gland, composed by three lineages of epithelial cells, i.e. luminal, myoepithelial and alveolar epithelial cells that derive from luminal progenitors and are differentiated for synthesizing milk proteins. The mammary gland undergoes a sequence of dynamic changes throughout the female life cycle (Gjorevski and Nelson, 2011), and the capacity of the mammary gland epithelium to expand and remodel during puberty and repeated cycles of pregnancy is highly suggestive of the existence of resident mammary gland stem cells (SCs) in the adult (Visvader, 2009).

The first evidence of the existence of mammary stem cells derived from experiments in which a transplantation of different dissected parts of the mammary gland into the fat pad of syngenic rodents, gave rise to fully developed and functional mammary glands (Hoshino, 1964). Other studies carried out with the purpose to identify a stem cell population able to regenerate the mammary gland (Gudjonsson et al., 2002; Rudland et al., 1997), but the isolation and characterisation of a true stem cell population from both humans and mice, has been obtained only few years later, using in vitro and in vivo reconstitution transplantation experiments (Dontu et al., 2003a; Dontu et al., 2003b; Stingl et al., 1998; Stingl et al., 2001; Stingl et al., 2006). Afterwards, the generation of the different mammary epithelial cells by resident mammary stem cells was demonstrated by two different groups through lineage-tracing experiments in mice, as depicted in the scheme of Figure 2 (Rios et al., 2014; Van Keymeulen et al., 2011; Visvader and Lindeman, 2011).
Figure 2. Model of the epithelial differentiation hierarchy in the mammary gland
There is recent evidence for a heterogeneous compartment of stem cells, in which the stem cell at the apex is multipotent and likely has higher self-renewing capacity than the unipotent stem cells (myoepithelial-stem cell, Myo-SC; luminal-stem cell, Lum-SC) and the ‘shorter-term’ mammary repopulating cell that emerges in pregnancy (Preg-SC). Alternatively, there may be one true stem cell and a hierarchy of descendant progenitor cells (indicated by overlap between the stem and progenitor cell compartments) that include long- and short-lived lineage-restricted progenitors (Visvader and Lindeman, 2011).

Adult stem stem cells are defined as undifferentiated and multipotent or bi/unipotent cells that have the potential to differentiate and generate the specialized end cells of that tissue. Moreover they are able to self-renew, so to maintain in this way the stem cells’ compartment for many passages, and to generate some committed progenitors with tissue maintaining potential. This ability is a consequence of their peculiar way of cell division. Indeed they are able to divide giving rise to one stem cell, identical to the initial stem cell, and a daughter committed progenitor cell, which undergoes a differentiation process. This type of division is called asymmetric. By contrast, in specific conditions, stem cells are able to divide also symmetrically and generate two stem cells, without the differentiated one, expanding in such way the stem cell pool (Liu et al., 2005; Stingl and Caldas, 2007).

Seminal work performed by Shackleton and Stingl has provided proper means to isolate and characterize a stem cell population capable of reconstituting a functional mammary gland in mice, called mammary repopulating units (MRUs) (Shackleton et al., 2006; Stingl et al., 2006). MRU cells are purified from the mammary gland after digestion and sorted by both excluding cells expressing hematopoietic and endothelial markers and including the expression of CD24, α6-integrin (CD49f), Sca-1 and β1-integrin (CD29). In particular, cells with a CD24med/CD49fhigh profile characterize the MRU population and it is present in a proportion of 1 MRU out of 1400 total cells in mice.
Moreover, by using an *in vitro* colony assay, other groups have demonstrated that a small subpopulation of mammary gland stem cells (MaSC) is able to give rise to luminal and myoepithelial lineages, suggesting the existence of bipotent stem cells (Dontu et al., 2003; Guo et al., 2012). Another *in vitro* experiment, that is now a widely used as a surrogate stem cell assay, is based on the ability of cells to generate clonal mammospheres in non-adherent, serum-free conditions and it evaluates the stem cells’ content in a breast cell line or tissue. Notably, these mammospheres are enriched in multi-lineage progenitors and display a transcriptional profile enriched in stemness factors and pathways (Dontu et al., 2003).

The ability to prospectively isolate and analyze the stem cell population of the mammary gland gives the opportunity to identify molecular signatures and factors required for their own maintenance. In fact, thanks to these methods, many groups had dissected the molecular bases of stem cells from both mouse and human breast tissues delineating the main factors involved in stemness (Ben-Porath et al., 2008; Dontu et al., 2003; Pece et al., 2010; Raouf et al., 2008; Spike et al., 2012).

In summary, a huge number of data allowed identifying a stem cell population that is able to generate, *in vitro*, mammospheres and colonies composed of both lineages and, more importantly, an entire functional mammary gland *in vivo*. Notably the isolation and characterisation of human mammary stem cells lacks a real *in vivo* validation, but the *in vitro* assays are well established and reliable.

### 3.1.3 Breast cancer stem cells

Breast cancer stem cell (CSCs) research is one of the most investigated fields in breast cancer research in the last years. Like normal stem cells that are able to regenerate an organ, also CSCs are considered to be responsible for re-generation of the tumour, as demonstrated by sequential transplantation assays. From a clinical point of view breast CSCs are associated to aggressiveness in breast cancer since they are chemoresistant, they have been linked to metastases formation and associated to the undifferentiated, poor outcome breast cancer subtypes (Stingl and Caldas, 2007; Valent et al., 2012; Visvader and Lindeman, 2012). Interestingly, the most aggressive and high-grade breast cancer subtypes display an enriched CSCs population and a stem cell-like signature (Ben-Porath et al., 2008; Pece et al., 2010). As a matter of fact, a better knowledge of their properties will be important in order to find possible strategies to curb their tumourigenic potential.
3.1.3.1 Breast CSCs: origin and history

There is increasing evidence that normal stem cells might be the targets of transformation during tumourigenesis. Tumours are believed to arise through a series of mutations that occur over many years. Adult stem cells are slowly dividing, long-lived cells, which by their very nature are exposed to damaging agents for long periods. There are two possible theories concerning the formation of CSCs. The first, also called the CSC hypothesis, claims that in some cancers CSCs have arisen from normal stem cells that, through acquisition of genetic and epigenetic modifications, become cancerous and display perturbed self-renewal. Another possibility is that terminally differentiated epithelial cells, or restricted transient amplifying progenitors, through clonal evolution, acquire tumourigenic features and, through reprogramming or acquisition of plasticity, they lose their committed fate and become more stem-like. Although no clear in vivo evidence of the CSC origin has been yet found, in favour of the first hypothesis comes the observation that normal stem cells and cancer stem cells share several important features (Dontu and Wicha, 2005; Liu et al., 2005; Stingl and Caldas, 2007; Visvader and Lindeman, 2012).

Using different experimental approaches, several investigators proved how only a minority of cells in human cancers are capable to self-renew. This has been most convincingly demonstrated by examining the ability of subpopulations of tumour cells identified by cell surface markers to form tumours when transplanted into immunosuppressed NOD/SCID mice. This approach was first successfully used to demonstrate the existence of leukemic stem cells (Dick, 1996) and, some years later, also for breast cancer stem cells (Al-Hajj et al., 2003). Using the cell surface markers CD44 and CD24, Al-Hajj and colleagues have been able to isolate a putative CSCs population since a small number of injected cells was able to generate a tumour in a host mice. There is now evidence for the existence of CSCs in many other tumours (Valent et al., 2012; Visvader and Lindeman, 2012), thus suggesting that CSCs are present in a wide number of cancers and, as a consequence, they could be considered as a hallmark of cancer.

The identification of pathways that govern CSCs’ maintenance is important to develop targeted therapies. The developmental Notch pathway, Bmi-1, Hedgehod and Wnt signalling, p53, c-myc, TAZ, ErbB2 and regulators of epithelial-to mesenchymal transition such as Slug, are the main regulators of stemness in breast cancer (Mani et al., 2008; Valent et al., 2012). Tumour-specific reactivation of some of these pathways or abrogation such as for p53, is able to re-program a committed or progenitor cell to a more dedifferentiated cell with stem cell features. Since most of these factors are also involved in cancer development it is evident how some genetic or
epigenetic mutations, that drive tumourigenesis, are able to promote formation of a stem cell population.

3.1.3.2 Breast CSCs isolation and functional characterisation

Nowadays no clear and unique assay has been validated and demonstrated to isolate breast CSCs. A multi-approach experimental detection and analysis of CSCs, indeed will be more predictive and reliable (Stingl and Caldas, 2007; Visvader and Lindeman, 2012). The most important and well-validated assay is the in vivo transplantation in immunodeficient mice. This assay evaluates the most peculiar trait of CSC, which is the ability of these cells to re-generate a tumour. For this reason CSC are often called tumour-initiating cells. In particular, while a CSC is able to generate a tumour, the differentiated counterpart of the tumour is unable to (Valent et al., 2012). This assay allows also to compare the CSCs’ content of different tumours: indeed, injection of low numbers of cells gives rise to tumours when derived from breast tumour/cell lines with a high content of putative CSCs, while the same amount of cells from tumours displaying a low CSCs’ content are not able to generate tumours with the same frequency. However, despite the reliability of this in vivo assay, there are some limitations: i) being host mice immunodeficient the innate and cell-based immune reactions as well as the content of cytokines is deranged, hence, depending on the injected cells, tumour development in these mice may be either facilitated or suppressed ii) the host mice lack the tumour microenvironment that is required as a supportive scaffold for tumours and CSCs, iii) the presence of both stem and progenitor cells requires second-generation recipient animals to better distinguish true stem cells from early progenitors which have the potential to generate some tumours in the first but not in the second transplanted animal (Valent et al., 2012).

The isolation of CSCs with specific markers is widely used but also this type of analysis has its own limitations. As a fact, no single marker to isolate/identify the CSC has yet been found. The first demonstration of the breast CSCs has been made by prospective immunophenotypic cell isolation using the surface markers CD44 and CD24. In particular, CSC are enriched in the CD44+CD24low/- population, as demonstrated by generation of tumours in host mice when injected in vivo. This immunophenotypic analysis is widely used but CD44 and CD24 cannot be considered as universal markers, indeed their usage does not allow to selectively evaluate and enrich for CSCs in ER- and triple negative breast cancers, as demonstrated by the fact that CSCs were found in both the CD44+/CD24- and CD44+/CD24+ (Meyer et al., 2010).
Ginestier and colleagues have discovered another breast CSC marker some years later. In particular they unveiled that both breast normal and cancer human mammary epithelial cells with increased aldehyde dehydrogenase activity (ALDH1) have stem/progenitor properties. Moreover, the ALDH1+ cells isolated from human breast tumours contain the CSCs population enabled with the capacity to self-renew and to generate tumours that recapitulate the original heterogeneity of the breast cancer from which they derive. Moreover, by performing a double flow-cytometry analysis of xenografted tumours with the stem cell markers ALDH1 and CD44+/CD24-, they identified only a small overlapping cell fraction, representing 1.2% of the entire population. This doubly recognized CSCs population displayed a higher tumourigenic capacity with respect to the single populations positive for one of the two markers. Thus, a double analysis of the same tumour population is strongly suggested to better define the CSCs subpopulation (Ginestier et al., 2007).

By taking advantage of the knowledge derived from neurospheres, Dontu and her colleagues established an in vitro assay called mammosphere assay. Analogously to primary neural cells, human mammary epithelial cells form spherical colonies, termed non-adherent mammospheres, when cultured in suspension on non-adherent surfaces in the presence of specific growth factors. These mammospheres are enriched in cells with functional characteristics of stem/progenitor cells. Transcriptional profiling of mammosphere-derived cells demonstrated significant overlap with the genetic programs of other stem cells. The use of this culture system facilitates the isolation and characterisation of mammary stem cells and it is useful to test their self-renewal ability as well as to understand the pathways that govern their self-renewal and differentiation (Dontu et al., 2003a). Almost all the research groups that have studied human and mouse stem cells from both normal and tumour breast tissues have extensively used this assay. Such assays have obvious caveats as factors important to the in vivo growth and self-renewal may not be provided by the in vitro conditions. As a result cells may be selected anomalously, or they are induced to differentiate or, more importantly, the true stem cells may be not selected. Moreover while CSCs have an unlimited proliferative potential, their growth in vitro is limited to some weeks/month. Therefore, results from mammosphere assays must be paralleled by in vivo evidence (Valent et al., 2012).

More recently other markers of human normal and breast CSCs have been described. The work was based on the intrinsic ability of CSCs to divide with lower frequency than their differentiated progeny. PKH-26 is a fluorescent dye that binds to cell membranes and segregates in daughter cells after each cell division, hence the intensity of staining inversely correlates with
the number of cell divisions. Notably PKH-26\textsuperscript{high}, slowly dividing cells were able to regenerate both an entire mammary gland and mammospheres with high efficiency. Transcriptional profiling of these cells, derived from human reductive mammoplasty, allowed determining a normal breast stem cell specific signature able to predict biological and molecular features of breast cancers. Indeed, in high-grade breast cancers the number of PKH26\textsuperscript{pos} cells is increased, indicating a higher CSCs content. By contrast, PKH-26\textsuperscript{neg}, highly dividing cells, were unable to generate mammary glands and mammospheres and they displayed markers of terminal differentiation. Thus, PKH26\textsuperscript{pos} cells are defined as stem cells (Cicalese et al., 2009; Pece et al., 2010).

Taken one by one, all the techniques used to isolate and analyze stem cells display some weaknesses; a combination of them will be more precise and clinically relevant for a better evaluation of the CSCs population. In addition, the \textit{in vivo} experiments are the most reliable ones and may facilitate the future development of methods for interrogating CSCs in patient samples for clinical purposes.

\subsection*{3.1.3.3 CSCs underlie heterogeneity of human breast cancers}

Breast cancer inter/intra-tumour heterogeneity represents a major problem in defining the fitting pharmacological regime for each patient. Indeed, despite classification of breast cancer subtypes has a prognostic value and can properly suggest the better therapy for each single patient, considerable heterogeneity in response to therapy still exists. As a result, there has been considerable effort in the breast cancer research community to identify biomarkers that more accurately predict patients outcome (Stingl and Caldas, 2007). For many reasons CSCs are suggested as responsible for this heterogeneity, as follows.

- Cancer stem cells boost tumour formation and give rise to a progeny with the same mutational features. Notwithstanding, CSCs and the more differentiated progeny display different phenotypic traits, as demonstrated for example by a different sensitivity to chemotherapeutic agents, contributing to intra-tumour heterogeneity. This aspect is even more exacerbated if more differentiated cells within a tumour, due to their genomic instability and increased proliferation, could acquire other epigenetic or genetic mutations. In this way the tumour will display different cell clones concurring to the intra-tumour heterogeneity, as depicted in Figure 3 (Stingl and Caldas, 2007; Kreso and Dick, 2014).
Figure 3. Unified model of clonal evolution and CSCs
Upper panel shows that acquisition of favorable mutations can result in clonal expansion of the founder cell. In parallel, another cell may gain a different mutation that allows it to form a new subclone. Over time, genetic mutations accumulate and subclones evolve in parallel. Lower panel shows that it may be that CSCs are not static entities but can evolve over the lifetime of a cancer as genetic changes can influence CSC frequency. Some subclones may contain a steep developmental hierarchy (left), where only few self-renewing CSCs exist among a large number of non-CSCs. Other subclones (middle) may contain an intermediate hierarchy, where the number of CSCs is relatively high but a hierarchy still exists. Some subclones may have the genetic alterations that confer high self-renewal potential, where most cells are tumorigenic. In this scenario, applying the CSC concept to such homogeneous subclones is not warranted because most cells can self-renew and few non-CSC progeny are generated (Kreso and Dick, 2014).

- It has been demonstrated that cancers can even harbour heterogeneous and biologically distinct populations of CSCs, as shown for leukaemia, colorectal and skin tumours (Goardon et al., 2011; Visvader and Lindeman, 2012). Intriguingly, distinct CSCs within a tumour have also the potential to interconvert, as demonstrated in skin squamous cell carcinomas. Since the leukaemia model is closely related to that of breast cancer, it is conceivable that also in breast cancers different pools of CSCs coexist and are able to interconvert (Visvader and Lindeman, 2012).

- As mentioned above, there is no great overlap between CSCs from the same breast cancer, when selected by different markers, suggesting presence of different types of CSCs highlighted the presence of genes associated with stemness features and, notably, increased aggressiveness and tumour grade can partially be ascribed to a higher CSCs content (Ben-Porath et al., 2008; Pece et al., 2010). Hence, these findings imply that increased percentage and heterogeneous CSCs may contribute to tumour heterogeneity explaining the different types of breast cancers.
3.1.3.4 CSCs are responsible for the failure of chemotherapeutic treatment

Clinically, chemoresistance is probably the major problem for treatment of breast cancers. Many studies have been carried out to better understand the molecular basis of chemoresistance in tumours. Collectively, these studies provide evidence that even within a single genetic clone, cancer cells are heterogeneous in their ability to survive chemotherapeutic insults, and point out both CSCs and tumour heterogeneity as major responsible for the failure of chemotherapeutic treatment (Figure 4) (Kreso and Dick, 2014; Dean, 2009; Dean et al., 2005).

In 1996 Goodell and colleagues, while staining murine bone marrow cells with the vital dye Hoechst 33342, discovered a population that contained low -or none- levels of fluorescence (Goodell et al., 1996). They called this population “side population” (SP), a “negatively stained” pool of cells that displayed hematopoietic stem cells traits since, upon transplantation into irradiated mice, they were able to reconstitute the bone marrow. Subsequent studies unveiled the presence of SPs in many tissues including brain, breast, lung, heart, pancreas, testes, skin, and liver that were enriched in stem cells. This technique leads to the identification of SP also in tumours and in particular in breast, lung, and neural tumours. The fact that this SP does not retain the dye is due to its intrinsic ability to exclude dyes through specific ABC transporters (ABCB1; ABCG2; ABCC1), promiscuous transporters of both hydrophobic and hydrophilic compounds such as dyes and drugs. Interestingly the SP in mammary tissues identify a stem cell population, suggesting that stem cells could promote efflux of dyes and, more importantly, drugs, underling a related chemoresistant properties of these cells. Indeed CSCs expressed high levels of these drug efflux pumps and are resistant to chemotherapy (Sarkadi et al., 2006).

The CSCs chemoresistance goes beyond the presence of ABC transporters. Although it is well known that chemoresistance could arise in any cell of a cancer through several mechanisms such as mutation, such features are more likely to be already present in CSCs. Indeed, CSCs, like normal stem cells, are relatively quiescent, resistant to apoptosis and they possess an efficient DNA-repair capacity (Dean et al., 2005).

It is evident that CSCs are the main responsible for the failure of chemotherapeutic treatment, since they already intrinsically possess a plethora of chemoresistance mechanisms. Even worse, despite treatment of breast cancer patients with chemotherapy causes an initial shrinkage of the tumour mass, a concomitant increase in the percentage of resistant cancer stem cells was observed. Notably, in vitro and in vivo experiments with cells obtained from these treated tumours demonstrated that these cells eventually regenerated an even more aggressive tumour,
phenocopying tumour recurrence after therapy (Figure 4) (Kreso and Dick, 2014; Dean, 2009; Dean et al., 2005; Korkaya et al., 2009; Yu et al., 2007).

Figure 4. CSCs within subclones impact response to therapy
Each clone (depicted by the different colors) contains a mixture of cells that vary with respect to their stemness and/or proliferative ability, including dormant or CSCs (depicted by a central dot in the cell). Together these factors represent the functional diversity present within single genetic subclones. Chemotherapy can reduce tumor burden by eliminating the highly proliferative cells within subclones, while sparing the CSCs; following therapy, these cells can seed a new cancer. Thereby, subclonal diversity can be altered with chemotherapy and can allow for the selection of cells with additional genetic mutations that confer a survival advantage. Adapted from Kreso and Dick, 2014.
3.1.3.5 EMT and metastases are linked to CSC traits

Aggressive and deadly tumours are characterized by metastatic dissemination and outgrowth of drug resistant tumours. The ability of the infiltrated tumour cells to survive during latency and to reinitiate growth at the secondary site, when conditions are favourable, was reminiscent of cells having a CSC phenotype.

