PTP1B Restrains Mammary Progenitor Cells Fate Commitment and Secretory Differentiation

Inauguraldissertation

Zur

Erlangung der Wuerde eines Doktors der Philosophie vorgelegt der

Philosophish-Naturwissenschaftlichen Fakultaet der Universitaet Basel

Von

Emanuela Milani

aus der Schweiz

Basel, 2013

Genehmigt von der Philosophish-Naturwissenschaftlichen Fakultät

Auf Antrag von:

Dr. Mohamed Bentires-Alj

Prof. Dr. Nancy E. Hynes

Prof. Dr. Gerhard Christophori

Dr. Robert Clarke

Basel, den 13. Dezember 2011

Prof. Dr. Martin Spiess

Dekan

TABLE OF CONTENTS

2. SUMMARY

Protein tyrosine phosphatase 1B (PTP1B) is a member of the classical tyrosine phosphatase family, which modulates diverse biological processes including proliferation, differentiation, apoptosis and migration. PTP1B is a major regulator of cell metabolism. It decreases insulin signaling by dephosphorylating the insulin receptor and IRS proteins, and attenuates leptin action by dephosphorylating JAK2. Identifying PTP1B as an important target in diabetes and obesity. PTP1B has received additional attention because several studies revealed a role for PTP1B in different types of cancer, including breast neoplasia. PTP1B is overexpressed in human breast cancer, and depletion of PTP1B delays mammary tumorigenesis in mouse models of mammary cancer induced by activated ERBB2. However, relatively little was known about the normal physiological role of PTP1B in the mammary gland before this study. PTP1B was suggested to play a role in normal mammary gland development by regulating the prolactin-mediated activation of STAT5, a key regulator of mammary gland development and differentiation. While informative, these findings were based on knockdown experiments in breast cancer cell lines, which may not accurately reflect the *in vivo* situation. Therefore, in this study we took advantage of a PTP1B knockout mouse model to gain a deeper knowledge on the role of this phosphatase plays in mammary cell fate and mammary gland development.

We found that loss of PTP1B increases the number of mammary alveolar progenitors, enhancing the pool of cells able to generate alveolar structures during pregnancy. Consistently, we observed an increase in alveolar density in glands from PTP1B$^{-/-}$ mice. We also found that deletion of PTP1B increased expression of milk proteins during late pregnancy. We showed that these phenotypes are mediated by precocious phosphorylation and activation of the transcription factor STAT5 and/or by increased expression of the progesterone receptor, two key regulators of mammary gland development and differentiation. These observations provide new insight into the signaling cascades that regulate mammary stem/progenitor cells differentiation and lineage commitment during mammary gland development.

In summary, we identified new functions for PTP1B in mammary gland alveologenesis and lactogenesis. The absence of PTP1B protects from, or delays HER2/Neu evoked mammary tumors in mice. Our studies raise the possibility that the change in mammary cell fate seen in glands lacking PTP1B may contribute to the observed cancer protective effect, a question that warrant further investigation.

3. INTRODUCTION

3.1 Mammary gland development and breast cancer

The mammary gland consists of a tree-like structure composed of hollow ducts and alveoli. It is composed of several cell types organized in two main compartments: the epithelium composed of luminal epithelial and myoepithelial cells forming the bilayered ductal lobular network; and the stroma, consisting mainly of adipocytes and fibroblasts. The mammary gland is the only organ that undergoes most of its development after birth and goes through repeated cycles of proliferation, differentiation and apoptosis during each round of pregnancy. These characteristics make the mouse mammary gland a suitable organ for biological research on developmental processes. Epidemiological studies have shown that early menarche, late menopause and late age of first pregnancy are all risk factors for developing sporadic breast cancer (Kampert, Whittemore et al. 1988). Clearly the hormonal milieu and breast development cycles, probably through changes in the differentiation state of breast stem/progenitor cells, affect the susceptibility of the breast to oncogenic transformation. Therefore, understanding the mechanisms regulating normal mammary gland development is of major importance for understanding breast tumorigenesis.

3.2 Mammary gland development

The human and rodent mammary gland essentially develops during three stages, in which different signaling networks are activated: embryonic, pubertal and adult (Fig 1). (Richert, Schwertfeger et al. 2000; Hennighausen and Robinson 2005; Brisken and O'Malley 2010)

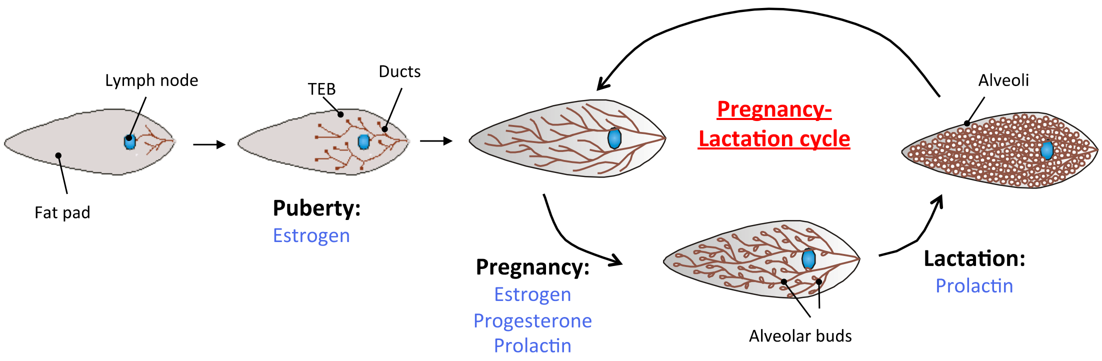


Figure 1. Stages of mouse mammary gland development. Schematic presentation of the different stages of mammary gland development and differentiation.

3.2.1 Embryonic and pubertal stages of mammary gland development

During embryogenesis a small, rudimentary ductal system arises as an appendix of the ventral skin, which grows after birth isometrically to the rest of the body until puberty. Several studies observed that the embryonic stage is independent of circulating hormones, but is regulated by a sequential and reciprocal cross-talk between the epithelium and the surrounding mesenchyme (Robinson 2007; Watson and Khaled 2008). During puberty the increased level of ovarian steroid hormones induces the formation of terminal end buds (TEBs) at the tips of the ducts. These are club-shaped structures with highly proliferative and migratory properties. The TEBs consist of an outer single layer of cap cells and an inner core formed by multiple layers of body cells. Under hormonal stimulation, the body cells proliferate inducing the elongation of the ducts in the surrounding stroma (also called fat pad) (Howlin, McBryan et al. 2006). Bifurcation of TEBs induces the formation of new primary ducts and together with lateral sprouting of secondary side-branches they fill the entire fat pad with a tree-like ductal network (Sternlicht 2006). This process is controlled by a number of paracrine, juxtacrine and autocrine factors; including hormones, growth factors, extracellular matrix molecules, matrix metalloproteases and morphogens (Sternlicht, Kouros-Mehr et al. 2006). A central role is played by the ovarian steroid hormone estrogen. Of the two estrogen receptor (ER) isoforms, ERα and ERβ, epithelial ERα is essential for ductal outgrowth, whereas ERβ and stromal ERα are dispensable for mammary gland development (Krege, Hodgin et al. 1998; Mallepell, Krust et al. 2006). $ER\alpha^{-/-}$ mice display an underdeveloped epithelial duct, but maintain the capacity for alveolar expansion during pregnancy (Bocchinfuso, Lindzey et al. 2000). ERα stimulates proliferation and morphogenesis in a paracrine fashion by inducing the expression of amphiregulin (AREG). AREG is a member of the epidermal growth factor receptor (EGFR) family of ligands that binds EGFR on neighboring cells stimulating their proliferation. Analysis of AREG-null mammary glands shows that AREG is essential for ductal elongation as well as estrogen-induced proliferation and terminal end bud formation (Ciarloni, Mallepell et al. 2007).

The adult mature virgin gland is essentially quiescent with the exception of the limited ductal side-branching and alveolar bud formation that occur as a result of the cyclic increase of ovarian hormones during the estrous cycle.

3.2.2 Pregnancy stages of mammary gland development

With the onset of pregnancy, extensive epithelial proliferation and tertiary side-branching occur with the formation and differentiation of a lobular alveolar system able to express and secrete milk during late pregnancy.

The complex and coordinated mechanisms governing the extensive morphological and cellular changes underlining mammary gland differentiation are mainly regulated by the steroid hormone progesterone, and the pituitary polypeptide hormone, prolactin.

Several studies demonstrated that progesterone induces extensive side-branching and formation of alveolar structures (process known as alveologenesis) during the early stages of pregnancy (Conneely, Mulac-Jericevic et al. 2007). Progesterone actions are mediated by the progesterone receptor (PR). In the absence of PR, the mammary gland displayed normal growth but failed to side branch and to form alveolar structures when exposed to hormonal stimuli (Lydon, DeMayo et al. 1995). PR occurs in two isoforms, PRA and PRB, which display differential transcription activities. Gene targeting experiments revealed that deletion of PRB displayed a phenotype similar to the complete PR mutant mammary gland, whereas absence of PRA did not impair mammary gland development. These results indicate PRB as the predominant mediator of progesterone action in mammary gland morphogenesis. The adult mammary gland displayed a nonuniform expression pattern of PR. It localizes in a scattered subset of epithelial cells along the ductal epithelium (Silberstein, Van Horn et al. 1996), indicating that not all epithelial cells are able to respond directly to progesterone. Analysis of cellular proliferation of the mammary gland in early pregnancy revealed that PR-positive cells located closely to proliferating epithelial cells (Russo, Ao et al. 1999), suggesting that progesterone-induced proliferation is mediated through paracrine activities. This observation was further confirmed by the elegant experiment in which a mixture of $PR^{-/-}$ and $PR^{+/+}$ cells was used to reconstitute wild type cleared fat pads. $PR^{-/-}$ cells, which by themselves cannot form side-branches and alveoli, can participate in the development of side-branches and alveoli when they are in close proximity to wild type cells (Brisken, Park et al. 1998). Therefore, PR seems to induce the expression of secreted factors, which induce the proliferation of neighboring cells. One candidate mediator of the progesterone response is WNT4, a secreted glycoprotein essential for regulating ductal branching (Brisken, Heineman et al. 2000). However, unlike PR-null mice, the morphogenic defects in WnNT4 knockout mice are overcome in late pregnancy, suggesting that additional progesterone downstream targets exist to mediate progesterone-response in the mammary gland. A further candidate is the receptor activator of nuclear factor kB (NF-kB) ligand (RANK-L, also known as OPGL), a member of the tumor necrosis factor (TNF) superfamily, which plays an important role in osteoclast development (Kong, Yoshida et al. 1999). Deletion of RANK-L or its receptor Rank from the mammary gland attenuated alveologenesis resulting in a lactational defect (Fata, Kong et al. 2000), a phenotype similar to PR knockout mice. RANK-L was proposed to mediate the parity-induced morphological changes by inducing activation of NF-kB and expression of cyclin D1 (Cao, Bonizzi et al. 2001; Fernandez-Valdivia, Mukherjee et al. 2009; Beleut, Rajaram et al. 2010). RANK-L expression was also shown to be regulated by the second key player of mammary gland differentiation: prolactin (PRL) (Ormandy, Naylor et al. 2003), revealing the existence of a complex interaction between different signaling pathways in the regulation of the extensive morphological changes underlining mammary gland differentiation. PRL is a peptide hormone expressed mainly by the pituitary gland (Freeman, Kanyicska et al. 2000), but also by several extrapituitary sites such as mammary gland, placenta and uterus (Ben-Jonathan, Mershon et al. 1996). Prolactin signaling is mainly mediated via the PrlR-JAK2-STAT5 pathway (Gouilleux, Wakao et al. 1994; Liu, Robinson et al. 1995). PRL binds and activates the prolactin receptor (PrlR), a class I cytokine receptor superfamily member (Boutin, Jolicoeur et al. 1988), which will then dimerize and lead to the activation of the associated kinase JAK2. JAK2 in turn phosphorylates the signal transducers and activators of transcription 5 (STAT5), which will dimerize and translocate to the nucleus where it can induce the transcription of genes involved in alveolar morphogenesis or lactation like the milk protein β-casein (Wartmann, Cella et al. 1996).

The role of PRL during mammary gland development and differentiation has been explored using PRL and PrlR knockout mouse models. The development of the mammary glands in these models exhibits normal proliferation, ductal elongation and bifurcation during puberty, suggesting that PRL and PrlR are not required for these morphological changes. However, ductal side branching, alveologenesis and lactogenesis are severely disrupted in both PRL$^{-/-}$ and PrlR$^{-/-}$ mammary glands (Horseman, Zhao et al. 1997; Ormandy, Camus et al. 1997; Brisken, Kaur et al. 1999; Vomachka, Pratt et al. 2000). Furthermore, JAK2 and STAT5 knockout mice phenocopied the failed lobuloalveolar development observed in PRL$^{-/-}$ and PrlR$^{-/-}$ (Liu, Robinson et al. 1997; Wagner, Krempler et al. 2004), indicating an essential role for the PRL-JAK2-STAT5 pathway in alveolar morphogenesis and milk secretion in the mammary gland.

The use of microarray technology allowed the identification of several PRL transcription targets shedding light on the complex regulatory network induced by

PRL signaling pathway (Ormandy, Naylor et al. 2003; Harris, Stanford et al. 2006). Among the different targets found to be regulated by the PRL pathway are the well known RANK-L and amphiregulin, the transcription factors GATA3 and ELF5 shown to regulate luminal cell differentiation (topic developed in the next session) and connexin 26, component of the gap junction complex shown to be necessary to the integrity of the forming alveoli. Signaling via the PRL-JAK2-STAT5 pathway culminates with the expression and secretion of milk proteins. Signaling via this pathway is, therefore, highly regulated by different enhancers and inhibitors acting to modulate PRL signaling. ERBB4 and β1-integrin have been shown to act as enhancer by regulating STAT5 phosphorylation (Long, Wagner et al. 2003; Naylor, Li et al. 2005). Negative feedback regulation is provided by members of the suppressors of cytokine signaling (SOCS) family of proteins, the expression of which is induced by PRL and acts by inhibiting the PRL signaling pathway via different mechanisms (Oakes, Rogers et al. 2008). Finally, Caveolin 1 was discovered to abrogate PRL signaling pathway by sequestering JAK2, such that it can no longer activate STAT5 (Park, Lee et al. 2002). Although different studies enabled the elucidation of several parts of the complex signaling network coordinating mammary gland differentiation, the complete picture underlying the molecular and cellular events is not completely understood.

3.2.3 Lactation and involution of the mammary gland

During lactation the expressed milk is secreted in the lumen of the alveoli allowing the feeding of the young (Neville, McFadden et al. 2002; Rudolph, McManaman et al. 2007). When the pups are weaned, the loss of suckling stimuli and milk stasis induce a complex remodeling program called involution (Green and Streuli 2004; Watson 2006). The initial phase of involution is characterized by a reversible induction of apoptosis of differentiated epithelial cells. After a prolonged absence of suckling, apoptosis is accompanied by the collapse of the alveolar structures, and an irreversible remodeling of the mammary gland into a virgin-like ductal structure (Lund, Romer et al. 1996). The involuted gland is now ready for a next round of pregnancy.

3.3 The mammary gland cellular hierarchy

The extensive regeneration potential of the mammary gland with each round of pregnancy suggested the presence of stem cell activity within this organ. Adult stem cells are long-lived, quiescent cells that are able to self-renew, maintaining the stem cell pool, and to give rise to a variety of differentiated cells (Reya, Morrison et al. 2001; Molofsky, Pardal et al. 2004). The first hints of the existence of a mammary stem cell (MaSC) were discovered around 50 years ago by DeOme and colleagues (Deome, Faulkin et al. 1959) with the observation that transplantation of tissue fragments of epithelium isolated from different regions of the mammary gland were able to reconstitute a complete, functional mammary ductal tree containing both epithelial and myoepithelial cells. Further studies demonstrated that MaSCs were present in all portions of the ductal network, independently of the developmental stage of the mammary gland (Smith and Medina 1988). Limiting dilution experiments and retroviral marking of mammary epithelial cells (Smith 1996; Kordon and Smith 1998) suggested the existence, besides of multipotent stem cells, of two further distinct progenitor cells with limited potential: the alveolar progenitors that give rise to the secretory lobules, and the ductal progenitors giving rise to the ductal network of the mammary gland. These results suggest the existence of a stem cell hierarchy in the mammary gland, which starts from stem cells with high proliferation, differentiation and self-renewal capacities, progressing via intermediate progenitor cells and ending in the differentiated epithelial cells, with limited self-renewal potential. Considerable progresses have recently been made in the isolation and characterization of functional MaSCs. Three complementary studies identified surface markers: CD24 (heat stable antigen), CD29 (β-integrin) and CD49f (α6-integrin), that are able to phenotype and functionally isolate single cells competent to reconstitute a complete, functional mammary gland when transplanted into cleared fat pad of recipient mice (Shackleton, Vaillant et al. 2006; Sleeman, Kendrick et al. 2006; Stingl, Eirew et al. 2006). Further characterization of these cells revealed that MaSCs belong to the basal compartment of the epithelium (showing low expression of CD24 like the basal myoepithelial cells) and that they were negative for the expression of ERα (Sleeman, Kendrick et al. 2007). These studies demonstrated that a population enriched for MaSCs is present in the mammary gland and is characterized by low expression of CD24 and high expression of CD29 and/or CD49f ($CD24^{+}CD29^{hi}$ or $CD24^{+}CD49f^{hi}$ cells). These and other studies provided evidence for a hierarchical model of epithelial cells in which MaSCs give rise to progressively restricted progenitors that culminate in the two differentiated epithelial and myoepithelial cells. Recent studies enabled the isolation and characterization of distinct progenitor cells by means of different surface markers. CD61 (β3-integrin) was shown to be a luminal epithelial progenitor marker (Asselin-Labat, Sutherland et al. 2007), c-Kit (CD117) marks early stage progenitor cells in the basal compartment and highly proliferative progenitor cells in the luminal compartment (Regan, Kendrick et al. 2011), and ALDH was shown to stain stem/progenitor cells (Ginestier, Hur et al. 2007).

Various models exist in the field for the mammary stem cell hierarchy. A possible model suggested by Visvader et al. proposed the existence of myoepithelial progenitors that give rise to myoepithelial cells, and a common luminal progenitor population that give rise to ductal luminal and alveolar luminal epithelial cells (Fig 2) (Visvader and Lindeman 2006; Asselin-Labat, Sutherland et al. 2007). Smith and coworkers suggested the existence of alveolar progenitors and ductal progenitors that give rise to both epithelial and myoepithelial cells in the two different structures of the mammary gland (Smith 1996). These models are not mutually exclusive; however, further studies are needed to understand how these findings can be combined in a common mammary stem cell hierarchy.

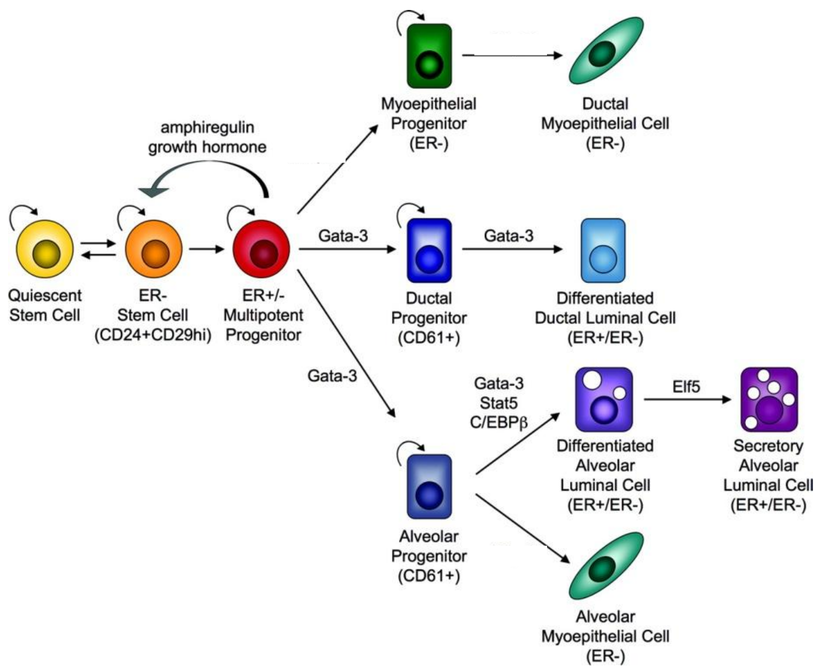


Figure 2. Mammary stem cell hierarchy. Regulation of mammary stem cells by hormones, growth factors, and transcription factors during lineage commitment. (adapted from LaMarca and Rosen 2008).

Several studies revealed that lineage commitment within the mammary stem cell hierarchy seems to be regulated by the action of different transcription factors.

GATA3 and ELF5 were shown to regulate luminal cell differentiation in the mammary gland (Asselin-Labat, Sutherland et al. 2007; Oakes, Naylor et al. 2008). Furthermore recent studies have shown that STAT5, besides its well known role in secretory differentiation and milk expression, also plays a crucial role in the establishment of luminal alveolar progenitor cells (Yamaji, Na et al. 2009). Loss of STAT5 resulted in a failure of the primitive SC/progenitor cells to generate alveolar luminal progenitor cells visible by a reduction in the proportion of luminal progenitor cells ($CD61^+$ cells) at the virgin stage. Although progress has been made in the elucidation of the MaSC hierarchy, the precise mechanisms that regulate stem/progenitor differentiation and lineage commitment during mammary gland development remain elusive. It is important to deeper understand the signaling mechanisms involved in cell fate commitment in order to determine how these signals are altered in mammary gland tumorigenesis.

3.4 Breast cancer

Breast cancer is the most frequently diagnosed cancer in women (Ferlay, Autier et al. 2007; Jemal, Bray et al. 2011) with an estimated 1.4 million new cancer cases diagnosed per year worldwide (23% of all cancers) (Jemal, Bray et al. 2011). Breast cancer arises from the epithelial compartment of the mammary gland and progresses into hyperplasia, atypical hyperplasia, ductal carcinoma *in situ* (DCIS) and invasive carcinoma (IDC) (Bombonati and Sgroi 2011). With each step the tumor becomes progressively aggressive and culminates in the often fatal invasive carcinoma were the epithelial cells invade the surrounding breast and metastasize to distant organs such as lung, bone, liver and brain (Nguyen, Bos et al. 2009).

Currently the different types of breast cancers are clinically characterized by the histological grade of the tumor (differentiation status), lymph node status and the expression of the hormone receptors ER, PR, and the tyrosine kinase receptor HER2 (ERBB2). However, due to the heterogeneity of breast cancers exhibiting different molecular alterations that drive their growth, survival and metastatic properties, the markers used are not exhaustive enough. Recently, genome-wide gene-expression profiles enabled the classification of breast cancer into 6 subgroups with different prognosis (Perou, Sorlie et al. 2000; Sorlie, Perou et al. 2001; Prat, Parker et al. 2010). The luminal A and luminal B subgroups account for 60% of breast cancers and are characterized by the expression of luminal markers like cytokeratin 8/18 and ERα with or without the coexpression of PR (Sims, Howell et al. 2007). A second subtype is the HER2-enriched breast cancer, characterized by the overexpression of HER2. It accounts for 20% of breast cancers and is associated with an aggressive disease and decreased survival (Slamon, Clark et al. 1987). The normal-like type of breast cancer shows an expression profile similar to normal breast samples. Finally the basal-like and claudin-low tumors, also known as triple-negative breast cancers (TNBC) due to the lack of expression of ER, PR and of amplification of HER2, account for 20% of the patients and correlate with the worst prognosis due also to the lack of effective therapy (Sims, Howell et al. 2007). This new classification of breast cancers enables a better prediction of tumor response to chemotherapy and opens the possibility to develop novel targeted therapies against specific subtypes of breast cancer.

3.5 How does mammary gland development and differentiation relate to human breast cancer?

Epidemiologic studies revealed that susceptibility of breast epithelium to develop a tumor is influenced by hormonal changes that affect normal mammary gland development (e.g. age of menarche, age of first pregnancy and age of menopause) (MacMahon, Cole et al. 1970; Brinton, Schairer et al. 1988; Kampert, Whittemore et al. 1988). These studies demonstrated that parous females have up to 50% reduction in lifespan breast cancer risk compared with nulliparous females, and that the timing of the first pregnancy plays a significant role in parity-induced protection. Several possible mechanisms mediating this protection have been suggested, including changes in the hormonal profile of parous women, or a more differentiated, and hence less susceptible mammary gland (Thordarson, Jin et al. 1995; D'Cruz, Moody et al. 2002; Sivaraman and Medina 2002; Russo, Moral et al. 2005). However, the molecular mechanisms underlying parity-induced protection remain elusive. Therefore, a deeper understanding of mammary gland differentiation is important to elucidate this effect.

In addition, the demonstration of the presence of a stem cell hierarchy in the mammary gland and human breast raised the hypothesis that the heterogeneity of breast cancers could be due to oncogenic transformation of cells with distinct differentiation states (Melchor and Benitez 2008). Therefore, investigation of the mechanisms regulating stem/progenitor cell renewal and commitment in normal mammary gland may provide new insights in breast cancer development.

Therefore, it is important to better understand the pathways regulating mammary gland development and cell fate commitment in normal tissue in order to shed light into tumorigenesis and breast cancer heterogeneity.

3.6 Protein-tyrosine phosphatases

Protein-tyrosine phosphorylation plays an important regulatory role in different biological processes including proliferation, differentiation, apoptosis and migration. Tyrosyl-phosphorylation is tightly regulated by the combined action of protein-tyrosine kinases (PTKs) and protein tyrosine-phosphatases (PTPs) (Fig 3). Not surprisingly, deregulated tyrosyl-phosphorylation underlies various diseases of deregulated growth and differentiation, including cancer.

Initially PTPs were thought to play simply a counteracting role to PTKs. However, it is now clear that they also play an active role in the regulation of many physiological processes. PTKs and PTPs act in a coordinated way in which PTKs regulate the amplitude of a signaling response, whereas PTPs are thought to play a role in controlling the rate and duration of a response (Heinrich, Neel et al. 2002; Hornberg, Bruggeman et al. 2005).

