Searching for a functional relationship between the breast cancer susceptibility gene BRCA1 and the progesterone receptor in breast cancer cells

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Dedicado muy especialmente a mi abuelo Delfín, y a mi abuela Isaura,
y, sobre todo, a mis padres Juan y Emilia

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No diré más porque no cabe pero vosotros/as ya sabéis que es más de lo que queda aquí escrito. Pero sirva de muestra.

Abstract

Germ-line mutations in the *breast cancer susceptibility gene* BRCA1 strongly increase the risk of developing breast and ovarian cancer in women. Different hypothesis have been proposed to explain this tissue specificity. One of the most argued hypothesis is the one that proposes a link between BRCA1 and ovarian hormones' action. Much data have been published in the last years pointing to an important role of progesterone receptor (PR) in inducing normal mammary development and also breast cancer formation. This study aimed to search for a functional relationship between BRCA1 and PR in breast cancer cells. We have found, by means of different approaches, that BRCA1 inhibits the transcriptional activity of PR. We have investigated in more detail the mechanism of this effect. BRCA1 and PR interact *in vivo* in a ligand-independent fashion. Most importantly, BRCA1 alters the ligand-independent and dependent degradation of PR protein through its ubiquitination and this might have a direct effect on the level of PR recruitment on regulated promoters. BRCA1 is recruited to the hormone-responsive regions of PR-target genes and affects the presence of histone deacetylase activity and the level of monoubiquitinated histone H2A, linking BRCA1 action with chromatin status. These findings support a connection between BRCA1, the principal tumour suppressor responsible for familial breast cancer, and the progesterone receptor transcriptional activity. This relationship can be hypothesized to be reflected in the BRCA1-related breast tumourigenesis.

Resumen

Mutaciones germinales en el gen breast cancer susceptibility gene BRCA1 aumentan altamente el riesgo de padecer cáncer de mama y ovario en mujeres. Se han propuesto diferentes hipótesis para explicar esta especificidad de tejido. Una de las hipótesis más argumentadas es la que propone una relación entre BRCA1 y la acción de las hormonas ováricas. En los últimos años se han publicado numerosos datos señalando al papel esencial del receptor de progesterona (PR) en la inducción del desarrollo normal de la mama y en la formación del cáncer de mama. Este estudio pretendía buscar una relación funcional entre BRCA1 y PR en células de cáncer de mama. A través de diferentes estrategias, hemos observado que BRCA1 inhibe la actividad transcripcional de PR. Hemos investigado en más detalle el mecanismo de este efecto. BRCA1 y PR interaccionan *in vivo* de una manera independiente de ligando. Y lo que es más, BRCA1 altera la degradación independiente y dependiente de ligando de PR a través de su ubiquitinización y esto podría tener un efecto directo en el nivel de reclutamiento de PR en promotores regulados. BRCA1 es reclutado a las regiones de respuesta a hormona de genes diana de PR y afecta la presencia de actividad histona desacetilasa y el nivel de histona H2A monoubiquitinada, estableciendo un enlace entre la acción de BRCA1 y el estado de la cromatina. Estos hallazgos apoyan una conexión entre BRCA1, el principal supresor de tumor responsable del cáncer de mama hereditario, y la actividad transcripcional del receptor de progesterona. Se puede hipotetizar que esta relación se ve reflejada en el proceso de tumorigénesis BRCA1-dependiente.

Preface

Since the discovery of BRCA1 as a prominent breast cancer susceptibility gene, several proposals have attempted to explain the tissue specificity consistently observed in BRCA1-mutant carriers. It was difficult to understand how such an essential and ubiquitous protein could be so specific in malignancy. As years passed by and the knowledge about the protein increased exponentially (some years being on the crest of the wave, some others going unnoticed), it seemed that, more than getting a clearer view of what is going on in the BRCA1-driven tumourigenesis process, things were getting more and more blurred. BRCA1 seemed to be involved in many diverse events, diversily interacting with proteins, getting involved in enzymatic reactions, and everything. To date, those questions and hypothesis are still up in the air, the image is still blur, things still look quite unconnected. We are just right at the point when images are framed but not focused. In this thesis we have attempted to focus the images into the frame to built up a view of BRCA1-related breast cancer tumourigenesis.

Index

INTRODUCTION

I.1 Mammary gland and breast cancer

I.1.1 Normal breast biology

I.1.1.1 Development

The breast is an unusual organ in that much of its development occurs in defined stages that are connected to sexual development and reproduction. The basics of the gland are developed during embryogenesis, when newly formed breast epithelial cells become inserted at the epithelial-stromal border and separate into 10-15 branches of epithelial ducts that open separately onto the epidermal surface at the nipple. After birth, mammary development is arrested until puberty. At puberty, the network of ducts leading from the nipple grows and divides into bundles of primary and secondary ducts lined with epithelial cells and ending with end bud structures (Terminal End Buds, TEBs). The adult female breast then consists of a branching, tree-like network of ducts lined by a double layer of epithelial cells and with end bud structures, which is surrounded by delimiting fibroblasts and embedded in an extracellular matrix. It is from the end buds and ductal side branches that the Terminal Ductal-Lobular Units (TDLU), or lobules, form (Fig. A). These lobules exist initially as alveolar buds that mature following menarche into a variable number of blind-ending, secretory sacs known as acini, alveoli or ductules. TDLUs become more elaborate with each successive ovulatory cycle, until the age of 35 years, approximately.

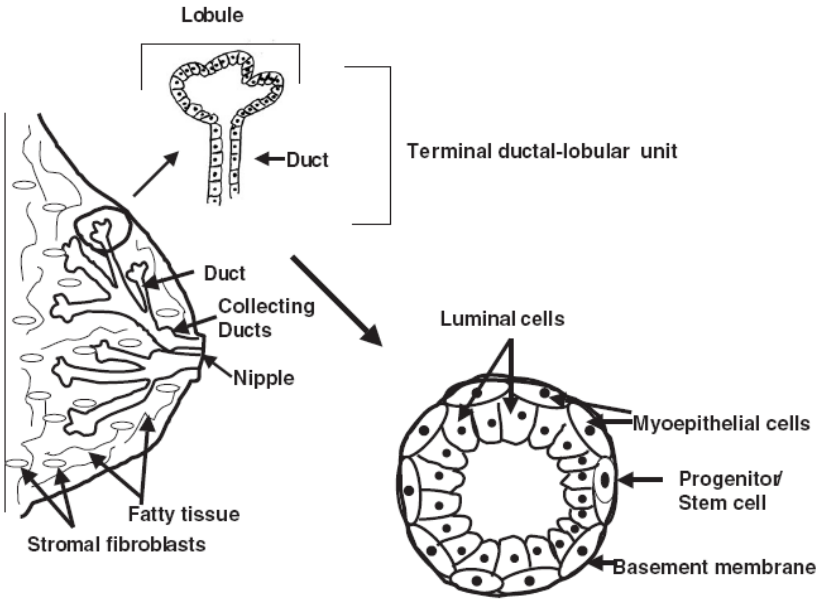


Fig A: ***Structure of the mammary gland****. The terminal ductal-lobular units (TDLUs) constitute the functional part of the adult mammary gland. They are composed by myoepithelial or basal cells, luminal cells and putative stem cells.*

The full development of the TDLUs is accelerated during pregnancy with the formation of tertiary branches, which terminate in alveolar buds, and the rapid proliferation of the luminal epithelium accompanied by differentiation and commitment to the secretory alveolar lineage. A lactogenic switch occurs during late pregnancy that is accompanied by the expression of milk proteins, whey acidic protein (WAP) and α-lactalbumin, and by the formation of lipid droplets.
Finally, following lactation, the lobules involute. Removal of the surplus alveolar cells is accomplished by cell death (apoptosis), the alveoli collapse and all components are remodelled to resemble those present in the non-pregnant gland, although they may retain a larger number of individual alveoli per lobule than before (Clarke, 2006; Watson and Khaled, 2008). Post-lactational regression, or involution, is the most dramatic example of physiologically regulated apoptosis in an adult tissue.

I.1.1.2 Mammary gland cell types

As mentioned before, the mammary gland is organized into a tree-like structure of hollow branches constituted by different epithelial cell phenotypes (Fig. A). The inner layer is formed by luminal epithelial cells that face the lumen and are surrounded by an outer layer of basal or myoepithelial cells and the basal lamina separating the mammary parenchyma from the stroma. Within the mammary arbor, the ductal cells are those that line the ducts of the mammary gland, which are surrounded by a continuous layer of contractile myoepithelial cells that contract in response to oxytocin stimulation during lactation.
In the non-pregnant gland, the myo- and luminal epithelial cells are distinguished not only by their relative positions, but also by the proteins that they express. The myoepithelium expresses a distinct subset of epithelial cytokeratins (CK 5 and 14), the Common Acute Lymphoblastic Leukaemia Antigen (CALLA) and smooth muscle actin. In contrast, the luminal cell type can be distinguished by the expression of a subset of epithelial cytokeratins (CK 8, 18 and 19), low (but detectable) levels of milk proteins and they may express nuclear receptors for the ovarian hormones estrogen (ER) and progesterone (PR).

The ability to replenish the mammary gland through cycles of pregnancy, lactation and involution throughout a woman's lifetime is attributed to stem cells that are proposed to reside in the mammary gland (Williams and Daniel, 1983; Novelli *et al.*, 2003; Tsai *et al.*, 1996). These cells are proposed to serve three functions: to give rise to the tissues of the adult mammary gland during development; to allow the enormous tissue expansion and remodeling that occurs in the mammary gland during multiple cycles of pregnancy, lactation and involution; and, rarely, to serve as a reserve for repair in the event of tissue damage.

The work of several laboratories has expanded the identification of distinct populations of stem/progenitor cells in the mammary gland. They showed that these stem/progenitor cells display different degrees of commitment and express distinguishable markers (Fig. B) (Woodward *et al.,*

2005). As the cell becomes more committed, the cell gradually loses its stemness.
The Long-Term Label-Retaining Cells (LTLRCs) might represent the most quiescent, primitive, template-retaining stem cells present in the stem cell niche. These stem cells are able to self-renew (process by which a stem cell produces a similar daughter cell by symmetric division) and proliferate within the niche. They are maintained in their undifferentiated state by cell-matrix and cell-cell interactions within the niche, involving integrin and cadherin proteins, respectively.
Upon stimulation, stem cells exit the niche by becoming Short-Term Label Retaining Cells (ST-LRCs). ST-LRCs actively cycle and are labelled by BrdU (a synthetic nucleoside analogue of thymidine, which is incorporated into newly synthesized DNA) but retain their original DNA template strand. They express stem cell markers such as p21, MSI-1 and CK19.
As they get further committed, they become the Transit-Amplifying progenitors (TAs), comprising the Side Population (SP) that are able to efflux the Hoechst dye (a characteristic used to identify potential stem cells). The SP/TAs express bipotential markers, such as $CK18^+$ and $CK14^+$, or EMA^- $CALLA^-$, and may be steroid receptor positive. These cells might correspond to the steroid receptor-positive cells found by Clarke *et al* to co-express several stem cell markers (CK19, p21, MSI-1, α6-integrin, among others) (Clarke *et al.,* 2005; Clarke, 2006).
The SP/TA cells eventually give rise to more committed progenitors that are $SCA1^+$. The $SCA1^+$ population differentiates into luminal and myoepithelial cells.

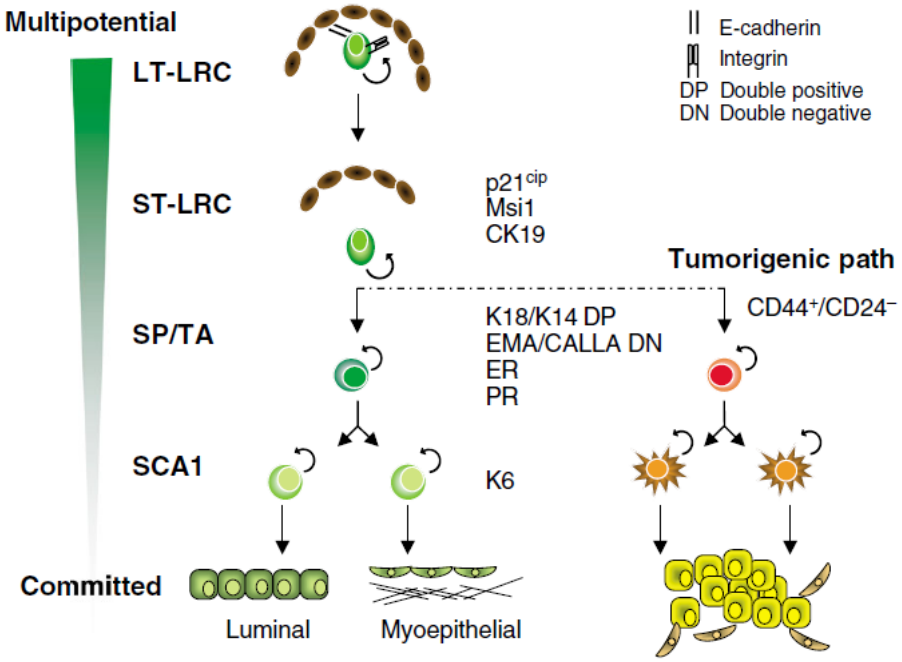


Fig B: ***Mammary gland stem/progenitor-cell fate.*** *The degree of stemness potentially decreases from top to bottom. The long-term label-retaining cells (LT-LRCs) represent the most primitive quiescent stem cells. In response to stimuli, they exit the niche by becoming short-term label retaining cells (ST-LRCs). These cells actively cycle and*

express markers such as p21, Msi1 and CK19. As they get further committed, they become the side population transit-amplifying cells (SP/TA). These cells express bipotential markers such as CK18+ and CK14+ or EMA- and CALLA-, and they may be steroid receptor positive. The SP/TA cells eventually give rise to more committed progenitors SCA1+. The SCA1+ cells differentiates into luminal or myoepithelial/basal cells. Tumorigenic mutations are presumably sustained in the SP/TA population. These cells give rise to tumourigenic progenitor cells CD44+CD24-. Adapted from Woodward et al., 2005.

I.1.1.3 Proliferative and differentiative control at the normal mammary gland

Systemic hormones (like progesterone, estrogen and prolactin), along with local growth factors like EGF and cytokines produced either in the stromal or epithelial compartment (RANKL, members of the hedgehog and TGFβ families, inhibin-βB) regulate stem cell fate and lineage commitment during mammary gland development, although the precise genetic mechanism remains undefined. The data by Clarke and colleagues suggest a model where scattered steroid receptor-positive stem cells are necessary for generating differentiated cells (luminal or basal epithelial cells) in a smaller patch of lobular epithelium in response to the cyclical release of ovarian hormones during menstrual cycles (Clarke *et al.,* 2005) (Fig. C).

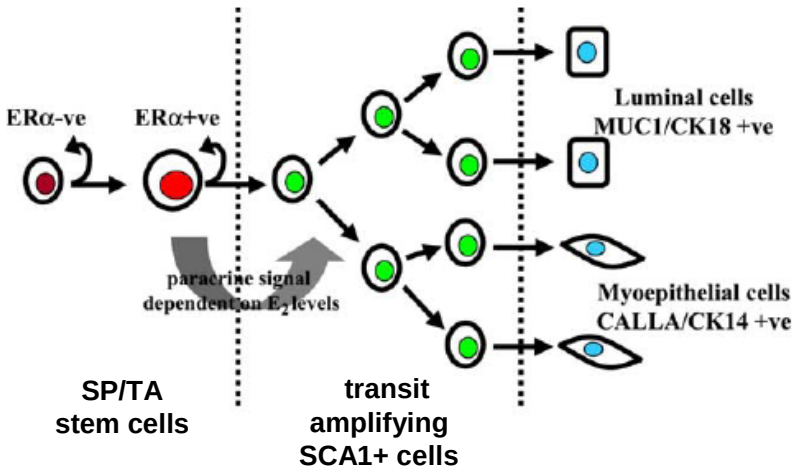


Fig C: *In the model proposed by Clarke and colleagues, steroid receptor-positive SP-TA stem cells undergo asymmetric division to give rise to transit amplifying SCA1+ cells. The proliferation of the transit amplifying cells would be regulated by paracrine factors released from steroid receptor-positive cells and it would lead to the final differentiation of these cells into myoepithelial/basal or luminal cells. Adapted from Clarke et al., 2005.*

Actually, Sartorius *et al* (Sartorius *et al*., 2005) observed that long-term treatment with progesterone leads to the appearance of cell subpopulations expressing basal markers CK5 and 6 among the cells of a tumour originated from T47D luminal epithelial ductal cancer cells (ER/PR and CK8, 18 and 19 positive). This would indicate that steroid hormones, in particular progesterone, can influence the differentiation state of proliferating tumours towards one type of cell population or another.

Indeed, ovarian hormones are absolutely necessary for the development, proliferation and differentiation of the normal human mammary gland. Evidence for ovarian control over mammary development first emerged 100 years ago, when Halban (1900) demonstrated that oophorectomy (ovary removal) caused mammary regression and that transplanted ovaries prevented this atrophy. The responsible bioactive compounds turned out to be the hormones progesterone and estrogen (Allen *et al*., 1924) and the effectors their respective receptors, PR (Sherman *et al.,* 1970) and ER (Jensen *et al.,* 1962). From the advent of menarche until menopause, both estrogen and progesterone are synthesized and secreted in a cyclical manner by the ovaries under the control of the pituitary gonadotrophins, while PR and ER are expressed at high levels in ovaries, uterus, mammary and pituitary glands.

Fig. D shows, schematically, the sequence of main hormones that control the development of the mammary gland (Hennighausen and Robinson, 2001). Epidermal growth factor (EGF) signals through the stroma and controls early ductal outgrowth before puberty. Together with estrogen (and with the induction of the progesterone receptor by estrogen), it also controls ductal elongation and branching during puberty. Progesterone and prolactin along with placental lactogens and the osteoclast differentiation factor signal alveolar proliferation and differentiation during pregnancy and possibly lactation. The signals inducing tissue remodeling during involution have not been defined.

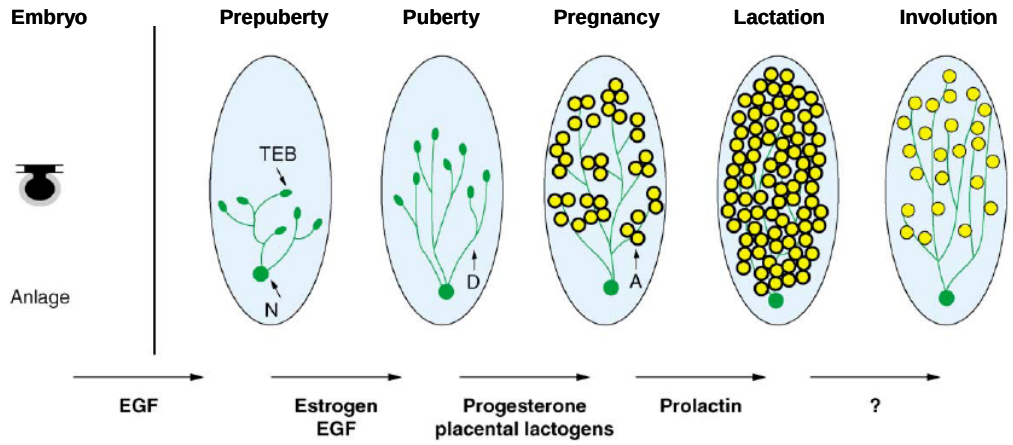


Fig D: ***Hormones that control development of the mammary gland****. The anlage at the embryo stage is composed of epithelium (black knob) and stroma (gray surrounding). The blue oval despicts the mammary fat pad (stroma). N stands for nipple, TEB for terminal end buds, D for ducts and A for lobuloalveolar structures. Adapted from Hennighausen and Robinson, 2001.*

The clinical and epidemiological evidence for an obligate role of **estradiol**, the main circulating estrogen, in mammary gland development is considerable. Estradiol deficiency demonstrates that the steroid is strictly

necessary, although not sufficient, to induce pubertal breast development (Anderson *et al.,* 2004).