The group of Weinberg found that both normal and cancer stem cells of the breast display features of epithelial-to-mesenchymal transition (EMT) features. EMT is a process by which epithelial cells lose their cell polarity and become mesenchymal cells to gain migratory and invasive properties. This process was first recognized as indispensable for embryogenesis. Indeed EMT, and its reverse process, the Mesenchymal-to-epithelial transition (MET) are critical for development of many tissues and organs in the developing embryo (Kong et al., 2011). The EMT is also involved in cancer, in particular during the metastatic process. Interestingly over-expression of EMT transcription factors (such as Slug, Snail and Twist) or the down-regulation of epithelial markers (such as E-cadherin) not only induce an EMT program but also cause a concomitant enrichment in stem cells, as detected by different in vitro assays (Mani et al., 2008). In addition, enforced EMT in terminally differentiated mammary cells promotes a reprogramming to a bipotent stem cell able to generate an entire and functional mammary gland. Accordingly, mammary stem cells have been shown to display mesenchymal traits instead of epithelial ones (Guo et al., 2012).

The precise identification of the molecular bases that govern the self-renewal of normal mammary stem cells is important because these same pathways are thought to be required for the induction and maintenance of the CSCs and their aggressive phenotype. Inhibition of such signalling pathways has been proposed to be a novel and promising therapeutic strategy. In particular there is a considerable effort in finding therapies that target pathways, mutations or histological features that are common to all CSCs, overcoming breast cancer heterogeneity. A possible candidate as a fine tuner of these stemness traits is the prolyl-isomerase Pin1.
3.2. The prolyl-isomerase Pin1: a critical hub of phosphorylation-dependent pathway cross-talk

Post-translational modifications (PTMs) represent a milestone process in biology (Prabakaran et al., 2012; Walsh, 2006). They are involved in many cellular processes, such as the control of cell cycle, apoptosis, transcription, cell commitment, metabolism and DNA repair. There are thousands of PTMs but, interestingly, phosphorylation on serine/threonine residues (phospho-S/T) is the most prevalent one and it is a common way to process information in cells (Khoury et al., 2011). Phosphorylation of a protein involves the enzymatically, and reversible, mediated addition of a phosphate group to its amino acid side chains. In particular, phosphorylation of serine or threonine preceding a proline, represents a key signalling mechanism involved in many physiological and pathological processes (Lu and Zhou, 2007). Phosphorylation of these residues is a prerogative of Proline-directed protein kinases including glycogen synthase kinase-3, p38 kinases, extracellular signal-regulated kinases and cyclin-dependent protein kinases. Proteins that have S/T-P sites phosphorylated adopt either a cis or a trans conformation, which implies that the same protein can perform different functions. The spontaneous conversion from one isomer to the other, i.e. uncatalyzed cis-trans isomerization is normally very slow and phosphorylation per se further decreases the already slow isomerization rate of prolines (Schutkowski et al., 1998). This reaction needs the intervention of the peptidyl-prolyl cis/trans isomerase Pin1 for a biologically relevant timescale cis/trans conversion to occur (Hunter, 1998).

Pin1 belongs to the parvulin family of Peptidil Prolyl cis/trans Isomerases (PPIases). PPIases are a class of enzymes that catalyze conformational changes centered around proline residues and that include three structurally distinct subfamilies: the cyclophilins, the FK506-binding protein family and the parvulins (Galat, 2003). Notably, the only known human isomerase that specifically regulates the cis-trans conformation of Prolines only upon phosphorylation of the preceeding Serine or Threonine residues, is the parvulin Pin1 (Lu et al., 1996; Ranganathan et al., 1997; Yaffe et al., 1997).

3.2.1 Pin1 structure and functional domains

Human Pin1 is a 18KDa protein of 163 amino acids and its gene is located in chromosome 19p13. Structurally, it is composed of two functional domains: an amino-terminal WW domain (amino acids 1-39) and a carboxy-terminal PPIase domain (amino acids 45-163) connected by a
short flexible linker region (Figure 5). For recognition and interaction with its targets on phospho-S/T-P sites, Pin1 requires the WW domain that is characterized by two conserved tryptophan residues and organized in a three-stranded antiparallel antiparallel β-sheet (Sudol and Hunter, 2000; Sudol et al., 2001). Subsequently the PPIase can interact with the phospho-S/T-P motifs and induce a cis-trans isomerisation of the peptide bond between the phosphorylated residue and the Proline. This enzymatic activity is mediated by a specific basic triad, composed by Lys63-Arg68-Arg69, that recognizes the negative charge present in the phospho-residues (Zhou et al., 2000).

**Figure 5. Pin1 structure and function**
The two-domain structure of Pin1 is shown in the upper part of the figure. The lower part of the figure shows the conversion of the target protein from cis to trans conformation and vice-versa (Yeh and Means, 2007).

Pin1 was first identified by a two-hybrid screening performed to identify proteins that interact with never in mitosis gene A (NIMA), a fundamental mitotic kinase in Aspergillus nidulans (Lu et al., 1996). Since overexpression of NIMA in yeast induces premature chromosome condensation and subsequently cell death and concomitant overexpression of Pin1 prevents this effect, it was suggested that Pin1 might be a regulator of mitosis.

Pin1 is an evolutionarily conserved protein and was found to be fundamental in *S. cervistiae*, *C. albicans* and *A. nidulans* but not in *X. laevis*, *D. melanogaster*, *M. musculus* where its depletion is linked to some developmental defects (Crenshaw et al., 1998; Fujimori et al., 1999; Hanes et
al., 1989; Hsu et al., 2001; Maleszka et al., 1996; Winkler et al., 2000). Pin1 knock-out mice (Pin1<sup>-/-</sup>) have been generated in two different backgrounds. In the mixed background 129SvJae/C57BL/6, Pin1<sup>-/-</sup> mice display premature aging and tissue defects, in particular decreased body weight, testicular atrophy, a dramatic retinal atrophy and alteration of the mammary gland that fails to undergo proliferation during pregnancy (Liou et al., 2002). Mice in the inbred C57BL/6 background, display a reduced number of germ cells due to an altered cell cycle in primordial germ cells (PGC), causing a decreased fertility both in males and females (Atchison and Means, 2003).

3.2.2 Regulation of Pin1 levels and activity

Even though no amplification or deletion of the PIN1 gene have been reported, the levels and functions of the protein are finely regulated by several layers of control under physiological and also pathological conditions. Some polymorphisms have been discovered, one of them, rs2233678 (-842G>C) in the Pin1 promoter that elicits reduced expression of the gene and is associated with a reduced risk of cancer in some human sub-populations (Han et al., 2010). Notably Pin1 expression is strongly associated with cell proliferation and cell cycle progression, since it is transcriptionally regulated by the E2F transcription factor in response to growth factor stimuli, Ras, and Her2/Neu (Ryo et al., 2002). In breast cancer cells, its mRNA levels are also upregulated by the Notch signalling pathway (Rustighi et al., 2009) and the Insulin-like growth factor (You et al., 2002). Accordingly, Pin1 mRNA and protein levels are increased in transformed cells (Bao et al., 2004). Recently, it has been reported that the apolipoprotein E4 is able to positively modulate Pin1 expression in the hippocampus and that this ApoE-Pin1 axis is involved in the pathogenesis of the Alzheimer disease (Lattanzio et al., 2014). Pin1 is also controlled by micro-RNA, in particular it is negatively controlled by miR-200b that promotes anoikis in breast cancer cells and is able to block metastatic progression (Zhang et al., 2013). It has become evident how pathways involved in pathologies such as cancer and Alzheimer mediate misregulation of Pin1 transcription. Consequently, Pin1 protein and mRNA levels are considered a prognostic and predictive marker for many diseases (Lu and Zhou, 2007).

At a post-translational level, the enzymatic activity of Pin1, its cellular localization and turnover are finely regulated. Indeed a lot of kinases affect Pin1 functions by modifying its phosphorylation status (Liou et al., 2011). Activation of PKA phosphorylates Pin1 on Ser16 in the WW domain and inhibits its function by impairing its ability to interact with protein target and changing the sub-cellular localization (Lu et al., 2002b). This inhibitory phosphorylation on
Ser16 is mediated also by treatment with 12-O-tetradecnoylphorbol-13-acetate (TPA), that promotes an interaction between the 90-kDa ribosomal protein-S6-kinase 2 and Pin1 (Cho et al., 2012). Another inhibitory phosphorylation occurs on Ser71 in the PPIase domain, and it is mediated by the Death-associated protein kinase1 (DAPK1). This phosphorylation blocks Pin1’s catalytic activity and nuclear localization (Lee et al., 2011). Despite a lot of kinases have an inhibitory effect on Pin1 activity, the mixed-lineage kinase 3 (MLK3) instead promotes Pin1 catalytic activity and nuclear localization by adding phosphate group to the Ser138 in the PPIase domain in breast cancer (Lee et al., 2011; Rangasamy et al., 2012). It is conceivable that an appropriate evaluation of the phosphorylation status of Pin1 in tissues will be more predictive of its activity rather than solely total protein levels.

SUMOylation is another important PTM that affects Pin1’s activity, in particular it inhibits its functions when it occurs on Lys6 or Lys63. This inhibitory effect is reverted by deSUMOylation by SENP1 which, in turn, increases Pin1 protein levels (Chen et al., 2013).

### 3.2.3. Biological functions of Pin1

As a consequence of its enzymatic nature, Pin1 requires a substrate to impact in any given cellular process. The peculiarity of a Pin1 target is the presence of a docking site constituted by a phosphorylated-Ser/Thr preceeding a Proline, which represents a key element in the signal transduction. Indeed Pin1 interacts with and modifies a large number of proteins, impinging on several cellular processes such as cell cycle control, transcription, chromatin remodelling, cell-fate commitment, DNA damage response, and metabolism. A growing body of evidence demonstrates that on one hand Pin1 acts as a hub that controls the dynamics of physiological cell behaviour by fine-tuning the cross-talk between phosphorylation signalling and cellular pathways. But on the other, its dysregulated expression in diseased conditions such as cancer, critically amplifies perturbed cell signalling, contributing to disease progression.

#### 3.2.3.1 Mechanisms through which Pin1 impinges on cellular processes

The conformational changes induced by Pin1 on its protein substrates can have profound effects mainly on their stability, but also modulation of the catalytic activity, other PTMs like de-phosphorylation, protein–protein interactions and subcellular localization have been described (Lu et al., 2002a).

Pin1 has been shown to control protein turnover of many substrates by either favouring or blocking their recognition by several E3 ubiquitin-ligases. The phosphorylation of Ser/Thr-Pro
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constitutes an important motif not only for Pin1 but also, when embedded within the so-called CDC4-phosphodegron, for specific recognition by the Skp1-Cullin-F-Box (SCF) complex that promotes the ubiquitination-dependent degradation of critical cellular substrates. Consequently Pin1 plays a major role in modulating the conformation of these proteins thereby affecting their interaction with the SCF complex and proteasomal degradation (Gutierrez and Ronai, 2006; Liou et al., 2011).

Fbxw7α is a component of SCF complex and is also known as CDC4, AGO, SEL10. It is an important E3 ubiquitin-ligase that controls many cellular processes such as cell proliferation, stemness and differentiation, metabolism and apoptosis (Buckley et al., 2012; Welcker and Clurman, 2008; Wertz et al., 2011). It regulates a plethora of targets in many tissues such as Notch1, Notch4, c-myc, cyclin E, c-jun, MCL-1 and SREBP (Cheng and Li, 2012; Welcker et al., 2004). Fbxw7α recognizes a phospho-epitope, termed CPD (for Cdc4 Phospho-Degron), contained within these substrates. Via its F-box domain, Fbxw7α recruits the remainder of an SCF ubiquitin ligase complex, thus promoting substrate ubiquitination and proteasomal degradation. The eight WD40 repeats at the C-terminal end form a phospho-epitope binding pocket that can recognize and bind to phosphorylated CPDs. CPDs consist of a central phosphorylated threonine immediately followed by a proline (pT-P). Moreover a priming phosphate in the +4 position is required, that is provided by a phosphorylated serine, or a phospho-mimetic residue, in order to phosphorylate the central threonine (Welcker and Clurman, 2008).

Once phosphorylated, the CPD might become a Pin1 binding site. Indeed, many of the Fbxw7α targets are also Pin1 substrates and, consequently, Pin1 is impinging on their ubiquitination and protein degradation mediated by this ubiquitin-ligase (Liou et al., 2011). Notably the role of Pin1 on the Fbxw7-mediated protein degradation is context- and tissue-dependent. Indeed it has been shown that on one hand Pin1 promotes protein stabilization of some targets, such as Mcl-1, on the other it promotes the degradation of c-Myc and cyclin E (Welcker et al., 2004). In addition it has been recently demonstrated, in colon cancer cells and fibroblasts, that Pin1 promotes auto-ubiquitination and degradation of Fbxw7 (Min et al., 2012). Thus the effect of Pin1 on Fbxw7 targets seems not to be unidirectional.

Besides the Pin1-Fbxw7α dualism in the control of protein degradation, Pin1 is able to control the stability of many other proteins, such as cyclin D1, NF-κB, β-catenin, p53 family members, Nanog, Oct4, ErbB2, Akt, Smad proteins and Tau by different mechanisms (Liou et al., 2011). Alteration of their protein levels was shown to affect their activities and their ability to interact and cross-talk with their partners and regulators. As a result Pin1, through modulation of protein
stability, is able to impinge on several cellular processes. The best-studied biological function of Pin1 is undoubtedly regulation of cell cycle progression. Important roles for this enzyme in the control of a variety of other phosphorylation-dependent processes have been unveiled, some of which have been studied by us and are described in depth below.

3.2.3.2 Cell-cycle control

Pin1 was discovered as a regulator of mitosis (Lu et al., 1996; Yeh and Means, 2007). Further studies unveiled a role for Pin1 in all phases of the cell cycle. In fact, during cell cycle progression there is a precise timing and sequence of activation and inactivation of CDKs which are known to be phosphorylated on Ser/Thr-Pro motifs and, indeed, Pin1 fits perfectly in this molecular organization (Lu and Zhou, 2007; Nigg, 2001). The G0/G1-S phase transition dictates the entrance in the cell cycle and a key regulator of this passage is cyclin D1. Notably Pin1 increases cyclin D1 levels in a bymodal manner, i.e. preventing its protein degradation and upregulating its transcription (Liou et al., 2002; Wulf et al., 2001). Another important step that occurs during the G1-S phase, is the coordination of centrosome duplication and DNA synthesis. Pin1 is localized in the centrosomes and induces their duplication; in breast cancer Pin1 over-expression promotes chromosome aneuploidy as a consequence of deregulated centrosome amplification. Conversely, reduction of Pin1 levels strongly impaired centrosome amplification (Suizu et al., 2006). Accordingly, its protein levels are higher during the G1-S transition (Ryo et al., 2002) while Pin1−/− mouse embryonic fibroblasts exhibit defects in the G1-S transition (Fujimori et al., 1999). The role of Pin1 in the regulation of G1-S transition is more complex since it promotes the degradation of cyclin E at a later stage of the S phase (van Drogen et al., 2006), thus suggesting that Pin1 is controlling the amplitude of the G1-S transition. During the S phase the RNA polymerase II is recruited on the promoters of transcribed genes while it is released in M phase. Notably Pin1 coordinates this dynamic association by directly binding the RNA polymerase II (Xu and Manley, 2007a). In particular, Pin1 controls also the G2-M transition, acting on the early mitotic inhibitor-1 and, consequently, on anaphase promoting complex (APC) thus coordinating the S and M phases (Bernis et al., 2007).

Mitosis is promoted by cyclin B-CDC2 that, also in this case, is controlled by Pin1. In this case Pin1 indirectly controls cyclin B, acting on CDC25C and WEE1, regulators of the cyclin B-CDC2 phosphorylation status (Okamoto and Sagata, 2007; Zhou et al., 2000). The impact of Pin1 in the M phase emerged also by the evidence that it interacts with topoisomerase IIα impinging on the G2-M phase transition (Xu and Manley, 2007b).
All this evidence supports the idea that Pin1 is a fine tuner of the cell cycle progression in which it calibrates the correct timing and quantity of factors that trigger initiation or termination of each single phase.

### 3.2.3.3 Pin1 at the crossroads between stemness and differentiation

The normal development of organisms and the maintenance of tissue integrity are guaranteed by a correct balance between differentiation and stemness (Chen and Daley, 2008; Keller, 2005; Yeo and Ng, 2013). During embryogenesis, the proper timing and specification of differentiation stimuli is fundamental for the commitment of embryonic stem cells to generate the three embryonic germ layers. A lot of studies have been done to understand the molecular basis of embryonic stem cells’ biology (ESCs) in light of a better comprehension of embryogenesis, of the pathways required by the CSCs and, recently, for their application in regenerative medicine (Yeo and Ng, 2013). In particular, manipulation of these molecular circuitries allowed the conversion of adult differentiated cells in pluripotent stem cells, called induced pluripotent stem cells (iPS) (Takahashi and Yamanaka, 2006).

It has been demonstrated that, beyond the up-regulation of important pro-stem factors such as Oct4, Nanog and Sox2 by transcriptional regulation and chromatin remodelling (Yeo and Ng, 2013), their post-translational modifications, in particular phosphorylation and ubiquitination, are essential for a correct shift from a pluripotent to a differentiated fate and vice versa (Brill et al., 2009; Buckley et al., 2012; Van Hoof et al., 2009). These evidences suggest that the ESC regulation is a plastic process governed by several layers of control in which small changes in ESC regulators are sufficient for a complete cell reprogramming (Yeo and Ng, 2013). In this scenario, the prolyl-isomerase Pin1, an important fine tuner of gene expression, protein levels and post-translational modifications (Liou et al., 2011; Lu and Zhou, 2007) emerged as an essential regulator of pluripotency and stemness both in ESC and iPS cells (Moretto-Zita et al., 2010; Nishi et al., 2011). In particular Pin1 is indispensable for the induction and maintenance of pluripotency in iPS by interacting with phosphorylated Oct4, preventing its ubiquitination, boosting its protein levels and activity. Accordingly, Pin1 levels are higher in iPS and ablation of its activity suppresses colony formation in murine ESC cells (Nishi et al., 2011). Similarly, Pin1 levels are higher in ESCs and its genetic or pharmacological ablation curbs their clonogenic potential. In ESCs Pin1 is boosting stemness by preventing the degradation of Nanog (Moretto-Zita et al., 2010).
The impact of Pin1 in cell fate commitment carries several clinical implications. In particular, the major problem of iPSSs is their intrinsic tumourigenic potential that has prevented their use in regenerative medicine (Takahashi and Yamanaka, 2006). This tendency could be controlled by modulation of Pin1 protein levels (Moretto-Zita et al., 2010; Nishi et al., 2011).

The regulation of the transition from an undifferentiated stem cell to a differentiated state and vice versa mediated by Pin1 is maintained also in adult tissues with several consequences for the organogenesis. It has been demonstrated that Pin1-/- mice develop normally but display some defects in many compartments (Atchison and Means, 2003; Fujimori et al., 1999; Liou et al., 2002; Shen et al., 2013). Different studies unveiled that these defects might be partially explained by a role of Pin1 as a master regulator of the cell commitment in many cellular types such as neurons (Ciarapica et al., 2014; Nakamura et al., 2012), skeletal muscle cells (Magli et al., 2010), primordial germ cells (Atchison and Means, 2003), adipocytes (Uchida et al., 2012), osteoblasts (Lee et al., 2013a) and dental pulp cells (Lee et al., 2013b). All this evidence pinpoints Pin1 as a cell-fate determinant.