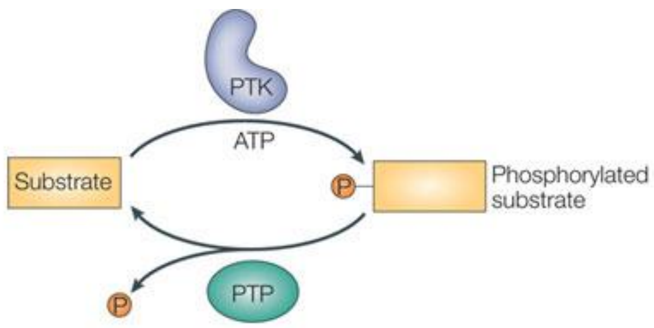


Figure 3. The coordinated action of PTKs and PTPs regulates tyrosine phosphorylation. Tyrosine phosphorylation is a key mechanism for signal transduction and for the regulation of many physiological

processes. Proteins are phosphorylated on tyrosine residues by PTKs and dephosphorylated by PTPs (Mustelin, Vang et al. 2005).

The human genome encodes ~107 PTPs that equal the diversity and complexity of PTKs (Alonso, Sasin et al. 2004). All members of the PTP family are characterized by the catalytic-site motif HCXXGXXR, which contains a conserved cysteine residue essential for catalysis (Andersen, Mortensen et al. 2001). Tyrosyl-phosphorylation occurs in a two-step mechanism. First the sulfur atom of the thiolate side chain of the cysteine nucleophilically attacks the phosphate while the conserved aspartic acid residue protonates the tyrosyl-leaving group of the substrate. This leads to the formation of a cystenil-phosphate catalytic intermediate, which will then be hydrolyzed by a glutamine and an aspartic residue, in the second step, causing the release of the phosphate group (Tonks 2003).

The PTP superfamily is divided into two categories: the classical phosphotyrosine-specific phosphatases, characterized by their specificity for phosphotyrosine residues; and the dual specificity phosphatases that can dephosphorylate phosphoserine/phosphothreonine residues as well as phosphotyrosine residues (Alonso, Sasin et al. 2004; Andersen, Jansen et al. 2004). The classical PTPs comprise 38 PTP members in rat and mouse, and 37 members in the human genome (OST-PTP is thought to be a pseudogene in human). This group can be further subdivided into two subgroups, the transmembrane receptor like proteins (RPTP) and the non-transmembrane, cytoplasmatic PTPs (Fig 4) (Andersen, Mortensen et al. 2001).

The transmembrane PTPs contain a variable extracellular domain responsible for cell-cell, cell-matrix and cell-ligand interaction, a single-pass transmembrane domain and an intracellular domain containing one or tandem PTP domains (with the major catalytic activity in the membrane-proximal domain) (Streuli, Krueger et al. 1990; Felberg and Johnson 1998). The cytoplasmatic PTPs are characterized by different non-catalytic, regulatory sequences important for the activity of the phosphatase; regulation mediated by direct interaction with the active site, by controlling substrate specificity or by controlling the subcellular localization of the phosphatase (Garton, Burnham et al. 1997; Pulido, Zuniga et al. 1998).

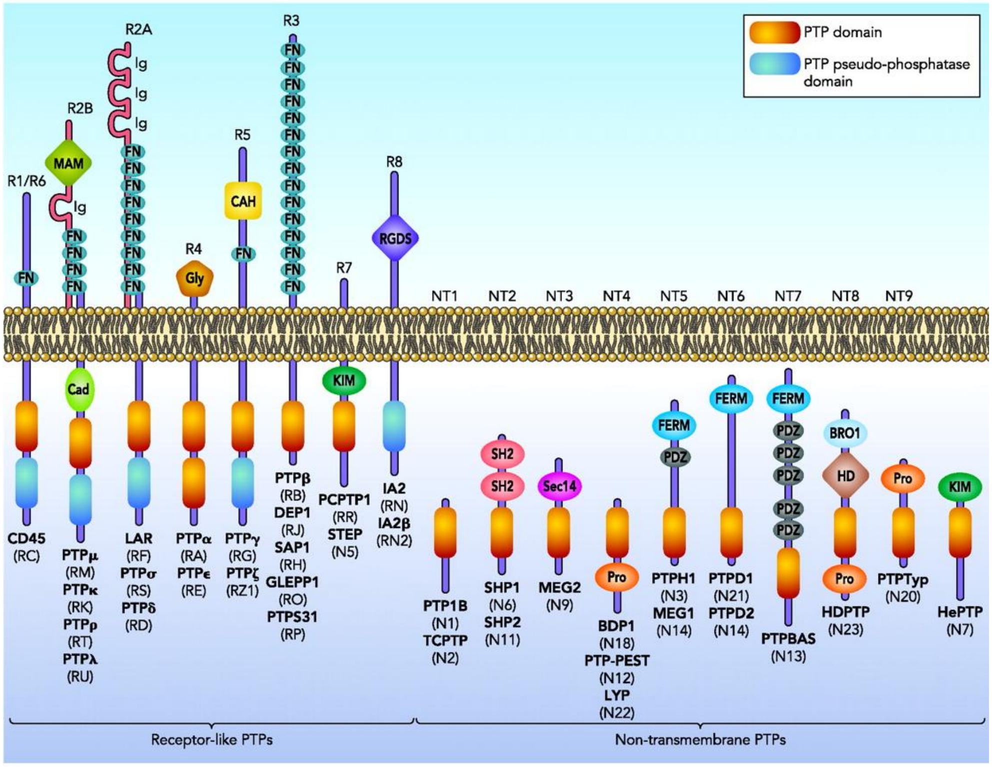


Figure 4. The family of classical PTPs. Classical PTPs can be categorized as transmembrane or non-transmembrane proteins (Soulsby and Bennett 2009).

3.7 Regulation of classical PTPs

Because the appropriate control of protein-tyrosine phosphorylation is essential for cellular homeostasis, PTPs are tightly regulated by a variety of mechanisms including gene expression, protein localization and post-translational modifications. With the last being a fast and often reversible mechanism to fine-tune tyrosine phosphorylation-dependent signaling pathways.

An important post-translational modification is reversible oxidation of PTPs (Meng, Fukada et al. 2002). The essential cysteine residue of the PTP catalytic motif is characterized by unusual low pKα (Groen, Lemeer et al. 2005; Salmeen and Barford 2005). Therefore, at neutral pH the cysteine is present predominantly as thiolate anion. This enhances the neutrophilic property of the cysteine and at the same time renders the PTP more susceptible to oxidation and thus to its inhibition. Reactive oxygen species (ROS), produced in a variety of physiological stimuli such as growth factors or antigen receptors, oxidize the thiolate anion of the essential cysteine into sulfenic acid (-SOH). In this form, the cysteine residue can no longer function as a nucleophile and therefore inhibits PTPs activity. Importantly, this oxidation and the concomitant inhibition of PTPs are reversible allowing a rapid and dynamic regulation of PTPs (Denu and Tanner 1998).

PTPs can be further regulated through phosphorylation, nitrosilation and/or sumoylation. Phosphorylation of tyrosine residues (e.g., in PTP1B, SHP1, SHP2 and PTPα) or serine residues (e.g., in PTPN12) affects their phosphatase activity and their affinity to substrates or interacting partners (Bennett, Tang et al. 1994; den Hertog, Tracy et al. 1994; Dadke, Kusari et al. 2001). In addition PTP1B can also be modified by the small ubiquitin-like modifier (SUMO), which was shown to decrease its activity (Dadke, Cotteret et al. 2007).

Proteolytic cleavage of regulatory domains of the PTPs is another post-translational mechanism suggested to regulate their activity. Upon calpain-mediated cleavage the catalytic activity of PTP1B, PTP-MEG1 and SHP1 were shown to be increased (Frangioni, Oda et al. 1993; Gu, Meng et al. 1996; Falet, Pain et al. 1998). In addition, the cleavage of the extracellular domain of the transmembrane PTPs like LAR, PTPκ and PTPμ was also shown to be important for regulating their phosphatase function (Streuli, Krueger et al. 1992; Anders, Mertins et al. 2006; Ruhe, Streit et al. 2006)

Transmembrane PTPs can be additionally regulated by binding to their ligands and/or by their dimerization. Ligand binding can have opposing effects on the phosphatase activity. For example the phosphatase function of LAR can be activated by binding to the transmembrane protein syndecan (Sdc) or inhibited by binding to the glycosylphosphatidylinositol-anchored protein dallylike (Dlp) (Fox and Zinn 2005; Johnson, Tenney et al. 2006).

3.8 PTPs and human disease

Several evidences have shown that members of the PTP superfamily are key components of tumorigenesis in various human cancers. PTPs were initially thought to act exclusively as tumor suppressors because of their counteracting role to PTKs, known to have relevant functions in cancer development. However, it is now clear that individual PTPs play also an active role in the regulation of many physiological processes; as such these PTPs may exert oncogenic functions. A well-known tumor suppressor among the 107 members of the PTP superfamily is the lipid phosphatase PTEN. PTEN was shown to dephosphorylate phosphatidylinositol(3,4,5)-trisphosphate (PIP_3) and thereby it opposes the activity of the oncogene PI3K. Somatic mutation analysis of human cancers showed a high frequency of mutations specifically for the PTEN gene resulting in an increased activity of the PI3K pathways associated with cell survival (Li, Yen et al. 1997; Myers, Pass et al. 1998; Wu, Senechal et al. 1998). Other PTPs have been suggested as tumor suppressors in breast cancer like PTPN12 (Sun, Aceto et al. 2011) PTPγ (Panagopoulos, Pandis et al. 1996; Zheng, Kulp et al. 2000) PTP-BAS (Bompard, Puech et al. 2002; Freiss, Bompard et al. 2004) MEG2 (Yuan, Wang et al.), GLEPP1 (Ramaswamy, Majumder et al. 2009) and PTPζ (Perez-Pinera, Garcia-Suarez et al. 2007) (for a detailed review (Ostman, Hellberg et al. 2006)).

By contrast, SHP2 (Chan, Kalaitzidis et al. 2008), PTPα (Ardini, Agresti et al. 2000; Zheng, Resnick et al. 2008), LAR (Yang, Zhang et al. 1999; Levea, McGary et al. 2000), PTPH1 (Zhi, Hou et al.) and PTP1B (discussed in section 3.11) were shown to have oncogenic role in breast cancers. SHP2 (PTPN11), for example, transduces mitogenic and pro-migratory signals from various types of receptors. SHP2 was shown to be hyperactivated either by mutations or downstream of oncoproteins in several types of tumors, like juvenile myelomonocytic leukemias, gastric carcinoma, anaplastic large cell lymphoma and glioblastoma (Tartaglia, Niemeyer et al. 2003; Chan, Kalaitzidis et al. 2008; Zhan, Counelis et al. 2009).

It is important to note that aberrant regulation of PTP-superfamily members is also associated with diseases other than cancer. Mutation of PTP Lyp (PTPN22) was shown to be a common risk factor for autoimmune diseases, including type I diabetes (Bottini, Musumeci et al. 2004). Early characterization of PTP1B illustrated its ability to antagonize insulin and leptin signaling pathways. Alteration in the level of expression or activity of PTP1B was shown to play a role in obesity and diabetes, waking up a great interest in the development of novel therapies against PTP1B to cure obesity and diabetes mellitus II.

In light of the implications of PTPs in several diseases it is of high importance to physiologically and functionally characterize the PTP superfamily in order to implement the development of PTP-based therapeutics.

3.9 Protein tyrosine phosphatase 1B

Protein tyrosine phosphatase 1B (PTP1B) was the first PTP to be purified (Tonks, Diltz et al. 1988) and the first, with its close homolog TC-PTP, to be cloned (Cool, Tonks et al. 1989; Chernoff, Schievella et al. 1990). PTP1B is the founding and prototype enzyme of the PTP superfamily; some futures of this family like structure, function and regulation were first discovered in the context of PTP1B and than proven for other members of this family.

PTP1B is a non-transmembrane, cytoplasmatic PTP encoded by the gene *PTPN1* located on chromosome 20 (region q13.1-q13.2) in human and on chromosome 2 (region H2-H3) in mouse (Brown-Shimer, Johnson et al. 1990; Forsell, Boie et al. 2000). PTP1B is a ubiquitously expressed 435-amino acid 50 kDa protein, composed by an N-terminal catalytic domain, two proline-rich motifs and a C-terminal hydrophobic region (Fig 5) (Stuible, Doody et al. 2008; Lessard, Stuible et al. 2010). The C-terminal hydrophobic domain anchors the protein to the cytoplasmic face of the endoplasmatic reticulum. The proline-rich motifs are binding sites for SH3-domain containing proteins such as p130CAS, GRB2 and many more. The N-terminal catalytic domain contains the active site with the essential cysteine amino acid involved in the dephosphorylation of PTP1B substrates. The crystal structure of this domain, the first to be solved for a member of the PTP superfamily (Barford, Flint et al. 1994), revealed a domain organization into eight α-helices and 12 β-strands with a 10-stranded mixed β-sheet. The signature motif, which forms the phosphate recognition site, is located at the base of a cleft, with the invariant aspartic acid and glutamine residues located on the side of the cleft. The depth of the cleft contributes to the specificity of PTP1B for phosphotyrosine substrates because the small phosphoserine and phosphothreonine residues are not able to reach the phosphate-binding site at the base of the cleft.

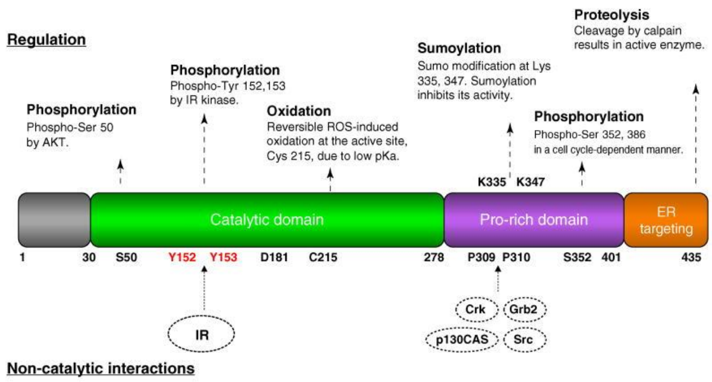


Figure 5. Structural domains and regulation of PTP1B. Schematic representation of the domain structure of PTP1B. Full-length human PTP1B is composed of an N-terminal catalytic domain (green) and C-terminal ER targeting domain (orange), flanking two proline-rich domains (purple), at least one of which is critical for protein–protein interactions. PTP1B is regulated by tyrosine phosphorylation, and serine phosphorylation at the indicated sites, oxidation of Cys215 at its active site, sumoylation at its PRD, and proteolysis by calpain (Yip, Saha et al. 2010).

The catalytic activity of PTP1B is tightly controlled. First, its localization to the ER may restrict its access to substrates often localized at the plasma membrane. Several studies suggested that PTP1B dephosphorylates ligand-activated receptor PTKs after their internalization (Haj, Verveer et al. 2002). Furthermore, PTP1B could directly dephosphorylate receptor PTKs at the plasma membrane due to the flexible nature of the endoplasmatic reticulum, which seems to extend under certain circumstance until the plasma membrane (Hernandez, Sala et al. 2006). PTP1B can be released from its location by calpain-mediated proteolytic cleavage. Depending on the context, PTP1B release allows it to access a larger set of substrates or to be targeted for degradation. PTP1B, like other PTPs, can be regulated by oxidation of the catalytic cysteine. Several external stimuli, including EGF, insulin and other growth factors, generate reactive oxygen species upon stimulation, which will lead to the oxidation and inactivation of PTP1B, thereby enhancing phosphotyrosine-mediated signaling (Meng, Buckley et al. 2004). PTP1B is further regulated by phosphorylation at multiple serine and tyrosine sites (Brautigan and Pinault 1993; Ravichandran, Chen et al. 2001; Tao, Malbon et al. 2001). However, the effects of phosphorylation on PTP1B activity are controversial. PTP1B can also be regulated by SUMO conjugation. Studies have shown that insulin transiently stimulates PTP1B sumoylation decreasing its dephosphorylation function (Dadke, Cotteret et al. 2007).

Besides post-translational modification PTP1B can be regulated at the transcriptional level. Several reports suggest that PTP1B levels are modulated by BCR-ABL, the tyrosine kinase responsible for chronic myelogenous leukemia (CML). BCR-ABL enhances PTP1B expression by binding to the p210 BCR-ABL responsive sequence (PRS) present in its promoter, in response to PTK stimulation (Fukada and Tonks 2001). A further candidate for the regulation of PTP1B expression is the transcription factor Y-box-binding protein 1 (YB-1), since analysis of PTP1B promoter characterized an enhancer sequence that serves as binding sites for YB-1 (Fukada and Tonks 2003). PTP1B mRNA levels are modulated in various types of cancer, for example it is downregulated in oesophageal cancer lesions and overexpressed in epithelial carcinomas, ovarian carcinomas and human breast carcinoma cells (reviewed in Dube and Tremblay 2004).

3.10 Substrates of PTP1B

Numerous substrates have been identified for PTP1B, involved in multiple cell processes such as glucose uptake, proliferation, differentiation, apoptosis, cell-cell adhesion, extracellular matrix attachment, motility and invasion (reviewed by (Stuible, Doody et al. 2008). They include receptor PTKs like EGFR (Flint, Tiganis et al. 1997; Haj, Markova et al. 2003), PDGFR (Haj, Markova et al. 2003; Dube, Cheng et al. 2004), IR and IGF-1R (Buckley, Cheng et al. 2002). Intracellular PTKs such as SRC (Bjorge, Pang et al. 2000), BCR-ABL (LaMontagne, Hannon et al. 1998), JAK2 and TYK2 (Myers, Andersen et al. 2001) are also substrate of PTP1B. In addition, adapter proteins for example p130CAS, cytoskeletal proteins like β-catenin (Balsamo, Leung et al. 1996) and transcription factors like STAT5 (Johnson, Peck et al. 2010) have also been shown to be targets of PTP1B. The identification of PTP1B substrates was achieved by the use of substrate trapping mutant, in which substitution of a cysteine in the catalytic center (Cys 215 for PTP1B) or the invariant asparagine (Asp 181 in PTP1B) generates a form of PTP1B that maintains a high affinity for substrates but doesn’t have catalytic activity (Flint, Tiganis et al. 1997). This mutant can form stable enzyme-substrate complexes, which allow the identification of substrates by immunoprecipitation. Single substrates were further validated by gene-targeting techniques *in vitro* and/or *in vivo*. One of the best understood PTP1B substrates are the insulin receptor (IR) and JAK2 proteins (Fig 6). Several *in vitro* and *in vivo* studies demonstrated that PTP1B regulates metabolic processes maintaining glucose homeostasis and body mass by dephosphorylating and inhibiting IR and JAK2 (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000). PTP1B knockout mice are healthy and exhibit insulin sensitivity, correlating with a decreased level of circulating insulin and an increased IR phosphorylation. They are lean and resistant to high fat diet-induced obesity, suggesting the involvement of PTP1B in the regulation of the leptin signaling. Furthermore, they are hypersensitive to leptin treatment, an effect associated with elevated phosphorylation and activation of JAK2 and its target STAT3 (Cheng, Uetani et al. 2002; Zabolotny, Bence-Hanulec et al. 2002).

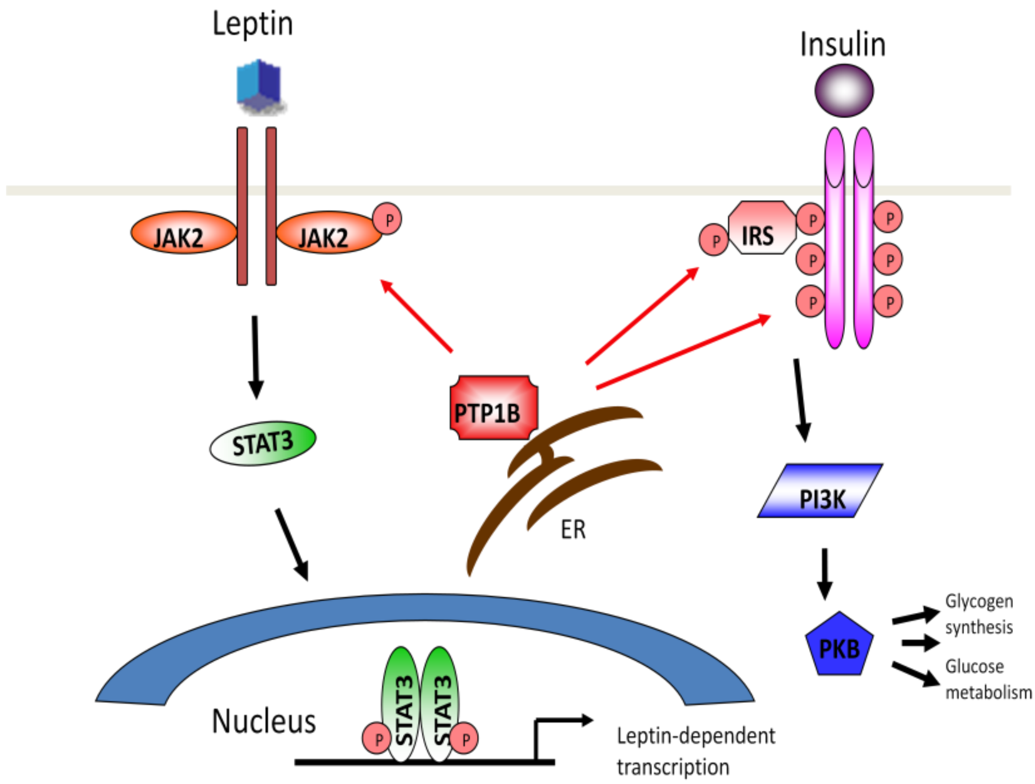


Fig 6. Metabolic signaling of PTP1B. PTP1B attenuates insulin signaling by dephosphorylating IR and IRS-1 and the leptin pathway by dephosphorylating JAK2.

In addition to its negative role on the IR, PTP1B can also dephosphorylate other receptor PTKs, including the EGF and platelet-derived growth factor (PDGF) receptors. Studies using fluorescence resonance energy transfer demonstrated that PTP1B dephosphorylated EGF and PDGF receptors after their internalization by endocytosis (Haj, Verveer et al. 2002), suggesting a role for PTP1B in the termination of the ligand-induced receptor PTK signaling. Further important substrates were found in PTP1B$^{-/-}$ mouse embryonic fibroblast cells (MEFs), including p62DOK and p120RasGAP which are known to regulate proliferation (Dube, Cheng et al. 2004).

The enzymatic substrates network of PTP1B is rendered more complex by the finding that, besides acting as an inhibitor of proliferative and metabolic signals, it can also have a positive signaling role in proliferation and other pathways. Indeed, PTP1B was shown to activate endogenous SRC by dephosphorylating the inhibitory tyrosine Y527 in breast and colon cancer cells (Bjorge, Pang et al. 2000; Arias-Romero, Saha et al. 2009). This activation was shown to promote growth and invasion of cancer cells suggesting a role for PTP1B as an oncogene.

Although increasing progresses have been made to characterize the substrates of PTP1B, the network of interactions and enzymatic substrates that have been discovered are not sufficient to fully explain the physiological phenotype observed by modulating PTP1B levels *in vitro* and *in vivo*. This is rendered difficult because PTP1B was shown to function in a tissue specific manner and because of its dual role in signaling, acting as signal activator or as signal inhibitor. Therefore, further studies are needed to delineate the signaling pathways regulated by PTP1B.

3.11 PTP1B and oncogenesis: tumor suppressor or oncogene?

Genetic analysis of human and mouse models of cancer revealed no loss of function or gene silencing of PTP1B gene. However, several evidences support the notion that PTP1B is a negative regulator of cellular growth. PTP1B plays an important role in dephosphorylating receptor PTKs known to be involved in oncogenesis. Studies in immortalized fibroblast revealed that exogenous expression of PTP1B prevents transformation by v-src, v-ras, v-crk, and Bcr-Abl (Woodford-Thomas, Rhodes et al. 1992; LaMontagne, Hannon et al. 1998; Liu, Sells et al. 1998). Furthermore, PTP1B was shown to promote apoptosis by increasing IRE1-mediated endoplasmic reticulum stress signaling (Gu, Nguyen et al. 2004), by downregulating pro-survival receptor PTKs (Gonzalez-Rodriguez, Escribano et al. 2007) or by facilitating the activity of Caspase 8/9 (Akasaki, Liu et al. 2006; Sangwan, Paliouras et al. 2006). All these observations suggest PTP1B as a tumor suppressor. The detection of high PTP1B expression in human tumors, most notably breast and ovarian cancers (Wiener, Hurteau et al. 1994; Wiener, Kerns et al. 1994), suggested a role for this protein in tumorigenesis. A breast cancer study suggested overexpression of PTP1B in 38% of the cases and that the expression of PTP1B positively correlated with HER2 expression, a well known oncogenic PTK frequently amplified in a subset of breast tumors (Wiener, Kerns et al. 1994). This correlation suggests a collaboration between these two proteins in tumorigenesis. Indeed, two independent groups discovered that a deficiency of PTP1B in a HER2/Neu-evoked breast cancer mouse model delayed tumor onset (Bentires-Alj and Neel 2007; Julien, Dube et al. 2007). Recent studies in our lab demonstrated that deletion of PTP1B specifically in the mammary epithelium delays HER2/NeuNT-induced breast cancer (Balavenkatraman, Aceto et al. 2011).

However, no effects on tumor progression were observed when the deletion of PTP1B occurred after the development of the breast cancer. These observations suggest a role for PTP1B in tumor onset but not in tumor growth progression, making PTP1B a therapeutic target for tumor prevention but not for treatment of advanced tumors.

In conclusion, PTP1B can act both as tumor suppressor and as oncogene depending on the substrate involved and the cellular context (Lessard, Stuible et al. 2010). The dual role of PTP1B in cancer highlights the need of a better understanding of its function prior to use PTP1B inhibitors to treat cancer.

4. RATIONALE OF THE WORK

The mouse mammary gland is a heterogeneous organ that undergoes most of its development after birth and goes through repeated cycles of proliferation, differentiation and apoptosis with every pregnancy. Although many studies were performed to elucidate the mechanism of mammary gland development and differentiation, the molecular and cellular events are not completely understood.