As for **progesterone***,* despite the significant advances made concerning the structural/functional analysis of PR *in vitro*, progress in the understanding of the physiological responses specifically attributable to this nuclear receptor has been hampered due to estrogen's overlapping functions, many of which depend on PR induction (Horwitz *et al.,* 1978).

Null mutation of the *Pgr* gene has provided evidence of a physiological role of the progesterone receptor in a variety of female reproductive and non-reproductive activities. The progesterone receptor presents two isoforms, A and B. Female mice lacking both *Pgr*-isoforms exhibit impaired sexual behaviour, neuroendocrine gonadotrophin regulation, annovulation, uterine dysfunction and impaired ductal branching morphogenesis and lobulo-alveolar differentiation of the mammary gland (Lydon *et al.,* 1995). Specific knockout mouse models for each *Pgr* isoform have confirmed functional differences between the two isoforms. Mice lacking the isoform A exhibit normal mammary gland and thymus development yet display severe uterine hyperplasia and ovarian abnormalities. Selective knockout of isoform B results in reduced mammary ductal side-branching and alveologenesis during pregnancy but does not affect ovarian, uterine or thymic responses to progesterone (Mulac-Jericevic *et al.,* 2003). Thus, the isoform A is both necessary and sufficient to elicit the progesterone-dependent reproductive responses required for female fertility, whereas the isoform B is required for normal proliferative responses to progesterone in the mammary gland (Conneely *et al.,* 2003; Humphreys *et al.,* 1997).

It is assumed that progesterone has a similar role in the human breast and stimulates TDLU formation and expansion during puberty and pregnancy.

In the adult, non-pregnant, non-lactating breast, epithelial proliferation is maximal approximately one week after ovulation, during the luteal phase of the menstrual cycle. This is when both estrogen and progesterone are being secreted by the corpus luteum. Nevertheless, estrogen is considered to be the prime inducer of breast epithelial cell proliferation that may regulate the cyclical variation in breast cell proliferation during the menstrual cycle, whereas progesterone may have a more significant role in the post-menopausal breast when estradiol levels are reduced (Anderson, 2001) and during pregnancy.

During pregnancy, progesterone and prolactin take over the mammary gland morphogenesis. These hormones activate the "alveolar switch", a genetic program that coordinates changes in mammary epithelial cell proliferation, migration, differentiation and deletion within the many tissue types of the mammary gland (Oakes *et al*., 2006). During the "*alveolar morphogenesis phase*", the increase in the levels of serum progesterone and prolactin triggers a rapid and global wave of proliferation of the epithelial cells within the ductal branches and developing alveoli. It increases both epithelial cell number and epithelial surface area, actions essential for sufficient milk production during lactation. The *Pgr*-knockout mouse demonstrated that progesterone is required for alveolar morphogenesis, and epithelial

recombination experiments demonstrated that PR in the mammary epithelium, not the stroma, was essential for epithelial cell proliferation. Progesterone has been found to increase DNA synthesis in normal mammary epithelium in organ culture (van Bogaert., 1978) and inhibit cell death in normal mammary epithelial cells *in vivo* (Berg *et al.,* 2002).
Cell differentiation becomes dominant as the gland moves into the "*secretory initiation phase"*. The alveolar cells become polarised and form a sphere-like single layer of epithelial cells that envelopes a circular lumen. Each individual alveolus is bordered by contractile myoepithelial (basal) cells. The myoepithelium of the alveoli is discontinuous so that the luminal cells directly contact the underlying basement membrane. After parturition, lactation starts and, following weaning, nearly all of the development induced by the alveolar switch is removed by programmed cell death during the "*involution phase"*, only to redevelop with the next pregnancy.

The data published by different groups (Clarke et al., 1997; Russo et al., 1999; Mallepell *et al*., 2006; Shoker *et al.,* 1999; Brisken *et al*., 1998) indicated that, in the pre-menopausal breast, approximately 5% of epithelial cells are proliferating in response to steroid hormones but, surprisingly, these cells do not express the receptors, ER and PR. However, these cells are often adjacent or in close proximity to those that do express the receptors. These receptor-positive cells are found within the luminal epithelium (a 25-30%), but not the myoepithelium or stromal cells. In normal human adult breast tissue, luminal cells co-expressing both ERα and PR-A and -B, along with prolactin receptor, are distributed evenly throughout the intra-lobular ducts and peripheral alveoli.

This dissociation between receptor expression and proliferation suggests a model where ovarian hormones stimulate proliferation via paracrine signals secreted by steroid receptor positive cells towards negative cells (Mallepell *et al*., 2006; Shoker *et al.,* 1999; Brisken *et al*., 1998). It should be noted that luminal epithelial cells in the mature mammary gland exhibit two distinct morphologies: tall, columnar-like and round, cuboidal-like. The former are non-proliferating, steroid receptor-negative cells, whereas the latter either express ER/PR or are proliferating. Although the functional significance of these luminal cell types remains unclear, it is evident that the precise patterning of steroid and prolactin receptors in the normal mammary gland is required to elicit the appropriate paracrine response to local growth factors (LaMarca and Rosen, 2008).

Further studies designed to identify this paracrine factor have implicated a number of factors in the paracrine mechanism: WNT proteins (Brisken *et al.,* 2000), epidermal growth factor (EGF) family of proteins (Chan *et al.,* 2002), cytokines such as RANKL (Martin *et al.,* 2001; Mulac-Jericevic *et al.,* 2003), LIF (Kritikou *et al.,* 2003) and members of the IGF (Hovey *et al.,* 2003), FGF (Palmieri *et al.,* 2003) and VEGF families (Liang *et al.,* 2005). The challenge is still to determine which are physiologically and clinically relevant.

In any case, the intention of such a mechanism of action would be to attenuate the sensitivity of the breast epithelium to steroid hormones (in

contrast to the endometrium) such that proliferation will occur only when a sufficient concentration of positive growth factors has accumulated. This might be achieved only after prolonged exposure to high levels of steroid and possibly other hormones, as occurs in early pregnancy, and may be a mechanism for preventing excessive proliferative activity at other times.

I.1.2 Breast cancer biology

Approximately 25% of new cancers arise in tissues whose development and function is strongly dependent on steroid hormones (breast, endometrium and prostate). Breast cancer is the most frequently diagnosed cancer among women in the western world.

I.1.2.1 Breast tumours classification

Breast cancers have been classified according to their gene expression profile into luminal A and B, basal-like, HER2+ and normal breast-like subtypes (Honeth *et al*., 2008).

More than 90% of breast tumours express cytokeratins distinctive of the luminal phenotype (CK18) and arise mostly from the TDLUs. About 75% express both estrogen and progesterone receptors (ER and PR). Another observation regarding the expression of the receptors is that the balanced expression of the two PR isoforms, PR-A and PR-B, is often disrupted. It is not common to see exclusive expression of either PR-A or PR-B in breast tumours, but rather a marked overexpression of one receptor form (most often PR-A) predominating over low expression of the other (Graham *et al.,* 2005; Mote *et al.,* 2004). Luminal tumours have a better prognosis and more diverse treatment. About two-thirds of these receptor-positive tumours regress after treatment with antiestrogens or aromatase inhibitors (Clarke *et al.,* 1997). Nonetheless, hormone resistance and consequent tumour recurrence remain a problem for ER/PR-positive breast cancers.

Basal-like and normal-like tumors are essentially all ER-negative, as are the majority of HER2+ tumors. Basal-like tumours are typically negative for ER/PR and for HER2 but positive for basal cytokeratins (CK5/6/14/17), for epidermal growth factor receptor (EGFR) and/or for c-Kit (Nielsen et al., 2004).

Multiple studies have demonstrated basal-like tumors to have a particularly poor prognosis (Rijn *et al.,* 2002; Sorlie *et al*., 2001), are more aggressive and of early onset. Premature relapse is common and a predilection for visceral metastasis is observed, and one should note that metastatic breast cancer is incurable. Besides, these tumours lack responsiveness to tamoxifen and aromatase inhibitors that target ER-positive luminal tumours, and herceptin that targets HER2-positive tumours. These tumours are commonly treated with two kinds of chemotherapies: DNA-damaging agents like cisplatin, etoposide or inhibitors of poly(ADP-ribose) polymerase, and microtubule-interfering agents like vinca alkaloids and taxanes (James *et al.,* 2007).

I.1.2.2 Breast cancer risk factors

Several breast cancer risk factors have been known for many years (Hankinson *et al.,* 2004). Increasing age is one of the strongest risk factors. Having a family history of breast cancer and inherited mutations in cancer-related genes (e.g. BRCA1, p53), an earlier age at diagnosis and greater number of affected relatives increases a woman's own risk. A personal history of benign breast disease, particularly with atypia (abnormal cells), and having dense breasts on a mammogram are associated with substantial increases in breast cancer. Height and post-menopausal body mass index are positively associated with disease, while pre-menopausal obesity is inversely associated, at least in western populations.

Both observational studies and randomized trials have confirmed and quantified the important role of ovarian hormones, exogenous and endogenous, in breast cancer etiology, and many known risk factors appear to operate through this pathway. The incidence of breast cancer in men is 1% of that seen in women. Factors that increase lifetime exposure to estrogens and progesterone, like early age at menarche, regular ovulation, and late age at menopause, also augment risk. Breastfeeding and being overweight during the woman's young adult life decrease the ovulatory frequency, and this probably accounts, at least in part, for their protective effects. Alcohol intake increases endogenous estrogen levels that may contribute to the observed increase in risk among regular drinkers. The modest increase in risk of breast cancer among current or recent users of oral contraceptives is probably due to their estrogenic (and progestational) effects. The use of post-menopausal estrogens also increases the risk, and the use of therapies, such as tamoxifen, that block the binding of estrogen to the estrogen receptor at the breast decreases the risk of disease. Progestins are added to estrogen in post-menopausal hormone replacement therapy (HRT) to counteract the increased risk of endometrial cancer associated with unopposed estrogen (Persson *et al.,* 1989). Advanced endometrial cancers are also treated with progestins therapeutically (Saegusa *et al.,* 1998). The success of these regimens fostered the assumption that progesterone would similarly counteract the proliferative effects of estrogen in breast cancers. However, combined estrogen-progestin HRT brought about a greater risk of breast cancer than estrogen alone (MWSC, 2003).

Pregnancy has a particularly complex influence on subsequent breast cancer risk. For about a decade after parity, risk is increased, probably due to the hormonal stimulation of already initiated breast epithelial cells. In contrast, risk is reduced over the long term, possibly by rendering the breast substantially less susceptible to somatic mutations. Increased parity and an earlier age at first pregnancy also is associated with a reduction in risk as it may shorten the time (from menarche to first birth) when the breast is particularly susceptible to mutations and, moreover, an early pregnancy reduces adult levels of mammary stem/progenitor cells (Siwko *et al*., 2008). Basal-like breast cancers, in contrast to luminal tumours, exhibit increased risk for parity and younger age at first full-term pregnancy. In addition,

increased age at menarque and breastfeeding seems to be more protective in basal-like cases (Millikan *et al.,* 2008) than in luminal tumours.

I.1.2.3 Breast tumourigenesis process

The process of breast tumourigenesis is thought to result from a "benign to malignant" progression in which the accumulation of multiple genetic changes allows evolution from normal breast epithelium through benign proliferative lesions to atypical proliferative lesions, and then to carcinoma in situ and frankly invasive tumours (Anderson *et al.,* 2004).

Breast cancer is initiated by carcinogenesis in a group of cells. The "stochastic model" of carcinogenesis proposes that, firstly, malignant transformation occurs by multiple mutations in a random single cell and, secondly, there is a subsequent clonal selection.

In contrast, the "hierarchical model" of carcinogenesis or "cancer stem cell hypothesis" upholds that the malignant transformation occurs in a subset of normal stem cells or progenitor cells, probably through the deregulation of self-renewal pathways (Melchor and Benítez, 2008). "Breast cancer stem cells" have been proposed to exist at TDLUs (Russo *et al.,* 1999; Smalley *et al.,* 2003; Dick, 2003; Al-Hajj *et al.,* 2003). The concept of "cancer stem cells" relies on the presence of a subpopulation of cells within tumours that drives tumourigenesis, as well as giving rise to a large population of differentiated progeny that constitutes the bulk of the tumour but lack tumourigenic potential (Ailles *et al.,* 2007). Since stem cells have long-term replicative capacity that is necessary for clonal expansion, they may accumulate and perpetuate the mutations that are required for tumourigenesis to occur. Tumorigenic mutations are presumably sustained in the SP/TA population, which give rise to the tumourigenic progenitor cells CD44+CD24- (Woodward et al., 2005).

It has been proposed a role for ovarian hormones/receptors in the tumourigenic process. If these putative breast cancer stem cells express or not steroid receptors might depend on the level of commitment of the precursor cell (Woodward *et al.,* 2005) and might be at the origin of the different breast cancer subtypes. It has been proposed that ER/PR+ breast cancers originate from ER/PR+ luminal progenitor cells, whereas ER/PR- cancers arise from ER/PR- basal stem cells (Melchor and Benitez, 2008). This hypothesis is supported by gene expression profiling of breast cancer subtypes, which shows that luminal tumours (the most abundant ones) are ER/PR+ and CK18+, whereas basal tumours are ER/PR- and CK5+.

While steroid receptor expression and proliferation occur in separate cells in the normal breast, proliferating breast tumour cells often express ERα and PR (Clarke *et al.,* 1997; Russo *et al.,* 1999). This change in the biology between normal and tumour epithelium is apparent at an early point in breast tumourigenesis as it can be detected in premalignant lesions such as epithelial hyperplasia of usual type or atypical ductal hyperplasia (Shoker *et al.,* 1999).

Estrogens, by their mitogenic effects on breast cells, appear to accelerate the development of breast cancer at many points along the progression from

early mutation to tumour metastasis (Russo *et al*., 2006). By increasing cell proliferation, estrogens may also increase the probability that DNA damage is not repaired, resulting in mutations (Preston-Martin *et al.,* 1990).

The role of **progestins** has been more controversial. In studies on cultured breast cancer cells, progesterone was shown to have biphasic effects: in the short term, it accelerates progression through the cell cycle but with continued treatment, progesterone causes cells to arrest in the G1 phase (Musgrove *et al*., 1997). Groshong *et al* (Groshong *et al.,* 1997) further developed this mechanism. They reported that a single pulse of progesterone stimulated one round of cell division in breast cancer cell lines expressing PR, and then arrests cells in late G1 of the second cycle, by sequentially raising the levels of p21 first and then of p27. Arrest in G1 may be accompanied by cellular changes that can be permissive for growth-stimulatory effects by other factors (Lange *et al.,* 1998). For instance, progesterone induces the expression of EGF and insulin receptor (Lange *et al.,* 1998; Papa *et al.,* 1990). The findings by Moore *et al* (Moore *et al.,* 2006) also support a generally stimulatory role of progestins in breast cancer, in which progestins increase the number of breast cancer cells by both stimulating the rate of proliferation and serving as survival factors, inhibiting apoptosis. Expression of the tumour suppressor protein p53 is decreased by progestins in T47D breast cancer cells (Hurd *et al.,* 1995), suggesting that progestins removes the anti-tumoural effects of this protein.

The activation of the c-Src/Ras/Erk signaling pathway by progesterone (Migliaccio *et al.,* 1998) may contribute to multiple aspects of breast cancer progression since the activation of c-Src in breast cancer cells is a critical event in tumour progression due to its association with cell invasion and metastasis of breast cancer cells (Carnevale *et al.,* 2007; Tan *et al.,* 2005; Rucci *et al.,* 2006).

Ablation of *Pgr* expression in PgrKO mice resulted in a significantly reduced incidence of mammary tumour growth in response to carcinogen challenge and a failure to develop preneoplastic mammary lesions in organ cultures of PgrKO glands exposed to chemical carcinogen (Soyal *et al.,* 2002; Ismail *et al.,* 2003). These findings are consistent with previous studies (Huggins *et al*., 1962) showing that exogenous progesterone given before a carcinogen is protective, whereas given after carcinogen exposure exacerbates tumour formation.

I.2 Progesterone/PR molecular biology

Progesterone plays a crucial role in the complex regulation of normal female reproductive function. Broadly speaking, the major physiological roles of progesterone in the mammal are:

1) in the uterus and ovary: release of mature oocytes, facilitation of implantation and maintenance of pregnancy, by promotion of glandular differentiation, stromal proliferation, development of predecidual cells and suppression of myometrial contractility

2) in the brain: mediation of signals required for sexually responsive behaviour

3) in the mammary gland: lobular-alveolar development in preparation for milk secretion and suppression of milk protein synthesis before parturition.

It was mentioned in the previous pages the implication of progesterone in the physiology of the normal breast and in the process of breast tumourigenesis. Now, we will learn about the mechanism of action of progesterone from a molecular point of view.

I.2.1 SHR family and domain organization

Steroid hormones like progesterone bind to steroid hormone receptors (SHR), the key mediators of steroid hormones' action. SHR and SHR-like receptors form the nuclear receptor (NR) subfamily 3 (NR3), which includes two receptors for estrogens (ERα and ERβ), two estrogen-related orphan receptors (ERRα/ERR1 and ERRβ/ERR2), a receptor for glucocorticoids (GR), mineralocorticoids (MR), androgens (AR) and progestins (PR).

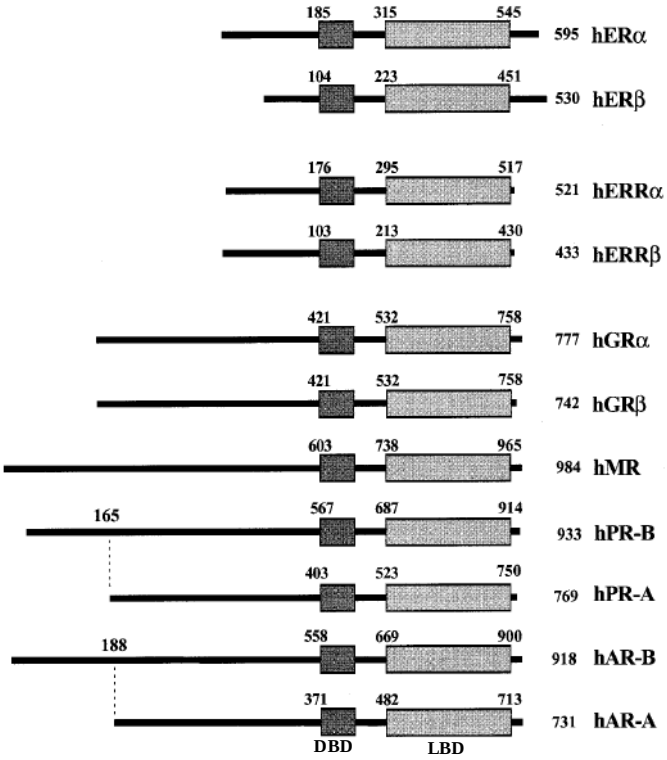


Fig E: ***The human steroid hormone receptor family*** *including isoforms and variants. Highlighted are the DNA binding domain (DBD) and the ligand binding domain (LBD). Numbers indicate amino acids. ERa/ERb= estrogen receptor. ERR= estrogen-related receptor. GR= glucocorticoid receptor. MR= mineralcorticoid receptor. PR= progesterone receptor. AR= androgen receptor. Adapted from Beato and Klug, 2000.*

The nuclear receptor superfamily shares a common domain organization (Fig. E) consisting of an N-terminal region, a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). The DBDs and LBDs possess considerable secondary structure while N-terminal regions differ extensively from one another in size, amino acid composition and structure (McEwan *et al.,* 2007). Between the DBD and LBD exists a flexible hinge region.