Interestingly, in adult tissues, Pin1 is not always linked to the maintenance of stem cells. Indeed, while in skeletal muscles it sustains the maintenance of undifferentiated cells, in the brain Pin1 drives neuronal differentiation. In particular, two different groups observed that Pin1 drives neuronal differentiation and its protein levels are higher in differentiated neurons during embryonic development, compared to less differentiated ones, without perturbing glia cells’ commitment (Ciarapica et al., 2014; Nakamura et al., 2012). This effect is mediated in a bimodal fashion: on one hand Pin1 interacts with β-catenin, a master regulator of neurogenesis, boosting its protein levels, on the other it cooperates with the protein kinase HIPK2 in antagonizing Gro/TLE1:Hes1, a protein complex involved in the inhibition of neuronal differentiation. As a result Pin1−/− mice display impaired neonatal motor activity.

The ability of Pin1 in the regulation of the cell-fate commitment is not only restricted to alterations of its proteins levels but, in some cases, also through its sub-cellular localization (Magli et al., 2010). In skeletal muscle cells Pin1 is localized in the nucleus and acts as an inhibitor of differentiation by promoting degradation of the Myocyte Enhancer Factor 2C (MEF2C). Interestingly, during muscle cells’ differentiation, there is a shuttling of Pin1 from the nucleus to the cytoplasm with a concomitant rescue of the stability and transcriptional activity of MEF2C that, consequently, induces a differentiation program.
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Based on these data, Pin1 can be clearly considered as a cell-fate determinant in the adult tissue, therefore alteration of its levels, activity or sub-cellular localization could compromise the integrity of many tissues.

3.2.3.4 Pin1 pinpoints the cellular response to environmental stresses

Cells exposed to genotoxic stress, chemical agents, or stimuli from the environment, undergo an adaptive response involving adjustments of the levels and activity of its genome-protecting protein machinery. When cell cycle checkpoints are switched on and stimulate DNA repair, cell cycle arrest, apoptosis or other processes are induced, depending on the stimulus and cell type. The p53 family members, in particular p53 and p73, are important tumour suppressors and are the major mediators of the cellular response to genotoxic insults and pharmacological treatment. Both p53 and p73 are subjected to proline-directed phosphorylation and are Pin1 targets (Mantovani et al., 2004; Mantovani et al., 2007; Zacchi et al., 2002; Zheng et al., 2002). In detail, Pin1 is able to prevent the interaction of p53 with its ubiquitin ligase Mdm2 thus enhances its half-life and, consequently, it increases cellular apoptosis upon genotoxic stress. The ability of Pin1 to potentiate p53 goes even beyond: on one hand Pin1 is required for a correct loading of p53 on its target genes and on the other it prevents the association of the apoptosis inhibitor iASPP (Mantovani et al., 2007). In addition, it has been recently demonstrated that Pin1, cooperating with HIPK2, promotes both p53 mitochondrial translocation and its transcriptional-independent apoptotic activity (Sorrentino et al., 2013). Moreover Pin1 boosts also p73 protein levels and transcriptional activity since it promotes p73 acetylation and subsequent protein stabilization; this circuitry has an important role in tumours cells lacking p53 and treated with chemotherapeutic agents (Mantovani et al., 2004). Interestingly Pin1 is not only able to control the DNA controllers, but it is directly involved in the regulation of DNA integrity by modulation of DNA double strand break (DSB) repair mechanisms in response to DNA damage. In particular Pin1 promotes non-homologous end-joining (NHEJ) at the expense of homologous recombination (HR), directly impinging on the DSBs resection (Steger et al., 2013).

The in vivo impact of these findings is still not so clear since many of these studies have been performed in primary fibroblasts and cells lines. It will be important to assess this issue, since Pin1 is often up-regulated in tumours (Bao et al., 2004) while p53 tumour suppressor activity is frequently abolished (Levine and Oren, 2009). The fact that Pin1 is required for a more efficient p53-dependent apoptosis following genotoxic stress may have clinical relevance in tumors with
concomitant high Pin1 levels and wild-type p53 status, since in these tumors its activity might be restored (Hoe et al., 2014).

Pin1 is not only able to drive the cellular response in genotoxic stress conditions, but also following oxidative stress. One of the major cellular mediators of this process is p66shc, which regulates cellular aging, apoptosis induction and the lifespan. As a consequence the knock-out mice models display longer lifespan and protection from oxidative stress (Migliaccio et al., 1999). It has been demonstrated that Pin1 potentiates the mitochondrial accumulation of p66shc thus inducing apoptosis in response to ROS (Pinton et al., 2007).

Thus, Pin1 is clearly an important modulator of the cellular response to environmental stresses acting through proteins of the DNA checkpoint and repair machinery and mediators of oxidative response.

Tumour chemoresistance is emerging as a major problem for the eradication of tumors. Despite clinical advances in the last years, chemoresistance still remains a major cause of death and an unresolved issue. The resistance could be intrinsic, since some tumour cells do not respond properly to the chemotherapeutic regime, or acquired, since they are initially responsive to the chemotherapy but become resistant later. In both cases cells display common features such as alteration of the pharmacological target, activation and expression of proteins that reduce the intracellular drug concentration, acquisition of the ability to change the cellular response by altering the apoptotic pathway or DNA repair machinery, or they being tolerant to genotoxic stresses (Dean et al., 2005).

Pin1 has been directly or indirectly linked to chemoresistance. First of all, Pin1 is overexpressed in tumours and its protein levels correlate with aggressiveness. Since the most aggressive tumours are often less responsive to chemotherapy it is conceivable that Pin1 is linked to chemoresistance (Ayala et al., 2003; Bao et al., 2004; Fukuchi et al., 2006; Wulf et al., 2001). Second, Pin1 is more expressed in some stem-cell subpopulations (Magli et al., 2010; Moretto-Zita et al., 2010; Nishi et al., 2011) which are chemoresistant by definition (Visvader and Lindeman, 2012) thus, as consequence, Pin1 is linked to a chemoresistant subpopulation. Third, Pin1 interacts with several factors that have been separately linked to chemoresistance such as mutant-p53 (Muller et al., 2014), Notch1 (Rustighi et al., 2009), Mcl-1 (Ding et al., 2008), Nanog and Oct4 (Hu et al., 2010; Moretto-Zita et al., 2010; Nishi et al., 2011). Fourth, in breast cancer cell lines Pin1 has been directly linked to specific chemoresistance mechanisms (Ding et al., 2008). Indeed, it was discovered that Pin1 protects cancer cells from paclitaxel treatment, one of the most used and effective chemotherapeutic agents against breast cancer, through up-
regulation of Myeloid cell leukemia-1 (Mcl-1), a Bcl-2–like antiapoptotic protein, involved in chemoresistance and tumourigenesis in several cancers. In particular, Pin1 is able to bind the Mcl-1 protein, upon phosphorylation by ERK, preventing its degradation and sustaining its protein levels. Notably Pin1 and Mcl-1 protein levels positively correlate in breast cancer patient samples. Despite all these publications, a real in vivo evidence of Pin1 in regulating the cellular response to environmental stresses is still missing.

This bivalent and opposed role of Pin1 in cooperating both with proteins that promote apoptosis or protect cells from chemotherapy is due to its enzymatic nature and its biological output is strongly dependent on the availability of its targets. Specifically, tumours often display down-regulation or loss of tumour-suppressors and concomitant activation of oncogenes and survival pathways, thus upsetting Pin1 to a more anti-apoptotic and tumour promoting role.

### 3.2.4. Pin1 in cancer

The first evidence of Pin1 in tumours has been reported in 2001 by two different publications in which Pin1 was found overexpressed in breast cancer (Ryo et al., 2001; Wulf et al., 2001). Later, high levels of Pin1 have been found in other tumours such as prostate, lung, colon, esophageal, human oral squamous cell, glioblastoma, ovary, cervical and melanoma cancers (Atkinson et al., 2009; Bao et al., 2004; Jin et al., 2011; Miyashita et al., 2003). All these studies indicated a prominent role of Pin1 in boosting tumourigenesis through up-regulation of key oncogenic pathways (Lu and Zhou, 2007). Pin1 was revealed not only as a marker of tumourigenesis but it was also shown to correlate with poor prognosis in some cancer types (Ayala et al., 2003; Fukuchi et al., 2006; Girardini et al., 2011; Tan et al., 2010; Wulf et al., 2001).

The first interactor of Pin1 described in cancer is cyclin D1 as demonstrated by in vivo and in vitro experiments (Wulf et al., 2001). The nuclear localization and proteasomal degradation of cyclin D1 are controlled by a phosphorylation on Thr-286-Pro that is promoted by glycogen synthase kinase-3beta (GSK-3β). Pin1 was shown to bind to this motif upon phosphorylation, preventing nuclear export and subsequent degradation, maintaining high cyclin D1 protein levels. Notably Pin1 knock-out and cyclin D1 knock-out mice share similar phenotypes in particular in the mammary epithelium, where both fail to undergo to the massive proliferative change during pregnancy (Liou et al., 2002).

The ability of Pin1 in boosting cyclin D1 levels goes even beyond an effect on protein stabilization. Indeed it has been demonstrated that the up-regulation of cyclin D1 induced by several signalling pathways such as the Ras, Her2/Neu, Wnt and NF-κB, requires the presence of
Pin1 which in turn is indispensable for full activity of these pathways (Liou et al., 2002; Lu and Zhou, 2007; Ryo et al., 2001; Ryo et al., 2003; Wulf et al., 2001). Pin1 can bind to phosphorylated c-Jun, β-catenin and NF-κB boosting their protein levels and transcriptional activity that is translated in high cyclin D1 gene expression. Moreover Pin1 is able to protect β-catenin and NF-κB from their negative regulators, APC (adenomatous polyposis coli gene product) and IκappaB, respectively.

Besides activation of growth factors receptors, one of the most important signalling pathways activated in cancer is the mitogen-activated protein kinase (MAPKs) cascade. In response to growth stimuli, Ras activates the Raf kinase, which in turn controls the MAPKs. As for other signalling pathways, this kinase cascade must be turned off by a negative feedback mechanism in which MAPKs phosphorylate and inactivate Raf. Notably Pin1 prevents this negative feedback, promoting Raf dephosphorylation with a consequent maintenance of an activated MAPKs cascade (Dougherty et al., 2005).

All these data point out Pin1 as a fine tuner of signalling pathways in cancer, regulating their activation, counteracting their inhibitors and thereby boosting their mediators. The proof that Pin1 is strongly required by these pathways emerged also by the discovery that, in turn, they induce Pin1 gene transcription and that genetic ablation of its gene block, in vivo, their oncogenic potential. In particular, Her2/Neu and Ras induce E2F transcription and progression of the cell cycle; E2F is then recruited on the Pin1 gene promoter inducing its transcription (Ryo et al., 2002). Since E2F is not only regulated by these pathways but it is a marker and promoter of the G1/S transition, it is conceivable that whatever the stimulus that induces cell cycle progression, Pin1 transcription can be induced.

Several reports have shown that Pin1 is also linked to aggressive traits of cancers. Indeed it has been demonstrated that Pin1 protein levels are higher in high-grade and more aggressive breast cancer subtypes (Wulf et al., 2001). In particular in triple negative breast cancer cell lines, Pin1 cooperates with two other important oncogenes such as Notch1 (Rustighi et al., 2009) and mutant-p53 (Girardini et al., 2011). Notch1 is a membrane bound heterodimer receptor, normally inactive, that becomes cleaved and activate by γ-secretase upon interaction with its specific ligand (Ranganathan et al., 2011). Pin1 interacts with the Notch1 receptor and increases the cleavage mediated by the γ-secretase, allowing full activation of the pathway and boosting its oncogenic activity both in vitro and in vivo. Also in this case the oncogene stimulated by Pin1 is able in turn to transcribe Pin1 itself, since Notch1 is directly recruited on the Pin1 gene.
promoter. As a consequence, in human breast cancer samples there is a strong correlation between high levels of activated Notch1 and Pin1 overexpression (Rustighi et al., 2009).

Pin1 has also been linked to metastasis, aggressiveness and poor prognosis in triple negative breast cancer through cooperation with mutant-p53, following oncogenic stress. In particular it has been described that Pin1 binds to a phosphorylated mutant-p53 in breast cancer cell lines where it co-regulates a transcriptional program, that promotes migration and metastasis formation in vivo and correlates with poor prognosis in breast cancer patients and. Ablation of Pin1 lowers the oncogenic gain of function of mutant-p53 with reduced formation of breast cancer metastases into the lung, suggesting interesting therapeutic opportunities (Girardini et al., 2011).

Another important Pin1 target involved in the metastatic process is the focal adhesion-associated non-receptor protein-tyrosine kinase (FAK) involved in cellular adhesion and cell migration. FAK becomes phosphorylated following oncogenic stress by a Ras-ERK axis on Ser910. This PTM recruits Pin1 on the FAK protein causing an association with the protein-Tyr phosphatase (PTP) (PTP-PEST) that in turn dephosphorylates FAK on Th397 inhibiting its activity. The result is the promotion of a migratory and invasive phenotype (Zheng et al., 2009).

Besides all the evidence that Pin1 is required for full activation of oncogenic pathways, it’s conceivable that its effects go even beyond. Phosphorylation plays a fundamental role in regulating many intracellular processes such as growth, proliferation, cell division, apoptosis and differentiation. It is clear that perturbations of the phosphorylation status in a cell are likely to drive many of the hallmarks of cancer. Thereby, many cancers display deregulation or mutational events in kinases and phosphatases, and many of the genes encoding for these proteins are oncogenes or tumour suppressors. Accordingly, phosphorylation sites mutated by single nucleotide variants are found in almost 90% of tumours and, moreover, 29% of these mutations directly abolish phosphorylation or modify kinases’ target sites (Reimand et al., 2013).

It is evident how Pin1 is strongly involved in this scenario, as a consequence of its way of action. It is unsurprising that modulation of Pin1 activity and levels could be comparable to deregulation or mutation in kinases and phosphatases, strengthening the idea that Pin1 is an oncogene. Accordingly, transgenic overexpression of Pin1 in mouse mammary glands leads to mammary hyperplasia and malignant mammary tumours (Suizu et al., 2006).
3.3. The Notch signalling pathway

The Notch pathway is a conserved signalling route indispensable for the development of multicellular organisms, based on interaction with ligands expressed on neighbouring cells that are required for its proper activation. Signals that activate this pathway boost initiation and amplification of molecular differences that promote the cell fate commitment (Artavanis-Tsakonas et al., 1999). Activation of the pathway promotes the release of the nuclear activated form of Notch that translocates into the nucleus and transactivates several target genes. This pathway is involved in the regulation of many cellular processes such as proliferation, cell death, metabolism, angiogenesis, stemness and cell fate specification during development and renewal of adult tissues. As a consequence, alteration of this pathway is associated with pathological situation, such as developmental defects, cardiovascular disease and cancer (Bray, 2006; Ranganathan et al., 2011).

3.3.1. Notch receptors and ligands

In 1917, Thomas Hunt Morgan discovered a particular mutant of Drosophila with notches at the end of their wings. This trait was attributed to a partial loss of function of what have subsequently been described as the Notch gene that transcribes for a type I trans-membrane receptor. The human Notch homologue gene is located in the 9q34.3 chromosome and was cloned in the 1985/6 by two groups (Kidd et al., 1986; Wharton et al., 1985). In Drosophila there are only one single Notch protein and two ligands (Delta and Serrate), by contrast mammals display four Notch proteins Notch 1-4 and five ligands (Delta-like-1,-3 and -4, and Jagged-1,-2) (Radtke and Raj, 2003) (Figure 6). Notch receptors are produced as monomeric precursors that are subsequently processed by Furin protease in the trans-Golgi (called S1 cleavage) and exposed on the cell surface as heterodimers (Blaumueller et al., 1997). The heterodimer consists of a non-covalent association between the extra-cellular domain (NECD) and the intracellular portion that goes across the membrane and protrudes in the extracellular space (NTCIM). The extra-cellular domain contains both 36 tandemly repeated copies of an epidermal growth factor-like motif, called EGF-like, required for the interaction with the ligands, a negative regulatory domain (NRR) composed by cysteins that prevents the inappropriate activation of the receptor in absence of its ligands and a heterodimerization domain (HD) that interacts with the extracellular part of NTMIC. The Notch intra-cellular domain, called NICD is released from the membrane and constitutes is the activated part of the cleaved heterodimer that mediates the majority of the
biological processes in which the Notch pathway is involved. NICD contains a RAM domain, necessary for interaction with transcriptional factors, seven ankyrin repeats, two nuclear localization signals, a trans-activation domain (TAD) and a PEST domain, enriched in proline, glutamate, serine and threonine, that regulates the stability of the protein (Andersson et al., 2011; Kovall and Blacklow, 2010).

Figure 6. Structure of Notch proteins and their ligands
a) Wing blade of a wild-type Drosophila melanogaster (left), and of a mutant with a partial loss of the Notch gene (right). The notches, which are absent in the wild type, but clearly visible at the border of the wing blade, have given the name to the implicated gene. b) Structure of Notch proteins and their ligands. Drosophila has one Notch receptor (dNotch) and vertebrates have four (Notch1–4), which are presented on the cell surface as heterodimers. The ectodomain of Notch receptors contains epidermal-growth-factor (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, also known as CDC10 repeats), two nuclear-localization signals (NLSs), followed by the transactivation domain (TAD) and a PEST sequence. c) 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). The ligands harbour an amino-terminal structure called DSL (Delta, Serrate and LAG-2), which is common to all family members, followed by EGF-like repeats. Serrate, Jagged1 and Jagged2 harbour a cysteine-rich domain (CR) following the EGF-like repeats (Radtke and Raj, 2003).
3.3.2. Notch pathway activation

Notch signalling is initiated by a receptor-ligand interaction between two neighbouring cells. Notably it has been recently demonstrated that also soluble forms of ligands forms exist, that can activate Notch signalling in non neighbouring cells, suggesting other possible levels of cellular communications (Lu et al., 2013a). The interaction between the ligand and the receptor is an essential step for the Notch signalling activation, since it induces a conformational change in the receptor that allows the cleavage and the subsequent release of a cytoplasmic activated form that translocates in the nucleus and activates its target genes. Following interaction with the ligand, the receptor is cleaved by the disintegrin and metalloprotease ADAM10 or ADAM17 that constitutes the S2 cleavage (Bray, 2006; Brou et al., 2000; Fortini, 2002). The subsequent cleavage within the trans-membrane domain of Notch is mediated by the multisubunit protease called gamma secretase that contains presenilin, nicastrin, PEN2 and APH1. This cleavage (S3 cleavage) results in the release of the Notch intra-cellular domain called NICD (Figure 3).

In the canonical pathway NICD translocates in the nucleus and cooperates with the DNA-binding protein CSL (CBF1, Su(H) and LAG-1) and its co-activator Mastermind to promote transcription of its target genes such as HES-1, HEY-L and cyclin D1 (Radtke and Raj, 2003). In the absence of NICD, CSL already bound to the promoters of Notch target genes, recruits co-repressors and histone deacetylases with a consequent inhibition of transcription (Kao et al., 1998). When NICD is activated and present in the nucleus it competes with the repressors for CSL binding, displaces them and converts CSL from transcriptional repressor to an activator (Bray, 2006; Fryer et al., 2002).

While Notch mediates a number of biological processes through the canonical pathway, ligand- or transcription-independent functions are emerging that may have and unprecedented impact in development and disease (Andersen et al., 2012).