Elucidating the mechanisms regulating normal mammary gland development is of major importance to better understand the development of diseases like breast cancer. Recently, PTP1B has received major interest due to the discovery of its oncogenic role in breast cancer development, suggesting PTP1B as a new target for the treatment of breast cancer. This work aims at understanding the role of PTP1B in mammary gland development and differentiation. We addressed this question by studying:

a) The role of PTP1B in cell fate commitment:

Recent studies demonstrated the existence of a mammary stem cell hierarchy, which is tightly regulated in each stage of mammary gland development to guarantee the correct formation of a functional mammary gland. Several transcription factors were shown to play an important role in the regulation of the commitment of mammary stem cells to progenitor cells and finally into differentiated epithelial cells. One of these is STAT5, shown to be a downstream target of PTP1B *in vitro*, raising the question whether PTP1B also plays a role in cell fate commitment.

b) The role of PTP1B in mammary gland development:

Previous *in vitro* studies suggested a role for PTP1B in normal mammary gland development by regulating the prolactin-mediated activation of STAT5, a key regulator of mammary gland development and differentiation. While informative, these findings were based on knockdown experiments in breast cancer cell lines, which may not accurately reflect the *in vivo* situation. We therefore, used a genetically ablated PTP1B mouse model to gain a deeper knowledge of the role PTP1B plays in mammary gland development and to define the mechanism regulated by this phosphatase.

5. Results

Research article published in Development:

Protein tyrosine phosphatase 1B restrains mammary alveologenesis and secretory differentiation

Emanuela S. Milani, Heike Brinkhaus, Regula Dueggeli, Ina Klebba, Urs Mueller, Michael Stadler, Hubertus Kohler, Matthew J. Smalley and Mohamed Bentires-Alj

Tyrosine phosphorylation plays a fundamental role in mammary gland development. However, the role of specific tyrosine phosphatases in controlling mammary cell fate remains ill defined. We have identified protein tyrosine phosphatase 1B (PTP1B) as an essential regulator of alveologenesis and lactogenesis. PTP1B depletion increased the number of luminal mammary progenitors in nulliparous mice, leading to enhanced alveoli formation upon pregnancy. Mechanistically, *Ptp1b* deletion enhanced the expression of progesterone receptor and phosphorylation of Stat5, two key regulators of alveologenesis. Furthermore, glands from *Ptp1b* knockout mice exhibited increased expression of milk proteins during pregnancy due to enhanced Stat5 activation. These findings reveal that PTP1B constrains the number of mammary progenitors and thus prevents inappropriate onset of alveologenesis in early pregnancy. Moreover, PTP1B restrains the expression of milk proteins during pregnancy and thus prevents premature lactogenesis. Our work has implications for breast tumorigenesis because *Ptp1b* deletion has been shown to prevent or delay the onset of mammary tumors.

Development ePress online publication date 15 November 2012

Development 140, 117-125 (2013) doi:10.1242/dev.082941

Protein tyrosine phosphatase 1B restrains mammary alveologenesis and secretory differentiation

Emanuela S. Milani[1,2], Heike Brinkhaus[1], Regula Dueggeli[1], Ina Klebba[1], Urs Mueller[1], Michael Stadler[1,3], Hubertus Kohler[1], Matthew J. Smalley[4] and Mohamed Bentires-Alj[1,*]

SUMMARY

Tyrosine phosphorylation plays a fundamental role in mammary gland development. However, the role of specific tyrosine phosphatases in controlling mammary cell fate remains ill defined. We have identified protein tyrosine phosphatase 1B (PTP1B) as an essential regulator of alveologenesis and lactogenesis. PTP1B depletion increased the number of luminal mammary progenitors in nulliparous mice, leading to enhanced alveoli formation upon pregnancy. Mechanistically, *Ptp1b* deletion enhanced the expression of progesterone receptor and phosphorylation of Stat5, two key regulators of alveologenesis. Furthermore, glands from *Ptp1b* knockout mice exhibited increased expression of milk proteins during pregnancy due to enhanced Stat5 activation. These findings reveal that PTP1B constrains the number of mammary progenitors and thus prevents inappropriate onset of alveologenesis in early pregnancy. Moreover, PTP1B restrains the expression of milk proteins during pregnancy and thus prevents premature lactogenesis. Our work has implications for breast tumorigenesis because *Ptp1b* deletion has been shown to prevent or delay the onset of mammary tumors.

KEY WORDS: PTP1B (Ptpn1), Stat5, Mammary gland, Stem cell, Progenitor cell, Mouse

INTRODUCTION

The epithelium of rodent and human mammary glands is hierarchically organized, encompassing cells at various differentiation stages (Stingl et al., 2006b; LaBarge et al., 2007; Visvader, 2009; Visvader and Smith, 2011). The results of serial transplantation of mammary gland fragments into cleared mouse mammary fat pad suggested the existence of mammary stem cells (Deome et al., 1959; Faulkin and Deome, 1960). Direct evidence was provided by the finding that serial transplantation of fragments from mouse mammary tumor virus-infected mammary glands yields clonal outgrowths with the same viral insertion site through five transplant generations (Kordon and Smith, 1998; Bruno and Smith, 2011). Other studies have used cell surface markers to enrich for, and isolate, mammary stem cells, progenitor cells, and more differentiated luminal and myoepithelial cells (Shackleton et al., 2006; Sleeman et al., 2006; Stingl et al., 2006a; Asselin-Labat et al., 2007; Regan et al., 2011). Notably, these cell subpopulations display different functional attributes: mammary stem cells [MaSCs, also called mammary repopulating units (MRUs)] are able to repopulate a cleared mammary fat pad. Progenitor cells display a high capacity for colony formation and proliferation *in vitro*. By contrast, terminally differentiated cells are not able to repopulate the mammary gland or to form colonies *in vitro* (Shackleton et al., 2006; Sleeman et al., 2006; Stingl et al., 2006a; Asselin-Labat et al., 2007). Recent lineage-tracing studies have questioned the existence of adult multipotent MaSCs and have instead suggested the existence of unipotent luminal and myoepithelial progenitor cells in the adult gland (Van Keymeulen et al., 2011).

[1]Friedrich Miescher Institute for Biomedical Research, Maulbeerstr. 66, 4058 Basel, Switzerland. [2]University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland. [3]Swiss Institute of Bioinformatics, Maulbeerstrasse 66, 4058 Basel, Switzerland. [4]European Cancer Stem Cell Research Institute, Cardiff School of Biosciences, Cardiff University, Cardiff CF1 3AX, UK.

*Author for correspondence (bentires@fmi.ch)

Accepted 1 October 2012

The mammary gland undergoes functional differentiation during pregnancy. In the early stages, epithelial cells undergo extensive proliferation and form alveoli (alveologenesis) (Brisken, 2002), while in later stages of pregnancy alveolar cells secrete milk proteins (lactogenesis) (Neville et al., 2002; Hennighausen and Robinson, 2005; Brisken and Rajaram, 2006). Progesterone induces the expansion of MaSCs and the formation of mammary alveoli via activation of the Rankl (Tnfsf11) pathway, which in turn elicits the proliferation of epithelial cells (Asselin-Labat et al., 2010; Joshi et al., 2010). Prolactin (Prl) controls alveologenesis and lactogenesis via binding to its receptor [prolactin receptor (Prl-R; Prlr)] and activation of the Jak2/Stat5 pathway. Activated Stat5 translocates to the nucleus and induces expression of its target genes (e.g. milk proteins) (Gouilleux et al., 1994; Wartmann et al., 1996; Groner, 2002). Although Prl-R, Jak2 and Stat5 are regulated by tyrosine phosphorylation, little is known about how protein tyrosine phosphatases regulate this pathway and affect breast cell fate and differentiation *in vivo*.

Protein tyrosine phosphatase 1B (PTP1B; also known as Ptpn1), a ubiquitously expressed phosphatase, is an established negative regulator of insulin and leptin signaling and a leading target for the treatment of diabetes and obesity (Elchebly et al., 1999; Klaman et al., 2000). PTP1B is also involved in breast cancer. Whole-body or mammary-specific deletion of *Ptp1b* delays or prevents mammary tumor onset evoked by Her2 (also known as Neu and Erbb2) (Bentires-Alj and Neel, 2007; Julien et al., 2007; Balavenkatraman et al., 2011).

In contrast to its well-studied involvement in metabolism and cancer, the role of PTP1B in breast development remains unclear. Early *in vitro* studies suggested that both Jak2 and Stat5 are PTP1B substrates (Myers et al., 2001; Aoki and Matsuda, 2002). In the present study, we asked whether PTP1B controls mammary cell fate commitment and/or alveologenesis and lactogenesis *in vivo*. Using *Ptp1b* knockout mice, we have found that PTP1B depletion increases the number of mammary progenitor cells in nulliparous mice, induces precocious formation of alveoli, and enhances the expression of milk proteins during pregnancy.

MATERIALS AND METHODS

Mice

All animal experiments were performed according to Swiss guidelines governing animal experimentation and were approved by the Swiss veterinary authorities. *Ptp1b*$^{-/-}$ mice (Klaman et al., 2000) were backcrossed to an FVB background for at least seven generations. Twelve-week-old female mice were mated and pregnancy scored by the observation of a vaginal plug and confirmed by the presence of fertilized eggs or embryos when mammary glands were collected at pregnancy days 3, 7 or 10. Mammary glands from nulliparous mice were collected when mice were in estrus, as determined by a vaginal plug after an overnight mating with a male.

Whole-mounts and histological analysis

For whole-mounts and histology, inguinal and thoracic mammary glands were dissected at the indicated time points. Following fixation with methacarn solution for 4 hours, tissues were hydrated, stained with Carmine Alum, and cleared with xylene. After analysis, the tissues were processed for paraffin sectioning and stained with Hematoxylin and Eosin (H&E).

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed on methacarn-fixed or 4% paraformaldehyde (PFA)-fixed, paraffin-embedded tissue sections using the following antibodies: Ki67 (Lab Vision), rabbit anti-milk serum (Marte et al., 1995), pStat5 (Cell Signaling Technology), Stat5 (Santa Cruz Biotechnology), estrogen receptor (ER; Esr1) (Santa Cruz Biotechnology) and progesterone receptor (PR; Pgr) (Thermo Scientific). Immunohistochemistry was carried out with the Discovery XT Staining Module (Ventana Medical Systems), except for ER and PR immunohistochemistry, which were performed manually. All sections were counterstained with Hematoxylin (J.T.Baker). Quantification of pStat5, PR and ER was performed by counting cells from at least 20 fields at a magnification of 20× and at least 2000 nuclei per sample. The number of positive cells was expressed as a percentage of the total number of Hematoxylin-stained cells. Quantification of epithelial density and proliferation index were performed on mammary gland sections stained with periodic acid Schiff (Ventana Medical Systems) and Hematoxylin, and scanned with Miramax Scan (Carl Zeiss). For epithelial density, the area covered by epithelial cells (excluding lumen and blood vessels) was measured and the ratio of epithelial area over total organ area was calculated using Definiens software as described (Stoelzle et al., 2009). The same protocol was followed for the proliferation index using the area covered by Ki67-positive epithelial cells over total area of epithelial cells. At least three mice per genotype were scanned for each developmental stage.

Immunofluorescence was performed on 4% PFA-fixed, paraffin-embedded tissue sections stained with Rankl; tissues were then incubated with Alexa Fluor 546 anti-goat IgG (Molecular Probes, Invitrogen), stained with DAPI (Boehringer Mannheim), mounted in ProLong Gold antifade reagent (Invitrogen), and analyzed with an LSM 700 scanning head and Zen 2010 software (Carl Zeiss).

Crystal Violet staining was performed on cells grown in 24-well BD Primaria plates (BD Biosciences). The numbers and sizes of Crystal Violet-stained colonies per well were quantified using ImagePro software (Media Cybernetics). Three wells per genotype were examined in four independent experiments.

Immunoblotting

Protein lysates were extracted from inguinal mammary glands using RIPA buffer [50 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EGTA, 10 mM NaF, 2 mM sodium orthovanadate, 2 mM PMSF and protease inhibitor cocktail (Pierce)]. Proteins (50 μg) were resolved on SDS-PAGE (Bio-Rad) and transferred to a PVDF membrane (Immobilon-FL, Millipore). Membranes were blocked in PBS with 5% skimmed milk powder and incubated with PTP1B (Klaman et al., 2000), pStat5 (Cell Signaling) and Stat5a (Transduction Laboratories) antibodies. Antibody binding was visualized by incubation of secondary antibodies comprising Alexa Fluor 680 anti-mouse IgG, Alexa Fluor 680 anti-rabbit IgG (Molecular Probes, Invitrogen), IRDye 800 anti-mouse IgG and IRDye 800 anti-rabbit IgG (Rockland), and examined with an Odyssey infrared imaging system (Li-Cor Bioscience).

Real-time PCR

Total RNA was isolated from frozen mammary glands using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and then treated with the TURBO-DNase Kit (Applied Biosystems). cDNA synthesis was performed using the Thermoscript RT-PCR system (Invitrogen). Real-time PCR was performed on 30-60 ng cDNA using the TaqMan Gene Expression Assay (Applied Biosystems) for *Wap* (Mm00839913_m1), β-casein (Mm00839664_m1), cytokeratin 18 (Mm01601702_g1), *Rankl* (Mm00441906_m1), *Pr* (Mm00435628_m1), *Er* (Mm00433149_m1) and *Gapdh* (Rodent Gapdh Control Reagents VIC Probe, Applied Biosystems) on an ABI Prism 7000 (Applied Biosystems) according to the manufacturer's instructions.

Mammary cell preparation, cell sorting and cell culture

Inguinal mammary glands were dissected from 10-week-old virgin females or pregnant mice at gestation day 10, mechanically disaggregated, and digested with collagenase (Sigma) and trypsin (Sigma) for 1 hour at 37°C (Sleeman et al., 2006). The resulting organoids were processed to single-cell suspensions by digestion with HyQTase (HyClone) for 10-15 minutes at 37°C and filtered through a 40-μm cell strainer (Falcon). Cells were stained as previously described (Sleeman et al., 2006) with the following antibodies: FITC-CD24, PE-CD49f (Itga6), PE-Cy7-CD45 (Ptprc) (Pharmingen), APC-Sca1 (Ly6a), biotinylated-CD61 (Itgb3) (Biolegend) and streptavidin-PE-Cy5.5 (eBioscience). FACS analysis and cell sorting were carried out on a MoFlo cell sorter (Beckman Coulter).

Colony-forming assays were performed by plating freshly sorted cells (500 cells) on irradiated 3T3-L1 feeder cells in Multiwell BD Primaria plates for 7 days in DMEM/Ham's F12 mix (Invitrogen) with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 5 μg/ml bovine pancreatic insulin (Sigma, cell culture tested solution) and 10 ng/ml cholera toxin (Sigma). Aldefluor assay was performed according to the manufacturer's instructions (Stemcell Technologies).

Hormone treatment

Six-week-old female mice were ovariectomized and treated 10 days later every 24 hours by subcutaneous injection of 17β-estradiol (Sigma; 4 ng/g body weight) in corn oil (Sigma) and sacrificed 48 hours later. For treatment with estrogen and progesterone, ovariectomized mice were injected with 17β-estradiol and 48 hours later injected with 17β-estradiol plus progesterone (Sigma; 100 μg/g body weight) daily for 72 hours.

Chemicals

NVP-BSK805 (Novartis, Switzerland) was freshly prepared in NMP/PEG 300/Solutol HS15 (5%/80%/15%). Twelve-week-old mice were treated every 24 hours by oral gavage (120 mg/kg body weight) for 5 consecutive days. Glands were collected and fixed 4 hours after the final treatment.

Microarray analysis

RNA was isolated from three biological replicates per condition using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was measured using a NanoDrop 1000 and the quality of the RNA assessed using the Agilent 2100 bioanalyzer and RNA Nano Chip. Aliquots (100 ng) of extracted total RNA were amplified using the Ambion WT Expression Kit and the resulting sense-strand cDNA was fragmented and labeled using the Affymetrix GeneChip WT Terminal Labeling Kit. Affymetrix GeneChip arrays were hybridized following the GeneChip Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix) with a hybridization time of 16 hours. The Affymetrix Fluidics protocol FS450_0007 was used for washing. Scanning was performed with Affymetrix GCC Scan Control version 3.0.0.1214 on a GeneChip Scanner 3000 with autoloader. Probe sets were summarized and probeset level values normalized with the justRMA() function from the R (version 2.12.0)/Bioconductor (version 2.6) package affy using the CDF environment MoGene-1_0-st-v1.r3.cdf (as provided by Bioconductor). Differentially expressed genes were identified using the R package limma (Gentleman et al., 2004) and by selecting genes with a minimum absolute $\log_2$ fold change of 1.5 and $P<0.05$, using the method of Benjamini and Hochberg for multiple testing correction. Microarray data are available at GEO under accession number GSE41768.

Statistical analysis

Statistical significance was determined by two-tailed Student's *t*-test. For FACS analysis, a paired two-tailed Student's *t*-test was performed.

RESULTS

Ptp1b deletion accelerates alveologenesis

Tyrosine phosphorylation plays an important role in mammary gland alveologenesis. To determine whether PTP1B regulates this process, we analyzed mammary glands of PTP1B-deficient and wild-type (WT) female mice at different developmental stages. Whole-mounts and histological analysis showed significant changes in the structure of PTP1B-depleted compared with control glands (Fig. 1A). In nulliparous mice at estrus, H&E and Ki67 staining revealed a twofold increase in epithelial cell density and threefold more Ki67-positive cells in glands lacking PTP1B than in WT glands (Fig. 1B-D). During early stages of pregnancy, *Ptp1b*$^{-/-}$ glands showed an overall increase in the number of epithelial cells and alveolar structures (Fig. 1B,C). These results demonstrate that PTP1B constrains cell proliferation and alveologenesis during estrus and early pregnancy.

Increased progenitor cell number in mammary glands from *Ptp1b*$^{-/-}$ mice

To test whether the enhanced epithelial density found in PTP1B-deficient mice is a consequence of an increase in the stem/progenitor cell subpopulations, we characterized mammary epithelial cells (MECs) from *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ mice phenotypically using Sca1, CD24 and CD49f markers (Sleeman et al., 2006; Stingl et al., 2006a), which have been shown to enrich for MaSCs (CD24^{lo} Sca1^{-} CD49f^{+}), luminal progenitor cells (CD24^{hi} CD61^{+}), more differentiated luminal cells (CD24^{hi} CD61^{-}) and myoepithelial cells (CD24^{lo} CD49f^{lo}) (Asselin-Labat et al., 2007; Sleeman et al., 2007). We found no significant differences in the proportion of MECs bearing the stem cell phenotype (CD24^{lo} Sca1^{-} CD49f^{+}) or in the proportions of total luminal epithelial cells (CD24^{hi} Sca1^{-} and CD24^{hi} Sca1^{+}) in *Ptp1b*$^{-/-}$ versus *Ptp1b*$^{+/+}$ mice (Fig. 2A; supplementary material Fig. S1A). Limiting dilution transplantation experiments showed no difference in the capacities of *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ MECs to repopulate the mammary gland and, thus, that *Ptp1b* ablation does not alter the properties of mammary repopulating cells (supplementary material Fig. S1B).

We then investigated whether *Ptp1b* deletion alters mammary colony-forming capacity (Stingl et al., 2006a). Freshly isolated MECs from *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ glands were cultured on feeder cells and the number of colonies quantified. *Ptp1b*$^{-/-}$ MECs formed approximately twice as many colonies as *Ptp1b*$^{+/+}$ MECs, which suggested an increase in progenitor cells in glands lacking PTP1B (Fig. 2B). Furthermore, the colonies formed by *Ptp1b*$^{-/-}$ MECs were larger (Fig. 2B), consistent with our results showing increased proliferation in glands from *Ptp1b*$^{-/-}$ compared with *Ptp1b*$^{+/+}$ mice (Fig. 1D). The increase in progenitor cell number was further tested by FACS analysis for CD61, an epithelial progenitor marker (Asselin-Labat et al., 2007). We found a significant increase in the CD24^{hi} CD61^{+} population in *Ptp1b*$^{-/-}$ MECs compared with *Ptp1b*$^{+/+}$ MECs (29.63% CD24^{hi} CD61^{+} cells in *Ptp1b*$^{-/-}$ MECs versus 19.86% in *Ptp1b*$^{+/+}$ MECs) (Fig. 2C).

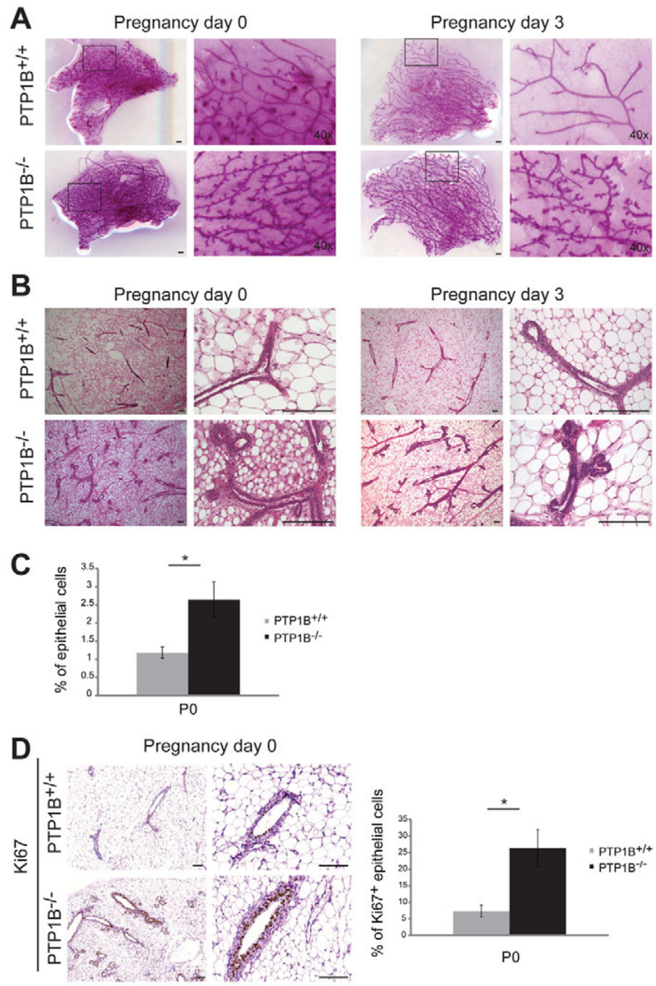


Fig. 1. Alveolar development is accelerated in PTP1B-deficient mammary glands. (**A**) Whole-mounts of *Ptp1b*$^{-/-}$ mammary tissues showing precocious alveolar formation compared with *Ptp1b*$^{+/+}$ mice. Boxed regions are magnified in images on the right. (**B**) H&E-stained histological sections of *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ mammary tissues. (**C**) The percentages of epithelial cells in *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ glands from nulliparous mice at estrus. (**D**) (Left) Ki67-stained histological sections. (Right) Proliferating (Ki67^{+}) cells as a percentage of total epithelial cells per gland. Pregnancy day 0 (P0) refers to nulliparous mice at estrus. Error bars indicate mean ± s.e.m.; $^{*}P<0.05$ by Student's *t*-test; C,D, $n=4$. Scale bars: 1 mm in A; 100 μm in B,D.

Several studies have suggested that high aldehyde dehydrogenase (ALDH) activity is a property of stem and/or progenitor cells in human and mouse mammary tissues (Ginestier et al., 2007; Cohn et al., 2010; Eirew et al., 2012). Using the Aldefluor assay, we found a higher ALDH activity in MECs from *Ptp1b*$^{-/-}$ than *Ptp1b*$^{+/+}$ mice (supplementary material Fig. S1C), further supporting an increase in the proportion of mammary progenitor cells in PTP1B-deficient glands. Together, these results show that PTP1B restrains the number of mammary progenitor cells in nulliparous mice.

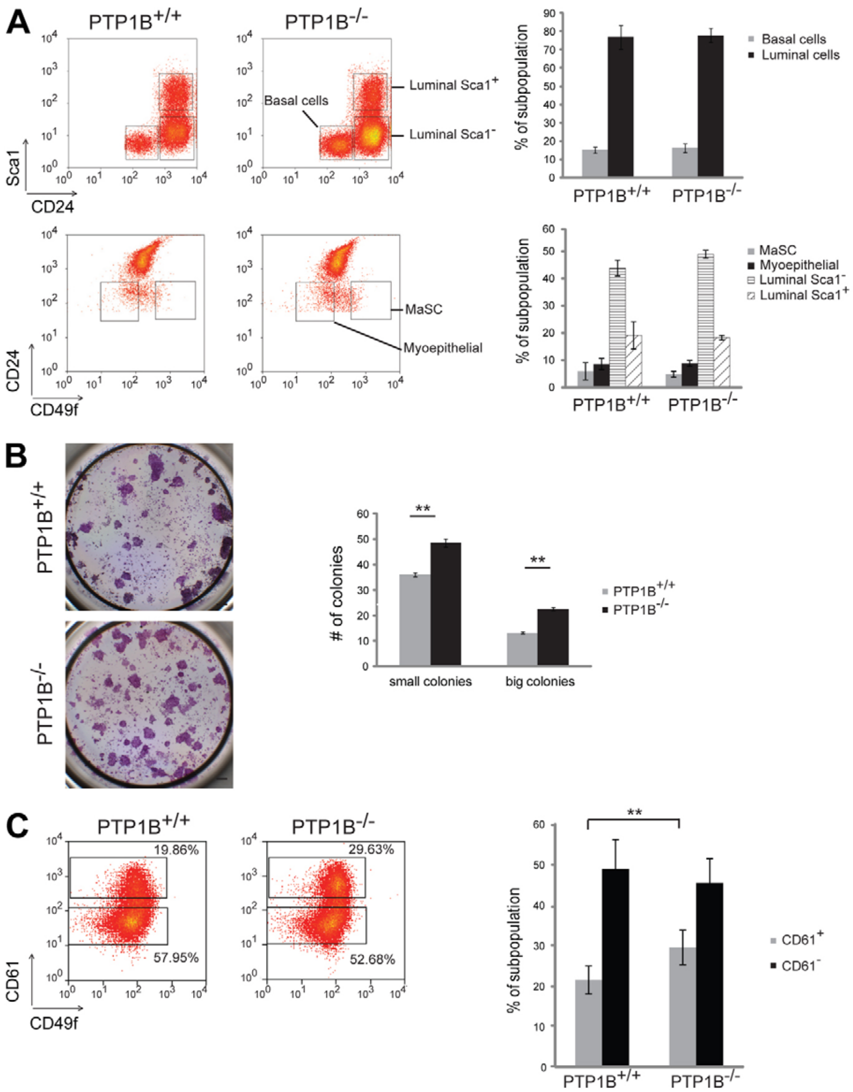


Fig. 2. PTP1B depletion increases the proportion of mammary progenitors. (**A**) (Top row, left) Flow cytometry dot plots of mammary epithelial cells (MECs) from nulliparous mice at estrus. Luminal cells: CD24^{hi} Sca1^{+} and CD24^{hi} Sca1$^{–}$. Basal cells: CD24^{lo} Sca1$^{–}$. (Top row, right) The percentages of luminal and basal cell subpopulations of MECs from *Ptp1b*$^{+/+}$ and *Ptp1b*$^{–/–}$ nulliparous mice at estrus. (Bottom row, left) Dot plots of myoepithelial (CD24^{lo} Sca1$^{–}$ CD49f$^{–}$) and mammary stem cell (MaSC) (CD24^{lo} Sca1$^{–}$ CD49f^{+}) populations. (Bottom row, right) The percentages of myoepithelial and MaSC populations of *Ptp1b*$^{+/+}$ and *Ptp1b*$^{–/–}$ glands from nulliparous mice at estrus. Error bars indicate mean ± s.e.m. (n=6). (**B**) (Left) Colony formation assay of MECs from nulliparous mice at estrus. Scale bar: 1 mm. (Right) The number of *Ptp1b*$^{+/+}$ and *Ptp1b*$^{–/–}$ colonies. Small refers to colonies <8000 μm^2 and big to colonies >8000 μm^2 (n=4, **P<0.01 by Student's t-test). (**C**) (Left) Flow cytometry dot plots of luminal cells (CD24^{hi} Sca1^{+} and CD24^{hi} Sca1$^{–}$) of MECs from nulliparous mice at estrus stained for the progenitor marker CD61. (Right) The percentages of CD61^{+} and CD61$^{–}$ populations of *Ptp1b*$^{–/–}$ and *Ptp1b*$^{+/+}$ MECs. Error bars indicate mean ± s.e.m. (n=4, **P<0.01 by paired two-tailed Student's t-test).