The progesterone receptor presents two isoforms, PR-A and PR-B (Fig. F). For humans, the two mRNA transcripts are generated from a single gene by differential promoter utilization (Conneely *et al.,* 2000).
Structurally, PR-B differs from PR-A only in that the B receptor contains an additional stretch of 164 amino acids at the N-terminus of the protein. As other nuclear receptors, both PR-A and PR-B contain a centrally located DNA-binding domain (DBD), which is flanked at the N-terminus by an activation function-1 (AF-1) and at the C-terminus by a hinge region containing nuclear localization signals (NLSs) as well as a ligand-binding domain (LBD) containing a second activation function (AF-2). Both PR isoforms show high affinity for the natural ligand progesterone and the synthetic agonist R5020. As regards the nuclear localization signals, the intracellular distribution of steroid receptors is the result of nuclear–cytoplasmic diffusion and ATP-dependent cytoplasmic–nuclear shuttling. At equilibrium, the majority of ER, AR and PR are found in the nucleus (Guiochon-Mantel *et al.,* 1991; Tyagi *et al.,* 1998). However, a small but significative fraction of progestin-responsive PR has been found associated with the inner side of the cell plasma membrane (Bagowski *et al.,* 2001; Bernauer *et al.,* 2001; Gerdes, 1998; Thomas, 2008; Ballare *et al.,* 2003). There are indications for the existence of an inhibitory region, called IF, which prevents the activity of the AF-1 in the context of PR-A (Hovland *et al.,* 1998). A third activation function (AF-3) is located within the N-terminal region specific to PR-B.

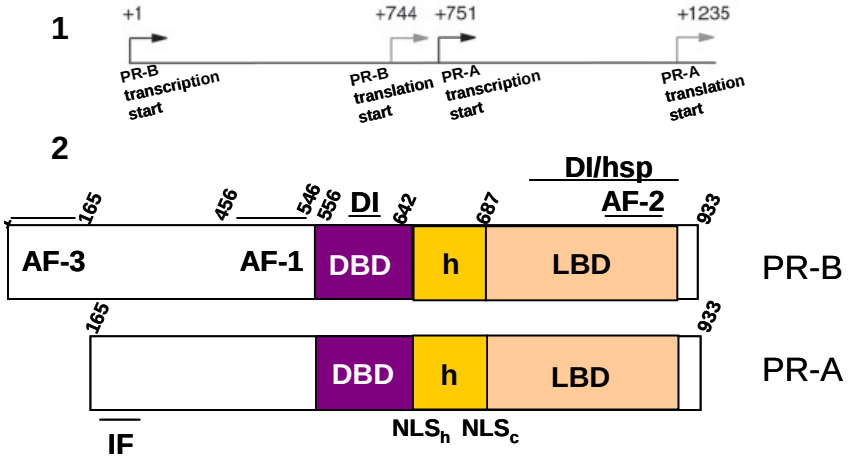


Fig F: ***Structure of PR isoform variants****. 1) diagram of transcriptional and translational start sites for human PR-A and PR-B. 2) domain organization of the human PR-A and PR-B isoforms. AF-1,-2 and -3 are transcription activation domains; DBD, DNA binding domain; h, hinge region; LBD, ligand binding domain; IF, inhibitory domain; DI,*

dimerization domain; hsp, heat shock protein binding region; NLS, nuclear localization signals hormone dependent (h) and constitutive (c)

I.2.2 Transcription factor mechanism of action

The main activity attributed to the progesterone receptor, and to the rest of steroid hormone receptors, is their function as transcription factors. The molecular mechanism by which progesterone regulates the transcription of target genes through PRs has been actively investigated over several decades.

❖ ligand-dependent mechanism of transactivation

The traditional ligand-dependent mechanism of receptor activation after hormone binding involves multiple steps. The receptor activation process is called *transformation*:

-First, **in the absence of hormone binding** (see Fig. K) or other activating signals, steroid receptors typically exist in heteromeric complexes with heat shock proteins (Hsp) and additional components of the molecular chaperone machinery: HSP70, DNAJ/HSP40, HSP90, HSP90-binding protein p23, HSP70/HSP90 organizing protein (HOP), HSP70 interacting protein (HIP), BAG-1 and others. Most classes of steroid receptors, including PR, are associated with the cell nucleus in the absence of hormone. A common role for steroid receptor-chaperone interactions has been the establishment and maintenance of the receptor's unstable hormone binding conformation (Pratt *et al.,* 1997). Another likely role for receptor-associated chaperones is to assist in the functional repression of receptors by inhibiting their abilities to bind DNA, dimerize, and interact with transcriptional coregulatory proteins in the absence of ligand binding or other stimulatory signal. Chaperone components can also modulate receptor affinity for ligand and influence the shuttling of steroid receptors between cytoplasmic and nuclear compartments, the recycling of activated receptors, and the subnuclear localization of receptors (DeFranco, 1999).

-Second, **progesterone is released***.* Progesterone is synthesized and secreted by endocrine cells of the ovaries. It travels via the blood stream to their target cells. It is believed that the lipophilic hormone, as well as synthetic compounds with agonistic or antagonistic effects, enters the target cell by simple diffusion and bind to the multiprotein complex of chaperones and PR. Binding of steroid in the steroid-binding pocket then favours continued folding of the LBD, destabilizing its interaction with HSP90. Binding of ligand leads to a tighter nuclear localization. Free from heat-shock proteins and with the new LBD conformation, the receptor is now able to dimerize and bind to target DNA response elements.

PR, GR, MR and AR bind to the same hormone-responsive elements, HREs (Fig. H), which originally were described as glucocorticoid responsive element, GREs (Scheidereit *et al.,* 1983; Karin *et al.,* 1984). HREs are composed of halves (AGAACA) arranged as inverted (palindromic) repeats and separated by three non-conserved base pairs (Beato, 1989; Truss *et al.,* 1991; Mangelsdorf *et al*., 1995). Steroid hormone- responsive elements are

usually found in multiple copies or clustered with other cis-acting elements. Thus, HREs often interact synergistically with other HREs or with unrelated cis-acting elements. SHRs can also activate genes lacking HREs by means of interaction with other sequence-specific transcription factors bound to their target sequences. However, even in genes with HREs, transactivation by SHRs often requires a synergistic interaction with other sequence-specific transcription factors (Beato *et al.,* 1995).

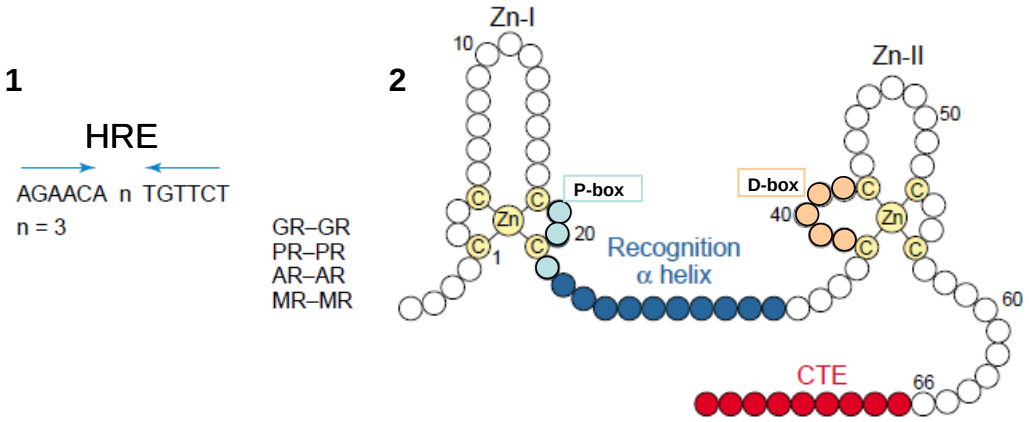


Fig H: *1) Symmetric repeats using the consensus half-sites 5'-AGAACA-3' are used by the glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR) and mineralcorticoid receptor (MR). The spacing between half-sites is a crucial determinant in response element recognition. The estrogen receptor (ER) binds similar symmetric sites but with consensur 5'-AGGTCA-3' half-sites. 2) The DNA binding domain (DBD) in the nuclear receptor family contains a conserved recognition α-helix (in blue) and a variable C-terminal extension (CTE, in red). It comprises two zinc fingers, the proximal P-box (important for recognition of the HRE) and the distal D-box (important for the dimerization of the DBDs). Adapted from Khorasanizadeh and Rastinejad, 2001.*

The DNA-binding domain of PR and SHRs in general, comprises ~80 amino acids enclosed at the DBD (conserved recognition α-helix) plus some 14 C-terminal amino acids of the hinge region (C-terminal extension) (Fig. H). The DBD contains two zinc fingers that are able to tetrahedrally co-ordinate a zinc atom in the form Cys_2-Cys_2 (Luisi *et al.,* 1991; Schwabe *et al.,* 1993). Only very few amino acids, termed the "proximal (P)-box", within the first zinc finger are responsible for specific recognition of the cognate hormone-responsive element. Another set of amino acids, called the "distal (D)-box" within the second zinc finger, forms the weak dimerization interface of the DNA-binding domain. Another less well defined dimerization interface overlaps with the LBD and seems to be dependent on hormone binding (Truss and Beato, 1993). Each receptor monomer recognizes a HRE half-site (Luisi *et al.,* 1991) contacting a narrow sector of one side of the DNA double helix in such a way that SHRs are able to bind to HREs organized in chromatin since the contacted side is exposed on the nucleosome surface (Beato and Eisfeld, 1997; Piña *et al.,* 1990). Some SHRs appear to form homodimers in solution, but binding to DNA stabilizes homodimer formation.

Once bound to DNA, transcriptional competence of PR is exerted by two independent activation function regions, a constitutively active one -the activation function-1, AF-1- and the ligand-inducible activation function-2 (AF-2). The two AFs act synergistically and connect the receptor to the transcription apparatus via direct interactions with basal transcription factors, sequence-specific transcription factors and/or transcriptional coactivators. According to the observations done on the 3D-structure of the ligand-bound LBD/AF-2 of PR (Tanenbaum *et al.,* 1998), the binding of the hormone produces a conformational change that generates new surfaces for the binding of coactivators to the LBD/AF-2. Recent evidence has demonstrated that the presence of the AF-3 (only in PR-B) allows the binding of a subset of coactivators to PR-B that are not efficiently recruited by progestin-bound PR-A (Giangrande *et al.,* 2000). In addition, it has been proposed that this additional region limits the conformational ensemble of PR-B to fewer more active conformers, accounting for its stronger transcriptional activity (Bain *et al.,* 2001).

In transient transfections, PR-B is generally a much stronger transcriptional activator than PR-A, whereas PR-A can act as a dominant inhibitor of PR-B, and of other nuclear receptors including the estrogen receptor, glucocorticoid, androgen, and mineralocorticoid receptors (Vegeto *et al.,* 1993; Wen *et al.,* 1994). The mechanism of transrepression involves N-terminal sumoylation and intramolecular communication within PR-A (Abdel-Hafiz *et al.,* 2002). Marked functional differences are also seen between the two receptors when they are stably introduced, individually, into cells. Breast cancer cells constitutively expressing only PR-A or PR-B show a remarkably non-overlapping profile of gene regulation (Richer *et al.,* 2002), which would be in accordance with the genetic studies in mice showing that PR-A and PR-B are responsible for a different set of progesterone-dependent biological functions (Conneely *et al.,* 2003; Humphreys *et al.,* 1997). However, the study by Graham *et al* (Graham *et al.,* 2005) showed that progestin-regulated transcriptional targets are largely insensitive to changes in PR-A:PR-B ratio, although small but significant changes are observed. The predominance of PR-A over PR-B, which mimics the ratios found in cancer, resulted in the acquisition of progestin responsiveness of a small but important subgroup of specific gene targets in signaling pathways that influence cell shape and adhesion. It is possible that some of the functional differences between PR-B and PR-A may be due to the inability of PR-A to interact with ERα and activate the endogenous c-Src/Ras/Erk signaling pathway in response to progestins.

-Following with the order of events in transcription activation, the **formation of the preinitiation complex** at a core promoter is a sequential process. First, TFIID recognizes the TATA box; for a TATA-less promoter, binding of an initiator protein may be required. Next, TFIIA and TFIIB bind independently to the TFIID-DNA complex. Importantly, when TFIIB enters the complex, the TFIIF-RNA polymerase II (RNAPII) complex can then enter to form the ABDF-polymerase-DNA complex. Lastly, the remaining general transcription factors assemble to form the complete preinitiation complex. It is

possible that the progesterone receptor could act on any of these steps to enhance formation of the stable preinitiation complex.

Although direct interactions of SHRs with components of the basal transcription machinery have been described (Beato and Sánchez-Pacheco, 1996), most recent efforts have been mainly devoted to identify and characterize SHR coregulators.

A plethora of PR coregulators have been reported in the literature. Considering that coactivators have a very short residence time at promoter sites, O'Malley and collaborators predicted that exists a kinetic pattern of multiple and distinct coactivator complexes which are recruited ''sequentially'' to the promoters of hormone-regulated genes (O'Malley, 2003). They speculate that a sequence of stochastic non-productive interactions of diverse coactivator complexes leads to an eventual productive interaction with a steroid receptor dimer in place at the promoter of a gene. When a specific and productive interaction occurs, transcription advances one step forward and then the receptor must seduce a new productive interaction with a different requisite coactivator complex that advances the process to transcription one step more. Each individual step in this process is likely replete with non-productive interactions, but since the half-life of coactivator interactions at promoters has been estimated to be less than 20 seconds, little time is lost in discharging an inappropriate complex and recruiting the next correct coactivator complex required to produce a translatable mRNA. Post-translational modifications of coactivators, such as phosphorylation, methylation, acetylation, ubiqui-tination/sumoylation and glycosylation (McKenna and O'Malley, 2002), increase or reduce the affinity of coactivators for a given set of transcription factors (Rowan *et al.,* 2000).

Coregulators are involved in diverse steps of transcription, from initiation to alternative RNA splicing, termination, elongation and even mRNA transport (Li *et al.,* 2004). They exhibit an ever-expanding diversity of enzymatic activities through which they reorganize protein-protein or protein-DNA contacts, chromatin remodeling and receptor-mediated transcription and they may modulate the function of transcription factors in a tissue-specific manner.

They can be divided into two generic classes depending on their enzymatic activity:

a) Histone-modifying cofactors:
- acetylation/deacetylation: histone acetyl-transferases (HATs) like CBP/P300, PCAF and the SRC/p160 family; histone deacetylases (HDACs) like HDAC 1, 2 and 3
- methylation/demethylation: histone methyl-transferases (HMTs) like CARM-1 and PRMT-1; demethylases like LSD-1 and Jumonji domain factors
- phosphorylation/dephosphorylation: kinases like RSK-2, MSK-1 and CDK-2
- poly(ADP)ribosylation

- ubiquitination/deubiquitination: ubiquitin ligases like RNF20, MDM2, Ring1B, 2A-HUB and BRCA1; deubiquitinases like USP7, USP21, UBP-M and 2A-DUB.

b) ATP-dependent chromatin remodeling complexes

a) Histone-modifying cofactors

Histone proteins are target of extensive post-translational modifications such as acetylation, phosphorylation, ADP ribosylation, methylation, and ubiquitination (Iizuka *et al.,* 2003; Fischle *et al.,* 2003). These modifications are thought to contribute to the changes in histone-histone and histone-DNA interactions that could lead to modulation of chromatin structure. These modifications can also act as signals for recruitment and binding platforms for other chromatin-modifying factors and complexes that lead to overall changes in chromatin architecture (Strahl *et al.,* 2000; Jenuwein *et al.,* 2001).

Histone acetylation has been studied extensively in the context of gene regulation. Histone acetylation is highly dynamic and occurs on lysine residues mainly within the N-terminal tail of histone proteins. Acetylation of positively charged lysine residues could alter the histone-DNA interactions, creating more open chromatin architecture (Grunstein, 1997) and may act as signals for recruitment of other proteins that contain bromo domains for the recognition of acetylated lysine residues (Dhalluin *et al.,* 1999; Yang *et al.,* 2004).

Histone acetylation is catalyzed by the enzymatic activities of histone acetyl-transferases (HATs) and removed by the action of histone deacetylases (HDACs). The majority of the literature has correlated histone acetylation with gene activation, and histone deacetylation with gene repression (Schubeler *et al.,* 2004). Indeed, many transcription coactivators contain intrinsic HAT activity, whereas many transcription corepressors complexes contain subunits with HDAC activities.

The family of **SRC/p160** proteins bind in a hormone-dependent manner to the AF-2 region of a broad range of nuclear receptors. **SRC-1** (NCOA-1) and **SRC-2** (Mukherjee *et al.,* 2006) interact with the ligand-bound PR and coactivates the receptor in the uterus and breast, primarily, while **SRC-3** (NCOA-3) acts mainly in the breast (Han *et al.,* 2006). They contain three LXXLL (L, leucine; X, any amino acid) motifs that are responsible for interaction with ligand-bound nuclear receptors (Heery *et al.,* 1997).

SRC-1 null mice exhibit partial hormone resistance in progesterone target tissues, such as mammary gland and uterus, further substantiating the importance of SRC-1 for the biological actions of progesterone (Xu *et al.,* 1998). It is likely that in most instances, a member of the SRC/p160 family forms the initial primary bond with steroid receptors to initiate transcription (O'Malley, 2003). The C-terminal domains of SRC-1 and SRC-3 contain HAT activities, raising the possibility that SRC coactivators may play a direct role during the process of PR-directed initiation of transcription, disrupting the local repressive chromatin structure by acetylating histones and possibly other chromatin-associated factors (Spencer *et al.,* 1997).

SRC coactivators may bind with secondary coactivators, like CBP and its relative p300 (Chen *et al.,* 1997) or CARM-1 (Koh *et al.,* 2001). BAF57, a subunit present only in mammalian SWI/SNF complexes, can also interact with SRC-2 and SRC-3 (Belandia *et al.,* 2002).

CBP and p300 function as coactivators for multiple nuclear receptors as well as p53 and NF-κB. Both possess intrinsic HAT activity. PR has been shown to interact with both proteins in a ligand-dependent manner (Li *et al.,* 2003; Aoyagi and Archer, 2007). Besides, CBP/p300 interacts with members of the SRC family and synergizes with SRC-1 in the transactivation of PR and ER (Smith *et al.,* 1996).