3.3.3 The Notch pathway regulation

Although the intracellular transduction of the Notch signal is remarkably simple, with no secondary messengers, there is an enormous complexity in its regulation to keep in check the amount and timing of its activation. Such regulation is essential since Notch controls both the transcription and the function of genes and proteins involved in many cellular processes. Indeed alteration of these checkpoints is associated with developmental defects and diseases (Grabher et al., 2006; Ranganathan et al., 2011).
Notch signalling is modulated in many ways during maturation and upon activation such as through post-translational modifications of both receptors and ligands, endocytosis, ubiquitination and receptor maturation (Le Borgne et al., 2005) (Figure 7). Numb, a cell fate determinant is a known inhibitor of the Notch pathway. Numb is an endocytic adaptor protein that acts as a suppressor of Notch signalling (Knoblich et al., 1995; Uemura et al., 1989). Mechanistically, Numb has been shown to recruit the E3 ubiquitin ligase Itch, the mammalian homolog of Drosophila Suppressor of deltex (Su(Dx)), to promote the degradation of the Notch receptor and to regulate post-endocytic sorting events for Notch (McGill et al., 2009; McGill and McGlade, 2003). Numb differentially affects various Notch receptors, since it negatively regulates Notch1 and Notch2 receptors, but not Notch3, during myogenic differentiation (Beres et al., 2011). In human, six alternatively spliced NUMB isoforms have been characterized to date. The two most recently identified isoforms, NUMB5 and NUMB6, are less potent antagonists of Notch signalling (Karaczyn et al., 2010), although it remains to be established if the difference in biological effects among the different isoforms is strictly due to different effects on Notch, as Numb also interacts with other signalling proteins, such as p53, Gli1, and Hedgehog pathway effectors (Colaluca et al., 2008; Di Marcotullio et al., 2006). There is emerging evidence that the relationship between Numb and Notch is not unidirectional. Indeed it has been shown that Notch also can influence Numb since high levels of Notch reduce Numb protein levels (Chapman et al., 2006).

Another important inhibitor of the Notch pathway is the E3-ubiquitin ligase and tumour suppressor Fbwx7α (Gupta-Rossi et al., 2001; Oberg et al., 2001). As mentioned before, activation of the Notch signalling promotes the cleavage and the translocation into the nucleus of the NICD that recruits Mastermind for a proper activation of its target genes. Notably, the trans-activation complex recruited by NICD on one hand promotes Notch target genes’ transcription, on the other it promotes its own turnover. Indeed Mastermind recruits the CDK8/CycC that promotes NICD phosphorylation thus triggering recognition by Fbwx7α and the subsequent proteasomal degradation (Figure 7) (Fryer et al., 2002; Gupta-Rossi et al., 2001; Oberg et al., 2001).
Introduction

Figure 7. Notch pathway activation and regulation

a-b) Notch is produced as a monomer and it is glycosylated by O-fucosyltransferase1 (OFUT1) at the endoplasmic reticulum (ER) thus into the Golgi for the subsequent cleavage mediated by Furin protease. c) This results in a heterodimeric receptor with non-covalently associated domains that is transported to the plasma membrane. d) Ligand binding initiates two successive proteolytic cleavages (S2 and S3). The first, mediated by an ADAM proteinase (a disintegrin and metalloproteinase domain), occurs in the extracellular domain. S2 cleavage allows access of the gamma-secretase complex, which is responsible for the second proteolytic cleavage (S3), which occurs within the transmembrane domain and liberates the intracellular domain (ICN). e) The ICN translocates to the nucleus, where it interacts with the CSL transcription factor. Numb suppresses Notch signalling, possibly by preventing nuclear localization and targeting the ICN for degradation through the E3 ligase Itch. g) The stability of nuclear ICN is regulated through its PEST domain (enriched in proline, glutamate, serine and threonine). Binding of MAML to p300 and cyclin-dependent kinase 8 (CDK8) promotes hyper-phosphorylation of the ICN PEST domain to facilitate ubiquitylation, through recruitment of Fbwx7α E3 ligases, which target the ICN to the proteosome (Grabher et al., 2006).

Fbwx7α is an important E3 ubiquitin-ligase that controls, besides N1-ICD, the turn-over of many other important transcription factors, by recognition of the phosphorylated CPD motif. The Notch1 CPD is located in the PEST domain, it is constituted by FLTPSPESP residues and it is centred on threonine 2512. Indeed substitution of this threonine with an alanine disrupts the
interaction of N1-ICD with Fbwx7a (Thompson et al., 2007). Since NICD is the final mediator of activated Notch signalling, the regulation of its amount is of utmost importance as emerged by the evidence that mouse models knock-out for Notch or Fbwx7a are similar since they die in utero for the same cardiovascular disease (Swiatek et al., 1994; Tetzlaff et al., 2004; Tsunematsu et al., 2004). In addition, in about 50% of human T-cell acute leukaemias and lymphomas (T-ALL) (Weng et al., 2004), some of which disrupt binding to Fbwx7a. Notably, in some cases, T-ALLs harbouring NOTCH1 activating mutations and treated with the gamma-secretase inhibitor (GSI), were resistant to the pharmacological treatment because of mutation on Fbwx7a that impaired its ability to bind N1-ICD (O’Neil et al., 2007; Thompson et al., 2007).

3.3.4. Biological functions of the Notch pathway

Notch biological functions are fundamental during both embryogenesis and regulation of adult tissues in many organisms, through transcription of its target genes and cooperation with other signalling pathways (Ranganathan et al., 2011).

While the genetic ablation of Notch1 or Notch2 induces premature embryonic death, the absence of Notch3 or Notch4 does not induce this phenotype suggesting a non-redundant role for Notch1/2 (Conlon et al., 1995; Krebs et al., 2003; Swiatek et al., 1994). During embryogenesis the cooperation with Wnt, SHH and TGFβ signals is required for promotion of an epithelial-to-mesenchymal transition (EMT) resulting in upregulation of the Snail/SLUG transcriptional factor and down-regulation of E-cadherin (Grego-Bessa et al., 2004; Timmerman et al., 2004).

Notch is one of the major cell-fate determinants in particular during neurogenesis. Indeed cells that over-express the Notch ligands will be committed to neuronal differentiation, while cells that express the receptors will acquire epidermal traits (Artavanis-Tsakonas et al., 1999). Another compartment in which Notch drives differentiation is the hematopoietic system, in particular in lymphocytes’ precursors, in which it promotes activation of PDK1 and mTOR with a consequent differentiation and proliferation as T cells (Kelly et al., 2007). By contrast lymphocytic precursors in which the Notch pathway is turned off, will acquire B cell features (Osborne and Minter, 2007). In addition Notch has a role in the cardiovascular system, regulating vasculo- and artherio-genesis by cooperation with VEGF and HIF1α, respectively (Holderfield and Hughes, 2008; Pear and Simon, 2005).

The ability of Notch signalling to drive cellular fate persists also during adulthood (Androutsellis-Theotokis et al., 2006; Molofsky et al., 2004). Indeed the stem cell compartment of many adult tissues, such as the hematopoietic, breast, muscular and intestinal one requires
Notch activation for a proper regulation and of cell-specification. This process is a consequence of the interplay between Notch with other signalling pathways and an inappropriate deregulation of this well controlled mechanism could cause an unbalance in the stem cell compartments, as occurs in cancer (Ranganathan et al., 2011).

The Notch regulators can have both positive and negative effects on the Notch pathway activation. In this context well known interactors of Notch are the p53 family members (Dotto, 2009). Notch and p53 counteract each other at multiple levels regulating many cellular processes. In addition, another member of the p53 family, p63 has been demonstrated to curb the Notch signalling both in mammary stem cells and in epithelial cells where it blocks the Notch-induced EMT (Yalcin-Ozuysal et al., 2010). On the contrary, enforced Notch activation impairs p63 cellular functions with a consequent transformation process (Mazzone et al., 2010).

The result is an involvement of the Notch signalling in the regulation of stemness, proliferation, apoptosis, differentiation, EMT, angiogenesis, tissue homeostasis, which, when altered, are associated with tumourigenesis pointing out Notch as an oncogene (Miele et al., 2006; Ranganathan et al., 2011).

### 3.3.5. Notch and cancer

The role of Notch in cancer is strongly dependent on the cellular context. Indeed, in the skin, Notch is an important tumour suppressor, mainly by blocking p63 functions (Dotto, 2008). Notably in many other tissues, Notch emerges as a master oncogene. The first evidence of a role of Notch in cancer, has been done in 1987, in human T-cell acute lymphoblastic leukemia (T-ALL) in which there was a translocation involving the Notch locus on chromosome 7 and the T-cell-receptor-beta on chromosome 9 (Reynolds et al., 1987). The consequence of this translocation is the aberrant transcription of a truncated Notch1 protein that corresponds to the nuclear active form, N1-ICD. Very frequent point mutations have subsequently been found mainly in T-ALL, in particular on the heterodimerization and PEST domains, causing ligand-independent activation and protein stabilization, respectively (O'Neil et al., 2007). In T-ALL, Notch1 regulates two important proteins involved in tumourigenesis such as c-Myc and PTEN, in an opposite fashion: on one hand Notch1 directly activates c-Myc transcription and on the other PTEN gene expression is dampened by the transcriptional repressor Hes1, one of the most important mediators of the Notch pathway (Palomero et al., 2008; Palomero et al., 2006; Weng et al., 2006). In this way, Notch controls multiple cellular processes such as differentiation, transformation, metabolism, apoptosis, boosting tumourigenesis. After the discovery of its
involvement in T-ALL, Notch signalling has been implicated in various solid tumours, including breast cancer, medulloblastoma, colorectal cancer, pancreatic tumour and non–small cell lung carcinoma (NSCLC) (Miele et al., 2006).

With exception of NSCLC, in all these solid tumours deregulated Notch activity results mainly from over-activation in absence of mutations. In particular it has been demonstrated that up-regulation of the Notch pathway in 30% of NSCLS is caused by loss of Numb expression, while in 10% of cases is caused by gain-of-function mutations of the NOTCH-1 gene (Westhoff et al., 2009) suggesting an important role of the Notch pathway in this tumour type.

The oncogenic potential of Notch activation in solid tumours was first observed in mouse mammary tumor virus (MMTV)–driven breast cancer. The integration of MMTV in specific loci of the host genome results in dysregulated expression of adjacent genes and subsequent outgrowth of tumorigenic clones (Dievart et al., 1999). Characterisation of one of these loci revealed expression of a truncated constitutively active form of Notch4 (Gallahan and Callahan, 1987; Gallahan et al., 1996). In mouse models, Notch activation can clearly drive mammary tumours, and in human breast cancers increased expression of Notch and Jagged1 correlates with poor prognosis (Reedijk et al., 2005). Loss of Numb expression causes over-activation of Notch signalling and tumourigenesis also in a large part of breast cancers (Pece et al., 2004). In breast tumours Notch has been demonstrated to functionally cooperate with several proteins for which a role in breast tumourigenesis has been well established such as ErbB2, Ras signalling, Pin1, cyclin D1 and c-Myc (Miele et al., 2006; Ranganathan et al., 2011).

The impact of Notch in breast tumours patients, derives from the evidence that Notch plays a major role in driving malignant phenotypes including EMT features, CSCs, metastases and chemoresistance, as assessed also by analyses of overall survival (Rizzo et al., 2008).

In normal breast stem cells the role of Notch1 is well established since it was shown to trigger luminal progenitors’ formation by several groups. Consistent with this function in normal mammary stem cells and progenitors, the Notch pathway controls also breast CSCs. It seems that while Notch1 is mainly expressed and required for the early progenitors, Notch4 in more expressed in the CSC pool (Bouras et al., 2008; Dontu et al., 2004; Harrison et al., 2010). Accordingly, genetic and pharmacological abrogation of the Notch pathway in breast cancer cells is effective in curbing the CSCs pool.

The ability of activated Notch receptors to promote also chemoresistance is not simply a consequence of an increased CSC/progenitor population. Indeed Notch has well-established roles in different mechanisms of chemoresistance as a consequence of the promotion of cell
survival factors such as BIRC5 (SURVIVIN), Bcl-2, and drug efflux pumps such as ABCG2 (Bhattacharya et al., 2007; Ranganathan et al., 2011). Thus abrogation of Notch signalling sensitizes both cancer cells and CSCs to chemotherapeutic agents (Espinoza et al., 2013).

Notch has been clearly linked to EMT features and metastases in breast cancers with *in vitro* and *in vivo* experiments. It has been demonstrated that Notch induces Slug gene transcription, with a consequent E-cadherin decrement, that results in an increased migratory and invasive ability (Leong et al., 2007). Recently it has also been demonstrated that activation of the Notch pathway affects the breast cancer secretome and promotes the metastases formation in the brain. Notably pharmacological ablation of Notch activation prevents this effect (Xing et al., 2013).

It is evident that Notch is a candidate druggable pathway in cancer since it is enzymatically controlled at many levels and it is linked to aggressiveness phenotypes such as chemoresistance, tumourigenesis and CSCs maintenance and involvement of this pathway in breast CSCs’ biology warrants further investigation.
A major issue of breast cancer management is the failure of therapeutic regimens and disease relapse. These features are widely suggested to be promoted by cancer stem cells’ subpopulations. It is conceivable that genetic or pharmacological ablation of molecular pathways required for breast CSCs induction and maintenance, will exhaust this cell population with a consequent gain in sensitivity to chemotherapy. Moreover, the abrogation of this molecular signalling will prevent reprogramming of terminal differentiated cells to a more stem-like phenotype, blocking the selection of a chemoresistant and aggressive cancer cell population.

The aim of my PhD work was to discover factors that might underlie all these aspects in breast CSCs. A putative candidate for such a role is the prolyl-isomerase Pin1 since i) it is indispensable for full activation of oncogenic signalling pathways ii) its protein levels are up-regulated in high-grade breast cancers which exhibit an elevated CSCs content and iii) Pin1 is a stemness factor in embryonic stem cells and some adult tissues.

The first part of this study will focus on the role of Pin1 in the maintenance of normal mouse mammary stem cells ex vivo and in vivo. As normal stem cells and CSCs display considerable similarities in their molecular regulators, the subsequent part will be aimed at dissecting how Pin1 controls and orchestrates breast CSCs number, maintenance and aggressive traits in vivo such as tumourigenesis and chemoresistance.

In the second part we will investigate which is the functional mediator of the Pin1 promoted CSCs maintenance. In particular this study will focus the attention on how Pin1 potentiates the Notch pathway by safeguarding Notch1 and Notch4 nuclear active forms from their E3-ubiquitin-ligase Fbxw7α. Thereafter we will investigate the impact of the Pin1-Notch1/4 axis in tumourigenesis, CSC maintenance with in vitro and in vivo analyses in mouse models and in human breast cancer samples.

These studies, collectively, might be relevant for providing the rational for a therapeutic strategy based on Pin1 inhibition to hit CSCs, restore chemo-sensitivity and inhibit metastatic spread.
Results

5. RESULTS

5.1 The prolyl-isomerase Pin1 is required for the self-renewal of normal mammary stem cells

Pin1 knock-out mice display a variety of developmental defects. In particular the mammary epithelium of these mice fails to undergo the dynamic changes required for its expansion during pregnancy (Liou et al., 2002). Since it has been demonstrated that some of these defects are a consequence of an impaired stem cell function (Atchison and Means, 2003), we hypothesized a possible function of Pin1 in the regulation the mammary stem cell compartment. For this aim we decided to evaluate the mammary stem cell number of wild-type (Pin1\(^{+/+}\)) and Pin1 knock-out (Pin1\(^{-/-}\)) mice. Mammary tissues from 8-10 weeks old virgin female mice were dissociated, prepared as single cell suspensions of purified, lineage-depleted epithelial cells (Stingl et al., 2006) and grown in suspension cultures to form secondary mammospheres (M2) (Dontu et al., 2003). We detected a significant reduction in the spheres’ formation in absence of Pin1, since cells obtained from Pin1\(^{+/+}\) mice formed an average of 22.9 (±1.44) M2 mammospheres per 100,000 seeded cells, while those from Pin1\(^{-/-}\) cells gave rise to only 13.53 (±2.49), indicating a 40% reduction of M2 formation (Figure 8A).

![Figure 8](image-url)

**Figure 8. Ablation of Pin1 reduced the mouse mammary epithelial stem cells number**

A) Left panel: Number of secondary mammospheres (M2) generated from primary mammary epithelial cells of indicated mice. Right panel: representative M2 microscope images with 200 µm scale bar. B) Serial replating of mammospheres (M1-M5) generated from Pin1\(^{+/+}\) mice treated with DMSO or PiB (1.5 µM). Means, standard deviations and p-values (t-test, n=4) are indicated in the histogram.

It is known that the microenvironment is important to control the stem cell compartment’s homeostasis (Valent et al., 2012). To determine if the reduced stem cell number in Pin1\(^{-/-}\) mice was due to an effect of Pin1 specifically in the mammary stem cells or in the microenvironment, we performed with Pin1\(^{+/+}\) mammary epithelial cells an *ex vivo* mammosphere experiment by blocking *in vitro* the enzymatic activity of Pin1. We serially replated cells from M2 to tertiary
Results

and quaternary mammospheres (M3, M4) in presence or absence of the Pin1 inhibitor PiB (Uchida et al., 2003) (Figure 8B).

As expected in these conditions, we observed a progressive decrease in mammosphere formation efficiency (MFE) at each passage, due to exhaustion of adult stem cells (Cicalese et al., 2009). Notably, this effect was exacerbated by almost 50% at the M4 stadium upon addition of the Pin1 small molecule inhibitor PiB. These data suggest that inhibition of Pin1 can directly inhibit the stem cell self-renewal of mammary stem cells.

This evidence indicates a role for Pin1 in determining self-renewal and replicative potential of mammary stem cells thus implying alterations of the mammary stem cell compartment in Pin1−/− mice. To better address this point we analyzed the mammary epithelial stem cells hierarchy in Pin1+/+ and Pin1−/− mice by detection of specific immune phenotype markers, CD24 and CD49f, that identify both stem/bipotent progenitors (CD24med/CD49fhigh or mammary repopulating units, MRU) and luminal progenitors (CD24high/CD49flow or mammary colony forming cells, Ma-CFCs) (Stingl et al., 2006). In line with our hypothesis, the MRU and Ma-CFC cell populations from Pin1−/− mammary glands were present at lower proportion as compared to Pin1+/+ mice (Figure 9A).

![Figure 9](image_url)

**Figure 9. Bipotent stem cell and luminal progenitor number is decreased in Pin1−/− mammary tissue**

Bipotent stem cell and luminal progenitor number is decreased in Pin1−/− mammary tissue. Left panel: representative FACS analyses of mammary epithelial cells from indicated mice. CD24/CD49f plots and gateings for MRU and Ma-CFC populations are indicated. Right panel: histogram of mean counts of MRU and MA-CFC populations from Pin1−/− normalized to Pin1+/+ mice. Means, standard deviations and p-values (t-test, n=3) are indicated.

To demonstrate that the analyzed populations were indeed MRUs and Ma-CFCs, we sorted these two populations by FACS and analyzed the expression of specific myoepithelial (SMA, CK14) or luminal (CK-18, CK-19) markers, that are mainly expressed in the stem cell and progenitor populations, respectively (Stingl et al., 2006). As shown in Figure 10, Ma-CFC and MRU cells
sorted from the mammary epithelial cell population expressed higher levels of CK-18, CK-19 or SMA and CK-14, respectively, when compared to unsorted cell population (TOTAL).

**Figure 10.** Molecular characterisation of the sorted MRU and Ma-CFC mammary epithelial cells.
Control qRT-PCR for myoepithelial (SMA, CK14) and luminal (CK18, CK19) markers of mRNA extracted from MRU and Ma-CFC (Stingl et al., 2006) sorted populations relative to the total population, shows accuracy of the FACS sorting procedure.

Many stem cells factors, such as Slug and Pin1 itself, are more expressed and active in the stem cell compartments (Guo et al., 2012) (Moretto-Zita et al., 2010; Nishi et al., 2011). For this reason we compared Pin1 mRNA and protein levels in the stem cells’ compartment with their differentiated counterpart. Interestingly, we found almost three times higher Pin1 levels in the MRU cell population as compared to the total of mammary epithelial cells (Figure 11).
This evidence confirmed our hypothesis and suggests a prominent role of Pin1 in sustaining the mammary stem cell compartment *in vivo*.