PTP1B negatively regulates ER activity

To investigate the molecular mediators of the observed increase in epithelial density and mammary progenitors in mammary glands from *Ptp1b*$^{–/–}$ mice, we performed gene expression profiling of *Ptp1b*$^{–/–}$ and *Ptp1b*$^{+/+}$ glands from mice at estrus. The absence of PTP1B increased the expression of several components of the cell cycle machinery: including cyclin B2, cyclin A2, cyclin-dependent kinase 1 and topoisomerase 2A (Fig. 3A; supplementary material Fig. S2A, Table S1). These results, combined with the increased proliferation observed by immunohistochemistry (Fig. 1D), support a role for PTP1B in the regulation of epithelial cell proliferation.

Further, analysis of the expression profiles of *Ptp1b*$^{–/–}$ and *Ptp1b*$^{+/+}$ glands revealed increased expression of several estrogen-responsive genes (supplementary material Fig. S2A): *Pr*, amphiregulin, *Expi* (*Wfdc18*), *Egr2* and *c-Myb*. Furthermore, quantitative RT-PCR and immunohistochemistry analysis revealed an increase in PR expression in glands lacking PTP1B (Fig. 3B,C). Similarly, we found increased expression of *Rankl*, an established downstream target of PR (Fata et al., 2000; Beleut et al., 2010), in glands deficient in PTP1B (Fig. 3B; supplementary material Fig. S2B). These data suggest that PR-induced expression of Rankl might account for the increased number of MECs observed in glands from *Ptp1b*$^{–/–}$ mice.

We then tested whether overexpression of ER and/or estrogen accounts for the increased transcription of ER targets in glands from *Ptp1b*$^{–/–}$ mice, but found no difference in ER expression between *Ptp1b*$^{–/–}$ and *Ptp1b*$^{+/+}$ glands (Fig. 3B,C) and no difference in plasma levels of estrogen between *Ptp1b*$^{–/–}$ and *Ptp1b*$^{+/+}$ mice at estrus (supplementary material Fig. S3A). Further analysis showed no differences in the plasma levels of progesterone in *Ptp1b*$^{–/–}$ and *Ptp1b*$^{+/+}$ mice (supplementary material Fig. S3A). Thus, *Ptp1b* deletion appears to increase mammary cell proliferation by enhancing the responsiveness of the mammary gland to normal levels of circulating estrogen and progesterone. To test this possibility directly, we assessed the effects of 17β-estradiol treatment alone or in combination with progesterone on *Ptp1b*$^{–/–}$ and *Ptp1b*$^{+/+}$ mice that were previously depleted of endogenous steroid hormones by ovariectomy. Treatment with 17β-estradiol for 48 hours increased the expression of PR in glands from *Ptp1b*$^{–/–}$ ovariectomized mice compared with ovariectomized WT littermates (Fig. 3D). These data show that *Ptp1b* deletion increases ER activity in nulliparous glands. Furthermore, treatment of *Ptp1b*$^{–/–}$ and *Ptp1b*$^{+/+}$ mice with 17β-estradiol and progesterone for 72 hours resulted in enhanced

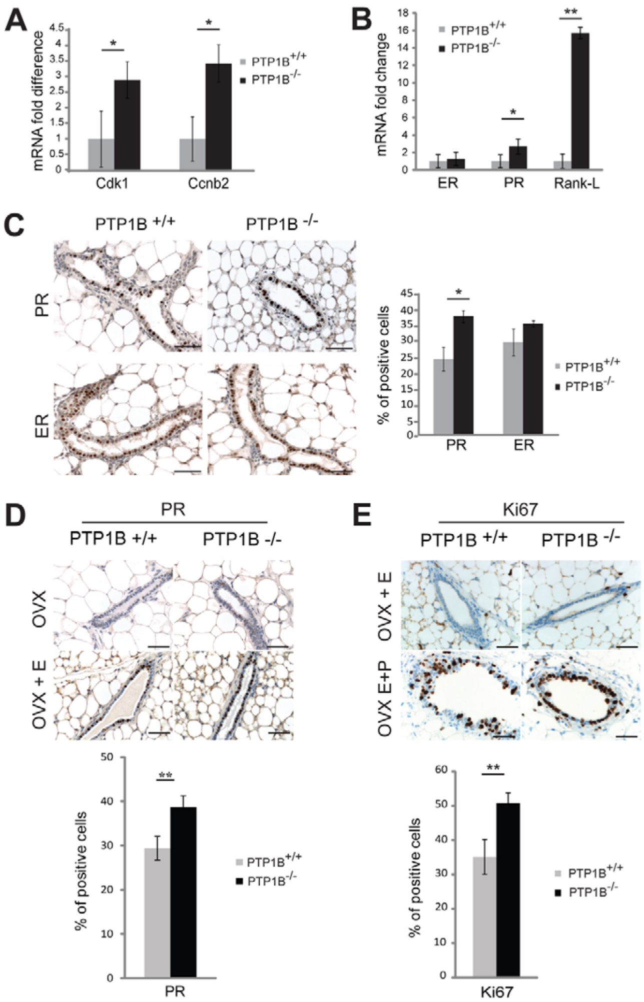


Fig. 3. Absence of PTP1B increases the expression of cell cycle and estrogen-responsive genes. (**A**) Fold changes in *Cdk1* and *Ccnb2* mRNA as assessed by quantitative real-time PCR. (**B**) *Er*, *Pr* and *Rankl* mRNA fold changes as assessed by quantitative real-time PCR. (**C**) (Left) ER- and PR-stained sections of mammary glands from nulliparous mice at estrus. (Right) The percentages of PR- and ER-positive epithelial cells. (**D**) (Top) Mammary glands from ovariectomized (OVX) mice treated (or otherwise) with 17β-estradiol (+E) for 48 hours and stained for PR. (Bottom) The percentages of PR-positive epithelial cells in 17β-estradiol-treated glands (22 images from two mice per genotype were quantified). (**E**) (Top) Mammary glands from ovariectomized mice treated with 17β-estradiol alone (+E) or together with progesterone (E+P) for 72 hours and stained for Ki67. (Bottom) The percentages of Ki67-positive epithelial cells in the 17β-estradiol plus progesterone-treated glands (30 images from four mice per genotype were quantified). Error bars indicate mean ± s.e.m.; *$P<0.05$, **$P<0.01$ by Student's *t*-test; A-C, $n=3$. Scale bars: 50 μm.

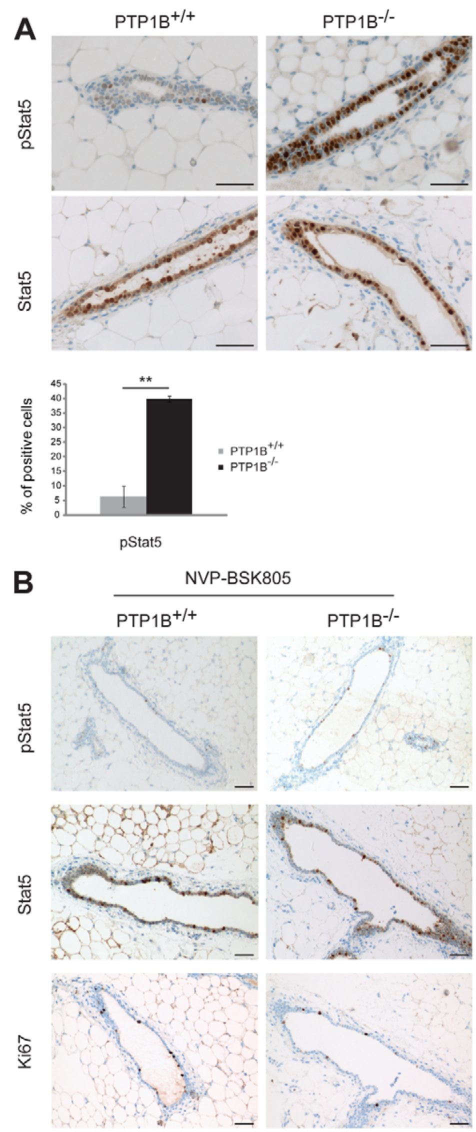


Fig. 4. Absence of PTP1B increases Stat5 phosphorylation. (**A**) (Top) Mammary gland sections from nulliparous mice at estrus stained for pStat5 and Stat5. (Bottom) The percentage of cells positive for nuclear pStat5. Error bars indicate mean ± s.e.m.; $n=4$; **$P<0.01$ by Student's *t*-test. (**B**) Mammary gland sections from NVP-BSK805-treated mice stained for pStat5, Stat5 or Ki67. Scale bars: 50 μm.

expression of Rankl and proliferation of *Ptp1b*$^{-/-}$ epithelial cells compared with WT (Fig. 3E; supplementary material Fig. S2B). Thus, PTP1B restrains epithelial cell proliferation by negatively regulating ER activity and PR expression.

PTP1B depletion increases Stat5 phosphorylation

Genetic depletion of Stat5 revealed that this transcription factor enhances the proliferation of epithelial cells in response to estrogen and progesterone stimuli, increases the number of mammary luminal progenitor cells, and promotes alveologenesis (Miyoshi et al., 2001; Cui et al., 2004; Yamaji et al., 2009). The *in vitro* data suggesting Stat5 as a potential PTP1B substrate (Aoki and Matsuda, 2002) raise the possibility that Stat5 is hyperactivated in glands lacking PTP1B. To test this, we stained control and *Ptp1b* knockout glands for Stat5 and phosphorylated Stat5 (pStat5) and found a dramatic increase in pStat5 in the absence of PTP1B (Fig. 4A). We then tested whether Jak2/Stat5 inhibition blocks the increased epithelial cell proliferation observed in *Ptp1b*$^{-/-}$ glands. Treatment of *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ mice with NVP-BSK805 [a selective Jak2 inhibitor that results in Stat5 dephosphorylation (Baffert et al., 2010)] inhibited Stat5 phosphorylation and, notably, significantly reduced proliferation in *Ptp1b*$^{-/-}$ glands (Fig. 4B).

We next investigated whether overexpression of Prl and/or Prl-R or hyperphosphorylation of Jak2 accounts for the increased pStat5 in glands from *Ptp1b*$^{-/-}$ mice. We found no difference in the plasma levels of Prl, in Prl-R expression or in Jak2 expression and phosphorylation between *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ glands (supplementary material Fig. S3A-D). This suggests that PTP1B acts via the Stat5 pathway in constraining epithelial cell proliferation.

PTP1B depletion accelerates mammary gland differentiation during pregnancy

We next assessed the consequences of *Ptp1b* deletion on mammary gland development at later stages of pregnancy. Similar to the phenotype at pregnancy day 3 (Fig. 1A,B), whole-mounts and H&E staining of glands showed that the absence of PTP1B also results in the increased formation of alveolar structures at pregnancy days 7 and 10 (Fig. 5A-C).

FACS analysis of MECs isolated from *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ mice at pregnancy day 10 showed an increase in the luminal CD24^{hi} Sca1^{-} population, which is enriched in milk-expressing cells (Sleeman et al., 2006). No changes were observed in the other subpopulations (Fig. 5D). Furthermore, histological analysis revealed that the alveolar structures in *Ptp1b*$^{-/-}$ but not *Ptp1b*$^{+/+}$ glands were precociously distended, displayed lipid droplets and expressed milk proteins, which are all characteristics of differentiated alveoli (Fig. 5A,B, Fig. 6A). Given the higher number of alveoli in glands lacking PTP1B, the observed increase in milk protein expression might be caused by enhanced expression and/or by an increase in the number of milk-producing cells. To distinguish these possibilities, we assessed expression of the genes encoding the early pregnancy milk protein β-casein and the late pregnancy milk protein whey acidic protein (Wap), normalized to the expression of epithelial markers cytokeratin 8 and 18 in glands from control and *Ptp1b*$^{-/-}$ mice. PTP1B-depleted alveoli not only expressed milk proteins precociously but also expressed a higher level of milk proteins per cell. PTP1B depletion resulted in 5.5-fold and 4.9-fold increases in the levels of β-casein and *Wap*, respectively (Fig. 6B; data not shown).

Next, we assessed the molecular mechanism underlying the precocious lactogenesis seen in *Ptp1b*$^{-/-}$ glands. Immunoblotting revealed increased phosphorylation of Stat5, a well-established inducer of milk protein expression during pregnancy (Wakao et al.,

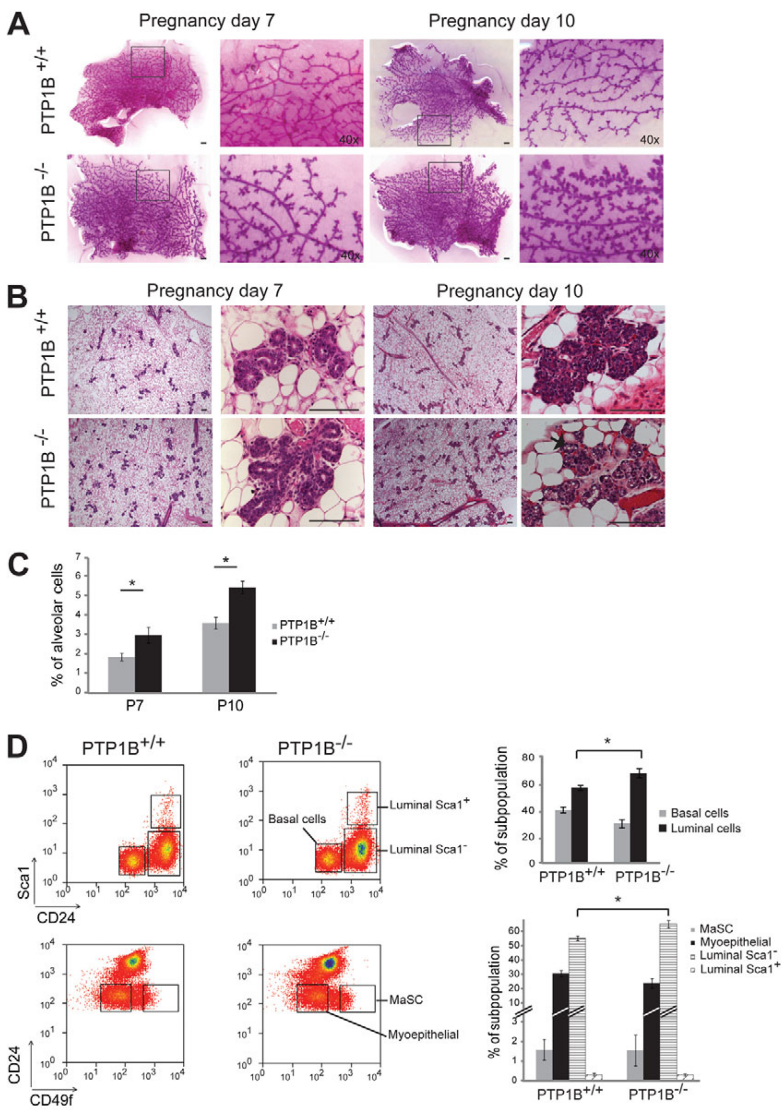


Fig. 5. Precocious secretory differentiation in PTP1B-deficient mammary glands. (**A**) Whole-mounts of *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ mammary tissues at pregnancy days 7 and 10. Boxed regions are magnified in images on the right. (**B**) H&E-stained histological sections of *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ mammary tissues at pregnancy days 7 and 10. The arrow points to lipid droplets. (**C**) The percentages of alveolar cells in *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ glands at pregnancy days 7 and 10. (**D**) (Top row) Dot plots (left) and percentages (right) of luminal (CD24^{hi} Sca1^{+} and CD24^{hi} Sca1^{-}) and basal (CD24^{lo} Sca1^{-}) cell populations from *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ glands at pregnancy day 10. (Bottom row) Dot plots (left) and percentages (right) of myoepithelial (CD24^{lo} Sca1^{-} CD49f^{-}) and stem cell (CD24^{lo} Sca1^{-} CD49f^{+}) populations. Error bars indicate mean ± s.e.m.; *P<0.05 by Student's *t*-test; C, *n*=4; D, *n*=3. Scale bars: 1 mm in A; 100 μm in B.

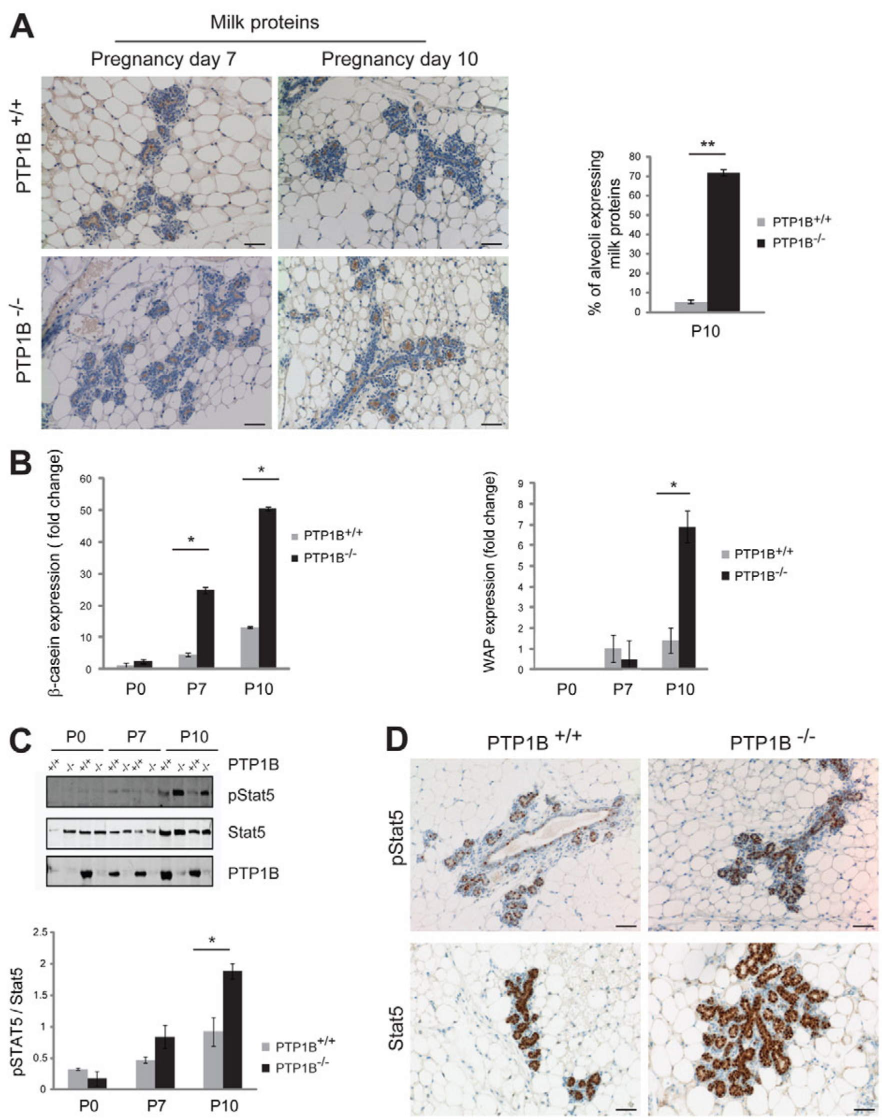


Fig. 6. Activation of Stat5 induces precocious lactogenesis in *Ptp1b*$^{-/-}$ glands. (**A**) (Left) Mammary gland sections stained for expression of total milk proteins. (Right) Milk protein-expressing alveoli as a percentage of total alveoli per gland. (**B**) Fold changes in β-casein and *Wap* mRNA from mice as assessed by quantitative real-time PCR. P0, nulliparous mice at estrus; P7 and P10, pregnancy day 7 and pregnancy day 10. (**C**) Immunoblots of lysates from glands as indicated (top) and the ratio of pStat5/Stat5 (bottom). (**D**) Mammary gland sections stained for Stat5 and pStat5 at pregnancy day 10. Error bars indicate mean ± s.e.m.; **P*<0.05, ***P*<0.01 by Student's *t*-test; A, *n*=4; B,C, *n*=3. Scale bars: 50 μm.

1994; Liu et al., 1997), in PTP1B-deficient glands compared with controls (Fig. 6C). To exclude the possibility that the observed changes in pStat5 were due to changes in the stroma and not in epithelial cells, we performed immunostaining against pStat5. We found that pStat5 in epithelial cells of glands lacking PTP1B was markedly increased (Fig. 6D). We then tested whether Jak2 expression and/or phosphorylation is altered in glands lacking PTP1B and found no difference in pJak2 between *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ glands at pregnancy day 10 (supplementary material Fig. S3C,D).

To investigate whether *Ptp1b* deletion affects involution, we analyzed glands from *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ mice 5 days after cessation of suckling and observed no differences (supplementary material Fig. S3E).

These data show that PTP1B depletion precociously increases Stat5 phosphorylation, thus triggering the expression of milk proteins. This suggests that PTP1B expression constrains lactogenesis during pregnancy.

DISCUSSION

Tight regulation of mammary alveologenesis and lactogenesis is fundamental for lactating species. In this study, we have shown that the tyrosine phosphatase PTP1B constrains these important processes. Alveologenesis is a developmental program characterized by the expansion and differentiation of mammary progenitor cells into alveolar cells. Loss of PTP1B increases the number of progenitor cells in nulliparous mice. This enhances the pool of cells able to generate alveolar structures and, thus, results in the increased alveolar density observed in *Ptp1b*$^{-/-}$ glands during early pregnancy.

Several factors influence mammary gland alveologenesis. Mechanistically, we found that lack of PTP1B induces the expression of several estrogen-responsive genes in nulliparous glands, including *Pr* and its downstream target *Rankl*. PR plays a key role in epithelial cell proliferation and alveolar formation during early pregnancy (Lydon et al., 1995; Brisken et al., 1998; Mulac-Jericevic et al., 2003; Obr and Edwards, 2012). Therefore, the precocious alveolar development observed in *Ptp1b*$^{-/-}$ glands might be mediated by the overexpression and activation of PR, which then precociously initiates alveologenesis.

Estrogen and progesterone have been shown to regulate the number and/or activity of MaSCs via a paracrine mechanism involving the Rank and Wnt pathways (Asselin-Labat et al., 2010;

Joshi et al., 2010; Axlund and Sartorius, 2012). In glands lacking PTP1B, we observed an increase in ER and PR activity associated with an increase in the number and activity of progenitor cells but not of MaSCs. The discrepancy between our results and those reported previously might be due to differences in the degrees of ER and PR activation in the two models.

In addition to increasing PR expression, PTP1B depletion increased the phosphorylation of Stat5, a key regulator of mammary luminal progenitor cells and alveologenesis (Yamaji et al., 2009), suggesting that PTP1B restrains the number of mammary progenitor cells and regulates alveologenesis via Stat5 dephosphorylation. Stat5 has been shown to promote the proliferation of epithelial cells in response to estrogen and progesterone stimuli, which indicated that they act in a common pathway (Miyoshi et al., 2001; Cui et al., 2004). Conceivably, PTP1B depletion increases ER activity and PR expression, which in turn activates Stat5 and leads to increased alveologenesis. *In vitro* studies have demonstrated that PTP1B can directly dephosphorylate Stat5 (Myers et al., 2001), raising an alternative possibility that lack of PTP1B independently increases PR expression via ER and Stat5 phosphorylation. These two possibilities are not mutually exclusive.

But how does lack of PTP1B increase ER activity? *In vitro* studies have shown that PTP1B dephosphorylates ER at tyrosine 537, a residue known to inhibit estrogen binding and to reduce transcriptional activity of ER when phosphorylated (Arnold et al., 1997). These data raise the possibility that PTP1B activates the estrogen pathway by regulating the phosphorylation of ER.

Our results also support a role for PTP1B in lactogenesis. PTP1B depletion induces the precocious expression of milk proteins due to an increase in Stat5 phosphorylation. Indeed, Stat5 is a well-established regulator of lactogenesis, mediating Prl-induced milk expression (Wakao et al., 1994; Liu et al., 1997). Taken together, our results suggest a role for PTP1B as a temporal regulator of mammary gland development that downregulates the progesterone and Stat5 pathway(s) and thus prevents the inappropriate onset of alveologenesis and lactogenesis during pregnancy.

Mammary gland development and differentiation are regulated by a complex mechanism involving several different pathways (Hennighausen and Robinson, 2005; Brisken and O'Malley, 2010). We cannot exclude the possibility that PTP1B acts via other pathways in constraining mammary gland alveologenesis and lactogenesis. For example, PTP1B is a well-known regulator of the insulin and leptin pathways in other organs, and mice lacking PTP1B are insulin and leptin hypersensitive (Elchebly et al., 1999; Klaman et al., 2000). In the light of reports of a role for insulin, Igf1 and Igf2 in mammary gland morphogenesis (Kleinberg et al., 2000; Brisken et al., 2002; Hovey et al., 2003; Berlato and Doppler, 2009), PTP1B might also regulate mammary gland morphogenesis via its inhibitory effects on these pathways.