Nuclear receptor corepressor (**N-CoR**) and silencing mediator of retinoid and thyroid receptor (**SMRT**) are both corepressors of numerous transcription factors, including steroid hormone receptors. Both N-CoR and SMRT interact with nuclear receptors (in the case of steroid receptors, in the presence of antagonists) and associate with HDAC-3 in large protein complexes, which is an important pathway for transcriptional repression (Li *et al.,* 1997). Besides, the N-CoR/HDAC-3 complex specifically recruits a histone H2A ubiquitin ligase, 2A-HUB, to a subset of regulated gene promoters, leading to the establishment of more marks of repression in histones (Zhou *et al.,* 2008).

Another modification recently related to gene regulation is the monoubiquitination of histones H2A and H2B (Table A). Early studies identified H2A as a target for ubiquitination in higher eukaryotes and, around the same time, ubiquitinated H2B was also detected in mouse cells. It soon became clear that only a single ubiquitin moiety is conjugated to H2A at Lys119 (uH2A) and H2B at Lys120 (uH2B) in mammals (Osley, 2006).

In mammals, there are potential proteins that may be the responsible for H2B monoubiquitination. As ubiquitin ligase enzymes, the sequence homolog to yeast Bre1, **RNF20** (Zhu *et al.,* 2005) and **MDM2** have the capability to ubiquitinate H2B *in vivo* (Minsky and Oren*,* 2004), as well as BRCA1 *in vitro* (Xia *et al.,* 2002). The sequence homologs to yeast Rad6, hHR6A and hHR6B (Koken *et al.,* 1991) and UbcH6 can function as ubiquitin-conjugating enzymes. Ubiquitin can be removed from target H2B by a class of thiol proteases known as ubiquitin-specific proteases (USPs), in particular **USP7** (Nijman *et al.,* 2005).

Histone H2B monoubiquitination has been linked to gene activation and transcription elongation. Evidence from *in vitro* transcription elongation experiments indicates that H2B ubiquitination might assist the histone chaperone FACT in stimulating the passage of RNAPII through a nucleosomal template (Pavri *et al.,* 2006). The FACT histone chaperone complex can displace an H2A/H2B dimer from a nucleosome core, enhancing transcription elongation on chromatin templates (Laribee *et al.,* 2007). Importantly, histone H2B monoubiquitination is a prerequisite por histone H3K4 and H3K79 di- and trimethylation (Dover *et al.,* 2002; Sun and Allis, 2002).

In humans, H2A monoubiquitination is mediated by at least two different ubiquitin ligases, Ring1B and 2A-HUB, both of which are associated with transcriptional silencing (Cao *et al.,* 2005; Wang *et al.,* 2004; Zhou *et al.,*

2008; Wei et al., 2006). Other potential ubiquitin ligases specific for H2A have been identified *in vitro*, although their role *in vivo* is unclear.
Knockdown of **Ring1B** in human cells largely reduces the level of uH2A, indicating that this enzyme is responsible for much of the H2A ubiquitination *in vivo* (Wang *et al.,* 2004). In flies and humans, uH2A localizes to the promoters of Polycomb-target genes, including the Hox genes, in a Ring1B-dependent manner (Cao *et al.,* 2005; Wang *et al.,* 2004; Wei *et al.,* 2006). Ring1B associates with three separate repressive complexes: Polycomb repressive complex-1 (PRC-1), E2F-6.com-1, and the FBXL10-BcoR complex (Sanchez *et al.,* 2007). The E2F-6.com-1 repressive complex is involved in the silencing of E2F- and Myc-responsive genes in quiescent cells (Ogawa *et al.,* 2002).
The H2A ubiquitin ligase **2A-HUB** (Zhou *et al.,* 2008) associates with the N-CoR/HDAC3 complex at the promoter of a subset of chemokine genes, where it represses transcription via inhibition of RNAPII elongation, by blocking FACT recruitment.
H2A ubiquitination has also been related to histone methylation. Ring1B-mediated H2A ubiquitination occurs downstream of H3K27 methylation, as knockdown of SUZ12, which reduces H3K27 methylation, reduces Ring1B and uH2A localization at silenced promoters (Cao *et al.,* 2005). Moreover, uH2A inhibits MLL3-mediated di- and trimethylation of H3K4, repressing transcription initiation, but not elongation, *in vitro* (Nakagawa *et al.,* 2008).
Some of the roles of uH2A in repression of transcription might relate to the finding that uH2A enhances the binding of the linker histone H1 to reconstituted nucleosomes *in vitro* (Jason *et al.,* 2005) and that uH2A deubiquitination cause the dissociation of linker histones from core nucleosomes (Zhu *et al.,* 2007). This idea is consistent with the structure of the nucleosome in which the C-terminus of H2A appears to interact with linker histones (Luger *et al.,* 1997). Thus, it is possible that uH2A contributes directly to transcriptional repression by regulating higher-order chromatin structure, in addition to inhibiting H3K4 methylation.
Recently, three major deubiquitinases specific for uH2A were identified: **UBP-M**, **2A-DUB**, and **USP21** (Joo *et al.,* 2007; Nakagawa *et al.,* 2008; Zhu *et al.,* 2007). The studies by Joo *et al* revealed that H2A deubiquitination by UBP-M is a prerequisite for subsequent phosphorylation of H3S10 and chromosome segregation when cells enter mitosis. They also demonstrate that UBP-M regulates Hox gene expression through H2A deubiquitination.
2A-DUB acts during transcription initiation and is required for gene activation at a subset of promoters (Zhu *et al.,* 2007). It interacts with the HAT enzyme PCAF and preferentially deubiquitinates hyperacetylated nucleosomes *in vitro*. USP21 is also involved in transcription initiation and can deubiquitinate uH2A *in vitro* (Nakagawa *et al.,* 2008).

Enzymes Involved in H2A and H2B Ubiquitination/Deubiquitination in Different Organisms							
	H2B Ubiquitination		H2B Deubiquitination		H2A Ubiquitination		H2A Deubiquitination
	E2	E3	Transcription	Silencing	E2	E3	
S. cerevisiae	Rad6	Bre1	Ubp8	Ubp10 (Dot4)	–	–	–
S. pombe	Rhp6	Brl1 (Rfp2/Spcc1919.15) Brl2 (Rfp1/Spcc970.10c)			?		
Drosophila	Dhr6	Bre1 (CG10542)	Nonstop	USP7		dRing (Sce)	
Mouse	mHR6A/mHR6B						
Human	hHR6A/hHR6B *UbcH6 ?* *Mdm2 ?*	RNF20 MDM2 BRCA1?	USP22 *USP3 ?*			Ring1B (Ring2/Rnf2) 2A-HUB (hRUL138) BRCA1?	Ubp-M (USP16) 2A-DUB (MYSM1) USP21 *USP3 ?*
Arabidopsis		HUB1		SUP32 (UBP26)			

Table A: ***Enzymes involved in H2A and H2B Ubiquitination/Deubiquitination in different organisms*** (adapted from Weake and Workman, 2008)

Core histones, specially histone H3 and H4, can be methylated. Lysine residues can be mono-, di- or tri-methylated and arginine residues can be mono- or di-methylated, enriching the epigenetic signals derived from it. Whereas methylation of histone H3K4, 36 and 79 has been associated with gene activation, methylation of histone H3K9 and 27, and histone H4K20 are associated with heterochromatization, although histone H3K9 di- and tri-methylation are found in the transcribed region of active genes (Shilatifard, 2006).

The histone methyl-transferases (HMTs) **CARM-1** and **PRMT-1** are associated with gene activation. Besides, they can interact with SRC-2 and enhance synergistically the activity of diverse nuclear receptors (Koh *et al.,* 2001; Stallcup *et al*., 2000).

Very few human demethylases have been described so far. **LSD-1** coactivates AR by catalyzing the specific removal of methyl groups from mono- and di-methylated histone H3K9. Another demethylase, the Jumonji C domain-containing protein **JMJD2C**, was also found to demethylate tri-methyl histone H3K9 and stimulate this way AR-dependent transcription (Wissmann *et al*., 2007).

Another modification that histone tails may suffer is phosphorylation (mostly on histones H3, H4 and H2A). By far, the most studied one is histone H3S10 phosphorylation. Serine 10 of histone H3 can be substrate of different kinases depending on the cell cycle phase. During mitosis, **Aurora B** is the responsible for its phosphorylation and it correlates with chromosome condensation prior to mitosis. At interphase, the phosphorylation of histone H3 does not affect the whole genome but a subset of genes. In this case, phosphorylation is carried out by kinases like **RSK2**, **MSK1** or **PKA** and correlates with transcriptional activation of genes (Prigent and Dimitrov, 2003). MSK1-mediated histone H3S10 phosphorylation has been involved in the initial steps of activation of the MMTV promoter by progesterone (Vicent *et al.,* 2006).

b) ATP-dependent chromatin remodeling machineries

SHRs can gain access to HREs exposed in the surface of nucleosomes (Piña *et al.,* 1990), but efficient binding of receptors to complex hormone regulatory regions and subsequent transactivation requires remodeling of chromatin by ATP-dependent complexes (Muchardt and Yaniv, 1993; Yoshinaga *et al.,* 1992). These complexes use the energy of ATP hydrolysis to make hidden HREs and other cis-regulatory elements accessible for binding of the cognate factors (Di Croce *et al.,* 1999), which act as nucleation points for recruitment of further coregulators and the basal transcriptional machinery (Kinyamu and Archer, 2004). In this sense, the human homologs of yeast **SWI/SNF** complex (**hBRM** for the Swi2 and **BRG-1** for Snf2 of yeast) have been linked to SHRs to exert this function of chromatin remodeling (Mymryk *et al.,* 1995; Rayasam *et al.,* 2005; Vicent *et al.,* 2006).

c) Other coregulators

Other proteins have been shown to regulate the transcriptional activity of PR: SNURF, HMG-1 and -2, the E3 ubiquitin ligases RPF-1, E6-AP and CUEDC2, PIAS3, UbcH7, PBP/DRIP205/TRAP220, TIP60, proline-rich nuclear receptor coregulatory protein 1, Cdc25B, GT198, JDP-2, nuclear receptor coactivator-62, ASC-2, APE2, steroid receptor RNA coactivator (SRA) and CDK2, among others.

Small nuclear RING finger protein (**SNURF**) binds to the DBD domain of steroid receptors and activates steroid receptor-dependent transcription. It forms a functional link between steroid- and Sp1- regulated transcription (Moilanen *et al.,* 1998).

High mobility group (**HMG**) **-1 and -2** are members of a family of non-histone chromatin proteins that binds to DNA in the minor groove, recognizing bends in DNA. PR appears to utilize HMG-1 or -2 proteins for high affinity interaction with DNA *in vitro* and for full transcription activity *in vivo*. The specificity of HMG-1/2 coactivation seems to be achieved by functionally substituting for the C-terminal extension and facilitating DNA binding by nuclear receptors (Boonyaratanakornkit *et al.,* 1998).

Two E3 ubiquitin ligases have been described as coregulators of PR activity: the receptor potentiating factor-1 (**RPF-1**) (McKenna *et al.,* 1999) and the E6-associated protein (**E6-AP**) (Nawaz *et al.,* 1999). The N-terminal receptor activation domains of E6-AP and RPF-1 are separable from their ubiquitin ligase domains that reside in their C-terminal HECT domain. It has been demonstrated by transient transfection assays that RPF1 and E6-AP could potentiate the transcriptional activity of PR, GR and other nuclear receptors, although their ubiquitin ligase activity was not required for the co-activation (Nawaz *et al.,* 1999).

Another ubiquitin pathway enzyme, **UbcH7**, was shown to bind to activated receptors, including PR. It was recruited to target promoters where it enhanced their transcriptional functions (Verma *et al.,* 2004). The enzymatic

activity of UbcH7 was required for its ability to potentiate transactivation by steroid receptors although the substrate is still unknown.
CUEDC2 (commented later at “Protein turnover) interacts with PR and repress the transcriptional activity and signaling induced by ligand-bound receptor.
PIAS3, a SUMO-E3 ligase (Man *et al.,* 2006) inhibits gene activation by PR-B in a manner that is independent of SUMO modification, through the inhibition of the DNA-binding and nuclear export of PR.

❖ ligand-independent mechanism of transactivation

In addition to the synergistic effect of phosphorylation on ligand-dependent transactivation, some agents that stimulate intracellular kinase pathways can also activate receptors in the "absence of ligand”. Signals like elevation of intracellular cyclic AMP (cAMP), a common second messenger for a number of hormones and a direct activator of protein kinase A (PKA), okadaic acid and dopamine can activate PR in the absence of progesterone (Tsai and O’Malley, 1994). Growth factors are able to activate certain steroid receptors. The EGF family member heregulin can stimulate nuclear localization, DNA binding, and transcriptional activity of PR in T47D breast cancer cells in the absence of hormone (Labriola *et al.,* 2003). This was accompanied by activation of MAPK and PR Ser294 phosphorylation (Qiu *et al.,* 2003).

❖ ligand-dependent mechanism of transrepression

Although they usually become positive regulators, PR can repress certain genes if the cis-elements are arranged appropriately. Repression of transcription by steroid hormones may be explained in different ways.
One mechanism may imply the competition between hormone receptors and other transcription factors for binding to essential sites on the promoter, as occurs in the case of the glucocorticoid receptor.
As documented in several examples of GR-regulated genes, transcriptional repression can be triggered by protein-protein interaction between GR and the components of the AP1 complex, JUN and FOS (Jonat *et al*., 1990).
An alternative mechanism for transcriptional repression by hormone receptors is the “squelching” of limiting factors (Truss and Beato, 1993). For instance, the repressive effect of progesterone on prolactin hormone induction of β-casein is thought to be due to a mutual interference between PR and STAT5A (Buser *et al.,* 2007).
Estradiol-dependent recruitment of corepressors like N-CoR and Repressor of estrogen receptor activity (REA) but also of coactivators like SRC-2 or CBP/p300 was associated with many of the negatively regulated ER-target genes, indicating that a complex array of mechanisms are utilized to repress gene expression (Karmakar *et al.,* 2009).

I.2.3 Modulation of PR activity

❖ Phosphorylation

Like other steroid hormone receptor family members, PR isoforms are heavily phosphorylated by multiple protein kinases. Phosphorylation occurs primarily on serine residues mostly concentrated within the amino termini (Weigel *et al.,* 1996; Takimoto *et al.,* 1996). PR contains a total of 14 known phosphorylation sites (Zhang *et al.,* 1995 and 1997) (Fig. I). Serines at positions 81, 162, 190, and 400 are defined as "basal" sites, constitutively phosphorylated in the absence of hormone, and may be enhanced in the presence of ligand. Serines 102, 294, and 345 are hormone-induced sites that are maximally phosphorylated 1–2 h after progestin treatment.

Specific kinases responsible for phosphorylation of selected sites have been identified, whereas others remain unknown. For example, serine 81 has been demonstrated to be phosphorylated by casein kinase II (Zhang *et al.,* 1994); serine 294 by MAPK (Lange *et al.,* 2000; Shen *et al.,* 2001); eight sites (serines 25, 162, 190, 213, 400 and Thr 430, 554, and 676) have been demonstrated to be phosphorylated by cyclin A/CDK2 *in vitro* (Zhang *et al.,* 1997). Five of these sites (serines 162, 190, 213, 400, 676) have been confirmed as authentic *in vivo* phosphorylation sites.

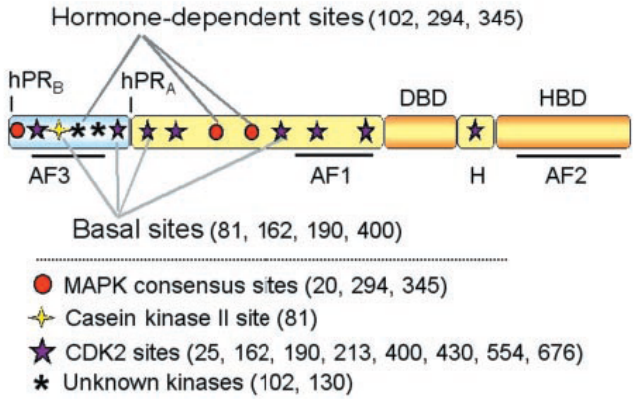


Fig I: ***Phosphorylation sites in human PR****. Fourteen residues have been shown to represent basal (constitutive) and hormone-induced phosphorrylation sites. Specific kinases responsible for selected sites phosphorylation have been identified (MAPK, casein kinase II, cyclinA/CDK2) whereas others remain unknown.From Lange, 2004.*

Phosphorylation is generally accepted as a positive regulator of steroid receptor function and serves to integrate signals initiated by growth factors with responses to steroid hormones in endocrine tissues. It may affect ligand-independent and dependent PR transcriptional activity. Unliganded phosphorylated PR may regulate genes via non-classical mechanisms (ligand-independent mechanism of transactivation) (Bamberger *et al.,* 1996). Narayanan *et al* (Narayanan *et al.,* 2005) found that PR activity is highest in S phase, lower in the G0/G1 phases and impaired during G2/M concomitant with lower PR phosphorylation. Progestins activate CDK2, and PRs are predominantly phosphorylated by CDK2, perhaps allowing for the coordinate regulation of PR action during cell cycle progression. In the absence of hormone, CDK2-dependent Ser400 phosphorylation seems to enhance the

basal transcriptional activity of PR, while in the presence of hormone CDK2 seems to alter PR function indirectly by increasing the recruitment and activity of the coactivator SRC-1. Besides, CDK2-induced Ser400 phosphorylation is required for increased PR nuclear localization (Pierson-Mullany *et al.,* 2004; Moore *et al.,* 2007). A third aspect known to be regulated by phophorylation is the receptor turnover (see below).

From all the PR phosphorylation sites, **Ser294** influence on PR function is the best characterized one. Ser294 forms part of a proline-directed MAPK consensus site (PXXSP) that becomes rapidly phosphorylated by the c-Src/Ras/Erk pathway, activated by the exposure to progestins or other growth factors (Zhang *et al.,* 1995; Lange *et al.,* 2000; Qiu *et al.,* 2003; Vicent *et al.,* 2006). Ser 294, although present at the two isoforms, is phosphorylated in PR-B but not in PR-A (Clemm *et al.,* 2000). This is likely due to the N-terminus of PRA adopting a distinct conformation that either hinders access of cellular kinases to this site or creates a unique active site domain for an interacting protein that blocks phosphorylation of Ser294. PR-A does not activate the c-Src/Ras/Erk pathway in response to progestins and does not interact with ERα, either (Migliaccio *et al.,* 1998).
MAPK-dependent PR Ser294 phosphorylation has been involved in the regulation of numerous functions of PR-B. Ser294 phosphorylation greatly increases transcriptional activity of liganded PR at PRE-containing promoters (mutant S294APR-B is about 10 times less active in the presence of progestins than wtPR-B) (Shen *et al.,* 2001). This phosphorylation site is also required for EGF-induced nuclear translocation and ligand-independent transcriptional activation of PR (Qiu *et al.,* 2003; Labriola *et al.,* 2003). Finally, this site is also very important to signal for ligand-induced receptor downregulation by the ubiquitin-proteasome pathway (Lange *et al.,* 2000; see below).

❖ Protein turnover

Modulation of protein turnover is emerging as a key means by which nuclear receptors are regulated. After ligand binding, PR undergo rapid downregulation (Nardulli *et al.,* 1988). Specific inhibitors of the 26S proteasome block this process, and ubiquitinated PR species accumulate in cells (Fig. J) (Lange *et al.,* 2000), meaning that PR is a substrate for the ubiquitin-proteasome pathway. PR stability is associated with nuclear retention or, conversely, nuclear export may be required for receptor downregulation (Qiu *et al.,* 2003).