**Figure 11.** Pin1 mRNA and protein levels are upregulated in the mammary stem cell compartment.
Left panel: qRT-PCR of endogenous Pin1 mRNA in MRU sorted populations relative to total population. Means, standard deviations and p-values (t-test, n=3) are indicated. Right panel: Western blot analysis of the same cell populations as in the left panel. Fold change in Pin1 protein levels determined by Image J software with respect to actin levels is indicated by a number, Molecular weights in KDa (Mr (K)) are shown on the right.
5.2 Pin1 inhibition impairs mouse and human cancer stem cells traits

Molecular pathways required for normal mammary stem cells’ maintenance are often indispensable also for CSCs (Valent et al., 2012; Visvader and Lindeman, 2012). Thus many of these pathways are up-regulated also in the CSCs. Since we demonstrated that Pin1 is indispensable for the maintenance of normal stem cells, we investigated whether Pin1 could have a role also in the regulation of breast CSCs.

For this aim we performed mammosphere formation with NOP6 mouse mammary cancer cells, harbouring a Her2/Neu amplification, for serial passages, in presence or absence of the Pin1 inhibitor PiB. Interestingly Pin1 inhibition strongly impaired the formation of mammospheres at each passage, indicating that Pin1 is required for the self-renewal of mouse mammary cancer stem cells (Figure 12).

![Figure 12. Pin1 inhibition curbs mouse mammary CSCs maintenance.](image)

Serial replating of mammospheres from M1 to M4 generated from NOP6 cells treated with DMSO or PiB (1,5µM). Mammosphere formation efficiency (%MFE) was calculated as percentage of mammospheres divided by the number of plated cells.

To verify if Pin1 is required also by human breast cancer stem cells, we infected the triple negative breast cancer cell line MDA-MB-231 with a doxycycline-inducible knockdown lentiviral construct for Pin1 (pLKO-TetO-shPin1) and tested their ability to generate mammospheres. As shown in Figure 9A, while MDA-MB-231 MFE remained constant throughout serial replating to M4, the formation of mammospheres from Pin1 silenced cells (+DOX) progressively decreased at each passage. Accordingly the content of putative stem cells was lower following Pin1 silencing or inhibition, as confirmed by the Aldefluor assay, that valuates the activity of Aldehyde dehydrogenase 1 (Aldh), a marker for breast CSCs (Ginestier et al., 2007) (Figure 13A). Accordingly, Pin1 pharmacological inhibition curbed not only MDA-MB-231 mammospheres formation but also the MFE of other breast cancer cell lines such as and BT-549 and SUM-159 (Figure 13B). These observations could be extended also to two patient-
derived aggressive breast cancers, whose CSCs were reduced by Pin1 inhibition (Figure 13C). All these data demonstrate that genetic or pharmacological ablation of Pin1 impairs breast CSC self-renewal and replicative potential in a broad spectrum of breast cancer cells.

Figure 13. Pin1 inhibition curbs human mammary CSCs traits.

A) Left panel: MFE of MDA-MB-231-pLKO-shPin1 control cells (Ctrl) compared to shPin1 induced cells (DOX) upon serial passages. Right panel: Quantification of Aldh-positive and Aldh-negative cells from control- and shPin1 induced M4, as assessed by FACS. B) Left: Serial replating of mammospheres (M1-M4) generated from MDA-MB-231 human breast cancer cells and treated with DMSO or PiB (1.5 µM and 3 µM). Mammosphere formation efficiency (%MFE) was calculated as percentage of mammospheres divided by the number of plated cells. Means, standard deviations and $p$-values are indicated (t-test, n=3, M4). Right: Percentage of M2FE of BT-549 and SUM-159 breast cancer cell lines treated with vehicle (DMSO) or PiB. Means, standard deviations and $p$-values are indicated (t-test, n=3). C) Secondary and tertiary mammosphere formation efficiency of a grade 2 (G2) and grade 3 (G3) patient-derived breast cancer cells treated with vehicle (DMSO) or 3 µM PiB.
Conversely, overexpression of Pin1 in MDA-MB-231 cells increased M2 formation by almost two fold and produced an increase of Aldh-positive cells, when compared to empty vector harbouring cells (Figure 14).

![Figure 14. Pin1 over-expression boosts human mammary CSCs traits.](image)

Left: Secondary mammosphere formation efficiency of MDA-MB-231 cells transduced with empty or HA-Pin1 expressing vectors. Representative microphotographs and scale bars are shown. Right: Histogram showing percentage of Aldefluor median fluorescence intensity difference (MFI).

To unveil the molecular mechanism underlying the role of Pin1 in promotion of CSCs, we evaluated the expression of several genes acting within pathways that govern the stemness phenotypes of breast CSCs (Leong et al, 2007; Yu et al, 2007, 2011; Polyak & Weinberg, 2009; Cordenonsi et al, 2011; Visvader & Lindeman, 2012) in Pin1-silenced quaternary mammospheres. Interestingly, as shown in Figure 11, no single pathway emerged alone, as the expression of all tested factors (Hes1, HeyL, Birc5, CTGF, Slug, ABCG2, Ptch, Bmi-1, HMGA2 and Klf4) decreased by Pin1 knockdown thus suggesting a common down-regulation of stemness signalling pathways following Pin1 depletion.

Epithelial-mesenchymal plasticity in breast carcinoma has recently been linked to acquisition of stem cell traits by tumor cells (Mani et al, 2008). We therefore also analysed the impact of Pin1 modulation on this process by analyzing markers of epithelial-to-mesenchymal transition (EMT). Of note, Pin1 downmodulation caused enhanced mRNA expression of the epithelial marker E-cadherin (CDH1) while that of mesenchymal markers Vimentin and Fibronectin (VIM1, FN) was reduced (Fig 2C, left panel), in parallel with a strong recovery of E-cadherin and decay of Slug and Vimentin at the protein level (Figure 15).
All together these results indicate that Pin1 is indispensable for breast CSCs maintenance and its depletion strongly impairs CSCs number and traits, through the modulation of a specific stemness and mesenchymal gene expression program.

5.3 Pin1 promotes breast CSCs maintenance mostly through up-regulation of the Notch signalling

As shown above, breast CSCs depleted of Pin1 display down-modulation of many signalling pathways. The majority of these genes are directly -or indirectly- controlled by the Notch signalling pathway (Lee et al., 2008; Li et al., 2012; Ranganathan et al., 2011). The role of the Notch signalling in breast CSCs and EMT induction is well established, hence we tested if Notch is the key mediator of Pin1-mediated stemness. In particular we analyzed the protein levels of both Notch1 and Notch4 since their role in breast CSC is well described (Dontu et al., 2004; Harrison et al., 2010; Ranganathan et al., 2011). Intriguingly, the levels of their active forms, N1-ICD and N4-ICD, were strongly deregulated in Pin1 depleted quaternary (M4) mammospheres (Figure 16A). In a previous work we established that that Pin1 potentiates the cleavage of the Notch1 receptor, boosting its nuclear levels (Rustighi et al., 2009). However, the very strong down-regulation of the Notch nuclear active forms observed in this case could not be totally ascribed to a reduced cleavage of the receptor. Since N-ICD levels are finely tuned by
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proteasomal degradation, we speculated another role for Pin1 in boosting Notch signalling, probably through protein stabilization.

**Figure 16. N1- and N4-ICD protein levels are decreased in Pin1-silenced mammospheres**

Left panel: Western Blot analysis of N1- and N4-ICD protein from MDA-MB-231-pLKO-shPin1 M4 control cells (Ctrl) and shPin1 induced cells (DOX). Molecular weights (Mr) are indicated in kDa. Right panel: histogram representing the percentage of band intensity with respect to actin levels.

To understand if the effect of Pin1 on breast CSC required high N1-ICD levels, we tested the ability of N1-ICD or of a constitutively stable N1-ICD mutant (dPEST) to boost M2 formation following Pin1 knockdown. This mutant lacks the cdc4-phosphodegron (CPD) constituting the consensus for the E3 ubiquitin-ligase Fbwx7α. As expected, Pin1 silencing (+DOX) reduced the M2FE of MDA-MB-231 pLKO (Figure 16B). Moreover ectopic expression of N1-ICD in control cells did not affect the formation of mammospheres, since in these cells the endogenous Notch pathway is already strongly activated (Harrison et al, 2010). Interestingly, N1-ICD overexpression was not able to rescue M2FE in Pin1 silenced cells. This evidence suggests that in this context the role of Pin1 in boosting the Notch cleavage seems to be dispensable, since the ectopically expressed N1-ICD is already cleaved and activated. Instead, overexpression of N1-ICD-dPEST was able to rescue M2FE. When we analyzed the protein levels of Notch nuclear active forms we found that protein levels of over-expressed N1-ICD, but not those of N1-ICD-
dPEST, were strongly decreased in Pin1 depleted mammospheres.

These data suggest that Pin1 critically supports breast CSC self-renewal by sustaining high intracellular levels of N1-ICD. To better address this point, we analysed the endogenous levels of both Pin1 and N1-ICD in breast CSCs. Indeed we sorted the Aldh-positive cells from MDA-MB-231 mammospheres or collected patient derived mammospheres and analysed their protein content. As shown in Figure 13, Pin1 and N1-ICD protein levels were always higher in the stem cell compartment compared to their differentiated counterpart. Moreover we found that also in vivo, in breast cancer tissues, the expression of Pin1 co-localized with Aldh-positive cells (Figure 17, right panel).

**Figure 17. Pin1 and N1-ICD are up-regulated in the CSC compartment.**
Left panel: Comparative Western blot analyses of Aldh-positive (stem) versus Aldh-negative cells (nonstem) sorted from MDA-MB-231 M2 (left panel) and patient-derived breast cancer secondary M2 mammospheres (stem) versus cells cultured in adherence (2D) (right panel). Relative fold changes in Pin1 or N1-ICD protein levels were determined by Image J software with respect to actin levels is indicated by a number.

Right panel: Representative bright field microphotographs at 100X magnification of immunohistochemical analyses of serial sections of a breast cancer with the indicated antibodies are shown. Part of the figure indicated with a square is shown by an inset at 400X magnification. Scale bar is indicated.

Over-expression of Pin1 is detected in breast cancers and it is known that ectopic over-expression of Pin1 is able to disrupt cellular polarity of breast epithelial cells (Ryo et al., 2002), but it was not investigated yet if Pin1 is able to promote both an EMT program and formation of secondary mammospheres in normal cells and if the Notch signalling is required for this aspect.

To answer to these questions, we ectopically over-expressed Pin1 in epithelial non transformed MCF10A cells and observed that Pin1 promoted an enrichment of the stem cell population, as assessed by the specific CD44high/CD24low immunophenotype (Al-Hajj et al, 2003). Moreover Pin1 overexpression triggered a huge formation of mammospheres and a concomitant EMT as
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demonstrated by down- and up-regulation of the two markers E-cadherin and Slug, respectively (Figure 18). Crucially, the levels of cleaved Notch1 and Notch4, normally undetectable in these cells, were up-regulated by Pin1 over-expression and were essential for mammosphere formation, since block of Notch cleavage by a gamma-secretase inhibitor (GSI) was sufficient to blunt this process and to reduce the EMT markers as depicted in Figure 18A.

Figure 18. Pin1 over-expression promotes an EMT-stem cells phenotype mostly though the Notch pathway. A) Upper panel: %M2FE of MCF10A breast epithelial cells transduced with empty (pLPC) or HA-Pin1 overexpressing vectors (pLPC-HA-Pin1) and treated with DMSO (-) or 10 mM GSI. Means, standard deviations and P-values (t-test, n = 3) are indicated. Middle panel: Representative microphotographs of M2 are shown, scale bar of 200 μm is indicated. Lower panel: Western Blot of cell lysates from corresponding MCF10A clones. White and black arrows indicate over-expressed and endogenous Pin1, respectively. Comparative Western blot analyses of Aldh-positive (stem) versus Aldh-negative cells (nonstem) sorted from MDA-MB-231 M2 (left panel) and patient-derived breast cancer secondary M2 mammospheres (stem) versus cells cultured in adherence (2D) (right panel). Relative fold changes in Pin1 or N1-ICD protein levels were determined by Image J software with respect to actin levels is indicated by a number. B) CD44/CD24 FACS analyses of MCF10A cells transduced with pLPC or pLPC-HA-Pin1. Percentage of the different CD44/CD24 populations is indicated on the right.

All together our results demonstrate that Pin1 is a bona fide stem cell factor by promoting EMT, maintaining a mesenchymal/stem cell fate and self-renewal of CSCs mainly through regulation of the Notch pathway.
5.4 Suppression of Pin1 sensitizes breast CSCs to chemotherapy in vitro and in vivo

There is increasing evidence that, while chemotherapy reduces the tumour mass by killing the sensitive cancer cells, it selects a subpopulation of cancer cells that are intrinsically chemoresistant or alternatively, it induces cells to acquire resistance to chemotherapy by genetic or epigenetic alterations (Dean et al., 2005). In accordance, it has been demonstrated that chemotherapy selects and strongly increases the breast CSCs population (Yu et al., 2007). We also found that uninduced MDA-MB-231-pLKO-shPin1 cells treated with different chemotherapeutic agents display a slight increase in MFE (Figure 19). Instead, when we induced Pin1 silencing with doxycyclin (+DOX), this effect was completely lost, since Pin1 depletion does not only prevent accumulation of CSC following chemotherapy but more importantly it regains chemosensitivity.

![Figure 19. Pin1 depletion sensitizes CSCs to chemotherapeutic agents](image)

Pin1 knockdown synergizes with chemotherapy treatment to block breast CSCs’ self-renewal. Percentage of M2FE of control MDA-MB-231-pLKO-shPin1 cells (Ctrl) compared to shPin1-induced cells (DOX) treated with indicated drugs or PBS. Means and standard deviations are indicated, P-values are * = 0.001, **< 0.0003 (t-test, n = 3).

To address the in vivo impact of these findings, we performed a xenograft experiment in immunocompromised mice by injecting MDA-MB-231 infected with a control- or a Pin1 shRNA. We allowed the tumour to grow till tumour mass became visible, then mice where randomized in different groups and treated with paclitaxel or PBS. When we analyzed the size of the tumours, either the treatment with paclitaxel or the silencing of Pin1 similarly reduced the tumour mass, while the combination had a strong synergistic effect (Figure 20A). Analysis of the cancer stem cells’ content by evaluating the activity of the Aldh enzyme evidenced that, while paclitaxel treatment alone reduced the tumour mass but heavily increased the CSC content, the concomitant Pin1 depletion prevented this effect (Figure 20B). As a result, Pin1 ablation on one hand is able to reduce the tumour mass and the CSCs content, on the other hand it re-sensitize CSC to chemotherapy. At the protein level, Western Blot analysis unveiled mesenchymal-to-
Results

epithelial transition in Pin1 depleted tumours as emerged by the reduction in Slug and Vimentin and the concomitant increase in E-cadherin (Figure 20C). Accordingly, N1-ICD levels were down-regulated in Pin1 silenced tumours. Moreover, the increased cleavage of caspase-3, an apoptotic marker, was significantly increased in Pin1-silenced tumours treated with paclitaxel underlining a synergistic effect between paclitaxel treatment and Pin1 ablation in inducing apoptosis. It has been demonstrated by another group that Pin1 depletion curbed Mcl-1 protein levels and promoted apoptosis in breast cancer cell lines treated with paclitaxel (Ding et al., 2008). To verify the impact of Mcl-1 in our condition, we analyzed its protein levels in our xenografts. As expected Mcl-1 levels decreased in Pin1 depleted tumours, but also in the paclitaxel treated one, suggesting that Mcl-1 is not directly involved in the CSC accumulation due to paclitaxel treatment. More importantly Mcl-1 levels did not further decrease in Pin1 silenced tumours treated with the drug, suggesting the involvement of another signalling pathway that, once inactivated, could give rise to the apoptotic process (Figure 20C).

![Figure 20. Pin1 depletion sensitizes CSCs to chemotherapeutic agents in vivo](image)

A) Pin1 knockdown synergizes with paclitaxel to block breast cancer growth. Tumor growth of MDA-MB-231 xenografts expressing the indicated shRNAs and treated with paclitaxel (grey bars) or left untreated (PBS) (black bars). Means and standard deviations are indicated, P-values are *** < 0.0003 (t-test, n = 12). B) Pin1 knockdown blocks chemotherapy-induced breast CSCs’ expansion in vivo. Histogram representing the Aldefluor mean fluorescent intensity (MFI) of cells from control- and shPin1 MDA-MB-231 xenografts treated with Paclitaxel or PBS. Means and standard deviations are indicated, P-values are * = 0.001 (t-test, n = 3 for each condition). C) Pin1 knockdown induces reversal of EMT and cell death in breast cancer xenografts in combination with paclitaxel. Western blot analyses of tumor xenografts from A).

Indeed, to verify if Notch was the involved signalling pathway, we performed mammosphere formation assays with MDA-MB-231 expressing control- or Pin1-specific shRNA and treated with paclitaxel. Notably, over-expression of N1-ICD-dPEST totally rescued mammospheres’ formation and chemoresistance (Figure 21).
All together these data demonstrate that Pin1 is required for the accumulation of chemoresistant breast CSCs during chemotherapy and that ablation of its levels or activity curbes the CSCs population with concomitant gain of chemosensitivity.

Figure 21. N1-ICDs-dPEST over-expression is able to rescue the chemoresistance in Pin1-depleted breast cancer cells.
Left: expression of stable N1-ICD-dPEST rescues resistance to paclitaxel treatment in Pin1 silenced cells. Percentage of M2FE of control (black bars) or Pin1 shRNA (grey bars) expressing MDA-MB-231 cells transduced with empty (-) or N1-ICD-dPEST (+) expressing vectors and treated with Paclitaxel (+) or PBS (-). Means and standard deviations are indicated, P-values are **0.0001, ***<0.00003 (t-test, n = 3). Right: the corresponding analysis of protein expression by Western blot is shown. Arrow indicates position of the specific band.

5.5 Pin1 protects N1- and N4-ICD from proteasomal degradation

All the data presented above indicate that N1-ICD protein levels are under direct control of the polyl-isomerase, moreover the stable mutant N-ICD-dPEST is unaffected by modulation of Pin1 levels, suggesting that Pin1 is impinging on N1-ICD turnover through regulation of its protein degradation. To test this possibility, we performed protein stability assays. As shown in Figure 22A, Pin1 depletion by siRNA in MDA-MB-231 breast cancer cells treated with gamma-secretase inhibitor to block Notch cleavage at the membrane (Rustighi et al, 2009), reduced the half-life of N1-ICD. Addition of the proteasome inhibitor Lacatacystin rescued N1-ICD levels in this context, unveiling that this reduced half-life was proteasome-dependent. Pin1 silencing curbed the half-life of over-expressed N1-ICD protein also in the SK-BR-3 breast cancer cell line that was strongly reduced from 2 h to 40 min upon Pin1 siRNA treatment with respect to control silencing (Figure 22B). Moreover, re-introduction of a siRNA resistant Pin1 construct (Pin1r) in Pin1 silenced cells prevented the reduction of N1-ICD protein levels, demonstrating that N1-ICD protein levels are specifically dependent on the polyl-isomerase Pin1.
Pin1 depletion impaired N1-ICD protein half-life

A) Pin1 knockdown accelerates the decay of endogenous N1-ICD. Western blot of endogenous N1-ICD following RNA interference (RNAi) with the indicated siRNAs and time points following GSI or GSI plus Lactacystin (+) chase is shown. B) Left panel: Western blot of over-expressed N1-ICD-myc along with indicated siRNAs and empty (-) or siPin1 resistant pCDNA3-HA-Pin1 (Pin1r) expression vectors, treated with DMSO (-) or CHX. Right panel: Graph indicating N1-ICD-myc protein levels versus time of CHX treatment in the three tested conditions (siCtrl, siPin1 and siPin1 + HA-Pin1r). Means, standard deviations and p-values (t-test, n=3) are indicated.