Epidemiological studies have shown that early menarche, late menopause and late age of first pregnancy are all risk factors for developing sporadic breast cancer (Medina, 2005). Conversely, early full-term pregnancy (<24 years) decreases lifetime breast cancer risk. Clearly, the hormonal milieu and breast development cycles, possibly through changes in the differentiation state of breast stem/progenitor cells, affect the susceptibility of the breast to oncogenic transformation. Our finding that *Ptp1b* deletion induces precocious differentiation of the mammary gland raises the possibility that the cells of origin of Her2-evoked mammary tumors are decreased in *Ptp1b*$^{-/-}$ mice. This would explain why deletion of *Ptp1b* delays or prevents mammary tumor formation in MMTV-NeuNT and MMTV-NDL1 mice (Bentires-Alj and Neel, 2007; Julien et al., 2007; Balavenkatraman et al., 2011). An exploration of this possibility is now warranted.

Acknowledgements
We thank J. Regan (Institute of Cancer Research, Breakthrough Breast Cancer Research, UK) for help with the MEC isolation and FACS sorting; B. Neel (BIDMC/Harvard Medical School, Ontario Cancer Institute) for providing *Ptp1b* knockout mice; T. Radimerski and C. Pissot-Soldermann (NIBR) for supplying NVP-BSK805; A. Doelemeyer (NIBR) for quantification of mammary epithelial density; S. Bichet (FMI) for immunohistochemistry; T. Rolof (FMI) for microarray analysis; and S. Sarret (FMI) as well as further members of the M.B.-A. laboratory for advice and discussions.

Funding
Research in the laboratory of M.B.-A. is supported by the Novartis Research Foundation and the European Research Council [ERC Starting Grant 243211-PTPsBDC].

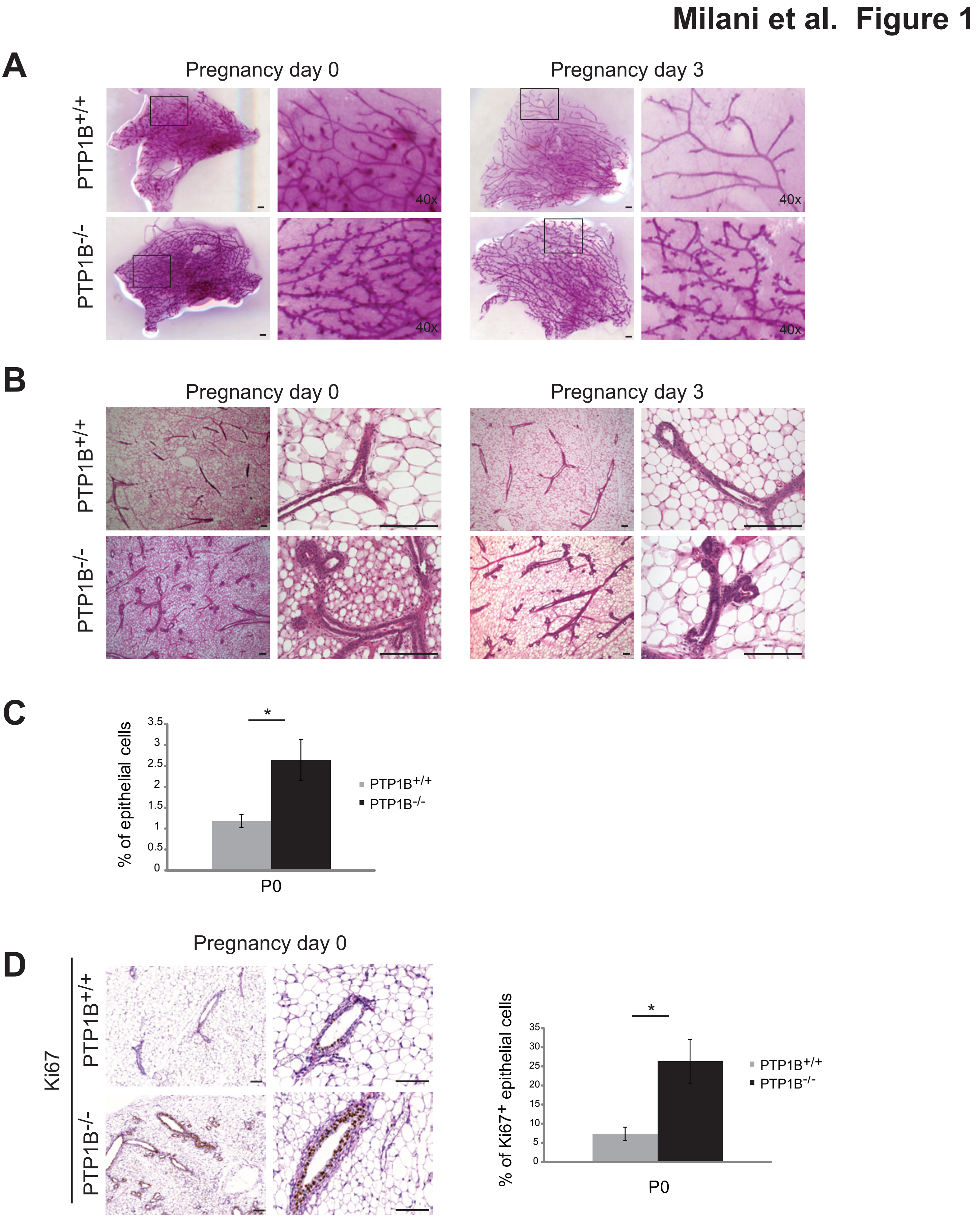
Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.082941/-/DC1

References
Aoki, N. and Matsuda, T. (2002). A nuclear protein tyrosine phosphatase TC-PTP is a potential negative regulator of the PRL-mediated signaling pathway: dephosphorylation and deactivation of signal transducer and activator of transcription 5a and 5b by TC-PTP in nucleus. *Mol. Endocrinol.* **16**, 58-69.
Arnold, S. F., Melamed, M., Vorojeikina, D. P., Notides, A. C. and Sasson, S. (1997). Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation. *Mol. Endocrinol.* **11**, 48-53.
Asselin-Labat, M. L., Sutherland, K. D., Barker, H., Thomas, R., Shackleton, M., Forrest, N. C., Hartley, L., Robb, L., Grosveld, F. G., van der Wees, J. et al. (2007). Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nat. Cell Biol.* **9**, 201-209.
Asselin-Labat, M. L., Vaillant, F., Sheridan, J. M., Pal, B., Wu, D., Simpson, E. R., Yasuda, H., Smyth, G. K., Martin, T. J., Lindeman, G. J. et al. (2010). Control of mammary stem cell function by steroid hormone signalling. *Nature* **465**, 798-802.
Axlund, S. D. and Sartorius, C. A. (2012). Progesterone regulation of stem and progenitor cells in normal and malignant breast. *Mol. Cell. Endocrinol.* **357**, 71-79.
Baffert, F., Régnier, C. H., De Pover, A., Pissot-Soldermann, C., Tavares, G. A., Blasco, F., Brueggen, J., Chène, P., Drueckes, P., Erdmann, D. et al. (2010). Potent and selective inhibition of polycythemia by the quinoxaline JAK2 inhibitor NVP-BSK805. *Mol. Cancer Ther.* **9**, 1945-1955.
Balavenkatraman, K. K., Aceto, N., Britschgi, A., Mueller, U., Bence, K. K., Neel, B. G. and Bentires-Alj, M. (2011). Epithelial protein-tyrosine phosphatase 1B contributes to the induction of mammary tumors by HER2/Neu but is not essential for tumor maintenance. *Mol. Cancer Res.* **9**, 1377-1384.
Beleut, M., Rajaram, R. D., Caikovski, M., Ayyanan, A., Germano, D., Choi, Y., Schneider, P. and Brisken, C. (2010). Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland. *Proc. Natl. Acad. Sci. USA* **107**, 2989-2994.
Bentires-Alj, M. and Neel, B. G. (2007). Protein-tyrosine phosphatase 1B is required for HER2/Neu-induced breast cancer. *Cancer Res.* **67**, 2420-2424.
Berlato, C. and Doppler, W. (2009). Selective response to insulin versus insulin-like growth factor-I and -II and up-regulation of insulin receptor splice variant B in the differentiated mouse mammary epithelium. *Endocrinology* **150**, 2924-2933.
Brisken, C. (2002). Hormonal control of alveolar development and its implications for breast carcinogenesis. *J. Mammary Gland Biol. Neoplasia* **7**, 39-48.
Brisken, C. and O'Malley, B. (2010). Hormone action in the mammary gland. *Cold Spring Harb. Perspect. Biol.* **2**, a003178.
Brisken, C. and Rajaram, R. D. (2006). Alveolar and lactogenic differentiation. *J. Mammary Gland Biol. Neoplasia* **11**, 239-248.
Brisken, C., Park, S., Vass, T., Lydon, J. P., O'Malley, B. W. and Weinberg, R. A. (1998). A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc. Natl. Acad. Sci. USA* **95**, 5076-5081.
Brisken, C., Ayyannan, A., Nguyen, C., Heineman, A., Reinhardt, F., Tan, J., Dey, S. K., Dotto, G. P. and Weinberg, R. A. (2002). IGF-2 is a mediator of prolactin-induced morphogenesis in the breast. *Dev. Cell* **3**, 877-887.

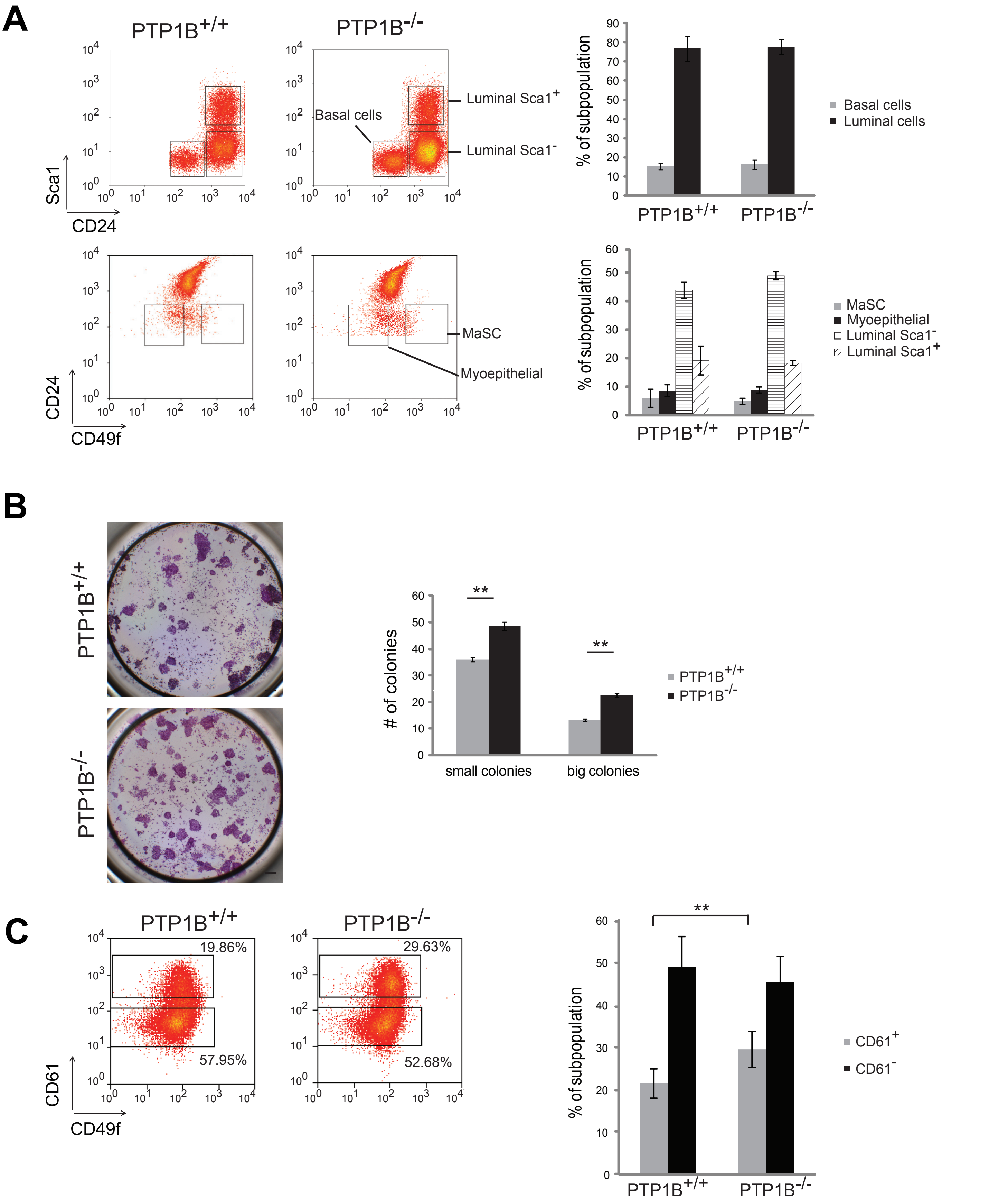
Bruno, R. D. and Smith, G. H. (2011). Functional characterization of stem cell activity in the mouse mammary gland. *Stem Cell Rev.* **7**, 238-247.
Cohn, E., Ossowski, L., Bertran, S., Marzan, C. and Farias, E. F. (2010). RARalpha1 control of mammary gland ductal morphogenesis and wnt1-tumorigenesis. *Breast Cancer Res.* **12**, R79.
Cui, Y., Riedlinger, G., Miyoshi, K., Tang, W., Li, C., Deng, C. X., Robinson, G. W. and Hennighausen, L. (2004). Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. *Mol. Cell. Biol.* **24**, 8037-8047.
Deome, K. B., Faulkin, L. J., Jr, Bern, H. A. and Blair, P. B. (1959). Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res.* **19**, 515-520.
Eirew, P., Kannan, N., Knapp, D. J., Vaillant, F., Emerman, J. T., Lindeman, G. J., Visvader, J. E. and Eaves, C. J. (2012). Aldehyde dehydrogenase activity is a biomarker of primitive normal human mammary luminal cells. *Stem Cells* **30**, 344-348.
Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A. L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C. C. et al. (1999). Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* **283**, 1544-1548.
Fata, J. E., Kong, Y. Y., Li, J., Sasaki, T., Irie-Sasaki, J., Moorehead, R. A., Elliott, R., Scully, S., Voura, E. B., Lacey, D. L. et al. (2000). The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* **103**, 41-50.
Faulkin, L. J., Jr and Deome, K. B. (1960). Regulation of growth and spacing of gland elements in the mammary fat pad of the C3H mouse. *J. Natl. Cancer Inst.* **24**, 953-969.
Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J. et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80.
Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., Jacquemier, J., Viens, P., Kleer, C. G., Liu, S. et al. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* **1**, 555-567.
Gouilleux, F., Wakao, H., Mundt, M. and Groner, B. (1994). Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO J.* **13**, 4361-4369.
Groner, B. (2002). Transcription factor regulation in mammary epithelial cells. *Domest. Anim. Endocrinol.* **23**, 25-32.
Hennighausen, L. and Robinson, G. W. (2005). Information networks in the mammary gland. *Nat. Rev. Mol. Cell Biol.* **6**, 715-725.
Hovey, R. C., Harris, J., Hadsell, D. L., Lee, A. V., Ormandy, C. J. and Vonderhaar, B. K. (2003). Local insulin-like growth factor-II mediates prolactin-induced mammary gland development. *Mol. Endocrinol.* **17**, 460-471.
Joshi, P. A., Jackson, H. W., Beristain, A. G., Di Grappa, M. A., Mote, P. A., Clarke, C. L., Stingl, J., Waterhouse, P. D. and Khokha, R. (2010). Progesterone induces adult mammary stem cell expansion. *Nature* **465**, 803-807.
Julien, S. G., Dubé, N., Read, M., Penney, J., Paquet, M., Han, Y., Kennedy, B. P., Muller, W. J. and Tremblay, M. L. (2007). Protein tyrosine phosphatase 1B deficiency or inhibition delays ErbB2-induced mammary tumorigenesis and protects from lung metastasis. *Nat. Genet.* **39**, 338-346.
Klaman, L. D., Boss, O., Peroni, O. D., Kim, J. K., Martino, J. L., Zabolotny, J. M., Moghal, N., Lubkin, M., Kim, Y. B., Sharpe, A. H. et al. (2000). Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. *Mol. Cell. Biol.* **20**, 5479-5489.
Kleinberg, D. L., Feldman, M. and Ruan, W. (2000). IGF-I: an essential factor in terminal end bud formation and ductal morphogenesis. *J. Mammary Gland Biol. Neoplasia* **5**, 7-17.
Kordon, E. C. and Smith, G. H. (1998). An entire functional mammary gland may comprise the progeny from a single cell. *Development* **125**, 1921-1930.
LaBarge, M. A., Petersen, O. W. and Bissell, M. J. (2007). Of microenvironments and mammary stem cells. *Stem Cell Rev.* **3**, 137-146.
Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A. and Hennighausen, L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.* **11**, 179-186.
Lydon, J. P., DeMayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Jr, Shyamala, G., Conneely, O. M. and O'Malley, B. W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev.* **9**, 2266-2278.
Marte, B. M., Jeschke, M., Graus-Porta, D., Taverna, D., Hofer, P., Groner, B., Yarden, Y. and Hynes, N. E. (1995). Neu differentiation factor/heregulin modulates growth and differentiation of HC11 mammary epithelial cells. *Mol. Endocrinol.* **9**, 14-23.
Medina, D. (2005). Mammary developmental fate and breast cancer risk. *Endocr. Relat. Cancer* **12**, 483-495.
Miyoshi, K., Shillingford, J. M., Smith, G. H., Grimm, S. L., Wagner, K. U., Oka, T., Rosen, J. M., Robinson, G. W. and Hennighausen, L. (2001). Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. *J. Cell Biol.* **155**, 531-542.
Mulac-Jericevic, B., Lydon, J. P., DeMayo, F. J. and Conneely, O. M. (2003). Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. *Proc. Natl. Acad. Sci. USA* **100**, 9744-9749.
Myers, M. P., Andersen, J. N., Cheng, A., Tremblay, M. L., Horvath, C. M., Parisien, J. P., Salmeen, A., Barford, D. and Tonks, N. K. (2001). TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. *J. Biol. Chem.* **276**, 47771-47774.
Neville, M. C., McFadden, T. B. and Forsyth, I. (2002). Hormonal regulation of mammary differentiation and milk secretion. *J. Mammary Gland Biol. Neoplasia* **7**, 49-66.
Obr, A. E. and Edwards, D. P. (2012). The biology of progesterone receptor in the normal mammary gland and in breast cancer. *Mol. Cell. Endocrinol.* **357**, 4-17.
Regan, J. L., Kendrick, H., Magnay, F. A., Vafaizadeh, V., Groner, B. and Smalley, M. J. (2011). c-Kit is required for growth and survival of the cells of origin of Brca1-mutation-associated breast cancer. *Oncogene* **31**, 869-883.
Shackleton, M., Vaillant, F., Simpson, K. J., Stingl, J., Smyth, G. K., Asselin-Labat, M. L., Wu, L., Lindeman, G. J. and Visvader, J. E. (2006). Generation of a functional mammary gland from a single stem cell. *Nature* **439**, 84-88.
Sleeman, K. E., Kendrick, H., Ashworth, A., Isacke, C. M. and Smalley, M. J. (2006). CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. *Breast Cancer Res.* **8**, R7.
Sleeman, K. E., Kendrick, H., Robertson, D., Isacke, C. M., Ashworth, A. and Smalley, M. J. (2007). Dissociation of estrogen receptor expression and *in vivo* stem cell activity in the mammary gland. *J. Cell Biol.* **176**, 19-26.
Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H. I. and Eaves, C. J. (2006a). Purification and unique properties of mammary epithelial stem cells. *Nature* **439**, 993-997.
Stingl, J., Raouf, A., Eirew, P. and Eaves, C. J. (2006b). Deciphering the mammary epithelial cell hierarchy. *Cell Cycle* **5**, 1519-1522.
Stoelzle, T., Schwarb, P., Trumpp, A. and Hynes, N. E. (2009). c-Myc affects mRNA translation, cell proliferation and progenitor cell function in the mammary gland. *BMC Biol.* **7**, 63.
Van Keymeulen, A., Rocha, A. S., Ousset, M., Beck, B., Bouvencourt, G., Rock, J., Sharma, N., Dekoninck, S. and Blanpain, C. (2011). Distinct stem cells contribute to mammary gland development and maintenance. *Nature* **479**, 189-193.
Visvader, J. E. (2009). Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev.* **23**, 2563-2577.
Visvader, J. E. and Smith, G. H. (2011). Murine mammary epithelial stem cells: discovery, function, and current status. *Cold Spring Harb. Perspect. Biol.* **3**.
Wakao, H., Gouilleux, F. and Groner, B. (1994). Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J.* **13**, 2182-2191.
Wartmann, M., Cella, N., Hofer, P., Groner, B., Liu, X., Hennighausen, L. and Hynes, N. E. (1996). Lactogenic hormone activation of Stat5 and transcription of the beta-casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity. *J. Biol. Chem.* **271**, 31863-31868.
Yamaji, D., Na, R., Feuermann, Y., Pechhold, S., Chen, W., Robinson, G. W. and Hennighausen, L. (2009). Development of mammary luminal progenitor cells is controlled by the transcription factor STAT5A. *Genes Dev.* **23**, 2382-2387.

5.2 Figures

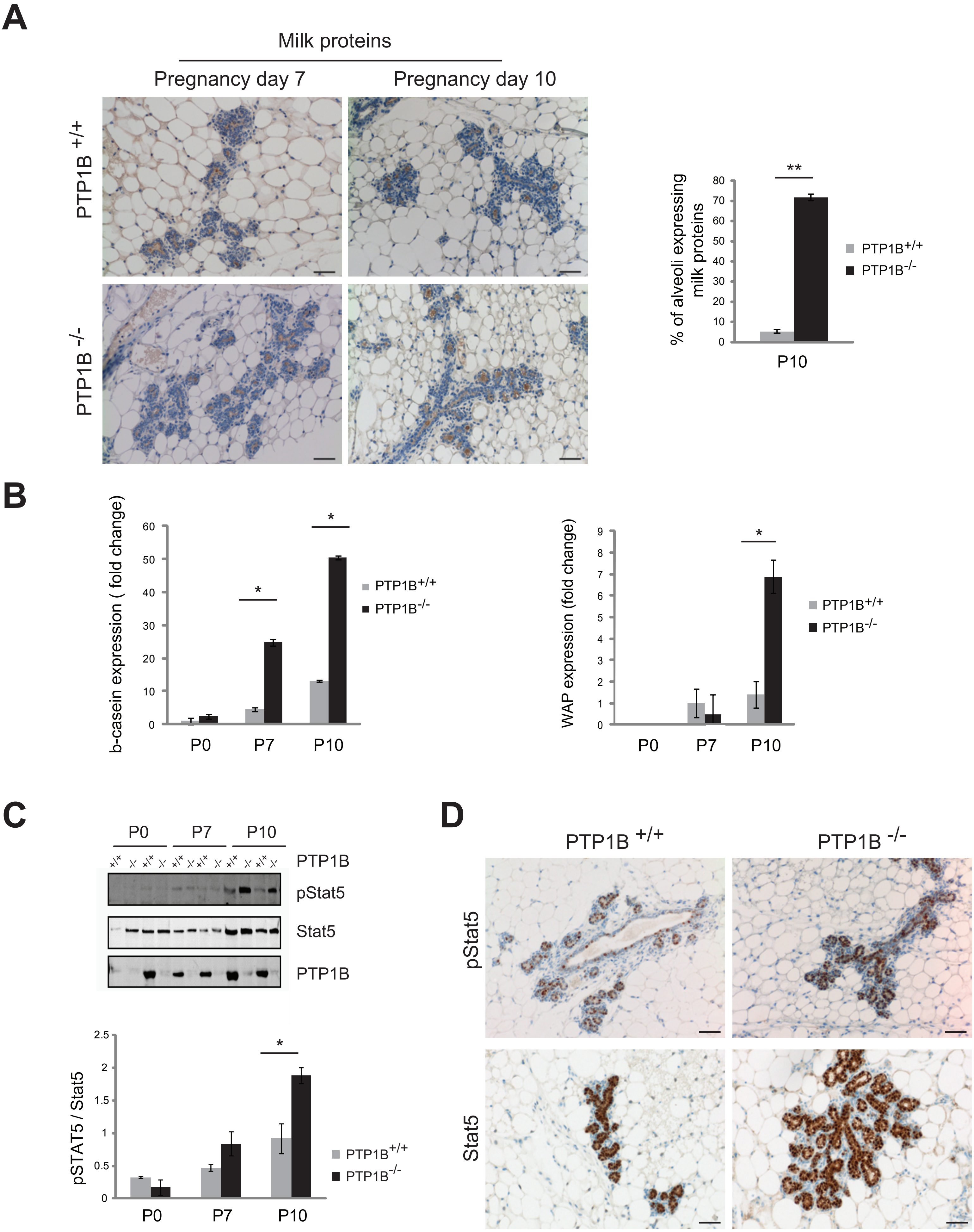


Milani et al. Figure 1
A
Pregnancy day 0
Pregnancy day 3
PTP1B+/+
PTP1B-/-
40x
40x
40x
40x
B
Pregnancy day 0
Pregnancy day 3
PTP1B+/+
PTP1B-/-
C
% of epithelial cells
3.5
3
2.5
2
1.5
1
0.5
0
*
PTP1B+/+
PTP1B-/-
P0
D
Pregnancy day 0
Ki67
PTP1B+/+
PTP1B-/-
% of Ki67+ epithelial cells
35
30
25
20
15
10
5
0
*
PTP1B+/+
PTP1B-/-
P0