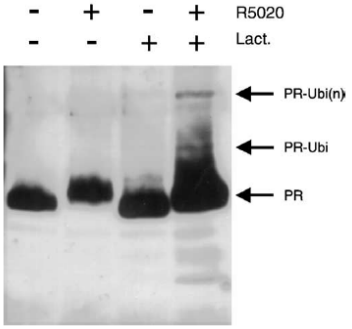


Fig J*: PR is a substrate for the ubiquitin pathway.* *T47D-YV cells expressing PR-B were pretreated for 4 h with +/- an inhibitor of the proteasome, lactacystin, followed by R5020 treatment. PR was immunoprecipitated and visualized by immunoblotting. High molecular weight ubiquitinated forms of PR in immunoprecipitates are indicated by arrows. From Lange et al., 2000.*

Until recently, even though PR was known to be ubiquitinated *in vivo*, there was no protein identified to be directly responsible for the ubiquitination of the receptor. Zhang *et al* (Zhang *et al.,* 2007) demonstrated that the protein **CUEDC2** promotes PR degradation through the ubiquitin-proteasome pathway, and it is indispensable for the ligand-triggered downregulation of PR. Lys388 is the target residue for ubiquitination, although Ser294 is also indispensable for the ligand-driven degradation of the receptor (Lange *et al*, 2000; Shen *et al*, 2001). CUEDC2 contains a CUE domain, known ubiquitin-binding motif (Shih *et al,* 2003). In addition, CUEDC2 interacts with PR and repress the transcriptional activity and signaling induced by ligand-bound PR.

It is unclear how unliganded PR is targeted for degradation. Unliganded PR exists in heteromeric complexes with several heat-shock protein (hsp), chaperones that maintain the unstable hormone binding conformationof the receptor (Pratt *et al*., 1997). A proposed model (Hernández *et al*., 2002) (Fig. K) despicts three distinct complexes that are formed prior to arriving at the mature PR complex with a functional hormone-binding domain. Firstly, HSP40 binds to PR, followed by the ATP-dependent recruitment of HSP70. Then, the complex assembles with HOP and HSP90 to form the intermediate complex. Binding of ATP to HSP90 is recognized by p23, who promotes the dissociation of the intermediate complex to a mature complex. The mature complex is stable enough to maintain the LBD of the receptor in a conformation capable of binding hormone. It should be noted that the mature PR complex is dynamic and is thought to dissociate and re-assemble continuously to maintain a high proportion of active PR (Cintron and Toft, 2006).

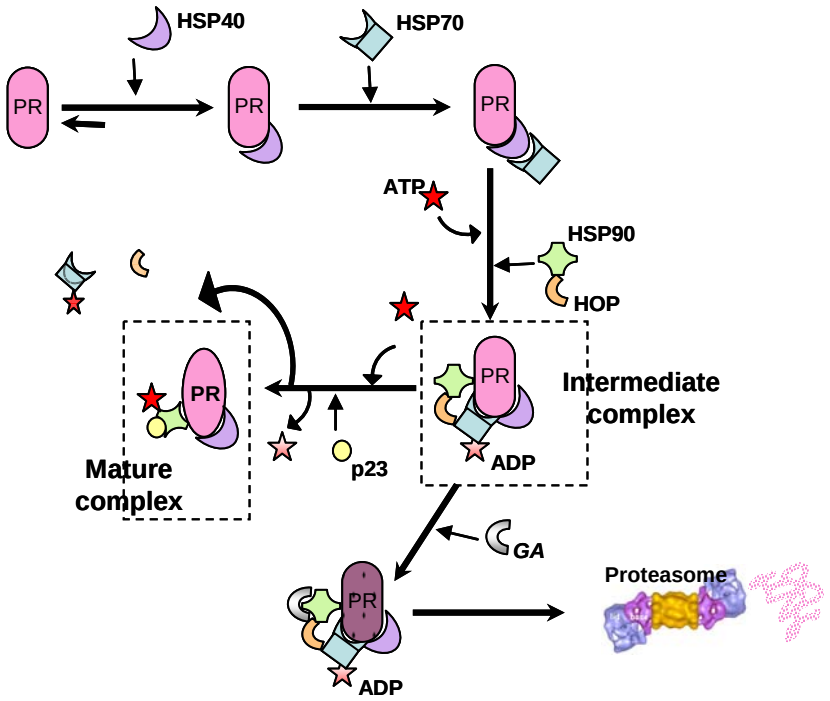


Fig K: ***A four-step model for the assembly of PR complexes****. The early complex is rapidly formed when PR first interacts with high affinity with HSP40. The pre-intermediate complex is formed when HSP70 is recruited to PR by an interaction with HSP40, followed by the ATP-dependent binding of HSP70 to PR, directly. The intermediate complex is formed*

when HOP and HSP90 join the PR complex. Binding of ATP to HSP90 is recognized by p23, who promotes the dissociation of the intermediate complex to form the mature complex, in which the LBD of PR is maintained in a conformation capable of hormone binding. The addition of the drug geldanamycin (GA) blocks the ATP binding to HSP90 and so p23 recruitment. This blocked inmature receptor is rapidly degraded through the proteasome.

Chaperones are primarily adapted to facilitate protein folding processes. Unlike a typical misfolded protein substrate, PR's folding is effectively arrested prior to hormone binding, thus extending indefinitely the chaperone-interaction phase that normally would be transitory during progressive protein folding. PR is specially adapted to remain "misfolded", and thus extend chaperone interactions that function efficiently in repressing PR's transcriptional activity while the receptor awaits an activating signal (Smith, 2002). Most importantly, chaperones also protect unliganded receptor from unwanted degradation (Bagatell *et al*., 2001).

The use of benzoquinone ansamycin drugs, particularly geldanamycin (GA), blocks the ATP binding to HSP90 and so p23 recruitment, disrupting this way the assembly of the mature complex. This blocking leads to the rapid proteasome-driven degradation of the receptor (Lange *et al.,* 1999). This induction to degradation has also been observed when disturbing with ansamycin drugs the normal function of HSP90 in the refolding of proteins, indicating that HSP90 function as a quality control system, sending proteins to refolding or maturation, or either to degradation if these processes can not be accomplished (Schneider *et al.,* 1996; An *et al.,* 2000). In a steady-state situation, PR degradation must be compensated by new PR synthesis to maintain receptor homeostasis, as happens with ER and GR (Laïos *et al*., 2005; Deroo *et al.,* 2002).

It is unknown if phosphorylation, as in the case of progestin-induced degradation, plays a role in the ligand-independent degradation of the receptor. Indeed, the mutant S294A PR-B is also rapidly degraded with geldanamycin, suggesting that the targeting mechanism for unliganded PR for degradation differs from that of liganded PR. Ser400, a basal phosphorylation site for CDK2 (Pierson-Mullany and Lange, 2004), has been proposed to be a regulator for the degradation of immature PR. It is possible that phosphorylation of PR at Ser294 may expose one destruction box involved in ligand-induced degradation, while Ser400 phosphorylation may expose a different site important for unbound receptor degradation.

❖ Other modifications

PR can be SUMO-modified (Abdel-Hafiz *et al.,* 2002; Daniel *et al.,* 2007; Chauchereau *et al.,* 2003). Modification is accomplished by the reversible attachment of SUMO (Small Ubiquitin related MOdifier) to the acceptor lysine residues located in the target proteins, similar to ubiquitination, with the help of a set of enzymes. Sumoylation does not promote protein degradation.

PRB sumoylation was strongly induced by PIAS3, a SUMO-E3 ligase (Man *et al.,* 2006). However, PIAS3 inhibits gene activation by PR-B in a manner that

is independent of SUMO modification, through the inhibition of the DNA-binding and nuclear export of PR. Lys388 was also described as the primary site of SUMO attachment, apart from Lys7 and Lys531 (Man *et al.,* 2006). Lys388 sumoylation then competes with ubiquitination and leads to autoinhibition and transrepression of PR (Abdel-Hafiz *et al.,* 2002) and increases PR stability (Man *et al.,* 2006).

I.2.4 Non-genomic functions

The signal-transducing properties of PR are not entirely limited to the classical genomic actions (transcription factor) of the receptor and certain responses to progestins may not even involve PR transcriptional activity. For the most part, rapid, non-genomic actions of progesterone and other steroids are mediated by interactions with cytoplasmic receptors. Membrane-associated, progesterone-specific receptors have been isolated and cloned from a range of tissues in a number of species, including human tissues (Bramley *et al.,* 2003; Zhu *et al.,* 2003).

❖ c-Src/Ras/Erk pathway

A rapid and transient activation of the c-Src/Ras/Erk pathway (also called Erk1/2 or p42/p44 MAPK pathway) (Fig. M) was observed upon short progestin treatment of cells (Migliaccio *et al.,* 1998). In breast cancer cells containing ERα, the progestin effect on the c-Src/Ras/Erk pathway is mediated by an interaction of the PR localized in the inner side of the plasma membrane with the ligand-binding domain of the ERα also located in the membrane. PR-B interacts with ERα through two independent domains located in the N-terminal half of PR-B, ERID-I, and ERID-II (Fig. L) (Ballare *et al.,* 2003). ERα, this way, gets active and interacts with c-Src, which triggers activation of the signaling pathways c-Src/Ras/Erk and the PI3K/Akt (Migliaccio *et al.,* 1996). PR-B can also function to activate the pathway by directly interacting with the SH3 domain of c-Src through a proline-rich region in PR-B (Boonyaratanakornkit *et al.,* 2001), which leads to the activation of the route. However, this activation is not as significant and does not reflect the physiological situation as much as in the presence of ERα (Ballare et al., 2003) (Fig. M).

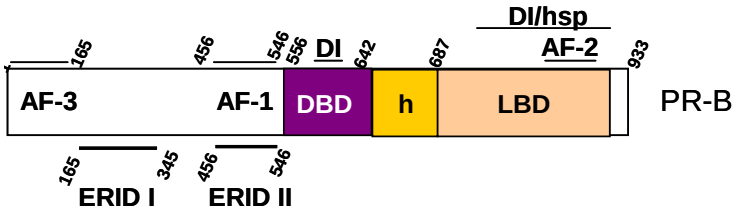


Fig L: ***Estrogen receptor-interacting domains (ERID) on PR-B protein.***

The ultimate targets of the active kinases are not known but likely include transcription factors and coregulators (Bjornstrom and Sjoberg, 2005). One of the targets is the PR itself, which can be detected to be phosphorylated at Ser294 as fast as five minutes after hormone induction (Vicent *et al.,* 2006). Unexpectedly, they also found that the downstream active kinases ERK and MSK were recruited to the hormone responsive-elements of the MMTV promoter and played an essential role in preparing the promoter chromatin for gene activation. These findings suggest an interesting link between signaling-mediated chromatin phosphorylation and gene regulation and find a connection between the nongenomic and genomic actions of steroid hormones (see Fig. O).

The eventual outcome of the activation of these signaling pathways are starting to be discerned. It was long known that crosstalk with the c-Src/Ras/Erk cascade is essential for cell proliferation in response to estrogens and progestins (Migliaccio *et al.,* 1998; Skildum et al., 2005; Carnevale et al., 2007).

The link found between signaling pathway activation and gene regulation (Vicent et al., 2006) suggested the possibility that, at least some target genes, would be regulated, to some extent, by the activation of the kinase pathways.

Effectively, the rapid and transient activation of the c-Src/Ras/Erk pathway is required for the induction of a number of progesterone target genes in breast cancer cells. Ballare *et al* found that 28% of the genes induced by progestins at 6h of hormone treatment in T47D cells exhibit a reduction in hormone induction when activation of ERK is blocked by PD (C. Ballare, B. Miñana, M.J. Melia, and M.Beato, unpublished data). Among these genes, they found rapid and transiently induced genes, like *c-FOS*, as well as genes induced with a more typical kinetic, such as *EGF*, *EGFR*, and *DUSP-1*. *Cyclin D1* expression was delayed by PD treatment and inhibition was only detectable at early time points (2 hr) after hormone induction. This gene is induced by progestin although no classical HRE can be identified within its promoter region (Herber *et al.,* 1994), so it is supposed that it gets induced as a result of signaling activation by
progestin (Skildum *et al.,* 2005).

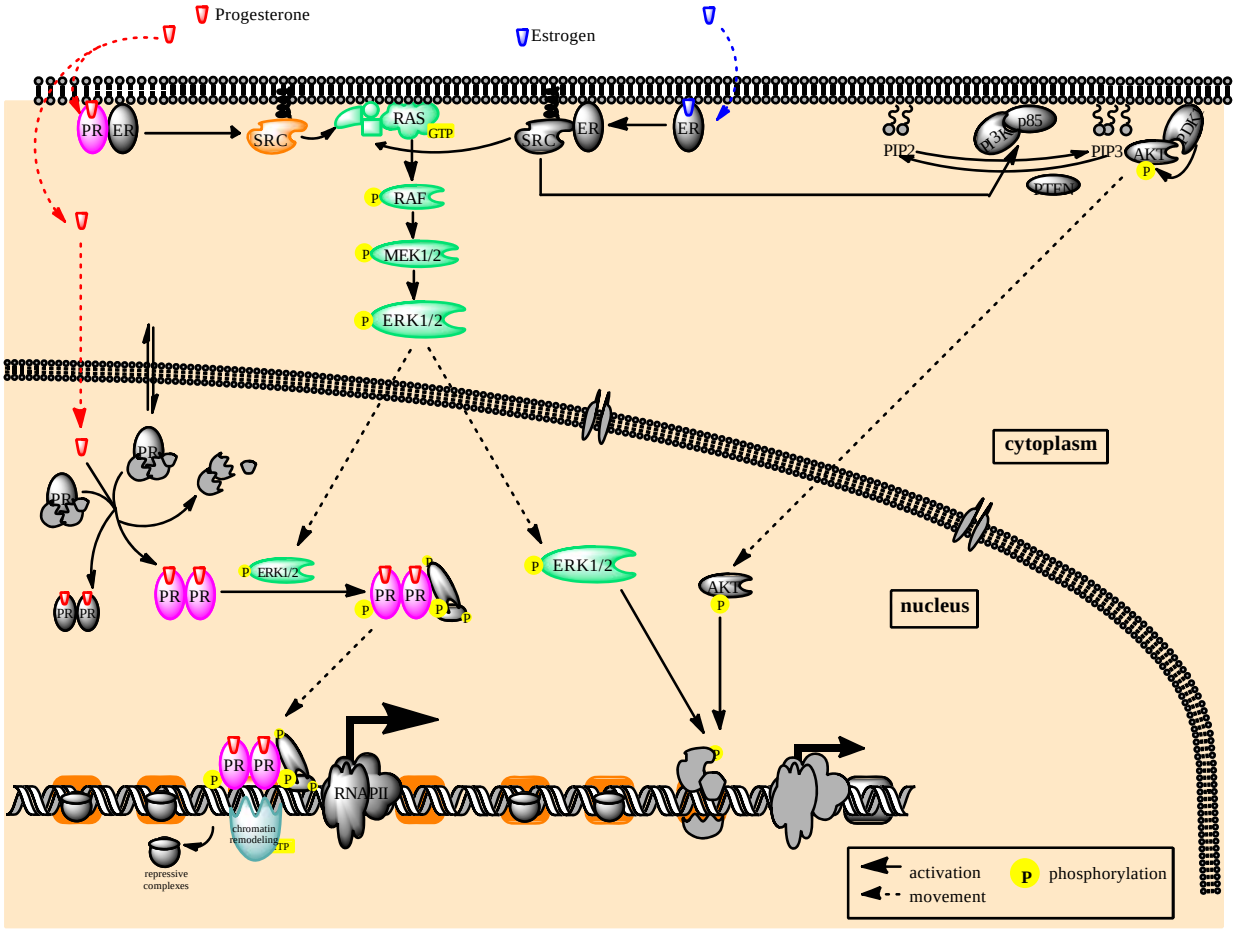


Fig M: *A rapid and transient* ***activation of the c-Src/Ras/Erk pathway*** *(also called Erk1/2 or p42/p44 MAPK pathway) is observed upon short progestin treatment of breast cancer cells. This effect is mediated by an interaction of PR localized in the inner side of the plasma membrane with ERα also located in the membrane. ERα gets active and interacts with c-Src, which triggers activation of the signaling pathways c-Src/Ras/Erk and the PI3K/Akt. PR-B can also activate the pathway by directly interacting with c-Src.*

I.2.5 PR target genes

In the last years, there has been a big advance in the identification of numerous PR target genes. In breast cancer cells, although some genes are regulated by progesterone through both PR isoforms, most genes are uniquely regulated through one isoform, predominantly through PR-B.
Kester *et al*. (Kester *et al.,* 1997) identified several PR-regulated genes in the T47D cell line: CD-9/MRP-1, $^{Na+/K+}$ATPase α1, desmoplakin, CD-59/protectin, FKBP51 and TSC-22 (a putative transcription factor), among others. Many of these genes are involved in regulation of transcription and cell differentiation. A significant number of genes are involved in membrane-initiated events, such as proteins involved in cell adhesion, membrane-bound receptors, calcium-binding proteins, and signaling molecules. These genes represent almost half of all progesterone-regulated genes identified in this study,

pointing to the cell membrane as an important target of progesterone action (Richer *et al.,* 2002). Some other genes regulated by PR are STAT4a, IRS-1 or C/EBPβ, which are important for breast cancer and mammary gland development. The up-regulation of the BCL-X_L gene tells about the role for progesterone in blocking apoptosis (Viegas *et al.,* 2004). Other PR target genes are involved in fatty acid, nucleotide, amino acid or cholesterol/steroid metabolism. It is known that progestins suppress the activity of other steroid hormones by upregulating enzymes that catalyze the inactivation of androgen (3α-hydroxysteroid dehydrogenase type IIb) or glucocorticoid (11β-hydroxysteroid dehydrogenase type 2 or 11β-HSD). The study by Graham *et al* (Graham *et al.,* 2005) demonstrated that the set of progestin-regulated genes was dependent on the length of progestin exposure. Genes involved in transcriptional regulation and cytoplasmic signaling pathways dominated the progestin targets at 6 h (genes like the PR chaperone FKBP54, the transcription factors SOX4, KLF4, vitamin D receptor and WT1). Prolonged progestin exposure for 48 h resulted in a relative shift toward cellular processes involved in basic functioning and homeostasis.

❖ The paradigm: the MMTV promoter

The mouse mammary tumour virus (MMTV) promoter is a well-documented example of transcriptional control by steroid hormones and of how hormone receptors alter chromatin structure to allow the expression of target genes (Beato *et al.,* 1995; Cato *et al*., 1986).

The MMTV promoter assumes a well-defined chromatin structure when stably integrated into a host genome. The promoter organizes into six phased nucleosomes termed A-F. The B nucleosome harbours the five PR binding sites and binding sites for other transcription factors like nuclear factor 1 (NF1) and the octamer transcription factor (Oct-1) (Fig. N) (Truss and Beato, 1993; Richard-Foy *et al*., 1987). For the hormonal activation of the promoter is required not only the HREs but also the NF1 binding site, indicating that both factors PR and NF1 synergize *in vivo* (di Croce *et al.,* 1999).