Pin1 is known to bind several substrates on phosphorylated Ser/Thr-Pro residues and to affect their stability (Liou et al, 2011). In vitro and in vivo binding assay demonstrated that Pin1 binds to N1-ICD and this interaction was direct. Moreover, domain-mapping analysis showed a marked reduction in Pin1 binding to N1-ICD when the C-terminal PEST domain containing the cdc4-phosphodegron was deleted (Figure 23A). Intriguingly, the residues T2512/P2513 within the phosphodegron in the PEST domain are known to be indispensable for recognition by the ubiquitin-ligase Fbwx7α when phosphorylated on T2512. To verify if this motif is also a Pin1 binding site, we performed in vitro GST-Pin1 binding assays with N1-ICD or a mutant construct in which we replaced the threonine residue 2512 with alanine (T2512A) (Figure 23B). This mutation impaired the interaction between Pin1 and N1-ICD. Accordingly, Pin1 overexpression was unable to affect the protein stability of this mutant as compared to wild-type N1-ICD (Figure 23C).
Figure 23. N1-ICD-T2512A mutation hampers binding to Pin1
A) Mapping of the Pin1-N1-ICD interaction. Upper: Schematic of pCDNA3-N1-ICD-myc C-terminal deletion constructs (from d2444 to d1991) used for mapping Pin1 binding domains in N1-ICD. TM: transmembrane, RAM: CSL interacting, ANK: ankyrin, STR: Serine-Threonine rich, TAD: transactivation, PEST: Pest domain. Numbering refers to Swissprot entry P46531. Interaction with Pin1 is indicated next to the constructs: +++ very strong, ++ strong, – no binding. A relevant drop in binding strength is observed by deletion of the PEST domain and the STR.

Lower: Representative anti-myc tag Western blot analyses of GST-Pin1 pull down assays of indicated proteins overexpressed in HEK 293T cells is shown. The borders around the panels demarcate juxtaposed parts of same or different gels aligned in function of the molecular weight.

B) Anti-myc tag Western blot analysis of GST-Pin1 or GST pull down assay and input levels of indicated proteins overexpressed in HEK 293T cells is shown. (F) N1-ICD-T2512A mutant half-life is unaffected by Pin1 levels.

C) Western blot of CHX chase experiment with N1-ICD-myc and N1-ICD-T2512A-myc mutant with empty (-) or HA-Pin1 expressing vectors. Molecular weights Mr(K) are indicated in KDa.
Finally, these results suggest that the T2512/P2513 motif, indispensable for Fbwx7α recognition, constitutes also the docking site for Pin1, which might exert N1-ICD stabilization by interfering with Fbwx7α-binding.

5.6 Pin1 protects N1- and N4-ICDs from degradation by the ubiquitin-ligase Fbwx7α

To test if Pin1 was counteracting N1-ICD degradation by hampering the interaction with the E3-ubiquitin-ligase Fbwx7α, we tested if Pin1 is able to affect N1-ICD ubiquitination. For this aim we performed an ubiquitination assay in COS-7 cells by a Ni-NTA pull down and, as expected, over-expression of Fbwx7α in N1-ICD transfected cells, caused an increase in N1-ICD poly-ubiquitination (Figure 24A). More importantly, when we depleted Pin1 from these cells by RNA-interference, we found an enhanced N1-ICD poly-ubiquitination. Thus, Pin1 interferes with Fbwx7α-mediated ubiquitination of N1-ICD. Accordingly when N1-ICD and Fbwx7α were co-ovexpressed in two different cellular contexts, the concomitant Pin1 over-expression completely rescued N1-ICD protein levels (Figure 24B-C). Since the final read-out of Notch pathway activation is the transcription of its target genes, we evaluated if Pin1 was able to boost Notch dependent transcriptional activity despite presence of high-levels of its negative regulator Fbwx7α. Interestingly, as emerged from luciferase assays, Pin1 fully rescued the ability of N1-ICD to promote gene transcription despite presence of its ubiquitin ligase (Figure 24D). These data clearly show that Pin1 is able to prevent Fbwx7α dependent degradation of N1-ICD, boosting its protein levels and transcriptional activity.
Figure 24. Pin1 protects N1-ICD from Fbw7α dependet degradation

A) Pin1 depletion enhances Fbw7α-dependent poly-ubiquitination of N1-ICD. Western blot analysis of high molecular weight N1-ICD-myc products (N1-ICD-myc) from a Ni-NTA pull-down in COS-7 cells transfected with the indicated vectors along with control- or Pin1 siRNA. Input levels of over-expressed proteins are shown.

B) Pin1−/− MEFs transfected with pCDNA3-N1-ICD-myc and increasing amounts of p3xFLAG-Fbw7α along with either empty vector (-) or pCDNA3-HA-Pin1. Borders around the panels demarcate juxtaposed parts of the same gel.

C) Pin1 overexpression rescues N1-ICD levels in presence of Fbw7α. Western blot analysis of lysates from SK-BR-3 cells over-expressing N1-ICD-myc, Flag-Fbw7α along with empty (-) or increasing amounts of HA-Pin1 expressing vector, normalized for co-expressed GFP protein.

D) Histogram of Luciferase reporter assays in SK-BR-3 cells co-transfected with pGL2-RBPjκ/LUC and empty vector (-) or pCDNA3-N1-ICD-myc, along with p3xFLAG-Fbw7 and increasing amounts of pCDNA3-HA-Pin1 as indicated by a wedge. Means, standard deviations and p-value (t-test, n=3) are indicated. Cell lysates were analyzed by Western Blot and are shown below.
Figure 16A showed that in Pin1 depleted mammospheres there is a strong decay also in N4-ICD levels. Since it has been demonstrated that also N4-ICD is a target of Fbwx7α (Wu et al., 2001), we speculated that also in this case Pin1 was able to prevent N4-ICD protein degradation. Interestingly, Pin1 silencing in SK-BR-3 strongly increased N4-ICD proteasome dependent degradation (Figure 25A) and, accordingly, Pin1 overexpression rescued both N4-ICD protein levels and transcriptional activity in presence of Fbwx7α as depicted in Figure 25B.

![Figure 25. Pin1 protects N4-ICD from Fbwx7α dependent degradation](image)

A) Western blot of a CHX chase in SK-BR-3 cells transfected with pCDNA4-N4-ICD-HA along with the indicated RNAi, in presence (+) or absence of proteasome inhibitor Lactacystin. B) Histogram of Luciferase reporter assays in SK-BR-3 cells co-transfected with pGL2-RBPjκ/LUC and pCDNA4-N4-ICD-HA alone or with p3xFlag-Fbxw7α and increasing amounts of pCDNA3-HA-Pin1. Means, standard deviations and p-value (t-test) are indicated for three independent experiments. Cell lysates were analyzed by Western blot and are shown below the histograms.

To confirm and to strengthen these evidences in vivo, we analyzed N1- and N4-ICD protein levels in the epithelial cells of the mammary gland from Pin1+/+ and Pin1−/− mice. As shown in Figure 26, from the immunostochemical and Western Blot analyses performed in Pin1−/− mice it emerged that the Notch pathway is strongly reduced, while the levels of Fbwx7α seemed not to be altered, when compared to wild-type mammary tissues.
Results

5.7 Pin1 protects N1- and N4-ICDs from interaction with Fbxw7α

Pin1 induces prolyl-isomerisation and conformational changes on its targets (Lu et al., 2002a). Thus we analyzed if Pin1 is able to disrupt the interaction between Fbxw7α with NICDs. For this aim we performed co-immunoprecipitation (Co-IP) experiments with both endogenous and over-expressed NICDs and Fbxw7α.

Co-IP in MDA-MB-231 unveiled that the binding between N1- and N4-ICD with Fbxw7α is increased when Pin1 activity was blocked with PiB (Figure 27A). Accordingly, we recapitulated these results in another cell line by over-expressing N1-ICDs and Fbxw7α following control- or Pin1-silencing (Figure 27B). Similar experiments performed in Pin1<sup>−/−</sup> fibroblasts unveiled that the catalytic activity of Pin1 was required for this effect. Indeed while over-expression of Pin1 is able to prevent the binding between N1-ICD and Fbxw7α, the catalytic inactive mutant Pin1<sup>S67E</sup> was unable to (Figure 27C).
Figure 27. Pin1 prevents the interaction between N1/N4-ICDs and Fbwx7α

A) Inhibition of Pin1 increases interaction between endogenous Fbwx7α and N1-ICD proteins. Western blot analysis of co-immunoprecipitation (Co-IP) experiments between endogenous Fbwx7α and both N1-ICD and N4-ICD from MDA-MB-231 cells treated with DMSO (-) or PiB (+). Anti-Fbwx7α or non-related antibody (NRA) immunoprecipitates (IP) were recognized with anti-N1-ICD (Val1744) and anti-N4-ICD (Val1432) antibody and after stripping with an anti-Fbwx7α antibody. Input levels are shown below. B) Left Panel: depletion of Pin1 increases Fbwx7α-N1-ICD interaction. Representative Western blot analysis of Co-IP experiments between over-expressed N1-ICD-myc and Flag-Fbwx7α in SK-BR-3 cells. Over-expressed N1-ICD-myc was immunoprecipitated (IP) and subjected to anti-Flag Western Blot to reveal Flag-Fbwx7α Co-IP. Input levels of over-expressed or silenced proteins are shown below. Right Panel: Graph depicting the mean of Flag-Fbwx7α Co-IP levels, normalized to immunoprecipitated N1-ICD. Standard deviations and p-value (t-test, n=3) are shown. C) Pin1 catalytic activity is required to uncouple N1-ICD from Fbwx7α. Co-IP as in (B) in Pin1−/embryo fibroblasts transduced with the indicated vectors. A)-C) Molecular weights Mr(K) are indicated in KDa.

All these data demonstrated that Pin1 is boosting Notch1/4 nuclear levels by impairing recognition and subsequent induced degradation by the E3-ubiquitin ligase Fbwx7α.
Results

Figure 28. PP2A is required for the Pin1-dependend N1-ICD detachment from Fbw7α.
A) Inhibition of PP2A enforces Fbw7α-N1-ICD interaction. Co-IP as above in figure 24 SK-BR-3 cells treated with DMSO (-) or okadaic acid and transduced with the indicated vectors. B) Inhibition of PP2A accelerates the half-life of endogenous N1-ICD. Western blot analysis of a GSI chase of MDA-MB-231 cells treated with vehicle (DMSO) or okadaic acid. Anti-p21Cip1Waf1 immunoblot was added as control for Okadaic acid functioning (Park et al, 2001). C) PP2A is required for Pin1-dependend N1-ICD detachment from Fbw7α. Co-IP as above in SK-BR-3 cells transduced with the indicated vectors and treated with DMSO (-) or okadaic acid.

Moreover we wanted to uncover the mechanism by which Pin1 is protecting N1/4-ICDs from Fbw7α. It is known that prolyl-isomerization of specific phospho-S/T-P sites leads, on certain substrates, to recognition and subsequent dephosphorylation by the trans-specific phosphatase PP2A (Liou et al, 2011). Insights from a nuclear Notch1 interactome revealed that PP2A binds to N1-ICD (Yatim et al., 2012). Hence, we investigated if, following isomerization by Pin1, the Notch cdc4-phosphodegron could be dephosphorylated by PP2A, thus eluding recognition by Fbw7α. To evaluate this possibility we performed co-immunoprecipitation assay between N1-ICD and Fbw7α in SK-BR-3 cells treated with the PP2A inhibitor okadaic acid. Interestingly the interaction between N1-ICD and Fbw7α increased when cells were treated with okadaic acid (Figure 28A). Accordingly, when we analyzed the protein half-life of N1-ICD in MDA-
MB-231 we found that okadaic treatment enhanced N1-ICD protein degradation (Figure 28B). We next analyzed the dynamics of this interaction following Pin1 overexpression in the same conditions as above. As shown in Figure 28C Pin1 expression consistently reduced the interaction between N1-ICD and Fbxw7α but only in the presence of functionally active PP2A, indicating that PP2A is required for Pin1-dependent N1-ICD accumulation.

5.8 Pin1 and Fbwx7α interplay affects Notch-dependent stem cells traits

Pin1 and Fbwx7α are able to tightly control the Notch signalling pathway at the nuclear level. Since the role of both Notch1 and Notch4 in the breast CSCs maintenance is well known, we investigated if the Pin1-Fbwx7α interplay on the Notch pathway can affect the CSC population. For this aim we performed mammosphere assays with MDA-MB-231 by over-expressing Pin1 and Fbwx7α. Interestingly Fbwx7α over-expression had a great effect in reducing the mammosphere formation, the percentage of putative CSCs as detected by the reduced number of Aldh+ cells and also the nuclear levels of both N1- and N4-ICDs and the transcriptional target HES1 (Figure 29). This inhibitory effect on the CSC populations mediated by Fbwx7α is almost completely abolished by a concomitant over-expression of Pin1 that, through stabilization of N1/N4-ICDs, recovered the mammosphere formation ability and the percentage of Aldh+ cells. To verify the contribution of the Notch signalling in this context, we treated cells in all the experimental points with the Notch inhibitor GSI, that abolished the CSCs population.
Results

Figure 29. Pin1 and Fbw7 modulate Notch breast CSCs activity in vitro
A) %M2FE of MDA-MB-231 cells overexpressing the indicated vectors. Black and grey bars indicate DMSO or GSI treated mammospheres, respectively. Means, standard deviations and P-values (t-test, n = 3), are indicated. B) Representative microphotographs of M2 are shown, 200 lm scale bar is indicated. C) Percentage of Aldh positive cells of clones from Figure 7A grown as mammospheres, calculated as Median fluorescence intensity (MFI) difference between samples containing BAAA and that of the same samples containing also DEAB. D) Western Blot of cell lysates from M2. White and black arrows indicate over-expressed and endogenous Pin1, respectively. Molecular weights (Mr) are indicated in kDa.

Since Fbw7α acts on several important targets (Wang et al, 2011), we assessed the relevance of Notch activity for this phenotype. As shown in Figure 30, down-modulation of Fbw7α caused a consistent increase of N1-ICD levels, boosted M2FE while treatment with GSI elicited a strong reduction of both mammosphere formation and Notch pathway activation, thus demonstrating that among several Fbw7α targets, Notch is the critical one in this cellular context.
Results

Figure 30. Fbw7 depletion increased mammospheres formation through Notch pathway

Left panel: Histogram showing %M2FE of MDA-MB-231 cells with the indicated siRNAs and treated with vehicle (black bars) or GSI (grey bars). Means, standard deviations and P-values (t-test, n = 3), are indicated. Right panel: Western Blot of cell lysates from M2. Molecular weights (Mr) are indicated in kDa.

We next addressed the Pin1-Fbw7α interplay in CSCs maintenance in an in vivo experiment by testing the tumour initiation properties. In particular, we performed a limiting dilution assay by injecting increasingly diluted MDA-MB-231 cells over-expressing Fbw7α, or Fbw7α plus Pin1, in the inguinal mammary glands of SCID mice. As shown in Table 1, while 300,000 injected control cells gave rise to a tumour in all mice, Fbw7α strongly impaired the tumour initiating properties, since only 2 out of 9 mice harboured palpable tumours. Notably, concomitant Pin1 over-expression significantly rescued the tumour initiation capability in Fbw7α over-expressing MDA-MB-231 to 7 out of 9 mice. At the subsequent dilution the same trend could be appreciated, demonstrating in vivo a potent counteraction of Pin1 and Fbw7α in controlling the CSCs population.

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<td>(1:485184-1:302989)</td>
<td>1.58 x 10^-6</td>
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<tr>
<td>Fbw7α + Pin1</td>
<td>1:466587</td>
<td>(1:302232-1:195771)</td>
<td>1.04 x 10^-2</td>
<td>0.0239</td>
</tr>
</tbody>
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Table 1. Mice were transplanted with decreasing numbers of MDA-MB-231 cells overexpressing empty, Fbw7α, or Fbw7α + Pin1 vectors (number of injected cells is indicated). Results are shown as the number of tumors per number of injected mice (upper panel). CSC frequencies (estimates and upper/lower limits) were calculated by limiting dilution analysis, as described in Materials and methods. Differences in CSC frequencies are indicated for each sample against the empty vector and for Fbw7α + Pin1 against Fbw7α only. Their significance is indicated by a P value (lower panel).
The content of CSCs in a tumour is linked to several aggressive features, such as tumour growth and metastasis formation. To gauge for these features, we performed xenograft experiments by injecting 1,000,000 of cells/flank with the same MDA-MB-231 clones used above. Notably Fbw7α exerted a potent tumour suppressor activity as emerged by the fact that, compared to empty vector infected cells, MDA-MB-231 over-expressing Fbw7α gave rise to small tumour with an impaired Notch signalling, a reversal of EMT, and a reduced number of metastases to the nearby lymph nodes and to the lungs. By contrast tumours that co-expressed Pin1 and Fbw7α were growing faster, with a re-activation of the Notch signalling, a rescue of mesenchymal markers and dissemination of metastases (Figure 31).

In summary, we uncovered a potent role of Fbw7α in reducing the breast CSC traits through a down-regulation Notch1/4 activity by promoting degradation of their nuclear active forms. In this context, concomitant Pin1 over-expression reverted all these effects boosting N1/4-ICDs protein stability and levels.
Results

Figure 31. Pin1 and Fbw7α modulate Notch breast CSCs activity in vivo

A) Tumour volume of orthotopic xenografts in SCID mice obtained from the indicated MDA-MB-231 cell clones.

B) qRT-PCR of Fbw7α and Fbw7α+Pin1 tumor xenografts relative to control tumors (empty) explanted at the end of the experiment.

C-D) Western blot of Fbw7α and Fbw7α + Pin1 tumor xenografts relative to control tumors (empty) explanted at the end of the experiment.

E) Representative images of colonized lymph nodes and hematoxylin and eosin stained pulmonary sections are shown. Rulers and Scale bars (1 mm) are indicated for calibration, arrows indicate metastatic areas.

A), B), E), F) Means, standard errors of the mean and P-values (t-test, n = 6), are indicated. C), D) Molecular weights Mr(K) are indicated in kDa.
5.9 Pin1 sustains Notch signalling in primary breast cancers in spite of Fbxw7a expression

According to CONAN, Cosmic and TCGA databases and recent publications, **FBXW7** and **NOTCH** a rarely mutated in breast cancer (Byrd et al., 2008; Ibusuki et al., 2011; Mao et al., 2008; Santarpia et al., 2012), but its known that Notch pathway is over-activated in many breast cancers (Farnie et al., 2007; Han et al., 2011; Pece et al., 2004; Reedijk et al., 2005; Rizzo et al., 2008; Rustighi et al., 2009; Xu et al., 2012). We have discovered a mechanism by which, despite absence of mutational events and presence of the ubiquitin-ligase Fbxw7a, N1- and N4-ICD are up regulated because of high Pin1 protein levels.

This hypothesis prompted us to analyze breast cancer tissues from patients to address if our mechanism occurs also in human patients. We took 43 breast cancer tissues from which the protein levels of N1-ICD had been already detected by IHC (Verzemovic et al., submitted). We extracted the total RNA from these tissues and analyzed the mRNA levels of Pin1 and Fbwx7a; notably it has been demonstrated that their mRNA levels provide a direct correspondence to protein levels. The tissues were grouped, based on the N1-ICD protein levels, into high or low expressing (Figure 32). Interestingly, 11/17 tissues displaying high N1-ICD protein levels, expressed also high **FBXW7α** mRNA levels and more importantly all of them expressed high **PIN1** levels. Moreover while **FBXW7α** did not correlate with any of the categories, **PIN1** levels were higher in the high N1-ICD expressing group. This evidence suggests that high N1-ICD protein levels can coexist with its negative regulator Fbwx7a because of high Pin1 expression.