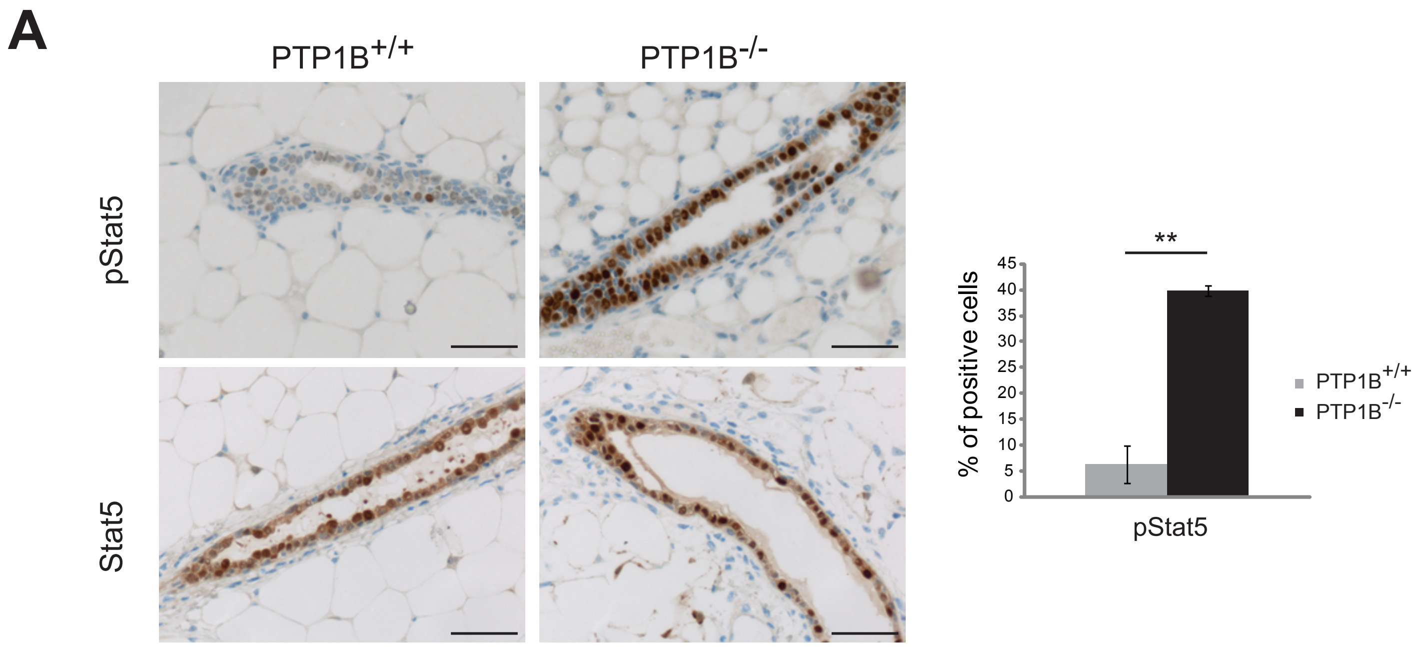
Milani et al. Figure 2

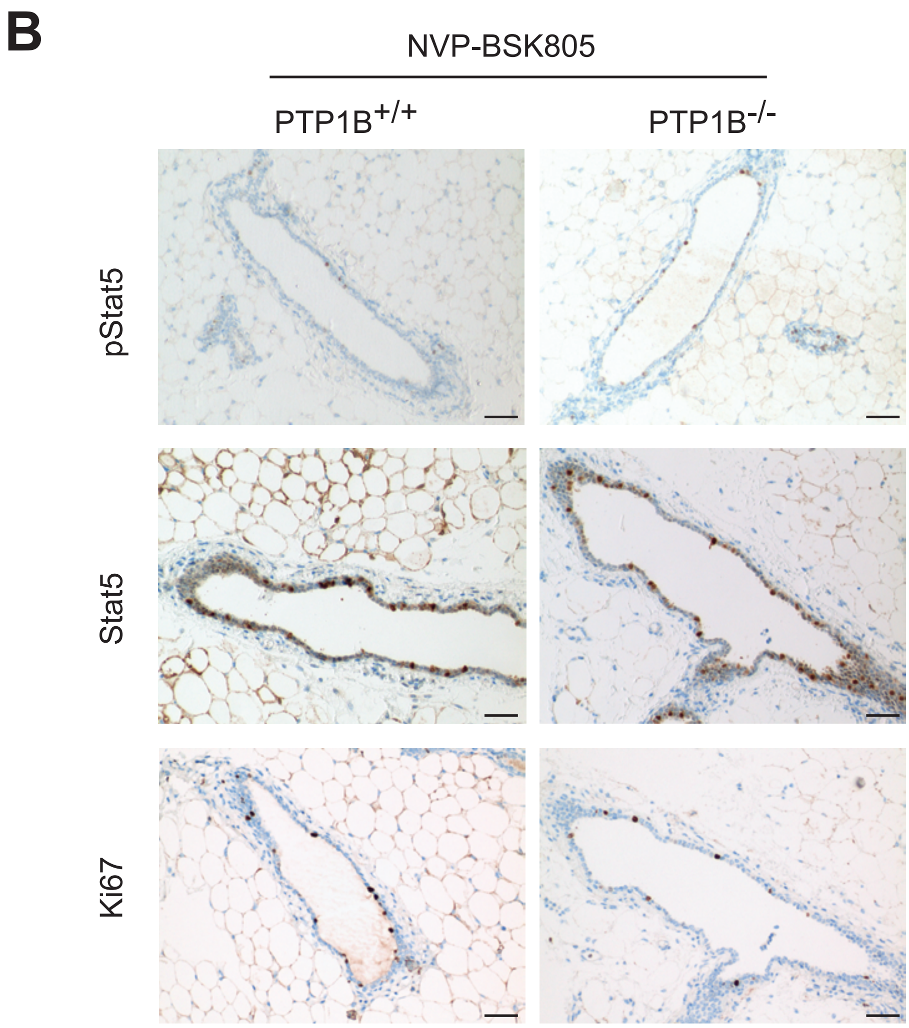


Milani et al. Figure 6

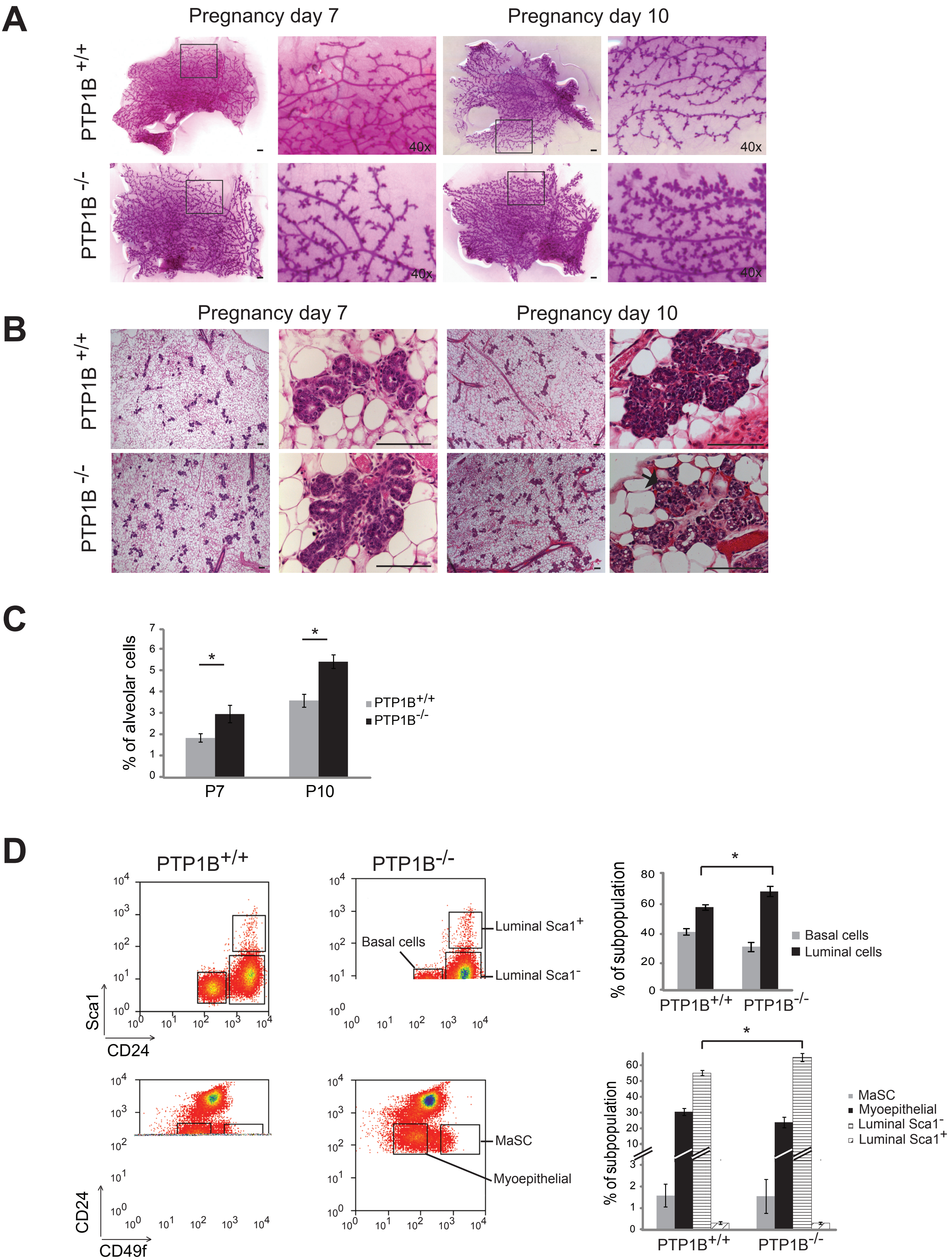


Milani et al. Figure 4

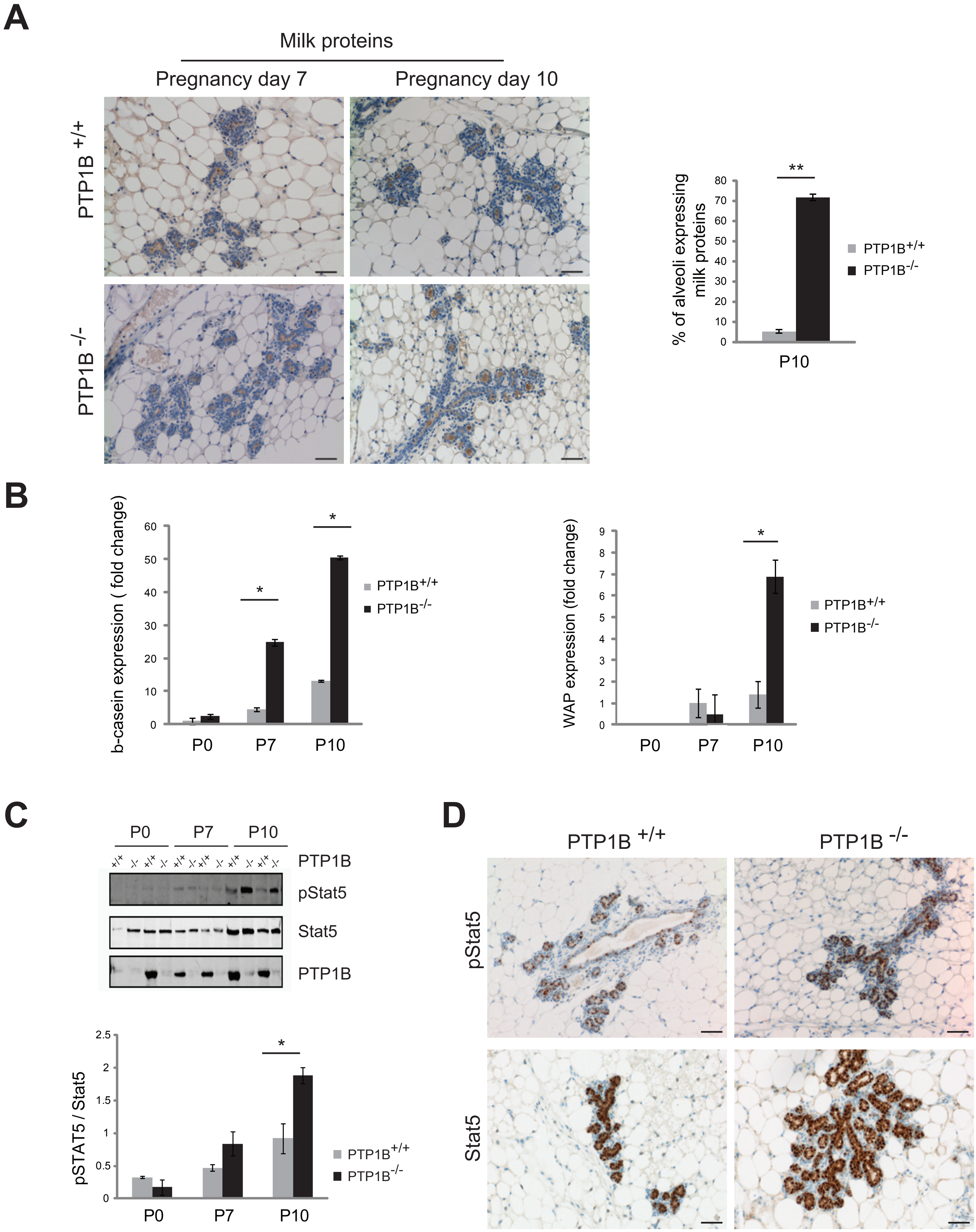




Milani et al. Figure 5



Milani et al. Figure 6



5.3 SUPPLEMENTARY INFORMATIONS

Supplemental Inventory

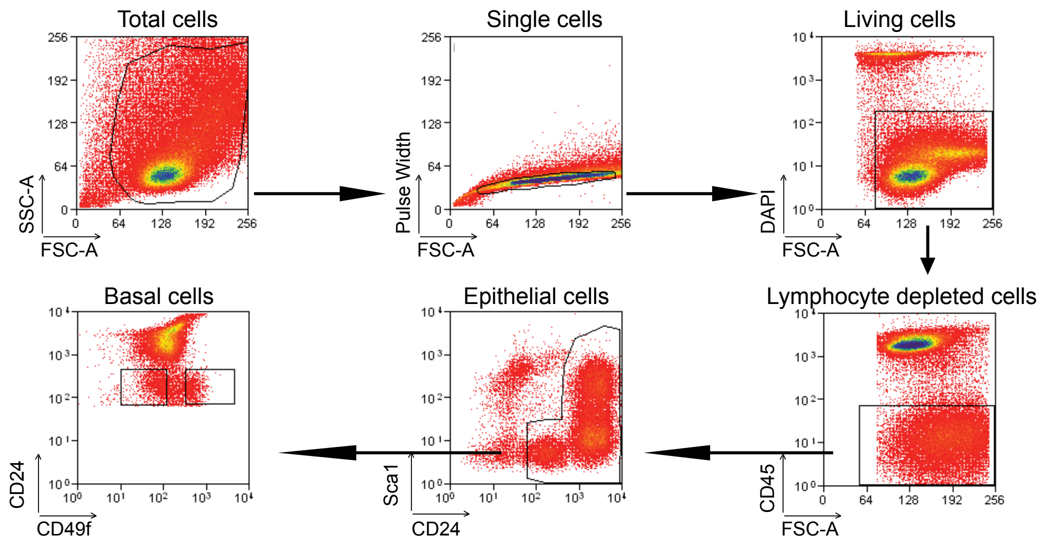
Figure S1: PTP1B depletion increases ALDH activity in mammary epithelial cells, related to Figure 2.

Figure S2: Absence of PTP1B increases expression of cell cycle genes and of the PR target Rank-L, related to Figure 3.

Figure S3: Absence of PTP1B does not affect plasma levels of estrogen in mice at estrous, related to Figure 3

Table S1: Top affected gene o ntology ter ms PTP1 B-/- vs. PTP1 B +/+ mammary g lands, related to Figure 3.

A



B

# epithelial cells	PTP1B WT	PTP1B KO	
10'000	3/3 ●●◕	3/3 ●●◕	
5'000	4/4 ●●◕◑	3/3 ●●◕	
2'000	3/3 ●●●	3/3 ●●●	
1'000	7/9 ●●◕◕ ◑◑◔	5/8 ●●● ◕◔	
500	4/9 ●◔◔◔	5/8 ●●● ◕◕	
MRU	1:663	1:838	p-Value 0.5

C

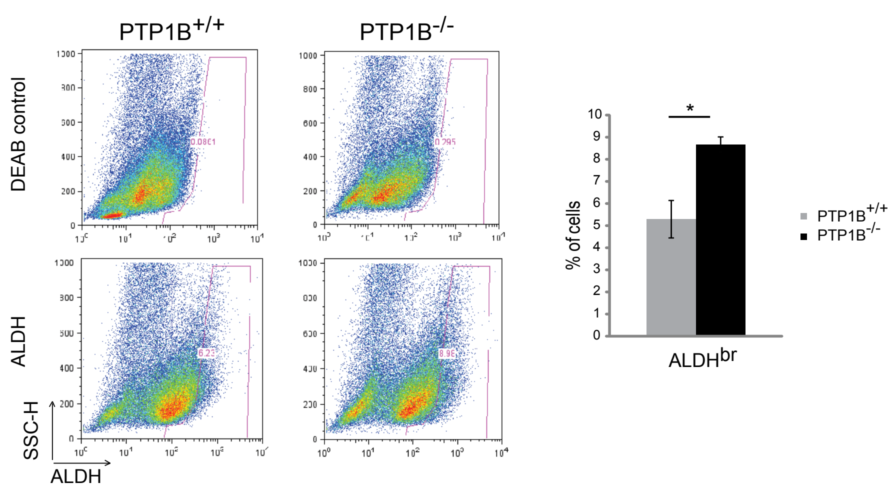


Fig. S1. PTP1B depletion increases ALDH activity in mammary epithelial cells. (**A**) Representative flow cytometry dot plots showing the gating strategy used to isolate mammary epithelial cell subpopulations. Cells were first gated on the basis of forward and side scatters (FSC-A and SSC-A), followed by the exclusion of doublets and higher-order cell clumps using a time-of-flight approach (pulse width). Dead cells were then gated out by DAPI staining, followed by the exclusion of leukocytes by CD45 staining. Finally, total epithelial cells were isolated based on their CD24 and Sca1 expression. Further isolation of MaSCs and myoepithelial cells was performed based on the expression of CD24 and CD49f. (**B**) Mammary repopulating activity of *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ MECs. 10,000, 5000, 2000, 1000 and 500 freshly isolated mammary epithelial cells (MECs) from *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ glands in estrus were injected into the cleared fat pad of FVB mice. The repopulation of the fat pad was scored 7 weeks after transplantation. The transplantation frequency analysis was performed using R and the statmod package as previously described (Shackleton et al., 2006). (**C**) (Left) Dot plot pattern of ALDH-positive cells showing an increase in ALDH activity in *Ptp1b*$^{-/-}$ MECs. (Right) The percentages of cells with high ALDH activity (ALDHbr) from *Ptp1b*$^{+/+}$ and *Ptp1b*$^{-/-}$ nulliparous mice at estrus. Values represent mean ± s.e.m. (n=3, *P<0.05 by Student's t-test).

A

Cell cycle genes upregulated in PTP1B -/-					
Acession #	Symbol	description	fold changes	p-value	Adj p-value
NM_009828	CCNA2	cyclin A2	2.46	0.0115	0.1768713
NM_011623	Topo2	topoisomerase (DNA) II alpha	2.30	0.0078	0.1592963
NM_007630	CCNB2	cyclin B2	2.14	0.0144	0.1916421
NM_011121	Plk1	polo-like kinase 1 (Drosophila)	2.14	0.0126	0.1836071
NM_007659	Cdc2a (CDK1)	cell division cycle 2 homolog A (S. pombe)	2.00	0.0104	0.1712105
NM_007691	Chek1	checkpoint kinase 1 homolog (S. pombe)	1.87	0.0244	0.2270902
NM_018754	SFN	stratifin	1.62	0.0005	0.062044
NM_001081117	Mki67	antigen identified by monoclonal Ab Ki 67	1.84	0.0418	0.2758198
NM_025415	CKS2	CDC28 protein kinase regulatory subunit 2	1.48	0.0403	0.272589

estrogen-responsive genes upregulated in PTP1B -/-					
Acession #	Symbol	description	fold changes	p-value	Adj p-value
NM_010118	Egr2	early growth response 2	3.00	2.28E-06	0.0039558
NM_009704	Areg	amphiregulin	2.88	9.55E-09	0.0003318
NM_007969	Expi	extracellular proteinase inhibitor	1.98	0.001761	0.0959992
NM_001081249	Vcan(cspg2)	vesican	1.94	0.001344	0.0889354
NM_010848	c-myb	myeloblastosis oncogene	1.93	3.95E-05	0.0176528
NM_008829	Pgr	Progesterone receptor	1.80	0.005725	0.1446963
NM_010849	c-myc	myelocytomatosis oncogene	1.59	0.002251	0.1048908
NM_011961	Plod2	procollagen lysine, 2-oxoglutarate 5-dioxy	1.57	0.005868	0.1450678
NM_010233	FN1	fibronectin 1	1.51	0.006227	0.1474954
NM_013650	S100A8	S100 calcium binding protein A8 (calgranul	1.46	0.009963	0.1695082
NM_033073	Krt7	keratin 7	1.43	0.007621	0.1586751
NM_019521	Gas6	growth arrest specific 6	1.41	0.002792	0.1120131
NM_008471	Krt19	keratin 19	1.41	0.020774	0.2154201

B

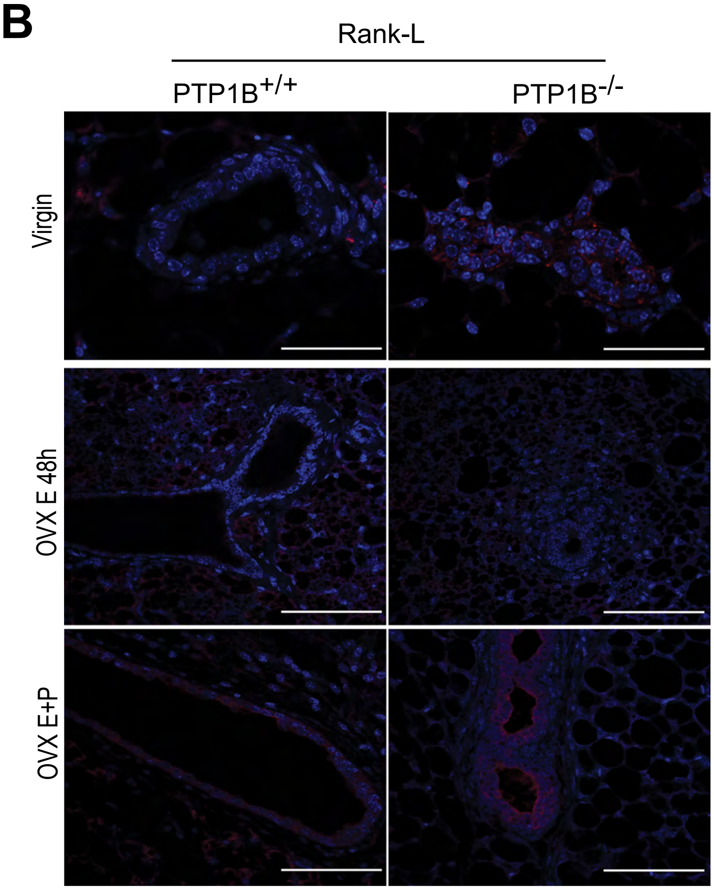


Fig. S2. Absence of PTP1B increases expression of cell cycle genes and of the PR target Rankl. (**A**) Upregulated cell cycle genes (top) and upregulated estrogen-responsive genes (bottom) in nulliparous *Ptp1b*$^{-/-}$ glands compared with wild-type littermates. (**B**) Representative images of mammary gland sections stained for Rankl. Scale bars: 50 μm.

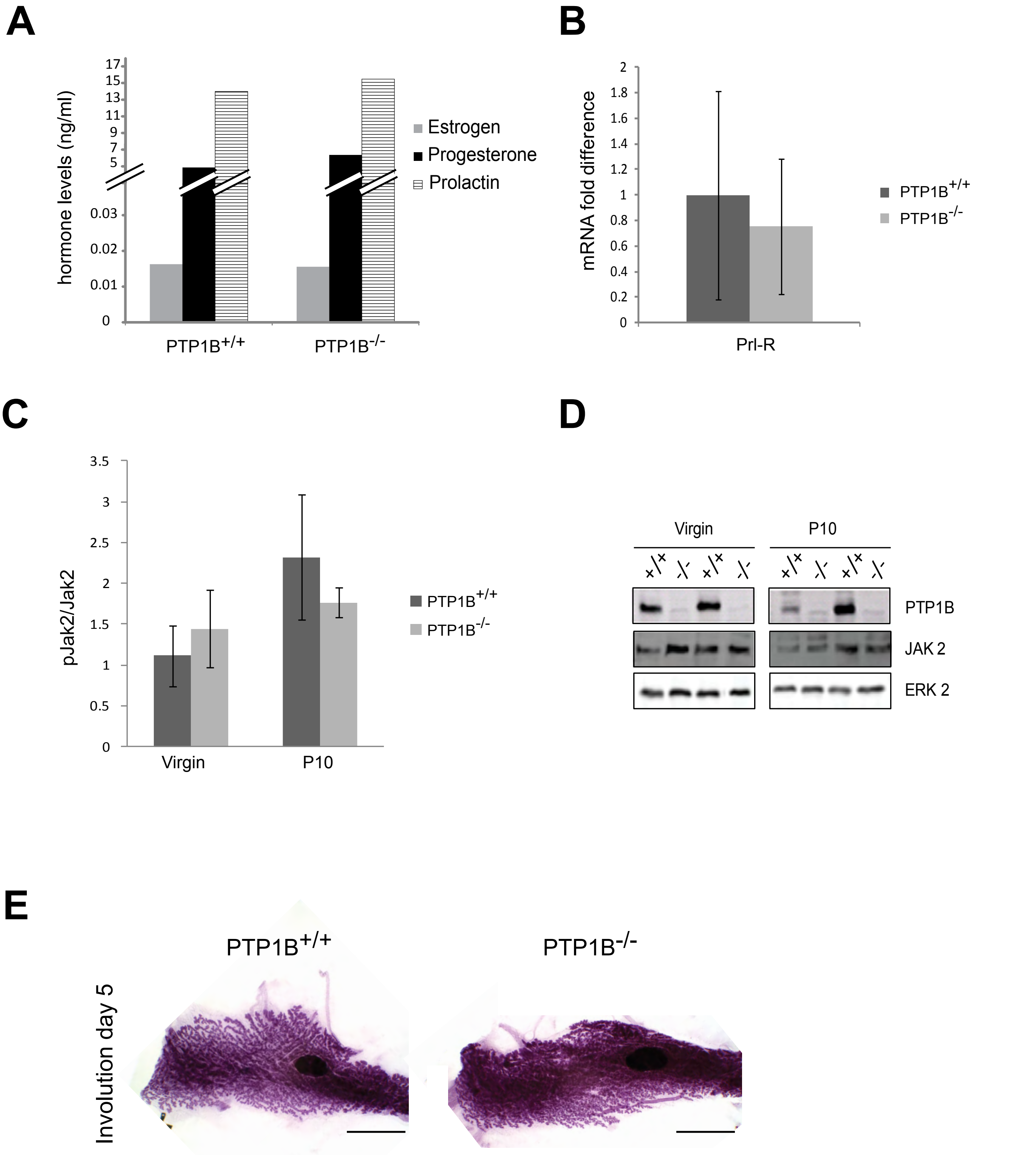


Fig. S3. Absence of PTP1B does not affect plasma levels of pregnancy hormones in mice at estrus and does not alter mammary gland involution. (**A**) Plasma levels of estrogen, progesterone and prolactin in nulliparous mice at estrus as assessed by ELISA (the plasma of three mice per group was pooled and analyzed using estradiol and progesterone ELISA from DRG Diagnostic and prolactin ELISA from Abcam). (**B**) Fold changes in Prl-R mRNA as assessed by quantitative real-time PCR. Values represent mean ± s.e.m. (n=3). (**C**) The ratios of pJak2/Jak2 in virgin and pregnant (day 10) mice. pJak2 levels were assessed using an ELISA assay (Tyr1007/1008, Invitrogen) and normalized to total Jak2 levels assessed by immunoblotting. Values represent mean ± s.e.m. (n=3, *P<0.05 by Student's t-test). (**D**) Immunoblots of lysates from glands in virgin and pregnancy day 10 mice (Jak2 antibody, Cell Signaling). (**E**) Representative images of whole-mounts of *Ptp1b*$^{-/-}$ and *Ptp1b*$^{+/+}$ mammary glands at involution day 5. Scale bars: 5 mm.