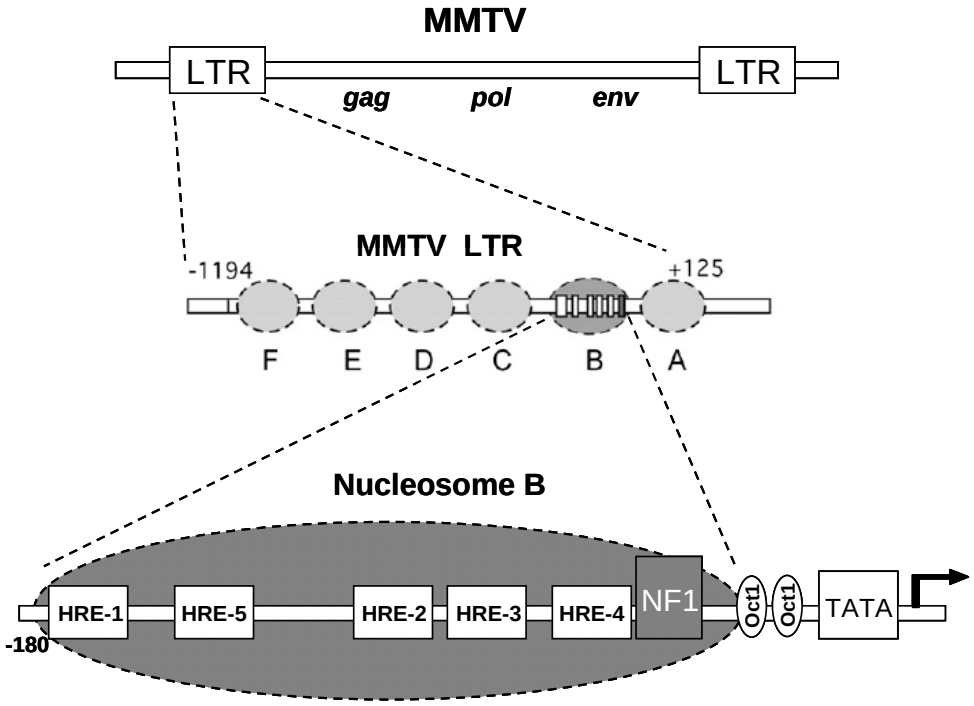


Fig N: *Schematic representation of nucleosomes over the MMTV-LTR and MMTV regulatory region on nuclesome B including hormone responsive elements (HREs), NF1 binding site and octamer motifs (Oct1). Adapted from Vicent et al., 2004 and 2006.*

In vitro experiments of nucleosome assembly on MMTV promoter DNA showed that the promoter adopts a precise rotational orientation on the surface that exposes HRE-1 and HRE-4 but leaves inaccessible HRE-2, -3 and -5, which are essential for hormone induction (Eisfeld *et al.,* 1997). This evidence suggested that the nucleosome may undergo changes during hormone induction to enable the binding of PRs and NF1 to their binding sites. Effectively, a DNAse I hypersensitive site appears after progesterone treatment in the HREs promoter region of the MMTV promoter integrated in the genome (Richard-Foy and Hager, 1987; Truss *et al*., 1995), indicating that changes in the chromatin structure of the promoter were taking place. Further *in vitro* experiments demonstrated that the promoter requires an ATP-dependent remodeling event when activated by progesterone (Di Croce *et al.,* 1999). This ATP-dependent remodeling machinery proved to be SWI/SNF, which was able to displace histones H2A and H2B from nucleosome B (Vicent *et al.,* 2004).

The dynamics of the nucleosome could also be regulated by the linker histone H1 due to its interaction with nucleosomal DNA. H1 is a structural component of chromatin that functions as a general repressor of transcription. H1 binds asymmetrically to the MMTV promoter, with preference for the distal 5'-end (Vicent *et al.,* 2002). In the presence of bound H1, SWI/SNF cannot remodel nucleosomes *in vitro* (Horn *et al.,* 2002). However, MMTV promoter transcription was enhanced in H1-containing minichromosomes, due to the better positioning of nucleosomes and better

binding of PR (Koop *et al.,* 2003). Once PR is bound to the promoter, H1 is phosphorylated and removed from the promoter on transcription initiation.

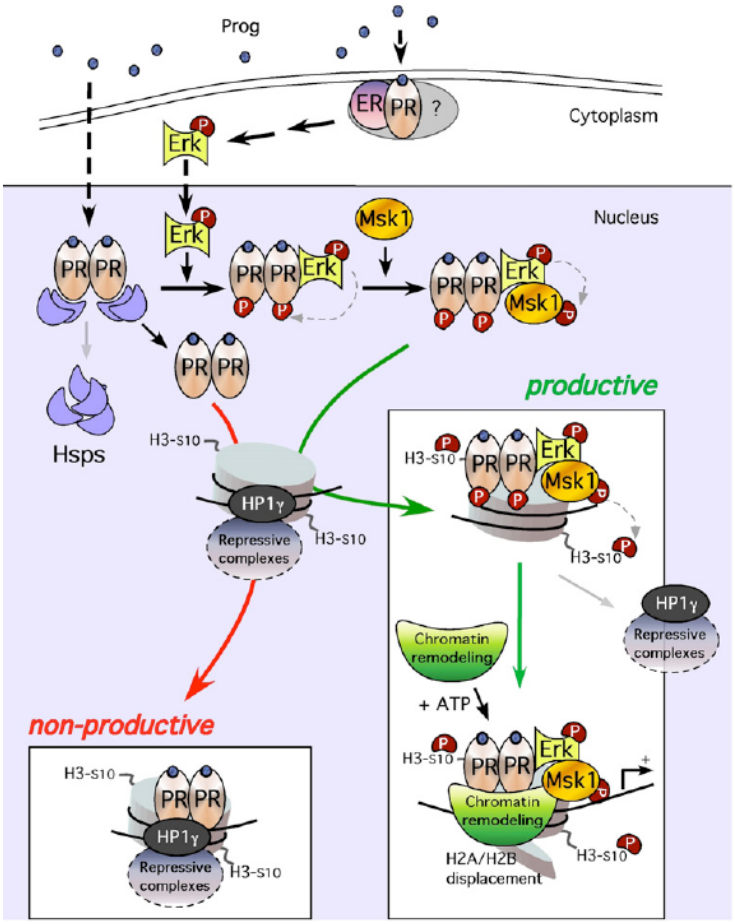


Fig O: ***Proposed model for the initial steps of MMTV promoter induction****. Upon hormone addition, the activation of the c-Src/Ras/Erk pathway leads to the accumulation of active phospho-ERK in the nucleus. Upon hormone binding, nuclear PR dissociates from Hsp chaperones and is ready for activation. A fraction of PR protein does not get phosphorylated and, although able to bind to exposed HREs of the MMTV promoter, is unable to induce transcription. The rest of PR homodimers gets phosphorylated and active and, along with phospho-Erk and phospho-Msk, is recruited to the promoter where it catalyzes the phosphorylation of H3S10 and the displacement of a HP1g-containing repressive complex (likely containing histone deacetylases). Subsequently, an ATP-dependent chromatin-remodeling complex is recruited and catalyzes the displacement of histones H2A/H2B, allowing for the binding of further PR molecules, coactivators and the basal transcriptional machinery, thus starting transcription. From Vicent et al., 2006.*

In an attempt to integrate the existing data, the sequence of events occurring at the promoter after hormone induction has been described in quite detail (Fig. O) (Vicent *et al.,* 2006). Five minutes after hormone addition, the activation of the c-Src/Ras/Erk pathway leads to the accumulation of active ERK in the nucleus. Ligand binding to the PR-Hsp complex leads to the formation of active and phosphorylated PR homodimers. ERK also activates MSK1 and then the three-protein complex (PR, ERK and MSK1)

are recruited to the nucleosome B of the promoter due to the affinity of PR for the exposed HREs (Piña *et al.,* 1990). Once bound to the promoter, MSK1 phosphorylates H3S10, an event possibly coupled to acetylation of H3K14, thus generating a signal that leads to the displacement of a repressive complex containing HP1γ. It is possible that, among the components of the repressive complex, there might be histone deacetylases like HDAC1 and HDAC3 (Aoyagi and Archer, 2007). After derepression, PR is able to recruit the ATP-dependent chromatin-remodeling complex SWI/SNF, which removes H2A/H2B dimers from nucleosome B, thus allowing the binding of further PR molecules, NF1, coactivators like SRC-1 and the basal transcriptional machinery, thus starting transcription.
A fraction of PR homodimers is not phosphorylated and binds to exposed HREs leading to unproductive complexes (Vicent et al., 2006).

I.3 Breast cancer susceptibility gene 1 (BRCA1)

I.3.1 Gene and expression regulation

I.3.1.1 BRCA1 locus

BRCA1 was first located to chromosome 17 via a genetic linkage analysis in 23 early-onset breast cancer families (Hall *et al.,* 1990), and was cloned and isolated in 1994 (Miki *et al.,* 1994). Further research localized it to 17q21. *BRCA1* is a large gene (100 kb-length), with a coding region of 5.5 kb and a total mRNA of approximately 8.0 kb and a gene product of 220 kDa. The gene has 24 exons, including 2 non-translating exons. There is little identifiable homology to known genes.

About a year after the identification of BRCA1, a second breast cancer susceptibility gene, *BRCA2*, was identified on chromosome 13q12 and cloned (Wooster *et al.,* 1995). The BRCA2 gene product is even larger than BRCA1 (380 kDa). Although BRCA2 is structurally distinct from BRCA1, they might be co-regulated during cell cycle progression and in response to DNA damage (Monteiro *et al*., 1996) and they may have overlapping functions (Connor *et al.,* 1997). Nevertheless, the functions of these genes are not identical since *Brca2* gene cannot take over all of the functions of the *Brca1* gene during early development in mutant embryos, and *vice versa* (Gowen *et al*., 1996).

The structure of the human *BRCA1* locus is complicated (Fig. P) in that it includes a partial duplication that results in a pseudo-*BRCA1* gene and two distinct genes (*NBR1* and *NBR2*) (Brown *et al.,* 1996; Xu *et al.,* 1997; Mueller and Roskelley, 2002) which are divergently transcribed from both the pseudo-gene and *BRCA1*. This structure is not found in the mouse, which may be partly responsible for the differences between human and mouse breast cancer models (i.e. heterozygous *Brca1* knockout mice do not develop breast cancer, whereas human carriers do). The *BRCA1* and *NBR2* genes are separated by 218 base pairs, and their transcription is divergent (Fig. Q). This region serves as the primary proximal promoter of *BRCA1*.

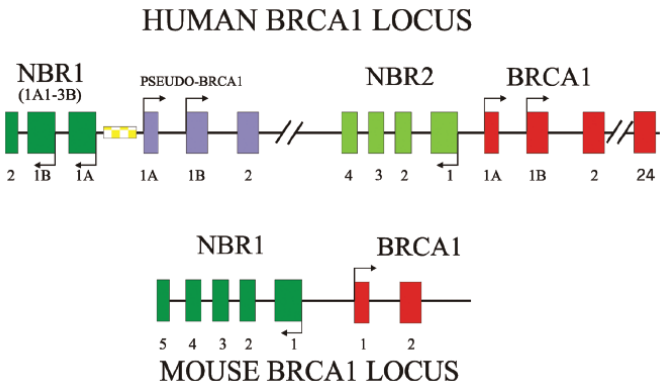


Fig P: ***The structure of the human and mouse BRCA1 loci*** *are shown. Transcription initiation sites are indicated by arrows. The exon structure is indicated by the variously shaded boxes. From Mueller and Roskelley, 2002.*

The *BRCA1* promoter harbours two distinct transcriptional start sites (exon-1A and exon-1B) (Xu *et al.,* 1995), however, translation of *BRCA1* mRNA always starts from the ATG codon located on exon-2. This 5' flanking region does not contain a TATA box but it does contain "initiator elements", which have been proposed to mediate transcription in TATA-less promoters and are frequently involved in basic "housekeeping" processes (Yang *et al.,* 2007).

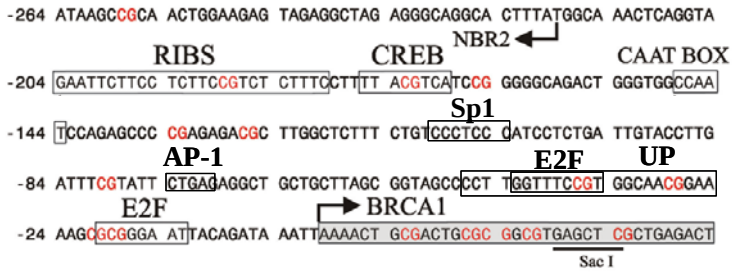


Fig Q: ***The sequence of the promoter region between the NBR2 and the BRCA1 genes*** *is indicated. The first exon of the BRCA1 gene is indicated by the shaded box. Binding sites for E2F, CREB and Sp1 are boxed as well as the AP-1-like, the RIBS and the repressor UP element. CpG dinucleotides that are potentially methylated are shown in red. Adapted from Mueller and Roskelley, 2002.*

Transcription factor binding sites for Sp1, PEA3, C/EBP, CREB, E4F1, Pu and NF-κB were identified in the 5' flanking regions of the exon 1a and 1b transcripts. The interaction of these DNA elements with transacting factors are likely to modulate the alternative use of the distinct transcription start sites and the expression of the *BRCA1* gene (Fig. Q). In cell culture, *BRCA1* expression increases in replicating cells (Gudas *et al.,* 1996) and this proliferation-mediated increase may act through an E2F site that has been characterized in the proximal promoter (Wang *et al.,* 2000). Conversely, p53 may inhibit *BRCA1* expression by preventing E2F binding to this site (MacLachlan *et al.,* 2000). Moreover, activating and repressive E2Fs simultaneously bind the *BRCA1* promoter at two adjacent E2F sites *in vivo*, and hypoxia induces a dynamic redistribution of promoter occupancy by these factors, leading to repression (Bindra *et al.,* 2005). It was identified a repressor element, referred to as the "UP site", that contains a binding site for the transcription factor GABP α/β. Both this sequence and an adjacent E2F recognition element are required for repressor activity (MacDonald *et al.,* 2007). Ets-2 and components of mammalian SWI/SNF were also found to form a repressor complex that negatively regulates the *BRCA1* promoter (Baker *et al.,* 2003). A CREB site was found to be a strong positive transcriptional element (Atlas *et al.,* 2001). Functional analysis of the *BRCA1* promoter revealed that the RIBS site (*EcoRI Band Shift*), which interacts with the transcription factor GABP α/β, is important for promoter activity, and appears to be differentially regulated in the MCF7 and T47D cell lines, being less active in T47D (Atlas *et al.,* 2000). There is also a estrogen-dependent activation of *BRCA1* transcription mediated by a GC-rich region, which is a binding target for Sp proteins (Hockings *et al.,* 2008). The Sp-binding motif is

located in close proximity to an AP-1-like site, which is a target for an ERα/p300 complex. Estrogen stimulates the recruitment of Sp1 and Sp4 to the GC-rich region (Jeffy *et al.,* 2005). They also provide evidence that the MAPK pathway activated by estrogens regulates the association of Sp1 and ERα with the *BRCA1* promoter.

I.3.1.2 Pattern of expression

❖ Cellular pattern of expression

Early studies established that BRCA1 expression fluctuates in a cell cycle-dependent manner. While the steady-state levels of BRCA1 polypeptides are low in resting (G0) cells and G1 cycling cells, these levels increase considerably as cycling cells enter S phase (Chen *et al.,* 1996). The induction of BRCA1 protein expression is preceded by a sharp increase in the levels of *BRCA1* mRNA at late G1 (Gudas *et al.,* 1996), which might be driven by E2F transcription factors, and then the steady-state levels of BRCA1 mRNA steadily decline as cycling cells divide and enter the subsequent G1 phase (Choudhury *et al.,* 2004). As BRCA1 levels rise at the G1/S boundary, there is a concomitant increase in protein phosphorylation, demonstrated by the shift to a slower migrating form of BRCA1 that is abrogated by treatment with phosphatases (Chen *et al*., 1996; Thomas *et al*., 1997). Immunoprecipitation/*in vitro* kinase assays revealed that several cyclins and associated cyclin-dependent kinases (cyclins D and A, CDK2) can associate with and phosphorylate BRCA1 on tyrosine residues in human mammary cell lines (Wang *et al*., 1997). A CDK2/cyclin A or E complex phosphorylates BRCA1 at a CDK consensus phosphorylation site (serine-1497) both *in vitro* and *in vivo* (Ruffner *et al*., 1999). In addition, Choudhury *et al* (2004) described another level of regulation of BRCA1 expression. They found that BRCA1 polypeptides are subject to ubiquitination and proteasome-mediated degradation at specific stages of cell cycle progression and that dimerization with its protein partner BARD1 serves to protect BRCA1 polypeptides from ubiquitination *in vivo*.

❖ Tissue pattern of expression

The aspect of cell cycle-dependent regulation correlates with its tissue pattern of expression, being ubiquitously expressed in human tissues but at maximal levels in rapidly proliferating cells like in testis, thymus, breast and ovary (Lane *et al.,* 1995; Marquis *et al.,* 1995). In cell culture, expression of BRCA1 decreases when breast epithelial or cancer cells are induced to become quiescent by confluence, serum starvation, or TGF-β treatment. Conversely, serum stimulation of quiescent cells caused re-expression of BRCA1 and resumption of cell proliferation (Rajan *et al*., 1996).

❖ Developmental pattern of expression

The developmental regulation of BRCA1 expression has been most extensively studied in the mouse (Marquis *et al.,* 1995; Lane *et al.,* 1995; Blackshear *et al.,* 1998). During early embryogenesis, Brca1 (in the mouse) is expressed diffusely in all three germ layers. Later, during foetal development, Brca1 expression is highest in condensed tissues undergoing considerable proliferative, differentiative and morphogenic changes. These include the following: spinal ganglia and the neuroepithelium of the eye (neuroectoderm derived); somites and cartilage (mesoderm derived); liver and lung (endoderm derived); lense of the eye; and basal proliferative layers of the epidermis (ectodermally derived).

This feature of developmental regulation is well illustrated during mammary gland development (Fig. R). At birth, the mouse mammary gland consists of a small number of epithelial ducts that radiate from the nipple a short distance into the underlying stromal fat pad. Before puberty, the mammary ducts elongate into the fat pad at a rate that is similar to the overall growth of the animal. Further development of the gland takes place at puberty and during adult cycles of pregnancy, lactation and involution (Hennighausen *et al.,* 1998). At puberty, the ends of the ducts swell to form the proliferative terminal end-buds, which strongly express Brca1 and initiate rapid ductal branching and elongation to the margins of the fat pad. After puberty, Brca1 levels fall in the quiescent adult virgin gland. With the onset of pregnancy, the terminal end-buds proliferate, branch and expand to form epithelial alveolar sacs. Throughout pregnancy, Brca1 is expressed at high levels in these developing alveoli. Later, during lactation, Brca1 levels fall gradually as alveolar cells cease proliferating, terminally differentiate and produce milk. After weaning, milk production ceases, Brca1 levels rise, and within a few days a massive apoptotic event is triggered. This drives involution and returns the gland to its quiescent state, whereupon Brca1 levels once again fall. Therefore, in the postnatal mammary gland epithelial Brca1 expression is induced in periodic waves that correspond to defined developmental periods in which intense proliferation, morphogenesis and/or apoptosis take place (Chodosh *et al.,* 1995).