Figure 32. Association of FBWX7Α or PIN1 expression in N1-ICD high and low breast cancers

A) Heatmap representing the expression of PIN1 and FBXW7 (detected at mRNA expression level) and N1-ICD (detected at protein level) in a cohort of 43 breast cancer patients. The color in each block represents if the mRNA or protein is above (red) or below (blue) the average value of the samples for each gene or was scored high or low by immunohistochemistry, respectively. B) Left panel: Box-plot representations of gene expression values (of FBXW7 and PIN1, upper and lower panels, respectively) in the breast cancer cohort of Figure S8A. Patients were binned in low or high categories for weak or strong N1-ICD staining (detected by IHC, see Methods for detail). Finally, to test the associations between N1-ICD staining and expression of FBXW7 and PIN1, Wilcoxon rank sum tests were performed. The results showed no association for FBXW7 (W = 192, p-value = 0.8998) and very strong association for PIN1 (W = 35, p-value = 2.967 x 10^-6). Right panel: Contingency table showing percentage of each category calculated on the precedent category of patients from A).
Results

To strengthen our observation we analyzed another cohort of breast cancer tissue from 38 patients (Figure 33). We selected in particular tissues of triple negative breast cancer subtypes, which have been described to be chemoresistant, highly metastatic and of grade 3, suggesting that they are also enriched in CSCs (Pece et al., 2010). We analyzed the protein levels of Pin1, N1-ICD ad Fbwx7α by immunohistochemical analysis. We found that 22/38 tissues display high N1-ICD nuclear protein levels and in 72.7% of them there was also a strong nuclear accumulation of Fbwx7α. Again, a large part of this group presented also high Pin1 protein levels as shown in Figure 32.

![TNBC patients](image)

**Figure 33. High N1-ICD levels in human breast cancers coexist with Fbwx7α thanks to high Pin1 expression**

Left panel: Heatmap representing the protein levels of Pin1, Fbwx7α and N1-ICD in a cohort of 38 breast cancer patients. The colours represent high (red) or low (blue) protein levels according to protein expression scores (see supplementary Methods). Right panel: Contingency table showing percentage of each category calculated on the precedent category of patients; chi-square test was performed for independence between the variables and the P-value = 105.

Finally we analyzed a medataset of a large cohort of 3254 breast cancer patients collected from 19 independent studies (see Material and Methods). We stratified patients into high or low levels of FBXW7α and PIN1 expressing patients (Adorno et al., 2009). Since the Notch mRNA is not a representative of N1-ICD protein levels, we generated a signature, that reflects the Notch pathway activation, since it is was created by selecting Notch direct transcriptional targets (NDT signature) (see Material and Methods). 48% of all samples exhibited high NDT expression and correlated with poor overall survival (Figure 34). Notably 51.7% of these samples displayed also high FBWX7α mRNA levels and again the majority of this group presented high PIN1 levels.
Figure 34. High N1-ICD levels in human breast cancers coexist with Fbwx7α thanks to high Pin1 expression

Upper panel: heatmap representing the contingency table frequencies of samples classified as having high or low levels of FBWX7A, of PIN1 and of the NDT gene signature. Number of samples in each category is indicated on the left. The association among high levels of NDT gene signature, PIN1, and FBWX7A resulted statistically significant (P < 0.001; chi-square test). Lower panel left: Contingency table showing percentage of each category calculated on the precedent category of patients. Expression correlation between NDT and PIN1 and FBWX7A mRNA levels. Average expression of NDT gene signature in breast cancer samples stratified according to high or low expression of PIN1 and FBWX7A mRNA. Data are shown as mean and standard error of the mean (s.e.m.). Lower panel right: Kaplan-Meier graphs representing the probability of survival in 1173 breast cancer patients from the meta-dataset of the Upper panel, with outcome information on survival. Tumor samples were classified as high or low Notch-dependent Direct Target (NDT) gene signature using the classifier described (Adorno et al., 2009). The log-rank test p value reflects the significance of the association between NDT gene signature low and longer survival.

In summary, all the 3 analyses confirmed our hypothesis that in breast cancer patients N1-ICD could coexist with its ubiquitin-ligase thanks to high Pin1 expression. Moreover the category displaying high NDT and Fbwx7α expression but low Pin1 is the less represented compared to
any other category. Accordingly, the average expression value of NDT was contingent on Pin1, while the Fbwx7α status was not influential, strengthening the idea that Pin1 is dominant, over Fbwx7α, in modulating Notch pathway in breast cancers (Figure 34).

In this work we discovered a functional interaction between Pin1, N1-ICD and N4-ICD in breast CSCs and aggressive breast tumour traits. Since all these features are connected to poor clinical outcome in patients, we analyzed in our cohort from figure 34 the survival of patients. Notably, as shown in Figure 35, we found that in the grade 3 breast cancers of this cohort, high Pin1 levels correlated with a worse outcome only in patients with activated Notch signalling. By contrast, in patients with low Notch signalling, Pin1 is unable to stratify patients, thus indicating that Pin1 requires Notch activation to promote aggressiveness and poor clinical outcome.

![Figure 35](image)

**Figure 35. Pin1 levels correlate with poor clinical outcome only in patients with high NDT signature.**

A) Kaplan–Meier survival curve is indicated for high NDT signature, grade 3 breast cancer patients of the metadataset in function of high or low PIN1 mRNA levels. P-value and the number of subjects at risk at each time point is indicated below. B) Kaplan–Meier survival curve is indicated for low NDT signature, grade 3 breast cancer patients of the metadataset in function of high or low PIN1 mRNA levels. P-value and the number of subjects at risk at each time point is indicated below.

To understand which molecular pathways are over-represented in patients with high Pin1/high NDT compared to patients with high Pin1/low NDT, we performed a Gene set enrichment analysis. Interestingly, as depicted in Table 2, we found enrichment of several stem cell pathway signature genes in this group of patients, supporting our findings of a pro-CSCs program induced by the cooperation between Pin1 and the Notch pathway.
Table 2. Enrichment of gene signatures in the list of genes preferentially expressed in NDT signature HIGH/PIN1 HIGH versus NDT signature HIGH/PIN1 LOW tumors in the metadataset (GRADE 3). Differentially expressed genes (n=1460, q-value <0.01 and fold change >1.5) were subjected to Gene set enrichment analysis (GSEA) as already described (Montagner et al., 2012). Enrichment has been determined using a Fischer’s exact test on all 254 signaling pathways in the database. P-values have been corrected using BH procedure.

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6. DISCUSSION

Cancer stem cells represent a major problem for breast cancer treatment, as they are responsible for tumour heterogeneity, recurrence, chemoresistance metastatic dissemination. Many studies have been carried out to understand the molecular bases of their sustenance, that have led to the identification of several involved signalling pathways, transcriptional factors and micro-environmental stimuli showing that CSCs may take advantage of the normal stem cell molecular machinery to maintain their self-renewal. Here we demonstrate that the prolyl-isomerase Pin1 is required for both normal and cancer stem cell biology. These Pin1 pro-stemness functions are mediated through the maintenance of both N1 and N4-ICD protein levels, protecting them from the tumour suppressor and E3-ubiquitin ligase Fbwx7α. Pin1 and Notch signalling promote each other since Notch1 directly induces Pin1 transcription (Rustighi et al., 2009). Thus the Pin1-Notch1/4 axis promotes a pro-CSCs program by up-regulating EMT markers, stemness signalling pathways and proteins involved in chemoresistance, regardless of Fbwx7α, as depicted by the model in Figure 36. As a consequence, genetic or pharmacological ablation of Pin1 suppresses CSC number and self-renewal, reduces the number of tumour initiating cells, the metastases dissemination and, more importantly, recovers sensitivity to chemotherapy both in vitro and in vivo.

![Diagram](image)

**Figure 36.** Schematic model that depicts the role of Pin1 in sustaining CSCs through Notch1 and Notch4 by antagonizing Fbwx7α-mediated destruction.

In G3 breast cancer patients, high Pin1 levels are associated to poor clinical outcome when Notch signalling is activated. Therefore, since it has been demonstrated that G3 are enriched in CSCs, it is conceivable that a major mechanism by which Pin1 is involved in cancer progression might be its promotion of the CSCs number. In addition, Pin1 has not yet been linked to any particular subtype or unique mutational event in breast cancer, nevertheless its levels increase in parallel with the tumour grade and it cooperates with several oncogenic pathways. In particular it cooperates with several oncogenes or oncogene-induced factors, that are involved in almost all
the breast cancer subtypes such as estrogen receptor alpha (ERα) tumors (Rajbhandari et al., 2012), progesterone receptor (PR) (Yi et al., 2005), Her2/Neu (Wulf et al., 2003), mutp53 (Girardini et al., 2011) and Notch1/4. Moreover Pin1−/− mice display reduction of the stem cell compartment without perturbing the luminal or myoepithelial fate commitment, as suggested by an unchanged proportion of the myoepithelial and luminal populations in Pin1−/− mice (Figure 9). Therefore, it seems that Pin1 goes beyond the heterogeneity of breast cancer, since it impinges on the heart of basic oncogenic mechanisms, and it could therefore be an important molecular target for breast cancer therapy. Indeed, ablation of Pin1 can reduce the CSCs population, the specific subtype driven breast cancer oncogenes and elicit sensitivity to chemotherapy.

A role for both Pin1 and Notch pathway in the promotion of chemoresistance has been well demonstrated (Ding et al., 2008; Ranganathan et al., 2011). Of note, the role of Pin1 is controversial, since on one hand it promotes apoptosis through p53 following genotoxic stress (Wulf et al., 2002; Zacchi et al., 2002), on the other it promotes chemoresistance acting on Mcl-1 stability (Ding et al., 2008). Nevertheless, to date no in vivo experiment has been done to address how Pin1 dictates the cellular response to pharmacological treatments. Here we provide a clear demonstration of the pro-survival role of Pin1 in presence of chemotherapy in breast cancers with in vitro and in vivo experiments. In particular breast tumour cells and CSCs depleted for Pin1 display reduction of the Notch signalling, of the drug efflux pump ABCG2 (Sarkadi et al., 2006), of the pro-survival factor SURVIVIN (Fukuda and Pelus, 2006), of the detoxification enzyme Aldh (Ginestier et al., 2009), and other factors for which a role in chemoresistance is already known. This evidence coupled with the fact Pin1 promotes exhaustion of CSC, point out Pin1 as an important mediator of chemoresistance in breast cancers through many mechanisms.

Notch1 and Notch4 receptors have been linked to many diseases such as cancer (Ranganathan et al., 2011). Notch1 has been associated for the first time to tumourigenesis in human T-cell acute lymphoblastic leukemia (T-ALL) due to mutational events that unleash high levels of N1-ICD in tumour cells and, accordingly, in 50% of T-ALL there are mutational events on NOTCH1 or FBXW7α (Ferrando, 2009). Moreover, in leukemia cells, high levels of Notch signalling are important for the CSCs maintenance (Liu et al., 2013). Intriguingly Notch proteins are also over-activated in breast cancer and important for normal and cancer stem cells; while Notch1 seems to be more important for luminal progenitors, Notch4 seems to be more related to bipotent stem cells (Dontu et al., 2004; Harrison et al., 2010; Raouf et al., 2008). Despite the high percentage
of mutational events in T-ALL, in breast cancer there are rare mutations on FBXW7α and NOTCH1 which can not provide molecular explanation for the great percentage of mammary tumours displaying high levels of N1/4-ICD protein levels. Our findings unveil a mechanism by which Pin1 is responsible for elevated Notch signalling in breast cancers without the need of mutational events on NOTCH and FBXW7α. Thus, an over-activated Notch-Pin1 axis can promote normal and cancer stem cells despite high levels of functional Fbwx7α.

New insights for the tumour suppressor Fbwx7α in breast cancer

There is only limited evidence in the literature that links Fbwx7 to breast tumours. Nevertheless this ubiquitin-ligase is a potent tumour suppressor in many other tumours. Overall, approximately 6% of tumours harbour mutations in FBW7, but there is substantial variation among tumour types. The highest mutation rates (approximately 30%) were found in cholangiocarcinoma and T-cell acute lymphoblastic leukaemias (T-ALL) whereas gastrointestinal cancers (pancreatic and gastric) as well as prostate and endometrial cancers had mutation frequencies in the range of 4–15% (Welcker and Clurman, 2008). By contrast, many tumour types do not exhibit or only rarely exhibit mutations in FBW7, in particular breast cancers. Rather, very often the oncogenic substrate proteins of Fbwx7, such as c-Myc or Notch1, have the CPD mutated in order to escape Fbwx7α-mediated recognition and proteasomal degradation.

In tumours with low mutations on Fbwx7 gene, deregulation of its protein levels is not documented and the role of Fbw7α inactivation by dominant oncogenes in human tumours is not known, with exception of human colon cancers where Fbwx7α protein levels were found decreased due to high levels of Pin1 (Min et al., 2012). By our observations in mice and human breast cancer samples, we did not find any correlation between Fbwx7α and Pin1 levels. Notably, and unexpectedly, also N1-ICD nuclear levels did not correlate with those of its ubiquitin-ligase Fbwx7. Notwithstanding, there might be a proportion of breast cancer patients where Pin1 and Fbwx7 are inversely correlated due to inactivation of Fbwx7 by Pin1, suggesting a further level of cooperation between Pin1 and several oncogenes in cancer. Here, we have described that a quite high number of breast cancer patients have high N1-ICD levels despite high levels of Fbwx7, due to the concomitant presence of Pin1 that protects N1/4-ICD from degradation. Ablation of Pin1 allowed Fbwx7 to interact with and turn-off the Notch pathway strongly impacting on tumourigenesis. Indeed in vitro and in vivo experiments demonstrated that
Fbxw7α over-expression strongly impaired the number and the self-renewal of breast CSCs, curbed tumourigenesis formation and metastases dissemination. Interestingly Fbxw7α controls the number and maintenance of stem cells in many other cells such as neurons, colon and hematopoietic cells mostly through regulation of the Notch signalling (Wang et al., 2012). It will be interesting to investigate if, in absence of mutational events on NOTCH or FBXW7 genes, also in these tissues Pin1 could cooperate with the Notch signalling pathways in the maintenance of normal and cancer stem cells.

**The interplay of the Notch-Pin1 axis with other signalling pathways**

The interplay between Pin1 and N1-ICD is not simply restricted to NICDs protein stabilization and Pin1-induced transcription. Indeed the Notch pathway is highly inter-connetected with several signalling pathways such as PI3K/Akt, Jak/STAT, NF-kappaB, ErbB2, Wnt, and HIF1 (Ranganathan et al, 2011). Strikingly, considering that most of them are also Pin1 substrates (Kim et al, 2008; Liou et al, 2011), this isomerase may contribute to increase Notch induced oncogenesis also by amplifying the signalling emerging from these cross-talks. In addition, the death associated protein kinase 1 (DAPK1), a tumour suppressor that negatively controls Pin1 activity (Lee et al., 2011), was shown to be repressed by N1-ICD (Li et al., 2008), suggesting that Notch signalling is not only boosting Pin1 transcription but also its activity.

Another important interactor of both Notch signalling and Pin1, is the tumour suppressor p53 (Beverly et al., 2005; Kim et al., 2007; Sun et al., 2011; Wulf et al., 2002; Zacchi et al., 2002). Interestingly wild-type p53 (wtp53) has a fundamental role in the regulation of the breast stem cell compartment, since its depletion leads to an uncontrolled symmetric expansion boosting the number of stem cells (Bonizzi et al., 2012; Cicalese et al., 2009; Insinga et al., 2013). Notably, pharmacologic inhibition of the Notch signalling in p53 depleted CSCs is able to revert the phenotype with a consequent reduction of CSCs number (Tao et al., 2011). The role of wtp53 in our findings is not evident, raising the question on whether in the mammary stem cell compartment Pin1 could act by modulating p53 and Notch functions in concert, unveiling novel intersections between these two pathways. While *in vitro* the activity of Pin1 on p53 has been well characterized in the context of genotoxic stress signalling acting on the wtp53, their interplay in the stem cell compartment remains to be elucidated. The fact that Pin1<sup>−/−</sup> mice have reduced mammary stem cells (Figures 1 and 2), in contrast with what has been observed in the TP53<sup>−/−</sup> genotype (Bonizzi et al., 2012; Cicalese et al., 2009), allows speculating a more complex scenario for a wtp53-Pin1 interplay in these cells.
On the other hand, also for oncogenic gain of function (GOF) mutant p53 proteins, there is growing evidence that supports a role in promoting cellular reprogramming and EMT and inciting expansion of mammary epithelial stem cells giving rise to mammary tumours (Chang et al., 2011; Dong et al., 2013; Lu et al., 2013b; Sarig et al., 2010). Nevertheless no in vivo experiment has been done yet to understand the molecular processes controlled by mutp53 in breast stem cells. In this context, we have recently demonstrated a key role of Pin1 in promoting mutp53 GOF in breast cancer (Girardini et al., 2011). On these bases, it is conceivable that in CSCs lacking wtp53 or expressing oncogenic mutp53, increased levels of Pin1, due to a hyperactivated Notch pathway, may foster stem cell traits by acting both on mutp53 oncogenic properties and on Notch itself. In support of this hypothesis, our analysis revealed that poorly differentiated grade 3 and NDT-high/PIN1-high breast cancers are enriched for the expression of stem cell and mutp53 signature genes.

In conclusion, this study has two major impacts. First, our findings provide explanation to understand how, in the absence of mutations in NOTCH genes, in a consistent proportion of breast cancer patients, activated Notch1 can exist in spite of the presence of its major constrain, the tumour suppressor Fbxw7α. The data presented, in fact, suggest that deregulated activity of Pin1, at least in breast cancer, where mutational events in NOTCH1 and FBXW7 are rare, could reduce the selective pressure for mutations providing an alternative mechanism for Notch oncogenic activation. Second, our findings pinpoint Pin1 as a crucial target in aggressive breast cancers, providing the rational for a therapeutic strategy based on Pin1 inhibition to hit CSCs, restore chemo-sensitiveness and inhibit metastatic spread.
7. MATERIAL AND METHODS

Cell lines and treatments.
MDA-MB-231, SK-BR-3, BT-549, and SUM-159 are human breast carcinoma cells, HEK 293T is a human embryonic kidney cell line with SV40 large T, immortalized Pin1-/- fibroblasts have been obtained by spontaneous immortalization from Mouse Embryo Fibroblasts of C57BL6/129Sv mixed background (Rustighi et al., 2009). COS-7 are monkey kidney cells immortalized with SV40 large T antigen. NOP6 is a mouse mammary tumor cell line (Yang et al., 2009). All cells were cultured in DMEM, supplemented with 10% Fetal Bovine Serum (Gibco) and Penicillin/Streptomycin, as described (Rustighi et al., 2009). For NOP6, in addition Insulin supplements were added (Yang et al., 2009). MCF10A cells were maintained in DMEM:F12 Ham’s (1:2; Sigma), supplemented with 5% horse serum (Gibco), insulin (10 µg ml⁻¹; Sigma), hydrocortisone (0.5 µg ml⁻¹) and EGF (20 ng ml⁻¹; Peprotech). Primary breast cancer cell lines were maintained in F12 Ham’s supplemented with 10% FCS and insulin, hydrocortisone and EGF as above. Transient transfections, retroviral or lentiviral infections were performed by standard procedures, as described (Rustighi et al., 2009). pEGFP was included as control for transfection efficiency. For creation of stable clones, a selection corresponding to the expressed vectors was applied for 2 weeks to infected cells at the concentrations of 2 µgr/ml for Puromycin and 0.5mg/ml for Blasticidin. Cycloheximide (Sigma) for chase experiments was used at 50 nM concentration. Treatment with gamma-secretase inhibitor DAPT (Sigma), Pin1 inhibitor PiB (Calbiochem) or proteasome inhibitor lactacystin (Sigma) have been described previously (Rustighi et al., 2009). Protein phosphatase inhibitor Okadaic Acid (Sigma) was dissolved in DMSO and used at a final concentration of 200 nM for 6h.