Table S1. Top 19 affected Gene Ontology terms in Ptp1b$^{-/-}$ versus Ptp1b$^{+/+}$ mammary glands

Gene Ontology term upregulated in Ptp1b–/–	Cluster frequency	Corrected P-value
Cellular process	244 of 359 genes, 68.0%	4.06E–26
Cell cycle	50 of 359 genes, 13.9%	3.85E–16
Cell division	30 of 359 genes, 8.4%	2.96E–15
Cell cycle phase	35 of 359 genes, 9.7%	4.94E–15
Cell cycle process	40 of 359 genes, 11.1%	7.08E–15
M phase	30 of 359 genes, 8.4%	2.50E–14
Biological regulation	168 of 359 genes, 46.8%	7.51E–14
M phase of mitotic cell cycle	24 of 359 genes, 6.7%	1.55E–12
Cellular component organization or	85 of 359 genes, 23.7%	2.32E–12
Cellular component organization	83 of 359 genes, 23.1%	2.34E–12
Nuclear division	23 of 359 genes, 6.4%	6.14E–12
Mitosis	23 of 359 genes, 6.4%	6.14E–12
Mitotic cell cycle	31 of 359 genes, 8.6%	6.45E–12
Organelle fission	23 of 359 genes, 6.4%	1.98E–11
Organelle organization	54 of 359 genes, 15.0%	3.02E–11
Regulation of biological process	157 of 359 genes, 43.7%	3.21E–11
Cellular component organization at cellular	68 of 359 genes, 18.9%	4.63E–11
Cellular component organization or biogenesis at cellular level	69 of 359 genes, 19.2%	1.29E–10
Metabolic process	159 of 359 genes, 44.3%	9.95E–10

Gene Ontology term downregulated in Ptp1b–/–	Cluster frequency	Corrected P-value
Alcohol metabolic process	10 of 62 genes, 16.1%	3.16E–06
Small molecule metabolic process	19 of 62 genes, 30.6%	3.18E–06
Glucose metabolic process	7 of 62 genes, 11.3%	5.47E–06
Carbohydrate metabolic process	10 of 62 genes, 16.1%	1.38E–05
Hexose metabolic process	7 of 62 genes, 11.3%	1.88E–05
Cellular carbohydrate metabolic process	9 of 62 genes, 14.5%	2.54E–05
Monosaccharide metabolic process	7 of 62 genes, 11.3%	6.20E–05
Cellular process	44 of 62 genes, 71.0%	0.00011
Carbohydrate transport	5 of 62 genes, 8.1%	0.00059
Skeletal muscle contraction	3 of 62 genes, 4.8%	0.00104
Localization	20 of 62 genes, 32.3%	0.00111
Transport	18 of 62 genes, 29.0%	0.00122
Striated muscle contraction	4 of 62 genes, 6.5%	0.0013
Establishment of localization	18 of 62 genes, 29.0%	0.00157
Biological regulation	33 of 62 genes, 53.2%	0.00245
Regulation of striated muscle contraction	3 of 62 genes, 4.8%	0.0026
Metabolic process	33 of 62 genes, 53.2%	0.0038
Protein homooligomerization	5 of 62 genes, 8.1%	0.00387

List of Gene Ontology terms upregulated and downregulated in Ptp1b$^{-/-}$ compared with Ptp1b$^{+/+}$ glands from nulliparous mice at estrus. Terms were obtained from the Process Ontology of gene_association.mgi (P<0.01).

6. DISCUSSION AND OUTLOOK

In this study we found that PTP1B plays an important role in mammary gland alveologenesis and lactogenesis. Using PTP1B knockout mice we determined that:

a) PTP1B controls mammary epithelial cell fate: it constrains the number of mammary alveolar progenitors in nulliparous mice and restrains alveoli formation during early pregnancy.
b) PTP1B also constrains milk expression during pregnancy.

6.1 Role of PTP1B in cell fate commitment and alveologenesis

Alveologenesis is a developmental program characterized by the expansion and differentiation of mammary progenitor cells into cells able to form alveoli. In this study we found that loss of PTP1B enhances the number of progenitor cells in nulliparous mice. This enhances the pool of cells able to generate alveolar structures during pregnancy and, therefore, results in the increased alveolar density observed in PTP1B$^{-/-}$ glands during early pregnancy.

Several factors influence mammary gland alveologensis. We found that lack of PTP1B induces the expression of several estrogen-responsive genes in nulliparous glands, including progesterone receptor and its downstream target RANK-L. PR plays a fundamental role in epithelial cell proliferation and alveolar formation during early pregnancy (Lydon 1995; Mulac-Jericevic 2003). Therefore, the precocious alveolar development observed in PTP1B$^{-/-}$ glands could be mediated by the overexpression and activation of PR, which is then able to initiate alveologenesis at an earlier time point than in the wild-type mammary glands.

In addition to PR, several transcription factors, like ELF5, GATA3 and STAT5, are important for alveolar development. These transcription factors regulate differentiation of mammary epithelial stem cells along the luminal epithelial lineage (Asselin-Labat 2007; Oakes 2008; Yamaji 2009). STAT5 is a hormonally responsive transcription factor that plays a key role in mammary gland alveologenesis and lactogenesis (Liu, Robinson et al. 1995; Liu, Robinson et al. 1997). Indeed, STAT5 increases the number of mammary luminal progenitor cells (Yamaji et al., 2009), enhances proliferation of epithelial cells, and promotes alveologenesis (Cui et al., 2004; Miyoshi et al., 2001). Similar phenotypes were also observed in mammary glands lacking PTP1B, suggesting STAT5 as molecular link, in addition to PR, between the loss of PTP1B and the precocious alveologenesis.

The findings that PTP1B depletion induces the expression of PR and the activation of STAT5 raise the possibility that they act in a common signaling pathway to regulate alveologenesis. Cui *et al.* showed that STAT5 required the hormonal stimulation of estrogen and progesterone to induce proliferation of epithelial cells in the mammary gland (Cui 2004). Furthermore, PR regulates expression of PrlR, a key regulator of STAT5 phosphorylation and activation (Ormandy 1992). These observations further suggest a molecular link between PR and STAT5 in PTP1B$^{-/-}$ mice.

In vitro studies have demonstrated that PTP1B can directly dephosphorylate STAT5 (Aoki 2002), opening the hypothesis that PTP1B may downregulate progesterone receptor expression and inhibit STAT5 activation in two independent pathways.

In conclusion, our studies suggest a role for PTP1B as a temporal regulator of mammary gland development, which downregulates the progesterone and STAT5 pathway(s) in order to prevent the inappropriate onset of alveologenesis.

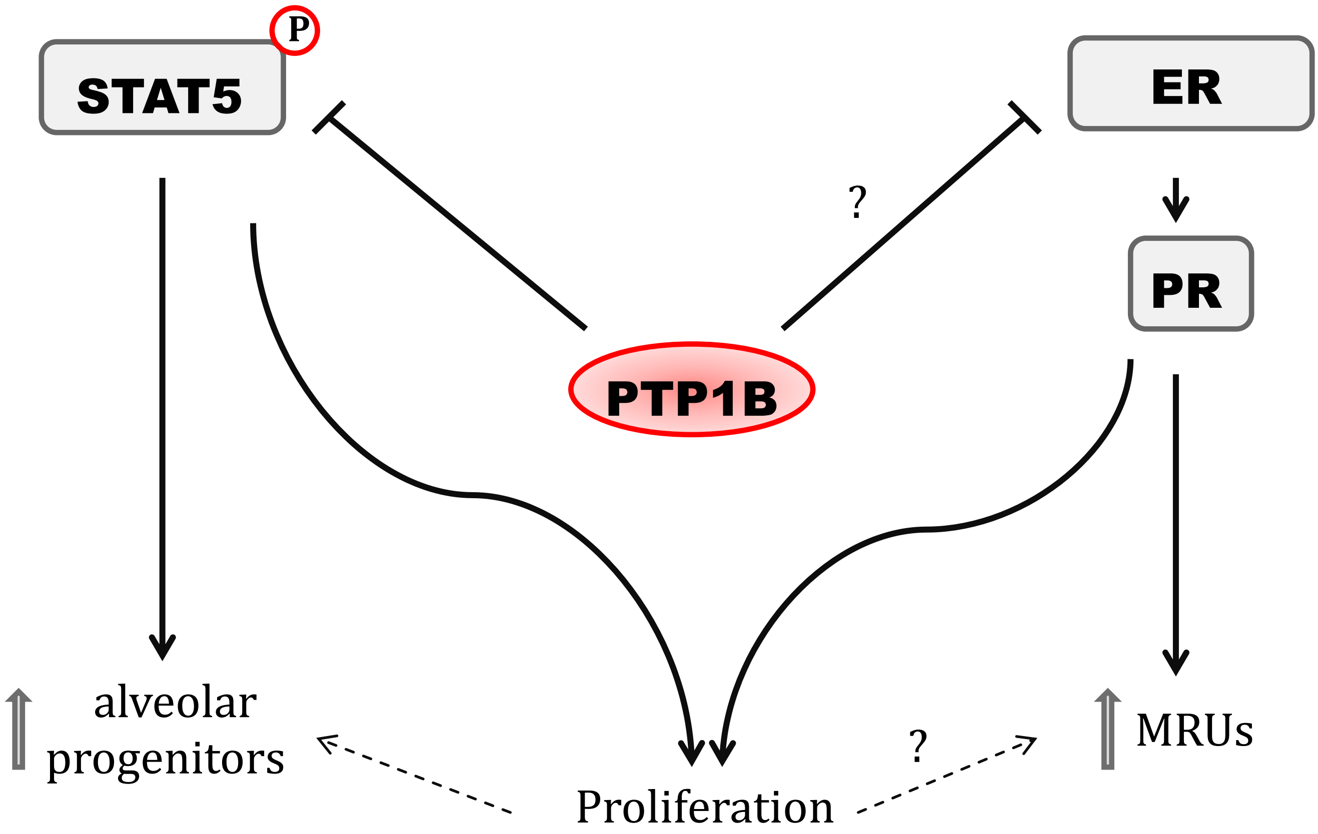


Fig 7 Model of the mechanism of action of PTP1B in the control of mammary epithelial cell fate.

6.2 Role of PTP1B in lactogenesis

Our studies also support a role of PTP1B in lactogenesis. We showed that PTP1B deletion induces precocious secretion of milk proteins during late pregnancy, due to an enhanced STAT5 phosphorylation and activation. This study suggests a role for PTP1B in the prevention of an inappropriate onset of lactogenesis during pregnancy.

6.3 Concluding remarks and future directions

Mammary gland development and differentiation is a complex mechanism regulated by several different pathways (Brisken 2010, Henninghausen 2005). Therefore, PTP1B may act via further pathways to constrain mammary gland alveologenesis and lactogenesis.

The increased activation of the ER and PR pathways seen in PTP1B$^{-/-}$ glands could be mediated by higher levels of hormones in the blood. However, we found no difference between estrogen, progesterone and prolactin levels in plasmas of PTP1B knockout and wild-type nulliparous mice. These results suggest that PTP1B constrains mammary alveologenesis by regulating the hormones responsiveness of the epithelial cells to normal circulating hormone levels.

Several studies showed that insulin, IGF 1 and IGF 2 are important in mammary gland morphogenesis (Kleinberg, Feldman et al. 2000; Brisken, Ayyannan et al. 2002; Hovey, Harris et al. 2003; Berlato and Doppler 2009). Since PTP1B is a well-known metabolic regulator that dephosphorylates the insulin and leptin pathways in other organs (Elchebly 1999, Klaman 2000), we cannot exclude that PTP1B may also act via the insulin pathways to constrain mammary gland morphogenesis. A hypothesis that warrants further investigations.

The mammary stem cell hierarchy is tightly regulated to guarantee the correct formation of a functional mammary gland. Deregulation in the mammary stem cell hierarchy may lead to breast cancer development. Therefore, the finding that PTP1B constrains the commitment of a primitive stem/progenitor cells to more differentiated progenitor cells open the hypothesis of a link between the role of PTP1B in mammary gland development and the involvement of PTP1B in tumorigenesis.

Epidemiological studies have shown that early menarche, late menopause and late age of first pregnancy are all risk factors for developing sporadic breast cancer (Kampert, Whittemore et al. 1988). The hormonal milieu and breast development cycles, probably through changes in the differentiation state of breast stem/progenitor cells, affect the susceptibility of the breast to oncogenic transformation. Our findings that PTP1B deletion induces precocious differentiation of the mammary gland raise the possibility that the cells of origin of HER2/Neu-evoked mammary tumors may be decreased in PTP1B$^{-/-}$ mice. This possibility would explain why deletion of PTP1B delays or prevents mammary tumor formation in MMTV-NeuNT and MMTV-NDL1 mice (Balavenkatraman et al., 2011; Bentires-Alj and Neel, 2007; Julien et al., 2007). This possibility merits further investigations.

7. REFERENCES

Akasaki, Y., G. Liu, et al. (2006). "A peroxisome proliferator-activated receptor-gamma agonist, troglitazone, facilitates caspase-8 and -9 activities by increasing the enzymatic activity of protein-tyrosine phosphatase-1B on human glioma cells." J Biol Chem **281**(10): 6165-74.

Alonso, A., J. Sasin, et al. (2004). "Protein tyrosine phosphatases in the human genome." Cell **117**(6): 699-711.

Anders, L., P. Mertins, et al. (2006). "Furin-, ADAM 10-, and gamma-secretase-mediated cleavage of a receptor tyrosine phosphatase and regulation of beta-catenin's transcriptional activity." Mol Cell Biol **26**(10): 3917-34.

Andersen, J. N., P. G. Jansen, et al. (2004). "A genomic perspective on protein tyrosine phosphatases: gene structure, pseudogenes, and genetic disease linkage." FASEB J **18**(1): 8-30.

Andersen, J. N., O. H. Mortensen, et al. (2001). "Structural and evolutionary relationships among protein tyrosine phosphatase domains." Mol Cell Biol **21**(21): 7117-36.

Ardini, E., R. Agresti, et al. (2000). "Expression of protein tyrosine phosphatase alpha (RPTPalpha) in human breast cancer correlates with low tumor grade, and inhibits tumor cell growth in vitro and in vivo." Oncogene **19**(43): 4979-87.

Arias-Romero, L. E., S. Saha, et al. (2009). "Activation of Src by protein tyrosine phosphatase 1B Is required for ErbB2 transformation of human breast epithelial cells." Cancer Res **69**(11): 4582-8.

Asselin-Labat, M. L., K. D. Sutherland, et al. (2007). "Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation." Nat Cell Biol **9**(2): 201-9.

Balavenkatraman, K. K., N. Aceto, et al. (2011). "Epithelial Protein-Tyrosine Phosphatase 1B Contributes to the Induction of Mammary Tumors by HER2/Neu but Is Not Essential for Tumor Maintenance." Mol Cancer Res **9**(10): 1377-84.

Balsamo, J., T. Leung, et al. (1996). "Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin." J Cell Biol **134**(3): 801-13.

Barford, D., A. J. Flint, et al. (1994). "Crystal structure of human protein tyrosine phosphatase 1B." Science **263**(5152): 1397-404.

Beleut, M., R. D. Rajaram, et al. (2010). "Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland." Proc Natl Acad Sci U S A **107**(7): 2989-94.

Ben-Jonathan, N., J. L. Mershon, et al. (1996). "Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects." Endocr Rev **17**(6): 639-69.

Bennett, A. M., T. L. Tang, et al. (1994). "Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor beta to Ras." Proc Natl Acad Sci U S A **91**(15): 7335-9.

Bentires-Alj, M. and B. G. Neel (2007). "Protein-tyrosine phosphatase 1B is required for HER2/Neu-induced breast cancer." Cancer Res **67**(6): 2420-4.

Bjorge, J. D., A. Pang, et al. (2000). "Identification of protein-tyrosine phosphatase 1B as the major tyrosine phosphatase activity capable of dephosphorylating and activating c-Src in several human breast cancer cell lines." J Biol Chem **275**(52): 41439-46.

Bocchinfuso, W. P., J. K. Lindzey, et al. (2000). "Induction of mammary gland development in estrogen receptor-alpha knockout mice." Endocrinology **141**(8): 2982-94.

Bombonati, A. and D. C. Sgroi (2011). "The molecular pathology of breast cancer progression." J Pathol **223**(2): 307-17.

Bompard, G., C. Puech, et al. (2002). "Protein-tyrosine phosphatase PTPL1/FAP-1 triggers apoptosis in human breast cancer cells." J Biol Chem **277**(49): 47861-9.

Bottini, N., L. Musumeci, et al. (2004). "A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes." Nat Genet **36**(4): 337-8.

Boutin, J. M., C. Jolicoeur, et al. (1988). "Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family." Cell **53**(1): 69-77.

Brautigan, D. L. and F. M. Pinault (1993). "Serine phosphorylation of protein tyrosine phosphatase (PTP1B) in HeLa cells in response to analogues of cAMP or diacylglycerol plus okadaic acid." Mol Cell Biochem **127-128**: 121-9.

Brinton, L. A., C. Schairer, et al. (1988). "Menstrual factors and risk of breast cancer." Cancer Invest **6**(3): 245-54.

Brisken, C., A. Heineman, et al. (2000). "Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling." Genes Dev **14**(6): 650-4.

Brisken, C., S. Kaur, et al. (1999). "Prolactin controls mammary gland development via direct and indirect mechanisms." Dev Biol **210**(1): 96-106.

Brisken, C. and B. O'Malley (2010). "Hormone action in the mammary gland." Cold Spring Harb Perspect Biol **2**(12): a003178.

Brisken, C., S. Park, et al. (1998). "A paracrine role for the epithelial progesterone receptor in mammary gland development." Proc Natl Acad Sci U S A **95**(9): 5076-81.

Brown-Shimer, S., K. A. Johnson, et al. (1990). "Molecular cloning and chromosome mapping of the human gene encoding protein phosphotyrosyl phosphatase 1B." Proc Natl Acad Sci U S A **87**(13): 5148-52.

Buckley, D. A., A. Cheng, et al. (2002). "Regulation of insulin-like growth factor type I (IGF-I) receptor kinase activity by protein tyrosine phosphatase 1B (PTP-1B) and enhanced IGF-I-mediated suppression of apoptosis and motility in PTP-1B-deficient fibroblasts." Mol Cell Biol **22**(7): 1998-2010.

Cao, Y., G. Bonizzi, et al. (2001). "IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development." Cell **107**(6): 763-75.

Chan, G., D. Kalaitzidis, et al. (2008). "The tyrosine phosphatase Shp2 (PTPN11) in cancer." Cancer Metastasis Rev **27**(2): 179-92.

Cheng, A., N. Uetani, et al. (2002). "Attenuation of leptin action and regulation of obesity by protein tyrosine phosphatase 1B." Dev Cell **2**(4): 497-503.

Chernoff, J., A. R. Schievella, et al. (1990). "Cloning of a cDNA for a major human protein-tyrosine-phosphatase." Proc Natl Acad Sci U S A **87**(7): 2735-9.

Ciarloni, L., S. Mallepell, et al. (2007). "Amphiregulin is an essential mediator of estrogen receptor alpha function in mammary gland development." Proc Natl Acad Sci U S A **104**(13): 5455-60.

Conneely, O. M., B. Mulac-Jericevic, et al. (2007). "Progesterone signaling in mammary gland development." Ernst Schering Found Symp Proc(1): 45-54.

Cool, D. E., N. K. Tonks, et al. (1989). "cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family." Proc Natl Acad Sci U S A **86**(14): 5257-61.

D'Cruz, C. M., S. E. Moody, et al. (2002). "Persistent parity-induced changes in growth factors, TGF-beta3, and differentiation in the rodent mammary gland." Mol Endocrinol **16**(9): 2034-51.

Dadke, S., S. Cotteret, et al. (2007). "Regulation of protein tyrosine phosphatase 1B by sumoylation." Nat Cell Biol **9**(1): 80-5.

Dadke, S., A. Kusari, et al. (2001). "Phosphorylation and activation of protein tyrosine phosphatase (PTP) 1B by insulin receptor." Mol Cell Biochem **221**(1-2): 147-54.

den Hertog, J., S. Tracy, et al. (1994). "Phosphorylation of receptor protein-tyrosine phosphatase alpha on Tyr789, a binding site for the SH3-SH2-SH3 adaptor protein GRB-2 in vivo." EMBO J **13**(13): 3020-32.

Denu, J. M. and K. G. Tanner (1998). "Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation." Biochemistry **37**(16): 5633-42.

Deome, K. B., L. J. Faulkin, Jr., et al. (1959). "Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice." Cancer Res **19**(5): 515-20.

Dube, N., A. Cheng, et al. (2004). "The role of protein tyrosine phosphatase 1B in Ras signaling." Proc Natl Acad Sci U S A **101**(7): 1834-9.

Elchebly, M., P. Payette, et al. (1999). "Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene." Science **283**(5407): 1544-8.

Falet, H., S. Pain, et al. (1998). "Tyrosine unphosphorylated platelet SHP-1 is a substrate for calpain." Biochem Biophys Res Commun **252**(1): 51-5.

Fata, J. E., Y. Y. Kong, et al. (2000). "The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development." Cell **103**(1): 41-50.

Felberg, J. and P. Johnson (1998). "Characterization of recombinant CD45 cytoplasmic domain proteins. Evidence for intramolecular and intermolecular interactions." J Biol Chem **273**(28): 17839-45.

Ferlay, J., P. Autier, et al. (2007). "Estimates of the cancer incidence and mortality in Europe in 2006." Ann Oncol **18**(3): 581-92.

Fernandez-Valdivia, R., A. Mukherjee, et al. (2009). "The RANKL signaling axis is sufficient to elicit ductal side-branching and alveologenesis in the mammary gland of the virgin mouse." Dev Biol **328**(1): 127-39.

Flint, A. J., T. Tiganis, et al. (1997). "Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases." Proc Natl Acad Sci U S A **94**(5): 1680-5.

Forsell, P. A., Y. Boie, et al. (2000). "Genomic characterization of the human and mouse protein tyrosine phosphatase-1B genes." Gene **260**(1-2): 145-53.

Fox, A. N. and K. Zinn (2005). "The heparan sulfate proteoglycan syndecan is an in vivo ligand for the Drosophila LAR receptor tyrosine phosphatase." Curr Biol **15**(19): 1701-11.

Frangioni, J. V., A. Oda, et al. (1993). "Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets." EMBO J **12**(12): 4843-56.

Freeman, M. E., B. Kanyicska, et al. (2000). "Prolactin: structure, function, and regulation of secretion." Physiol Rev **80**(4): 1523-631.

Freiss, G., G. Bompard, et al. (2004). "[PTPL1, a proapoptotic protein tyrosine phosphatase in breast cancers]." Bull Cancer **91**(4): 325-32.

Fukada, T. and N. K. Tonks (2001). "The reciprocal role of Egr-1 and Sp family proteins in regulation of the PTP1B promoter in response to the p210 Bcr-Abl oncoprotein-tyrosine kinase." J Biol Chem **276**(27): 25512-9.

Fukada, T. and N. K. Tonks (2003). "Identification of YB-1 as a regulator of PTP1B expression: implications for regulation of insulin and cytokine signaling." EMBO J **22**(3): 479-93.

Garton, A. J., M. R. Burnham, et al. (1997). "Association of PTP-PEST with the SH3 domain of p130cas; a novel mechanism of protein tyrosine phosphatase substrate recognition." Oncogene **15**(8): 877-85.

Ginestier, C., M. H. Hur, et al. (2007). "ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome." Cell Stem Cell **1**(5): 555-67.

Gonzalez-Rodriguez, A., O. Escribano, et al. (2007). "Levels of protein tyrosine phosphatase 1B determine susceptibility to apoptosis in serum-deprived hepatocytes." J Cell Physiol **212**(1): 76-88.

Gouilleux, F., H. Wakao, et al. (1994). "Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription." EMBO J **13**(18): 4361-9.

Green, K. A. and C. H. Streuli (2004). "Apoptosis regulation in the mammary gland." Cell Mol Life Sci **61**(15): 1867-83.

Groen, A., S. Lemeer, et al. (2005). "Differential oxidation of protein-tyrosine phosphatases." J Biol Chem **280**(11): 10298-304.

Gu, F., D. T. Nguyen, et al. (2004). "Protein-tyrosine phosphatase 1B potentiates IRE1 signaling during endoplasmic reticulum stress." J Biol Chem **279**(48): 49689-93.

Gu, M., K. Meng, et al. (1996). "The effect of overexpression of the protein tyrosine phosphatase PTPMEG on cell growth and on colony formation in soft agar in COS-7 cells." Proc Natl Acad Sci U S A **93**(23): 12980-5.

Haj, F. G., B. Markova, et al. (2003). "Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatase-1B." J Biol Chem **278**(2): 739-44.