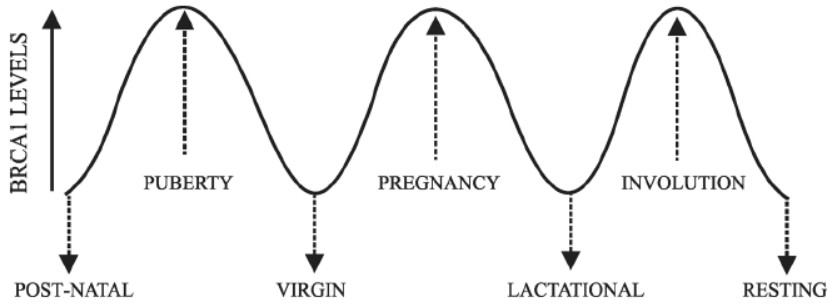


Fig R: ***Brca1 expression during mouse mammary gland development****. Levels are low in quiescent periods before puberty, in the virgin adult, lactational terminal differentiation and resting gland. Levels are high during the proliferative periods at puberty and pregnancy, and during the apoptotic involution after weaning. From Chodosh et al., 1995.*

Given this pattern of expression, it is not unreasonable to expect that the molecular regulators of these developmental changes might also regulate Brca1 expression, directly or due to the proliferative effect. Hormones acting on the mammary gland like estrogen and prolactin (Favy *et al.,* 1999) have been shown to upregulate Brca1 expression. Laminin, an extracellular matrix protein required for lactational differentiation, may be a negative regulator of Brca1 expression in differentiated mammary epithelial cells, and this negative regulation could be released during involution, when laminin is degraded (O'Connell *et al.,* 2000).

I.3.2 Protein structure

Initial reports also provided evidence for a complex pattern of alternate splicing and the potential for translation of a number of BRCA1 protein isoforms (El-Shamy *et al.,* 2004; Wilson *et al.,* 1997; Thakur *et al.,* 1997; Lixia *et al.,* 2007). The gene encodes for a predominant full-length protein of 1863 amino acids and 220 kDa molecular weight in humans, and 1812 amino acids in mice. It also encodes for at least two more protein products of smaller size due to alternative splicing. One of the variants, BRCA1-Δ11, is identical to the full-length form except for the absence of exon 11 and has a molecular weight of 97 kDa. The other is BRCA1-IRIS, which is a 1399-residue polypeptide encoded by an uninterrupted open reading frame that extends from codon one of the known *BRCA1* open reading frame to a termination point 34 triplets into intron 11 (molecular weight 150 kDa).

BRCA1 sequence conservation between mammalian species is weak (mouse and human BRCA1 share a 57% overall identity) with the exception of two highly conserved domains located in the N- and C-terminal regions of the protein, which include a RING domain located in the N-terminus and two tandem BRCT motifs at the extreme C-terminal end (Fig. S). Cancer-predisposing missense and truncating mutations are found within the RING domain and BRCT motifs indicating that the function of both N and C-terminal regions are of critical importance for tumour suppression (Friedman *et al.,* 1994).

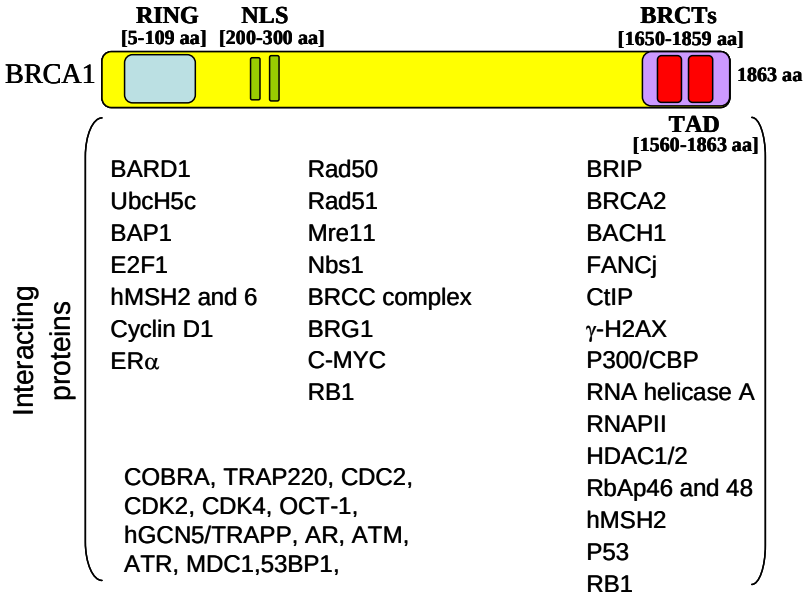


Fig S: ***Domains present at BRCA1 protein sequence and some of the described BRCA-interacting proteins.*** *Highly conserved RING domain and BRCT motifs are indicated. Putative nuclear localization signals (NLS) and C-terminal transcriptional activation domain (TAD) are also shown.*

The N-terminal RING domain was identified as soon as the *BRCA1* gene. It was cloned on the basis of homology with similar domains found in proteins that interact directly or indirectly with DNA, typically transcription factors. This domain is also found in E3 ubiquitin ligases. The RING domain of BRCA1 encompasses the first 109 amino acids. Within this region of the protein a characteristic core of approximately 50 amino acids (spanning exons 2 to 5) contains a conserved pattern of seven cysteine residues and one histidine residue arranged in an interleaved fashion to form a structure responsible for co-ordinating the binding of two Zn^{2+} ions (Brzovic *et al.,* 2001). Unlike some RING domains, this motif in BRCA1 does not bind directly to DNA, rather it forms an interaction surface responsible for heterodimerization with the structurally related protein BARD1.

The BRCT motif is an approximately 100-amino-acid domain that is present in a number of other DNA repair and DNA damage-response proteins, including 53BP1, MDC1, XRCC1 and budding yeast Rad9. Structural analyses of the two BRCT motifs in BRCA1 revealed that the individual motifs form a similar structure to each other that are packed together in a head-to-tail configuration. Many of the tumour-derived missense mutations in this region of BRCA1 map to the interface between the two BRCT motifs and result in the destabilization of the BRCT structure (Williams *et al*., 2001). These BRCT motifs can function as phosphopeptide-binding sites that can mediate protein–protein interactions with phosphoproteins (Manke *et al*., 2003; Clapperton *et al*., 2004).

Apart from the BRCT motifs, The C-terminal region contains an acidic domain (Miki *et al*., 1994) that can function as a transcriptional activation domain (TAD) in yeast and mammalian cells, when linked to a DNA-binding domain. TAD function requires the last 300 amino acids approximately (aa 1,560–1,863) for maximal activity. However, partial transcriptional activity is retained by a ‘‘minimal TAD’’ (aa 1,760–1,863). Deletion of a short segment or cancer-associated point mutations in the minimal TAD lead to ablation of activity.

BRCA1 main isoform (i.e. 220 kDa form) is, mainly, a nuclear protein. Two putative nuclear localization signals (NLS) were identified, both in exon 11, although only one of the two appears to be required for nuclear transport of BRCA1. Since these signals are located on exon 11, the 97 kDa isoform BRCA1-Δ11 is considered to be mostly cytoplasmatic (Thakur *et al.,* 1997), although some studies suggest this isoform can still localize to the nucleus and retains some BRCA1 functional activity (Huber *et al*., 2001). The BRCA1 protein was also found to contain a functional HIV Rev-type nuclear export signal (NES) within its N-terminus that facilitates its nuclear exit via the CRM1/exportin pathway (Rodriguez and Henderson, 2000). In an earlier study, it was demonstrated that heregulin could induce the phosphorylation of BRCA1 through HER2/neu-mediated activation of the serine/threonine kinase c-AKT (protein kinase B) (Altiok *et al*., 1999). The c-AKT-mediated phosphorylation of BRCA1 occurred on T508, which is immediately adjacent to the NLS1. The phosphorylation of T508 resulted in cytoplasmic accumulation of BRCA1. Taken

together, these findings suggest that BRCA1 may shuttle back and forth between the nucleus and cytoplasm in a physiologically regulated fashion, and so implementing the levels of regulation of BRCA1.

I.3.3 Activities and functions

It is possible that the diverse functions of BRCA1 manifest through its ability to interact with many different proteins (Fig. S). Indeed, BRCA1 has been reported to interact with tumour-suppressor genes (p53, RB1, BRCA2), oncogenes, transcriptional activators and repressors (p300, CBP, BRG1, RbAp46, RbAp48, histone deacetylases-1 and -2, CtIP), components of the basal transcription machinery (RNA helicase A, RNAPII), DNA-damage checkpoint components (Rad51, Rad50, hMSH2), cell-cycle regulators (E2F1, cyclins (D1, A, B1), cyclin-dependent kinases (CDC2, CDK2, CDK4), ubiquitination factors (BARD1, E2 ubiquitin-conjugating enzymes), steroid hormone receptors and other transcription factors (c-MYC, OCT-1, NF-YA). However, the biological significance of many of the reported interactions is yet unclear.

- ❖ E3 ubiquitin ligase activity

The RING domain present in a subset of proteins of the RING-domain family confers E3 ubiquitin ligase activity (for a more extensive review about the ubiquitin enzymatic cascade refer to enclosed **Box**).The study by Lorick *et al*. (Lorick *et al*., 1999) and Ruffner *et al*. (Ruffner *et al*., 2001) revealed that an N-terminal fragment of BRCA1 comprising its RING domain possessed E3 ubiquitin ligase activity *in vitro*. Several groups (Hashizume *et al*., 2001; Xia *et al*., 2003; Baer and Ludwig, 2002) found that the associated E3 ubiquitin ligase activity of BRCA1 is dramatically enhanced when combined with the BRCA1-associated ring domain protein 1 (BARD1).

Of the many BRCA1-protein-interaction partners identified, the significance of its association with BARD1 is beyond question and is supported by numerous *in vitro* and *in vivo* observations. BARD1 and BRCA1 interact to form a heterodimer. In fact, most cellular BRCA1 proteins are found in association with BARD1 (Yu *et al*., 2000). Like BRCA1, BARD1 also possesses an N-terminal RING domain and two BRCT motifs at the C-terminus. Three-dimensional structural analysis of the BRCA1/BARD1 RING heterodimer complex indicates that these proteins interact through an extensive four-helix bundle formed by helices that flank the core RING motif (amino acids 24–64) (Baer and Ludwig, 2002) (Fig. T). Indeed, missense mutations in five of the critical Zn^{+2}-binding residues in the BRCA1 RING domain have been found in tumours and functional analyses have shown that many of these mutations reduce or abolish heterodimerization (Jasin, 2002). The heterodimerization is also important for stability of the two proteins *in vivo* (Choudhury *et al*., 2004).

In the last years, a few details about the activity of the heterodimer have been disclosed. Mutation analysis experiments revealed that UbcH5c, an E2 conjugating enzyme, is of critical importance to the ubiquitination

function of BRCA1/BARD1 *in vivo* since the mutation of a single I26 residue in BRCA1, responsible for the recognition and binding with UbcH5c, abolished the ubiquitin ligase activity of BRCA1/BARD1 (Brzovic *et al*., 2003). It has also been shown that the heterodimer catalyzes different types of bonds in the ubiquitin chains, through Lys48 and Lys63 secondarily and primarily through Lys6- which is quite an unconventional type of linkage-, at least for the autoubiquitination of the heterodimer (Wu-Baer *et al.,* 2003).

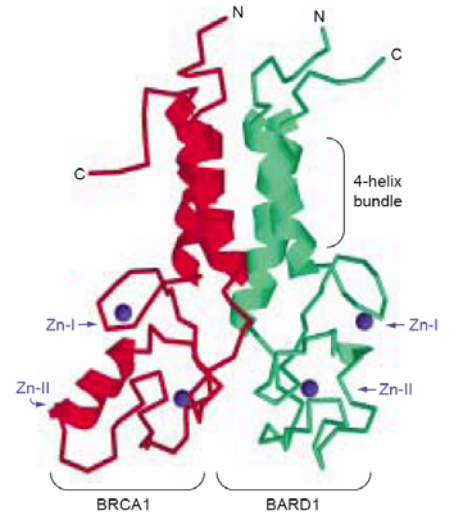


Fig T: ***Three-dimensional structural analysis of the BRCA1/BARD1 RING heterodimer complex*** *indicates that the proteins interact through an extensive four-helix bundle formed by helices that flank the core RING motifs. Missense mutations in five of the Zn+2 binding residues of BRCA1 reduce or abolish heterodimerization and are found in tumours. From Baer and Ludwig, 2002.*

It has been suggested that the ubiquitin ligase activity of BRCA1/BARD1 contributes to many of the biological functions of the BRCA1 protein, including its breast and ovarian cancer suppressor activity (Baer and Ludwig, 2002). It was shown that this activity is abolished by tumour-derived mutations (like C61G) in the conserved Cys residues within the RING domain (Hashizume *et al*., 2001), further supporting the notion that this is a biologically relevant function of BRCA1. In fact, it was recently demonstrated that conditional *Bard1*- and *Brca1*-mutant mice develop breast carcinomas that are indistinguishable from each other, probably indicating that the tumour suppressor activity of both genes is mediated through the BRCA1/BARD1 heterodimer (Shakya *et al.,* 2008). However, the principal protein targets of the BRCA1/BARD1 ubiquitin ligase *in vivo* are just starting to be identified.

The first substrate found to be ubiquitinated by BRCA1/BARD1 was the heterodimer itself (Mallery *et al.,* 2002). This autoubiquitination reaction was found to stimulate the E3 ubiquitin ligase activity of the heterodimer approximately 20-fold. Moreover, the ubiquitinated BRCA1/BARD1 have an increased affinity for binding to DNA repair intermediates (Simons *et al.,* 2006).

Another substrate found recently is topoisomerase IIα (Lou *et al*., 2005). The ubiquitination stimulates topoisomerase activity, which implies a better untangling of DNA concatenates after replication and so easier condensation of chromosomes prior to mitosis. The BRCA1 ubiquitin ligase also appends ubiquitin moieties on γ-tubulin and other centrosome proteins, and labels the centrosome as post-duplicated preventing a second round of duplication. It

also inhibits centrosomes from nucleating microtubules (Starita *et al*., 2004). Starita *et al*. described that a subpopulation of RNA polymerase II (RNAPII) is Ser-5 phosphorylated after DNA damage and subsequently ubiquitinated by BRCA1/BARD1 and sent to degradation by the proteasome (the target is the largest subunit of the RNAPII, Rpb1) (Starita *et al.,* 2005). The ubiquitination of RNAPII by BRCA1 has also been found to affect the process of polyadenylation (Kleiman *et al.,* 2005) and transcription (Horwitz *et al.,* 2007). Another well recognized substrate is CtIP (Yu *et al.,* 2006). Ubiquitinated CtIP is not targeted for degradation and, instead, it binds to chromatin following DNA damage and is likely involved in DNA damage checkpoint control. The DNA damage checkpoint control also promotes the association of BRCA1 and E2 enzyme on chromatin after DNA damage (Poloanowska *et al.,* 2006), and this is needed for the ubiquitination events taking place at stalled replication forks and double strand breaks (DSB) repair sites *in vivo* (Morris *et al.,* 2004).
BRCA1/BARD1 is also able to ubiquitinate *in vitro* nucleosome core histones H2A, H2B, H3 and H4 (Mallery *et al.,* 2002), FANCD, nucleoplasmin, p53 and estrogen receptor (ER), but its significance *in vivo* remains elusive.

BOX- THE UBIQUITIN ENZYMATIC CASCADE

The ubiquitin enzymatic cascade is comprised of ubiquitin, a three-enzyme ubiquitination complex, the intracellular protein target, and may also include the proteasome that is the organelle of protein degradation. The ubiquitination machinery is present in both the cytosol and the nucleus.

Eukaryotic **ubiquitins** (Ub) share an identical sequence. This stringent evolutionary conservation of ubiquitin underscores the fundamental importance of the ubiquitin pathway in basic cellular physiology. Ubiquitin is a 76–amino acid protein (9 kDa molecular weight) folded into a tightly packed globular conformation and is found either as a free monomer in the cytosol or covalently linked to itself and other proteins. The amino acid glycine 76 (G76), at the extreme C-terminal end of the peptide, protrudes from the protein core to serve as the site for covalent amide conjugation to other proteins.

A single enzyme, E1, initiates ubiquitination by activating the ubiquitin peptide monomer (Fig. 1b). Two isoforms of human **E1 ubiquitin-activating enzyme** arise by translation from alternate protein start sites on the same mRNA and have been found in both the cytoplasm and the nucleus. The essential nature of the protein is indicated by the finding that inactivation of the yeast E1 gene, *UBA1*, is lethal.
Ubiquitin activation begins with the formation of a ubiquitin-adenylate intermediate. One ATP molecule is expended for each E1-ubiquitin linkage. The intermediate serves as the donor of ubiquitin to a conserved cysteine residues in the E1 active site, where it is exchanged for adenosine monophosphate. In a subsequent transthiolation reaction, the activated ubiquitin moiety is passed from the E1 cysteine residue to the active-site cysteine of the E2 ubiquitin-conjugating enzyme.

There are at least 25 mammalian genes for **E2 ubiquitin-conjugating enzymes**, which are the first determinants of substrate specificity in the ubiquitin pathway. All E2 enzymes share a conserved core UBC domain of approximately 150 amino acids. Centrally located within the UBC domain is the conserved ubiquitin-binding cysteine residue. E2 enzymes may attach ubiquitin either to target proteins directly or to E3 ubiquitin ligases, depending on the kind of E3 involved. However, most ubiquitination seems to require both E2 and E3 enzymes.

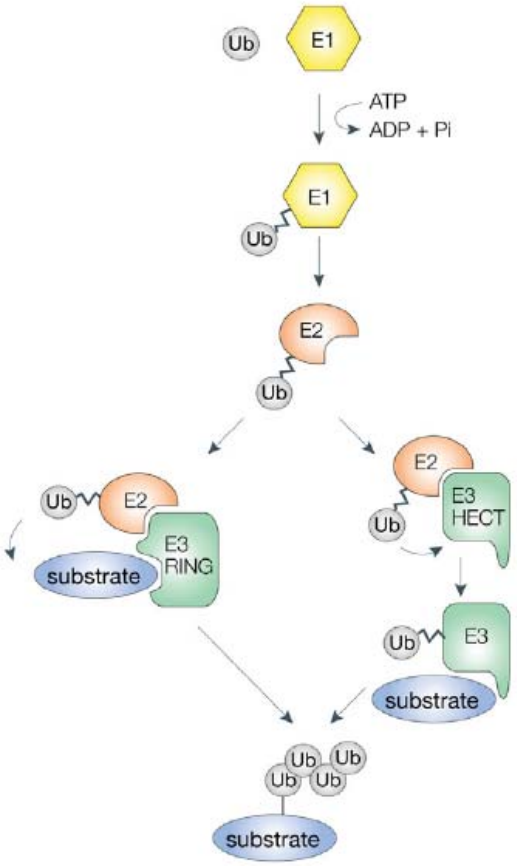


Fig 1b: ***Schematic represent-tation of the ubiquitination process****. A hierarchical set of three types of enzymes is required for substrate ubi-quitination: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) en-zymes. From Woelk et al., 2007.*

The **E3 ubiquitin ligase** family comprise a large and heterogeneous family of proteins that work by a variety of different mechanisms and vary in their interactions with E2 enzymes. The E3 enzymes are the components that bind to specific protein substrates and promote the transfer of ubiquitin from a thioester intermediate to amide linkages with internal lysines of proteins or polyubiquitin chains. They include two main classes of enzymes: HECT domain ligases, homologous to E6-associated protein (E6-AP), and RING domain ligases. In the first class of E3 ligases, the charged ubiquitin is transferred from the E2 to a Cys residue in the HECT domain. RING finger E3s contain a characteristic structure composed of conserved histidine and cysteine residues in complex with two central Zn^{2+} ions. The RING E3s serve as docking sites that bring together the target substrate and E2 enzymes to

mediate transfer of the ubiquitin moiety but do not form thioester bonds with ubiquitin.