Isolation and purification of mammary epithelial cells.
Mammary glands from 8 to 12-week-old virgin female mice were enzymatically digested and single cell suspensions of purified mammary epithelial cells were obtained, as described (Sleeman et al, 2006; Stingl et al, 2006). Briefly, Mammary glands from 8 to 12-week-old virgin female mice were digested for 1–2 h at 37°C in Epi-Cult-B medium (StemCell Technologies Inc, Vancouver, Canada) with 600 U/ml collagenase (Sigma-Aldrich, St. Louis, MO, USA) and 200 U/ml hyaluronidase (Sigma). After lysis of the red blood cells with NH4Cl, the remaining cells were washed with PBS/0.02% w/v EDTA to allow cell-cell contacts begin to break down. Cells were then dissociated with 2 ml trypsin 0.25%/w/v, 0.2% w/v EDTA for 2 min by gentle pipetting, then incubated in 5 mg/ml Dispase II (Sigma) plus 1 lg/ml DNase I (Sigma) for 5 min followed by filtration through a 40 IM cell strainer (BD Falcon, San Jose, CA, USA). Mammary epithelial cells were then purified using the EasySep Mouse Mammary Stem Cell Enrichment Kit (StemCell Technologies Inc).
**Flow cytometric analyses and sorting (FACS)**

Mouse mammary epithelial lineage-depleted cells, pre-enriched using the EasySep Mouse Mammary Stem Cell Enrichment Kit (see above), were analysed by FACS and sorted to near purity (85%) with antibodies against CD49f and CD24. FACS analysis and sorting from mammospheres based on aldehyde dehydrogenase (Aldh) activity were performed using the Aldefluor kit (StemCell Technologies Inc) following the manufacturer’s instructions and are detailed in Supporting Information. Briefly, cells were incubated with active substrate (BAAA) in presence or absence of a specific aldehyde dehydrogenase inhibitor (DEAB) for 30-60 min at 37°C, to allow conversion of BAAA into a fluorescent product (BAA). Fluorescence was measured by flow cytometry (FACS Calibur) using adjusted FSC and SSC voltages to center the nucleated and viable cell population. DEAB treated vs. non treated cells were used to identify Aldefluor positive cells. Median fluorescence intensity (MFI) was derived from each sample and the baseline fluorescence, used to identify the Aldefluor positive and negative populations, was calculated by the difference between the MFI of sample containing BAAA and the MFI of the same sample containing also DEAB. Sorting of the populations of interest was performed on ARIA II cell sorter (Beckton Dickinson) to near purity (85%). CD44/CD24 flow cytometric analysis was performed with mouse anti-human PE conjugated anti-CD44 and FITC conjugated anti-CD24 antibodies (BD).

**Plasmids, transfection, retroviral and lentiviral transfection**

For DNA trasfection Lipofectamine 2000 (Invitrogen) was used and for siRNA trasfection double-stranded RNA oligos (10pmol/cm²) were transfected using Lipofectamine RNAiMax (Invitrogen) according to manufacturer’s instructions. Retroviruses were made by transient trasfection of 293GP packaging cells with the appropriate plasmids with pMD2-ENV coding for envelope proteins, using standard calcium-phosphate metod. After 48h incubation at 32°C, the supernatants containing viral particles were collected and infection was performed as described (Rustighi et al., 2009) pCDNA3-N1-ICD-myc, pCDNA3-N1-ICD-deltaPEST(d2444)-myc, pCDNA3-N1-ICD-T2512A-myc, were generated by PCR and standard cloning procedures starting from pCDNA3-NdeltaE-myc (Rustighi et al., 2009) constructs as templates. pCDNA3-HA-Pin1 and pCDNA3-HA-S67E, retroviral pLPC-HA-Pin1, pCDNA3-HA-Pin1r, were already described (Rustighi et al., 200; Girardini et al., 2011). Retroviral pMSCV-3X-Flag-Fbxw7α was obtained by PCR and subcloning from p3X-Flag-Fbxw7α coding for the human alpha isoform. pCDNA4-N4-ICD-HA was a kind gift of I. Prudovsky and was already described (MacKenzie et al., 2004). For lentiviral infection an shPin1 corresponding to the siRNA sequence Pin1#1 was cloned into the Doxycyclin-inducible pLKO-Tet-ON (Addgene) following the “All-in-one” system described by D.Wiederschain (dmitri.wiederschain@novartis.com). For preparation of viral particles psPAX2 and pMD2.G were used in HEK 293T cells.
Material and Methods

Oligonucleotides for cloning and mutagenesis:

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siRNA sequences:

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</table>

Mammosphere cultures

To obtain mammospheres, cells from monolayer cultures were enzymatically disaggregated (0.05% trypsin–EDTA, Gibco) to a single cell suspension, passed though a 40 μm cell strainer (BD Falcon), plated at clonogenic density (2500 cells/cm2), and grown in nonadherent culture conditions, as described (Dontu et al, 2003). In detail, cells were grown for 7–10 days in DMEM:F12 (1:1) supplemented with B27 (Invitrogen Corporation, Carlsbad, CA, USA), 20 ng/ml EGF (PROSPEC, East Brunswick, NJ, USA), 20 ng/ml bFGF (BD Biosciences, San Jose, CA, USA), 4 μg/ml heparin (StemCell Technologies Inc.), 0.5 μg/ml hydrocortisone (Sigma) and 5 μg/ml Insulin (Sigma) in low attachment 24 or 96 well plates (Coroning) in a humified incubator at 37°C, 5% CO2. Primary mammospheres (≥200 μm) were obtained, collected, counted and again enzymatically disaggregated as above to re-plate cells at clonogenic densities to obtain secondary mammospheres. The same procedure was applied starting from secondary mammospheres to proceed to tertiary and quaternary mammospheres. Percentages of mammosphere forming efficiencies (%MFE) were calculated as number of mammospheres divided by the plated cell number and multiplied by a hundred. Mammospheres were counted with a 20× objective on an Olympus CK30 microscope (Olympus Italia Srl, Milan, Italy).

Quantitative Real Time PCR (qRT–PCR) analysis

Total RNA from cell lines and xenografts was extracted with QIAzol Lysis Reagent (Qiagen Srl-Italy, Milan, Italy). Total RNA from formalin-fixed, paraffin-embedded samples of breast cancer patients was extracted starting from 2 to 3 20 μm slices with the HighPure RNA paraffin Kit (Roche SpA, Monza,
Italy) and cDNA was transcribed with QuantiTect (Qiagen) in accordance with the manufacturer’s protocols, then amplified on a StepOne Plus cycler (Applied Biosystems, Life Technologies Europe BV, Monza, Italy), using SYBR Green Universal PCR Master Mix (Applied Biosystems). Histone H3 and GAPDH mRNA were used as internal controls.

Oligonucleotides for quantitative real-time PCR:

All oligonucleotides were supplied by MWG.

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Antibodies for Western blot, Far Western, Immunoprecipitations, and Immunohistochemistry.

The following antibodies were used: rabbit and goat polyclonal anti-Notch1 (C-20: sc-6014, and S-20: sc-23304, SantaCruz), rabbit polyclonal anti-N1-ICD Val1744 (#2421, Cell Signalling), rabbit polyclonal anti-N4-ICD Val1432 (SAB4502023, Sigma), rabbit polyclonal anti-Notch4 (H-225, sc-5594), rabbit polyclonal anti-Pin1 (Rustighi et al., 2009) and mouse monoclonal anti-Pin1 (G-8: sc-46660, Santa Cruz), rabbit polyclonal anti-HES-1 (#AB5702, Millipore), rabbit polyclonal anti-Slug (#9585S, Cell Signaling), mouse monoclonal anti-Vimentin (ab8069, Abcam), mouse monoclonal anti-E-cadherin (610182, BD), rabbit monoclonal anti-cleaved Caspase-3 (5A1E, Cell Signaling), rabbit polyclonal anti-Mcl-1 (S-19: sc-
819, Santa Cruz), for immunoprecipitation and Western blot of endogenous Fbxw7α mouse monoclonal (Abnova MO2, 3D1) and rabbit polyclonal (Abcam ab12292) antibodies were used, respectively. For tagged proteins rabbit polyclonal and mouse monoclonal (9B11) anti-myc (#2272 and #2276 respectively, Cell Signaling), mouse monoclonal anti-Flag clone M2 (F3165, Sigma), anti-GFP rabbit polyclonal serum was raised against GST-GFP fusion protein expressed in bacteria, affinity purified and used 1:1000, mouse monoclonal (12CA5) and rabbit polyclonal (Y-11, sc-805 Santa Cruz) anti-HA. For immunohistochemical stainings anti-cleaved Notch1 (Abcam 8925) anti-Notch1 (Santa-Cruz C-20), anti-Notch4 (Santa Cruz H-225), anti-Fbxw7α (Abnova MO2, 3D1), and anti-Pin1 home made anti-rabbit (Rustighi et al. 2009) were used.

**Western blot, in vitro binding, immunoprecipitation.**

In vitro binding assays, immuno- and co-immunoprecipitations and Western blot analyses were performed by standard procedures, as described (Rustighi et al., 2009). Briefly, for GST and GST-Pin1 pull-down analysis using cells were lysed in GST pull-down buffer (150 mM NaCl, 50 mM Tris/HCl pH 7.5, 10% glycerol, 0.1% Nonidet P-40, Sigma), supplemented with inhibitors of phosphatase (1mM sodium orthovanadate, 5mM NaF, Sigma) and protease (phenylmethylsulfonylfluoride (PMSF) 1mM and chymostatin, leupeptin, antipain, pepstatin 10 µg ml-1 each, Sigma). For Notch1 immunoprecipitations cells were treated in all cases with proteasome inhibitor lactacystin for 8 hrs and collected in GST pull-down buffer as above. 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 (Sigma), 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). For β-mercaptoethanol stripping of the primary antibody membranes were shortly boiled in 0.1 mM β-mercaptoethanol, 1% SDS, 50 mM Tris/HCl pH 6.9, then washed by shaking at RT with PBS, followed by blocking in Bлотto-tween (PBS, 0.2% Tween-20, not fat dry milk 5%) or with TBST (Tris/HCl 25 mM pH7.5) plus 5% BSA (Sigma) depending on the antibody.

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.

**Immunohistochemical analyses.**

Mammary tissue from Pin1+/+ and Pin1−/− mice was collected, formalin fixed and embedded in paraffin. 3 µm sections were stained with anti-Notch1 (Santa Cruz C-20, 1:50), anti-Notch4 (Santa Cruz H-225, 1:100), anti-Fbxw7α (Abnova MO2, 3D1, 1:250), and anti-Pin1 (home made anti-rabbit, 1:200, Rustighi et al. 2009) antibodies. For immunohistochemical analysis of human breast cancers, anti-cleaved Notch1
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(Abcam 8925, 1:200), anti-Pin1 (Santa Cruz mouse monoclonal G-8: sc-46660) and the abovementioned anti-Fbxw7α antibody were used. Briefly, stainings were performed according to standard procedures for paraffin embedded tissue. Slides were deparaffinized, rehydrated and antigen retrieved in a calibrated steam pressure cooker with citrate buffer (pH 6.0). Endogenous peroxidase was blocked with peroxidase block from an EnVision™ Kit (DakoCytomation, Glostrup, Denmark) for 15 minutes. The slides were blocked in 5% nonfat dry milk or PBS + 5% BSA for 20 minutes to 1 hour at room temperature to minimize nonspecific binding due to hydrophobic interaction. The slides were then incubated in blocking buffer without any antibody (negative control) or with the indicated primary antibodies for 1 hour at 37°C or overnight at 4°C. After washing, slides were incubated with secondary universal antibody (Vectastain) for 45 minutes at room temperature. Colorimetric detection was completed with diaminobenzidine and hydrogen peroxidase for 6 minutes and counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA). The immunostaining was scored semiquantitatively. In the evaluation of activated/cleaved Notch1 and Fbxw7α only nuclear staining was considered. The scores for IHC were: 0, no staining; 1, few nuclear staining of tumor cells (<5% of positive tumor cells); 2, 5-10% of positive tumor cells; 3, >10% positive tumor cells. For pairwise comparisons, the scores were collapsed to low (score, 1–2) versus high (score, 3) expression, excluding not interpretable samples.

Data acquisition, Image processing, Equipment and settings.

Western blot films were scanned with an Epson Stylus DX7450 scanner at a grey scale 600 d.p.i. resolution and saved as Tiff files. Single panels were cropped to obtain individual layers by Photpshop software. Densitometric values of protein levels in Western blot analyses were obtained by Image J software. Where necessary, for the sake of clarity, parts of the same or different gels with equal molecular weights were cropped and juxtaposed, demarcated with borders and indicated in the figure legend. Images of mouse and human immunohistochemical analyses were obtained using Leica DM4000B Microscope with DFC420C photocamera. Pictures of Lymph node metastases were taken with an Olympus Super bright Zoom Lens F1.8, C-4040200M, 4.1 Megapixel, 7.5 digital zoom. Images of haematoxylin and eosin stained sections of pulmonary metastases were obtained with an Olympus BX40 microscope and Leica DFC295 CH-9435 camera and were used for Computer-aided assessment of percentage of lung tissue area occupied by metastases. Microscope image files were obtained through Leica Application Suite LAS 4.1 software.

Breast cancer data collection and processing

We collected 21 datasets comprising microarray data of breast cancer samples and annotations on patients’ clinical outcome. All data were measured on Affymetrix arrays and have been downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). The complete list of datasets is provided in Table S1. Prior to analysis, we reorganized the datasets eliminating duplicate samples and samples without outcome
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information and renamed any original study after the medical center where patients were recruited. Briefly, the original studies have been modified as follows:

- Stockholm has been used as is and re-named as KI_Stockholm (Karolinska Institutet Stockholm);
- EMC-286 and EMC-58 have been merged into EMC-344 (Erasmus Medical Center);
- MSK has been used as is and re-named as MSKCC (Memorial Sloan-Kettering Cancer Center);
- Uppsala-Miller, Ivshina-Miller, and Loi datasets (GSE3494, GSE4922, and GSE6532) included samples derived from Uppsala University Hospital, John Radcliffe Hospital in Oxford, and Guys Hospital in London. Moreover, a comparison of the hybridization dates on the raw files and of the patients’ clinical information revealed that, although deposited twice, GSE3494 and GSE4922 were identical. As such, the 3 series have been split into KI_Uppsala, comprising all 253 unique patients of the Uppsala University Hospital, OXF composed of the 178 samples collected at the John Radcliffe Hospital in Oxford, and GUY composed of the 87 samples from the Guys Hospital in London;
- Sotiriou is entirely included in GSE6532;
- Desmedt has been used as is and renamed as TRANSBIG (after the consortium of cancer centers where samples have been collected);
- Schmidt has been used as is and re-named as Mainz (Johannes Gutenberg University in Mainz);
- Veridex has been used as is and re-named as Veridex_MultiCenter (after the three European and one US institutions where samples have been collected);
- Tamoxifen has been added to GUY;
- Chin and Zhou have been merged into UCSF (University of California, San Francisco) after removing samples deposited in both GEO (GSE7378) and ArrayExpress (E-TABM-158);
- TOP TRIAL has been re-named as IJB_TOP (Institut Jules Bordet /Trial of Principle) after removal of 13 samples lacking of outcome information;
- GSE19615 has been used as is and re-named as US_NCI (US National Cancer Institute);
- IPC has been re-named as CRCM (Centre de cancérologie de Marseille) after removal of 14 samples lacking of outcome information;
- KFSYSCC has been used as is and re-named as KOOF (Koo Foundation SYS Cancer Center);
- GSE31519 has been used as is and re-named as Goethe (Goethe-University, Frankfurt) after removal of 2 samples lacking of outcome information;
- Hatzis included samples derived from 4 cohorts, i.e., I-SPY-1 (Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and Molecular Analysis), LBJ_INEN_GEICAM (Lyndon B. Johnson Hospital, Instituto Nacional de Enfermedades Neoplásicas, and Grupo Español de Investigación en Cáncer de Mama), USO-02103 (US Oncology), and MDACC (M. D. Anderson Cancer Center, Houston). The original data has been split into I-SPY-1 comprising 83 samples, LBJ_INEN_GEICAM comprising 58 samples, MDACC comprising 313 samples, and USO-02103 comprising 54 samples. This re-organization resulted in a meta-dataset comprising 3254 unique samples from 19 independent cohorts (Table S1). According to Cordenonsi et al. (2011), we standardized clinical information among
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the various datasets redefining the outcome descriptions based on the clinical annotations of each individual study. Specifically, we defined survival as death because of cancer and includes overall survival, disease free survival, and disease specific survival. Raw expression data (i.e., CEL files) obtained from different platforms have been integrated using an approach inspired by geometry and probe content of HG-U133 Affymetrix arrays (Fallarino et al., 2010). Briefly, probes with the same oligonucleotide sequence, but located at different coordinates on different type of arrays, may be arranged in a virtual platform grid. As for any other microarray geometry, this virtual grid may be used as a reference to create a virtual Chip Definition File (virtual-CDF), containing the probes shared among the various HG-U133 platforms and their coordinates on the virtual platform, and a virtual-CEL files containing the fluorescence intensities of the original CEL files properly re-mapped on the virtual grid. Once defined the virtual platform through the creation of the virtual-CDF and transformed the CEL files into virtual-CELS, raw data, originally obtained from different HG-U133 arrays, are homogeneous in terms of platform and can be preprocessed and normalized adopting standard approaches, as RMA (Irizarry et al., 2003). Specifically, expression values were generated from intensity signals using the virtual-CDF, obtained merging HG-U133A, HG-U133AAofAV2, and HG-U133 Plus2 original CDFs, and the transformed virtual-CEL files. Intensity values for a total of 21981 meta-probe sets have been background adjusted, normalized using quantile normalization, and gene expression levels calculated using median polish summarization (RMA). The entire procedure has been implemented as an R script.

To identify two groups of tumor samples with either high or low levels of the Notch-dependent Direct Target (NDT) gene signature we used the classifier described in Adorno et al (2009). Briefly, we defined a classification rule based on summarizing the standardized expression levels of each gene in NDT signature into a combined score with zero mean. Tumors were then classified as NDT signature Low if the combined score was negative and as NDT signature High if the combined score was positive. Similarly, we defined tumors as expressing high or low levels of Fbxw7 and Pin1 mRNA if the standardized expression signal of Fbxw7 and Pin1 probes was positive or negative, respectively. This classification was applied to log2 expression values obtained using RMA on the meta-dataset described above.

**Survival analysis**

To evaluate the prognostic value of the NDT signatures, we estimated, using the Kaplan-Meier method (Kalbfleisch and Prentice), the probabilities that patients would remain free of death (survival). To confirm these findings, the Kaplan-Meier curves were compared using the log-rank or Mantel-Haenszel test (Harrington and Fleming). P-values were calculated according to the standard normal asymptotic distribution. When comparing “NDT signature High” and “NDT signature Low” groups, the group with low NDT levels displayed a significantly higher probability (at a significance level $\alpha=5\times10^{-2}$) of a reduced survival (Figure S8B). Survival analysis and Kaplan-Meier plots were obtained using R
survcomp package. Kaplan-Meyer curves have been compared using the log-rank test of the surv_test function (coin R package).

Breast cancer re-organized cohorts comprised in the meta-dataset analyzed in this study.

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Notch-dependent Direct Target (NDT) gene signature.

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8. BIBLIOGRAPHY


Bibliography


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During the period of my PhD I have been collaborating in the following publication:


(*= these authors equally contributed to the work)
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