Haj, F. G., P. J. Verveer, et al. (2002). "Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum." Science **295**(5560): 1708-11.

Harris, J., P. M. Stanford, et al. (2006). "Socs2 and elf5 mediate prolactin-induced mammary gland development." Mol Endocrinol **20**(5): 1177-87.

Heinrich, R., B. G. Neel, et al. (2002). "Mathematical models of protein kinase signal transduction." Mol Cell **9**(5): 957-70.

Hennighausen, L. and G. W. Robinson (2005). "Information networks in the mammary gland." Nat Rev Mol Cell Biol **6**(9): 715-25.

Hernandez, M. V., M. G. Sala, et al. (2006). "ER-bound PTP1B is targeted to newly forming cell-matrix adhesions." J Cell Sci **119**(Pt 7): 1233-43.

Hornberg, J. J., F. J. Bruggeman, et al. (2005). "Principles behind the multifarious control of signal transduction. ERK phosphorylation and kinase/phosphatase control." FEBS J **272**(1): 244-58.

Horseman, N. D., W. Zhao, et al. (1997). "Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene." EMBO J **16**(23): 6926-35.

Howlin, J., J. McBryan, et al. (2006). "Pubertal mammary gland development: insights from mouse models." J Mammary Gland Biol Neoplasia **11**(3-4): 283-97.

Jemal, A., F. Bray, et al. (2011). "Global cancer statistics." CA Cancer J Clin **61**(2): 69-90.

Johnson, K. G., A. P. Tenney, et al. (2006). "The HSPGs Syndecan and Dallylike bind the receptor phosphatase LAR and exert distinct effects on synaptic development." Neuron **49**(4): 517-31.

Johnson, K. J., A. R. Peck, et al. (2010). "PTP1B suppresses prolactin activation of Stat5 in breast cancer cells." Am J Pathol **177**(6): 2971-83.

Julien, S. G., N. Dube, et al. (2007). "Protein tyrosine phosphatase 1B deficiency or inhibition delays ErbB2-induced mammary tumorigenesis and protects from lung metastasis." Nat Genet **39**(3): 338-46.

Kampert, J. B., A. S. Whittemore, et al. (1988). "Combined effect of childbearing, menstrual events, and body size on age-specific breast cancer risk." Am J Epidemiol **128**(5): 962-79.

Klaman, L. D., O. Boss, et al. (2000). "Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice." Mol Cell Biol **20**(15): 5479-89.

Kong, Y. Y., H. Yoshida, et al. (1999). "OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis." Nature **397**(6717): 315-23.

Kordon, E. C. and G. H. Smith (1998). "An entire functional mammary gland may comprise the progeny from a single cell." Development **125**(10): 1921-30.

Krege, J. H., J. B. Hodgin, et al. (1998). "Generation and reproductive phenotypes of mice lacking estrogen receptor beta." Proc Natl Acad Sci U S A **95**(26): 15677-82.

LaMarca, H. L. and J. M. Rosen (2008). "Minireview: hormones and mammary cell fate--what will I become when I grow up?" Endocrinology **149**(9): 4317-21.

LaMontagne, K. R., Jr., G. Hannon, et al. (1998). "Protein tyrosine phosphatase PTP1B suppresses p210 bcr-abl-induced transformation of rat-1 fibroblasts and promotes differentiation of K562 cells." Proc Natl Acad Sci U S A **95**(24): 14094-9.

Lessard, L., M. Stuible, et al. (2010). "The two faces of PTP1B in cancer." Biochim Biophys Acta **1804**(3): 613-9.

Levea, C. M., C. T. McGary, et al. (2000). "PTP LAR expression compared to prognostic indices in metastatic and non-metastatic breast cancer." Breast Cancer Res Treat **64**(2): 221-8.

Li, J., C. Yen, et al. (1997). "PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer." Science **275**(5308): 1943-7.

Liu, F., M. A. Sells, et al. (1998). "Transformation suppression by protein tyrosine phosphatase 1B requires a functional SH3 ligand." Mol Cell Biol **18**(1): 250-9.

Liu, X., G. W. Robinson, et al. (1995). "Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue." Proc Natl Acad Sci U S A **92**(19): 8831-5.

Liu, X., G. W. Robinson, et al. (1997). "Stat5a is mandatory for adult mammary gland development and lactogenesis." Genes Dev **11**(2): 179-86.

Long, W., K. U. Wagner, et al. (2003). "Impaired differentiation and lactational failure of Erbb4-deficient mammary glands identify ERBB4 as an obligate mediator of STAT5." Development **130**(21): 5257-68.

Lund, L. R., J. Romer, et al. (1996). "Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways." Development **122**(1): 181-93.

Lydon, J. P., F. J. DeMayo, et al. (1995). "Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities." Genes Dev **9**(18): 2266-78.

MacMahon, B., P. Cole, et al. (1970). "Age at first birth and breast cancer risk." Bull World Health Organ **43**(2): 209-21.

Mallepell, S., A. Krust, et al. (2006). "Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland." Proc Natl Acad Sci U S A **103**(7): 2196-201.

Melchor, L. and J. Benitez (2008). "An integrative hypothesis about the origin and development of sporadic and familial breast cancer subtypes." Carcinogenesis **29**(8): 1475-82.

Meng, T. C., D. A. Buckley, et al. (2004). "Regulation of insulin signaling through reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B." J Biol Chem **279**(36): 37716-25.

Meng, T. C., T. Fukada, et al. (2002). "Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo." Mol Cell **9**(2): 387-99.

Molofsky, A. V., R. Pardal, et al. (2004). "Diverse mechanisms regulate stem cell self-renewal." Curr Opin Cell Biol **16**(6): 700-7.

Mustelin, T., T. Vang, et al. (2005). "Protein tyrosine phosphatases and the immune response." Nat Rev Immunol **5**(1): 43-57.

Myers, M. P., J. N. Andersen, et al. (2001). "TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B." J Biol Chem **276**(51): 47771-4.

Myers, M. P., I. Pass, et al. (1998). "The lipid phosphatase activity of PTEN is critical for its tumor supressor function." Proc Natl Acad Sci U S A **95**(23): 13513-8.

Naylor, M. J., N. Li, et al. (2005). "Ablation of beta1 integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation." J Cell Biol **171**(4): 717-28.

Neville, M. C., T. B. McFadden, et al. (2002). "Hormonal regulation of mammary differentiation and milk secretion." J Mammary Gland Biol Neoplasia **7**(1): 49-66.

Nguyen, D. X., P. D. Bos, et al. (2009). "Metastasis: from dissemination to organ-specific colonization." Nat Rev Cancer **9**(4): 274-84.

Oakes, S. R., M. J. Naylor, et al. (2008). "The Ets transcription factor Elf5 specifies mammary alveolar cell fate." Genes Dev **22**(5): 581-6.

Oakes, S. R., R. L. Rogers, et al. (2008). "Prolactin regulation of mammary gland development." J Mammary Gland Biol Neoplasia **13**(1): 13-28.

Ormandy, C. J., A. Camus, et al. (1997). "Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse." Genes Dev **11**(2): 167-78.

Ormandy, C. J., M. Naylor, et al. (2003). "Investigation of the transcriptional changes underlying functional defects in the mammary glands of prolactin receptor knockout mice." Recent Prog Horm Res **58**: 297-323.

Ostman, A., C. Hellberg, et al. (2006). "Protein-tyrosine phosphatases and cancer." Nat Rev Cancer **6**(4): 307-20.

Panagopoulos, I., N. Pandis, et al. (1996). "The FHIT and PTPRG genes are deleted in benign proliferative breast disease associated with familial breast cancer and cytogenetic rearrangements of chromosome band 3p14." Cancer Res **56**(21): 4871-5.

Park, D. S., H. Lee, et al. (2002). "Caveolin-1-deficient mice show accelerated mammary gland development during pregnancy, premature lactation, and hyperactivation of the Jak-2/STAT5a signaling cascade." Mol Biol Cell **13**(10): 3416-30.

Perez-Pinera, P., O. Garcia-Suarez, et al. (2007). "The receptor protein tyrosine phosphatase (RPTP)beta/zeta is expressed in different subtypes of human breast cancer." Biochem Biophys Res Commun **362**(1): 5-10.

Perou, C. M., T. Sorlie, et al. (2000). "Molecular portraits of human breast tumours." Nature **406**(6797): 747-52.

Prat, A., J. S. Parker, et al. (2010). "Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer." Breast Cancer Res **12**(5): R68.

Pulido, R., A. Zuniga, et al. (1998). "PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif." EMBO J **17**(24): 7337-50.

Ramaswamy, B., S. Majumder, et al. (2009). "Estrogen-mediated suppression of the gene encoding protein tyrosine phosphatase PTPRO in human breast cancer: mechanism and role in tamoxifen sensitivity." Mol Endocrinol **23**(2): 176-87.

Ravichandran, L. V., H. Chen, et al. (2001). "Phosphorylation of PTP1B at Ser(50) by Akt impairs its ability to dephosphorylate the insulin receptor." Mol Endocrinol **15**(10): 1768-80.

Regan, J. L., H. Kendrick, et al. (2011). "c-Kit is required for growth and survival of the cells of origin of Brca1-mutation-associated breast cancer." Oncogene.

Reya, T., S. J. Morrison, et al. (2001). "Stem cells, cancer, and cancer stem cells." Nature **414**(6859): 105-11.

Richert, M. M., K. L. Schwertfeger, et al. (2000). "An atlas of mouse mammary gland development." J Mammary Gland Biol Neoplasia **5**(2): 227-41.

Robinson, G. W. (2007). "Cooperation of signalling pathways in embryonic mammary gland development." Nat Rev Genet **8**(12): 963-72.

Rudolph, M. C., J. L. McManaman, et al. (2007). "Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine." Physiol Genomics **28**(3): 323-36.

Ruhe, J. E., S. Streit, et al. (2006). "EGFR signaling leads to downregulation of PTP-LAR via TACE-mediated proteolytic processing." Cell Signal **18**(9): 1515-27.

Russo, J., X. Ao, et al. (1999). "Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland." Breast Cancer Res Treat **53**(3): 217-27.

Russo, J., R. Moral, et al. (2005). "The protective role of pregnancy in breast cancer." Breast Cancer Res **7**(3): 131-42.

Salmeen, A. and D. Barford (2005). "Functions and mechanisms of redox regulation of cysteine-based phosphatases." Antioxid Redox Signal **7**(5-6): 560-77.

Sangwan, V., G. N. Paliouras, et al. (2006). "Protein-tyrosine phosphatase 1B deficiency protects against Fas-induced hepatic failure." J Biol Chem **281**(1): 221-8.

Shackleton, M., F. Vaillant, et al. (2006). "Generation of a functional mammary gland from a single stem cell." Nature **439**(7072): 84-8.

Silberstein, G. B., K. Van Horn, et al. (1996). "Progesterone receptors in the mouse mammary duct: distribution and developmental regulation." Cell Growth Differ **7**(7): 945-52.

Sims, A. H., A. Howell, et al. (2007). "Origins of breast cancer subtypes and therapeutic implications." Nat Clin Pract Oncol **4**(9): 516-25.

Sivaraman, L. and D. Medina (2002). "Hormone-induced protection against breast cancer." J Mammary Gland Biol Neoplasia **7**(1): 77-92.

Slamon, D. J., G. M. Clark, et al. (1987). "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene." Science **235**(4785): 177-82.

Sleeman, K. E., H. Kendrick, et al. (2006). "CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells." Breast Cancer Res **8**(1): R7.

Sleeman, K. E., H. Kendrick, et al. (2007). "Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland." J Cell Biol **176**(1): 19-26.

Smith, G. H. (1996). "Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype." Breast Cancer Res Treat **39**(1): 21-31.

Smith, G. H. and D. Medina (1988). "A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland." J Cell Sci **90 (Pt 1)**: 173-83.

Sorlie, T., C. M. Perou, et al. (2001). "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications." Proc Natl Acad Sci U S A **98**(19): 10869-74.

Soulsby, M. and A. M. Bennett (2009). "Physiological signaling specificity by protein tyrosine phosphatases." Physiology (Bethesda) **24**: 281-9.

Sternlicht, M. D. (2006). "Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis." Breast Cancer Res **8**(1): 201.

Sternlicht, M. D., H. Kouros-Mehr, et al. (2006). "Hormonal and local control of mammary branching morphogenesis." Differentiation **74**(7): 365-81.

Stingl, J., P. Eirew, et al. (2006). "Purification and unique properties of mammary epithelial stem cells." Nature **439**(7079): 993-7.

Streuli, M., N. X. Krueger, et al. (1992). "Expression of the receptor-linked protein tyrosine phosphatase LAR: proteolytic cleavage and shedding of the CAM-like extracellular region." EMBO J **11**(3): 897-907.

Streuli, M., N. X. Krueger, et al. (1990). "Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein tyrosine phosphatases LCA and LAR." EMBO J **9**(8): 2399-407.

Stuible, M., K. M. Doody, et al. (2008). "PTP1B and TC-PTP: regulators of transformation and tumorigenesis." Cancer Metastasis Rev **27**(2): 215-30.

Sun, T., N. Aceto, et al. (2011). "Activation of multiple proto-oncogenic tyrosine kinases in breast cancer via loss of the PTPN12 phosphatase." Cell **144**(5): 703-18.

Tao, J., C. C. Malbon, et al. (2001). "Insulin stimulates tyrosine phosphorylation and inactivation of protein-tyrosine phosphatase 1B in vivo." J Biol Chem **276**(31): 29520-5.

Tartaglia, M., C. M. Niemeyer, et al. (2003). "Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia." Nat Genet **34**(2): 148-50.

Thordarson, G., E. Jin, et al. (1995). "Refractoriness to mammary tumorigenesis in parous rats: is it caused by persistent changes in the hormonal environment or permanent biochemical alterations in the mammary epithelia?" Carcinogenesis **16**(11): 2847-53.

Tonks, N. K. (2003). "PTP1B: from the sidelines to the front lines!" FEBS Lett **546**(1): 140-8.

Tonks, N. K., C. D. Diltz, et al. (1988). "Purification of the major protein-tyrosine-phosphatases of human placenta." J Biol Chem **263**(14): 6722-30.

Visvader, J. E. and G. J. Lindeman (2006). "Mammary stem cells and mammopoiesis." Cancer Res **66**(20): 9798-801.

Vomachka, A. J., S. L. Pratt, et al. (2000). "Prolactin gene-disruption arrests mammary gland development and retards T-antigen-induced tumor growth." Oncogene **19**(8): 1077-84.

Wagner, K. U., A. Krempler, et al. (2004). "Impaired alveologenesis and maintenance of secretory mammary epithelial cells in Jak2 conditional knockout mice." Mol Cell Biol **24**(12): 5510-20.

Wartmann, M., N. Cella, et al. (1996). "Lactogenic hormone activation of Stat5 and transcription of the beta-casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity." J Biol Chem **271**(50): 31863-8.

Watson, C. J. (2006). "Post-lactational mammary gland regression: molecular basis and implications for breast cancer." Expert Rev Mol Med **8**(32): 1-15.

Watson, C. J. and W. T. Khaled (2008). "Mammary development in the embryo and adult: a journey of morphogenesis and commitment." Development **135**(6): 995-1003.

Wiener, J. R., J. A. Hurteau, et al. (1994). "Overexpression of the tyrosine phosphatase PTP1B is associated with human ovarian carcinomas." Am J Obstet Gynecol **170**(4): 1177-83.

Wiener, J. R., B. J. Kerns, et al. (1994). "Overexpression of the protein tyrosine phosphatase PTP1B in human breast cancer: association with p185c-erbB-2 protein expression." J Natl Cancer Inst **86**(5): 372-8.

Wiseman, B. S. and Z. Werb (2002). "Stromal effects on mammary gland development and breast cancer." Science **296**(5570): 1046-9.

Woodford-Thomas, T. A., J. D. Rhodes, et al. (1992). "Expression of a protein tyrosine phosphatase in normal and v-src-transformed mouse 3T3 fibroblasts." J Cell Biol **117**(2): 401-14.

Wu, X., K. Senechal, et al. (1998). "The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway." Proc Natl Acad Sci U S A **95**(26): 15587-91.

Yamaji, D., R. Na, et al. (2009). "Development of mammary luminal progenitor cells is controlled by the transcription factor STAT5A." Genes Dev **23**(20): 2382-7.

Yang, T., J. S. Zhang, et al. (1999). "Leukocyte common antigen-related tyrosine phosphatase receptor: increased expression and neuronal-type splicing in breast cancer cells and tissue." Mol Carcinog **25**(2): 139-49.

Yip, S. C., S. Saha, et al. (2010). "PTP1B: a double agent in metabolism and oncogenesis." Trends Biochem Sci **35**(8): 442-9.

Yuan, T., Y. Wang, et al. "Protein-tyrosine phosphatase PTPN9 negatively regulates ErbB2 and epidermal growth factor receptor signaling in breast cancer cells." J Biol Chem **285**(20): 14861-70.

Zabolotny, J. M., K. K. Bence-Hanulec, et al. (2002). "PTP1B regulates leptin signal transduction in vivo." Dev Cell **2**(4): 489-95.

Zhan, Y., G. J. Counelis, et al. (2009). "The protein tyrosine phosphatase SHP-2 is required for EGFRvIII oncogenic transformation in human glioblastoma cells." Exp Cell Res **315**(14): 2343-57.

Zheng, J., S. K. Kulp, et al. (2000). "17 beta-estradiol-regulated expression of protein tyrosine phosphatase gamma gene in cultured human normal breast and breast cancer cells." Anticancer Res **20**(1A): 11-9.

Zheng, X., R. J. Resnick, et al. (2008). "Apoptosis of estrogen-receptor negative breast cancer and colon cancer cell lines by PTP alpha and src RNAi." Int J Cancer **122**(9): 1999-2007.

Zhi, H. Y., S. W. Hou, et al. "PTPH1 cooperates with vitamin D receptor to stimulate breast cancer growth through their mutual stabilization." Oncogene **30**(14): 1706-15.

8. ABBREVIATIONS

CD24	heat stable antigen
CD29	b-integrin
CD49f	a6-integrin
CD61	b3-integrin
ER	estrogen receptor
HER2	epidermal growth factor receptor 2
IGF 1	insulin-like growth factor 1
IGF 2	insulin-like growth factor 2
IR	insulin receptor
JAK2	janus kinase 2
MaSC	mammary stem cell
MEC	mammary epithelial cells
MRU	mammary repopulating units
PR	progesterone receptor
PrlR	prolactin receptor
PrlR	Prolactin
PTK	protein-tyrosine kinases
PTP	protein tyrosine-phosphatases
PTP1B	protein-tyrosine phosphatase 1B
RANK-L	activator of nuclear factor kB (NF-kB) ligand
ROS	reactive oxygen species
STAT5	signal transducers and activators of transcription 5

9. Acknowledgements

I would like to show my gratitude to Momo, who gave me assistance and support throughout my PhD. Especially, the tremendous effort he put in into teaching me how to get over my initial “fear” of mice.

A special thanks goes, to all the members of my thesis committee for the support and suggestions given me during these years. Greatly to Nancy Hynes and Robert Clarke, who followed my project since the beginning of my thesis. Sincerely to, Gilbert Smith who wasn’t able to come to my thesis defense, but was always ready to discuss my project during different conferences. Profoundly to Georg Holländer, who was not able to attend my thesis defense due to a severe illness, but was an invaluable help during my thesis. I am especially grateful to Gerhard Christofori who took over the position of co-referee at the very last moment.

I would like to thank Heike, Nicola and Dominique with whom I shared the privilege in building up this lab from scratch. Together we shared many experiences professionally and personally. Gratitude goes also to the former and current lab members: Simon, Kamal, Abdullah, Nina, Janice, Fabienne, Ina, Shany, Stephan, Adrian and Jeff. Last but not least I would like to thank Laura and Heike, who played a big role during my ups and downs in these years.

I am indebted to my FMI colleagues that made it possible for the completion of my project.

Finally, I am heartily thankful to my family and all my friends who bore with me through the good and bad times during these years.

Emanuela Sara Milani

Adress	Binzmühlestrasse 78 8050 Zürich Switzerland
Phone	0041 76 391 62 79
e-mail	emanuela.milani@fmi.ch
Birthplace	Pforzheim (Germany)
Nationality	Swiss

EDUCATIONAL DETAILS

2011-2012 **Friedrich Miescher Institute for Biomedical Research (FMI), Basel, Switzerland**

Post-Doctor

2011 **Basic in Project Management, University Basel, Switzerland**

2006-2011 **Friedrich Miescher Institute for Biomedical Research (FMI), Basel, Switzerland**

PhD in biochemistry, International PhD program

Title of PhD thesis: **“Role of PTP1B in mammary gland development and secretory differentiation”**. Supervisor: Dr. Mohamed Bentires-Alj.
Earned the PhD title *Cum Laude*

Learned and developed broad number of techniques and analysis skills. Developed good ability to design and performed confirmatory and exploratory experiments in addition to knowledge in statistical analysis. As one of the founding members of our group I also contributed to the set-up of new laboratory space and tutored new lab members (students and scientists).

2000-2005 **Swiss Federal Institute of Technology Zürich (ETH Zürich), Switzerland**

Degree in Biochemistry and Molecular Biology

Diploma subjects include Biochemistry, Molecular Biology and Biophysics, Cell Biology, Genetic and Immunology

Title of Semester work and Diploma work: **“Characterization of dURI in Schneider’s Drosophila S2 culture cells”.** Supervisor Prof. Dr. Wilhelm Krek, Institute of Cell Biology, ETH Zürich.

Developed excellent knowledge of key concepts of the major fields of biological science, especially biochemistry, molecular and cell biology. Worked in small groups to plan, carry out and conduct different laboratory experiments.

Completed the university studies with 5.82 mark (over 6)

LABORATORY SKILLS

Microscopy: fluorescence and confocal microscopy.
Cell culture: cell lines and primary cells culturing, adherent and suspension cells, Co-culture epithelial and stromal cells.
FACS sorting: 6 color FACS sorting.
Biochemistry: protein purification, antibody purification, immunoprecipitation, protein quantification, Western blot, electrophoresis: agarose gel, SDS-PAGE, 2D gel
Molecular Biology: PCR, PCR primer design, RT-PCR, Sequencing, Bacteria transformation transient transfection, DNA isolation, RNA isolation, dsRNA preparation, cloning, stable infection.
Immunostaining: immunohistochemistry, immunofluorescence, freezing microtome, paraffin microtome, laser dissection, whole mounts.
Animal experiments: Preparation and extraction of mammary gland, dissection and isolation of primary cells from mammary gland, intraperitoneal drugs injection, animal operation.
Human tissue: isolation and culturing of primary cells from human reduction mammoplasty and tumor biopsy.

COMPUTER SKILLS

Windows: MS Office (Word, PowerPoint, Excel, Outlook), Adobe Illustrator, Photoshop, GraphPad.
Image optimization and analysis: use of several programs to analyze and quantify data. Photoshop, Image Pro Plus, Fiji, Axiovision, Miramax, Odyssey, FlowJo, Summit (Dako).

Molecular Biology: BLAST, Gene set enrichment analysis (GSEA), Expressionist, Ingenuity Systems Pathway Analysis, Vector NTI.
Data mining: in Pubmed and NCBI sequence databases swissprot, Endnote/Reference Manager.

SCIENTIFIC EXPERIENCES

November 2006 Practica at the Institute of Cell Biology ETH Zürich in the group of Sabina Werner. Developed good knowledge in genetic and histology techniques and acquired good ability to solve problem.

April-May 2006 Help assistant at the institute of Cell Biology, ETH Zürich.

2005 Laboratory assistant in biochemistry for students from the first years. Successfully trained first year student to be able to plan and develop an experiment in biochemistry and to analyze and solve problem when experiments were not successful.

2004 Help assistant at the Institute of Biochemistry, ETH Zürich.

POSITIONS AND RESPONSIBILITY

2009-2011 **Student representative** at the Friedrich Miescher Institute for Biomedical Research (FMI), Basel, Switzerland. Organized seminars and invited outstanding scientific speakers and Nobel Prize winners, partnered with and another scientific institute to organize a joint PhD conference in the Pyrenees mountains, Spain. Organized and collected founding for the PhD conference.

OTHER WORK EXPERIENCES

2002-2008 Voluntary service for disabled adults. Accompanied and motivated disabled adult to discover the Swiss mountains.

Summer 2006 Voluntary service in an orphanage in San Salvador. I assisted children, taught English and instructed basic computer skills. Provided an introduction in basic spoken Spanish.

Summer 2000 Voluntary service in Madagascar. Organized and collected founding for the construction of an infirmary in Madagascar. Volunteered in the construction of part of the infirmary, increasing my team work skills and augmenting the creativity in developing instruments with what the surrounding nature was offering.

1996-2000 Swimming instructor. Taught children to swim and planed training sessions to improve their swimming techniques.

OTHER SKILLS AND INTERESTS

Language: Italian mother tongue

	Understanding		Speaking	Writing
	Listening	Reading		
German	Excellent	Excellent	Excellent	Excellent
English	Excellent	Excellent	Fluent	Fluent
French	Excellent	Excellent	Fluent	Fluent

Interests: Sport like swimming, ballet and jogging. Participated to the half marathon 2010 in Basel Activity like scouts, assistance to disabled persons, voluntary services.

PUBLICATIONS

Development 140, 117-125

Protein tyrosine phosphatase 1B restrains mammary alveologenesis and secretory differentiation
Emanuela Milani, Heike Brinkhaus, Ina Klebba, Regula Dueggeli, Urs Mueller, Hubertus Kohler, Matthew Smalley, Mohamed Bentires-Alj

POSTERS

June 2008/2010	Gordon Research Conference in Mammary Gland Biology, Il Ciocco (Barga), Italy. **Protein Tyrosine Phosphatase 1B (PTP1B) Restrains Alveolar Development and Secretory Differentiation.**
April 2009	European Network of Breast Development and Cancer labs, Weggis, Switzerland. **Role of PTP1B in mammary gland development**

REFERENCES

Dr. Mohamed Bentires
Friedrich Miescher Institute
for Biomedical Research
Maulbeerstrasse 66,
CH-4058 Basel
Switzerland
Tel.: Tel.: +416169774048
mohamed.bentires-alj@fmi.ch

Prof. Dr. Nancy Hynes
Friedrich Miescher Institute
for Biomedical Research
Maulbeerstrasse 66,
CH-4058 Basel
Switzerland
Tel.: +41616978089
nancy.hynes@fmi.ch

Attached:
Phd certificate
Diploma/Master in Biochemistry and Molecular Biology