To mediate transfer of ubiquitin from E2s to specific protein substrates, the E3 enzymes are sensitive to distinct signals in the substrate. Several modes of recognition by E3 ubiquitin ligases are well characterized. One mode of recognition is governed by the "*N-end rule*", based on the finding that the *in vivo* half-life of a protein is related to the properties of its amino-terminal residue. Short-lived proteins commonly have basic or bulky hydrophobic residues at their N-terminus, and more stable proteins have one of the amino acids cysteine, alanine, serine, threonine, glycine, valine, or methionine at the N-terminus. The paradigm of this rule is the mitotic cyclins. They contain at the N-terminus what was called the "destruction box", a 9-amino acid motif conserved among A- and B-type cyclins. The "destruction box" consensus sequence consists in RxxLxxxxN, with some variations, and they usually share a downstream region enriched in lysines and neighbouring sequences play also a role in the recognition of the target site (King *et al.,* 1996).

However, most proteins are targeted for ubiquitination by more complex mechanisms. For example, *post-translational modifications*, such as phosphorylation, are common signals for ubiquitination. A number of important transcription factors are affected by phosphorylation-dependent ubiquitination. Phosphorylation might also prevent substrate recognition/interaction by the E3 ligase.

Some short-lived proteins contain a *PEST sequence*, which is a site enriched in the four amino acids proline, glutamic acid, serine, and threonine (Rogers *et al*., 1986).

Substrate proteins can be modified by Ub in different ways (Woelk *et al.,* 2007) (Fig. 2b). *Monoubiquitination* is the attachment of one Ub moiety to a single lysine residue. This modification is a reversible, non-proteolytic signal involved in endocytosis, endosomal sorting, histone regulation, DNA repair, virus budding and nuclear export. A variation of this modification occurs when several lysine residues of a substrate are modified by a single Ub molecule, giving rise to multiple monoubiquitination that plays a role in receptor internalization and endocytosis.

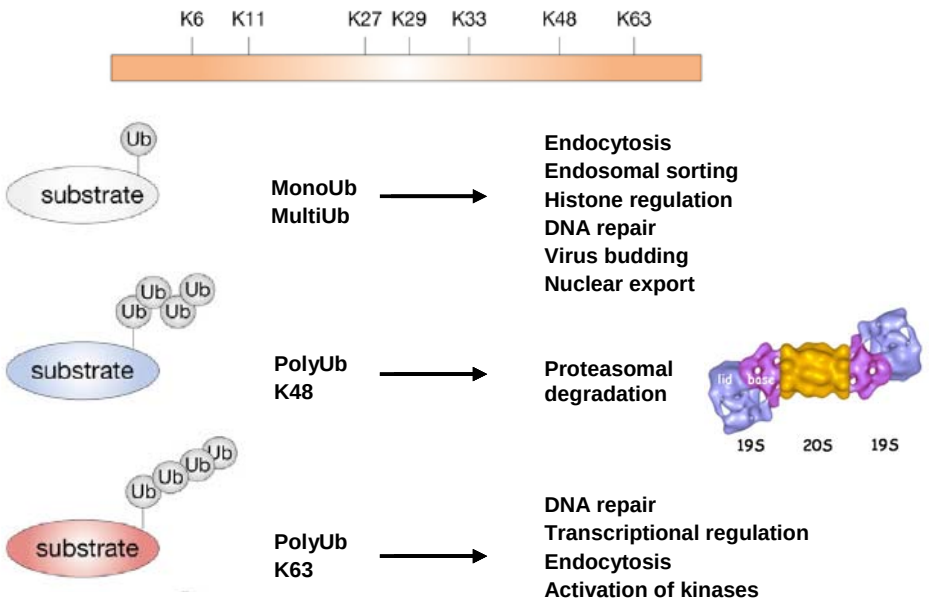


Fig 2b: ***Schematic representation of the different Ub-modifications with their functional role****. Very little is known about the precise function and topology of chains linked through K6, K11, K27, K29 and K33.*

Ubiquitin itself contains seven lysine residues that can be potentially used as acceptors for the attachment of other Ub molecules, allowing the formation of different types of *Ub-chains*. Lysine linkage is a central issue, since different poly-Ub chains contribute to a diversity of signals that cells interpret and translate into specific biological responses. However, it is currently not known if all of the linkages have a specific function.

Ub chains formed through *Lys63* have been subjected to intense study. Similarly to monoubiquitin, such chains generate a non-proteolytic signal involved in DNA repair, transcriptional regulation, endocytosis and activation of protein kinases. NMR data have confirmed the existence of a conformational difference between Lys48- and Lys63-linked chains, with the latter adopting an extended, linear conformation of Ub units arranged head to tail. This type of structure suggests that Lys63 chains might be recognized as a signal topologically similar to monoubiquitin.

Much less is known about the precise function and topology of chains that are linked through *Lys6, Lys11, Lys27, Lys29 and Lys33.*

The best-studied examples are chains of four or more Ub moieties linked through *Lys48*. This form of chain targets proteins for degradation via the 26S proteasome. The **26S proteasome** is a large multisubunit organelle, site for the ATP-dependent degradation of ubiquitin-tagged proteins. The structure and function of the proteasome are highly conserved from archaebacteria to eukaryotes, and the proteasome is essential for cell and organism viability in eukaryotes. The 26S proteasome is composed of two major subunits that can assemble in an ATP-dependent manner, the 20S catalytic component and the 19S regulatory component.

The *20S subunit* is the core of the proteasome and is made up of four heptameric protein rings stacked like four doughnuts. The two inner β-rings

harbor catalytic sites -contain trypsin, chymotrypsin and postglutamyl-like hydrolytic activities- that face into the hollow center of the ring structure. The two α-rings sandwich the β-rings. The amino terminus of the α-subunits blocks access to the proteolytic chamber. Thus, the inner cavity of the proteasome is only accessed through the narrow pores on either end of the cylinder.

The *19S regulatory component* is located at each side of the 20S subunit. It acts as a gate agent to limit entry to the proteasome to targeted proteins. This subunit is also essential for proteolytic activity because the 20S subunit alone is inactive. Each 19S particle is composed of numerous subunits, including six ATPases that most likely provide the energy necessary for substrate unfolding that is required before entry into the 20S chamber. The outer-lid of the 19S component is involved in the recognition and Ub-chain processing before substrate translocation and degradation.

Traditionally, it is thought that virtually all proteins that are degraded by the proteasomes must be ubiquitinated. However, several examples of ubiquitin-independent degradation by the proteasomes have been reported. The best-documented case is that of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis. Accumulation of polyamines stimulates synthesis of antizyme, which binds to ODC and facilitates its degradation by the 26S proteasome without ubiquitination (Murakami *et al.,* 1992). There are several additional examples for ubiquitin-independent degradation of proteins by the proteasome. Degradation of retinoblastoma protein (RB1) induced by cytomegalovirus pp71 occurs without detectable polyubiquitination and under conditions that lack a functional ubiquitin-conjugating system in cells harboring a thermo-labile E1 ubiquitin-activating enzyme (Kalejta *et al*., 2003). Experiments performed with c-Fos and c-Jun have shown that their degradation in cells does not require polyubiqutination on internal lysines or N-terminal ubiquitination (Bossis *et al.,* 2003; Jariel-Encontre *et al.,* 1995). P53, besides its ubiquitin-dependent degradation, can be degraded by the 20S proteasome in a ubiquitin-independent manner (Asher *et al.,* 2002).

Proteins are degraded in a processive manner by the proteasome; thus, a single protein is hydrolyzed to final products before the next substrate enters. Cleavage products in the proteasome average six to 10 amino acids in length and intact recyclable Ub molecules. Eventual hydrolysis to individual amino acids occurs in the cytosol.

Ubiquitination can be reverted by **deubiquitinating enzymes** (Ventii *et al*., 2008). Deubiquitination can occur at any time during the addition of ubiquitin moieties to a protein, underscoring the complexity of the balance between protein survival and degradation.

❖ Transcription regulation

A role for BRCA1 in transcription was suggested by the finding that BRCA1 had a *conserved acidic domain* at C-term with transcriptional activity in yeast and mammalian cells (Miki *et al*., 1994; Monteiro *et al.,* 1996).

Subsequently, it was found that BRCA1 regulates a variety of transcriptional activities.

BRCA1 transcription regulatory activity may be mediated, in part, by the *interaction with the basal transcriptional machinery* (RNA helicase A and RNAPII) (Anderson *et al.,* 1998). BRCA1 has been proposed to stimulate (Horwitz *et al*., 2006) or inhibit (Horwitz *et al*., 2007) initiation of transcription by regulating the formation of the RNAPII complex *in vitro* (Horwitz *et al*., 2006). They propose an interesting model, yet to be proven *in vivo*. In this model (Fig. U), BRCA1/BARD1 is recruited to specific promoters by DNA-binding factors. Depending on the interaction of an E2 ubiquitin-conjugating enzyme (a process known to be regulated (Polanowska *et al*., 2006)) with BRCA1/BARD1, the heterodimer would stimulate (in the absence of E2) or it would act as an E3 ubiquitin ligase enzyme and ubiquitinate RNAPII, which would sterically block the formation of the RNAPII complex or rather send it to degradation and so, inhibit transcription. BRCA1 has also been suggested to play a role in post-initiation events (Krum *et al*., 2003), since BRCA1 interacts preferentially with the hyperphosphorylated form of RNAPII (the post-initiation form).

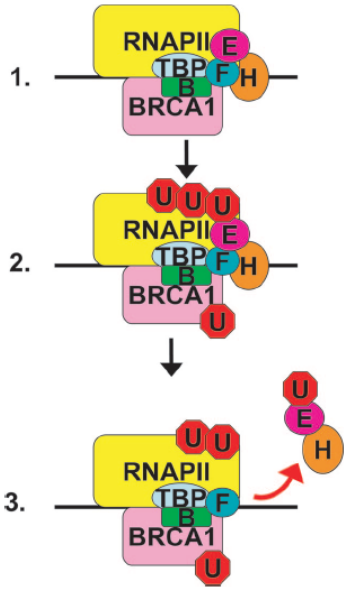


Fig U: ***Model diagram of BRCA1 regulation of initiation of transcription through the interaction with the basal transcriptional machinery.*** *Horwitz et al propose that BRCA1 is brought to specific promoters by DNA-binding factors. Once at the promoter, BRCA1 can stimulate (in the absence of E2) or inhibit (in the presence of E2) initiation of transcription by means of the ubiquitination of RNAPII and TFIIE. From Horwitz et al., 2007.*

BRCA1 also affects transcription through the *interaction with chromatin remodeling complexes and regulatory proteins*. BRCA1 interacts directly with the BRG1 subunit of the SWI/SNF chromatin remodeling complex (Bochar *et al*., 2000) and the function of the remodeling complex is necessary for the coactivation of p53 by BRCA1. BRCA1 was found to mediate a large-scale chromatin-unfolding activity through the recruitment of COBRA, a component of the negative elongation factor (NELF) (Ye *et al*., 2001).

TRAP220, a member of the TRAP/DRIP mediator complex, interacts with BRCA1 (Wada *et al.,* 2004). This complex stimulates the transcriptional activity of nuclear receptors such as peroxisome proliferator activated

receptors (PPAR) or thyroid hormone receptor (TR), and also coactivates BRCA1 transactivation functions. BRCA1 has been shown to interact with hGCN5 and TRRAP in a histone acetyltransferase (HAT) complex that required the presence of wild-type BRCA1 for transcriptional activation (Oishi *et al*., 2006).

Finally, the interaction of BRCA1 with various relatively generic transcriptional regulatory proteins may also contribute to its transcriptional regulatory activity, like the HATs p300 and CBP (Pao *et al.,* 2000), the retinoblastoma protein (RB1) (Yarden *et al.,* 1999), RbAp46 and 48 and histone deacetylases HDAC1 and 2 (Yarden *et al*., 1999).

By means of all these different mechanisms and interactors BRCA1 has been described to *modulate the activity of several transcription factors*.

BRCA1 interacts with and coactivates p53, and redirects its transcriptional activity to genes involved in DNA repair and/or cell cycle arrest (Zhang *et al*., 1998; MacLachlan *et al*., 2002). BRCA1 was found to interact with and inhibit Myc-mediated transcription (Wang *et al.,* 1998). It has been described to affect the activity of other transcription factors like STAT1, NF-κB, Oct-1, ELK-1, ZBRK1, among others.

BRCA1 was also found to regulate the activity of steroid hormone receptors. In several studies, BRCA1 was found to interact directly with the androgen receptor (AR) and stimulate its activity (Park *et al.,* 2000). BRCA1 upregulated the AR-mediated expression of the G1 cell cycle inhibitor p21WAF1 and enhanced dihydrotestosterone (DHT)-induced cell death in human prostate cancer cells (Yeh *et al.,* 2000).

BRCA1 was shown to inhibit the ligand-independent activity of the AF-1 domain of the estrogen receptor (ERα) (Zheng *et al*., 2001) and the ligand-dependent activity of the AF-2 domain of ERα (Fan *et al.,* 1999 and 2001; Xu *et al*., 2005) in breast and prostate cancer cell lines. A series of truncated BRCA1 proteins and cancer-associated mutants failed to or showed reduced ability to repress ER-α activity.

BRCA1 was found to interact with ERα in breast cancer cell lines (Fan *et al.,* 2001). The BRCA1:ERα interaction was mapped to the N-terminus of BRCA1 and AF-2 domain of ERα and did not require the presence of hormone (Fan *et al.,* 2001). A more detailed study of the BRCA1:ERα interaction revealed two potential contact sites for BRCA1 on ERα (the major site within amino acids 338–379 and a minor site within amino acids 420–595) and two contact sites for ERα on BRCA1 (amino acids 67–100 and 101–134) (Ma *et al.,* 2005). However, to date, there is no evidence for a unique mechanism to describe the inhibitory effect of BRCA1 over ERα transcriptional activity. The effect of BRCA1 on the transcription of the pS2 gene by ERα was suggested to be due to reduction of the recruitment of the receptor to the promoter (Wang *et al.,* 2005) while the effect on the transcription of the *CyclinD1* gene was not due to a change in ERα recruitment but of cofactors (Wen *et al.,* 2008). Wen *et al* (Wen *et al*., 2008) reported that BRCA1 affected the recruitment of the SMRT repressor, besides interfering in the recruitment of coactivators like SRC-3 and CBP, as a part of the mechanism of inhibition of ERα activity. Fan *et al* (Fan *et al.,*

2001 and 2002) also described the downregulation of p300 by BRCA1 and the competition for binding to the AF-2 domain of ERα as a possible explanation for the interference on ERα activity. Another proposed mechanisms for the inhibition of ERα ligand-independent activity was the interaction of BRCA1 with HDAC1 and 2 (Zheng *et al.,* 2001).

❖ Mammary gland development

We have already reviewed the regulated pattern of expression of *Brca1* following the different phases of mammary gland development in the mice (see section I.3.1.2). Selective disruption of *Brca1* in mouse mammary epithelial cells results in increased apoptosis and aberrant ductal development during pregnancy, lactation and involution (Xu *et al*., 1999). Furuta *et al* (Furuta *et al*., 2005) demonstrated that loss of functional BRCA1 causes a failure of mammary acinus formation but enhances the proliferation of mammary epithelial cells using an *in vitro* 3D culture system. This implies that BRCA1 is involved in the process of differentiation of mammary epithelial cells.

❖ Maintenance of genomic integrity

Clues to a major function for BRCA1 in the maintenance of genomic integrity have come from various types of experiments demonstrating that BRCA1: (1) plays a role in several highly specialized types of DNA repair; (2) is an essential component of several DNA damage-responsive cell cycle checkpoint mechanisms; and (3) is required for the proper replication and functioning of centromeres. BRCA1 interacts with various proteins to form a BRCA1-associated genome surveillance complex (BASC) that contains proteins involved in mismatch repair (MSH2, MSH6, and MLH1), DNA double-strand break (DSB) repair (ATM and the Rad50-Mre11-p95NBS1 (RMN) complex), DNA replication (RFC) and recombination (BLM) (Wang *et al*., 2000).

❖ DNA repair

Several studies have documented roles for BRCA1 in two highly specialized DNA repair processes: transcription coupled DNA repair (TCR) and homology-directed repair (HDR).
BRCA1 competent cells exhibit a greater ability to repair the transcribed strand than the non-transcribed strand; whereas BRCA1 deficient cells showed equal repair of both strands (Abbot *et al*., 1999) indicating that BRCA1 is involved in *transcription coupled DNA repair (TCR)* mechanisms. BRCA1 interacts with the familiy of hMSH proteins, members of the DNA mismatch repair cascade that is essential for the TCR pathway (Wang *et al*., 2001).
Chromosomal double strand DNA breaks (DSBs) can be repaired by two processes: *homology-directed repair (HDR),* in which the chromosomal DNA is restored to its original state; and non-homologous end joining (NHEJ).

Several studies indicate that BRCA1 deficient cells have a severe defect in HDR, which is partially rescued by providing an exogenous *BRCA1* gene (Moynahan *et al.,* 1999). A clue to the mechanism by which BRCA1 participates in DSB repair is the finding that BRCA1 interacts directly with BACH1, a member of the DEAH helicase family (Cantor *et al.,* 2001). The DEAH box family includes DNA and RNA helicases, which participate in DNA repair, meiotic recombination, and various aspects of RNA processing and editing.

BRCA1 may also participate in a form of nucleotide excision repair (NER) referred to as *global genomic repair (GGR).* The ability of BRCA1 to stimulate NER may be due, in part, to its ability to mediate p53-independent induction of several NER genes: XPC, DDB2, and Gadd45 (Hartman *et al.,* 2002).

BRCA1 colocalizes with Rad51, a DNA recombinase, during mitotic and meiotic phases (Scully *et al.,* 1997). The colocalization of BRCA1 and Rad51 implicates BRCA1 in *genetic recombination* events occurring during meiosis.

❖ Cell cycle checkpoints

The ability to control precisely the ordering and timing of cell cycle events is essential for maintaining genome integrity and preventing mutations that can disrupt normal growth control. Cells treated with DNA damaging agents coordinately arrest their cell cycle progression at the G1/S phase, the S phase and the G2/M phase to allow times for repairing the damage. Cellular machineries that mediate cell cycle arrest are called *cell cycle checkpoints*, which monitor DNA status and ensure the completion of the previous phase in the cell cycle before advancing to the next phase.
BRCA1 is associated with numerous proteins that may play important functions in all cell cycle checkpoints:

- G1/S cell cycle checkpoint: overexpression of wild-type BRCA1 in tumour cells inhibited cell proliferation (Holt *et al*., 1996). A study attributed this inhibitory activity to the interaction of BRCA1 with hypophosphorylated RB1 protein (Aprelikova *et al.,* 1999), which prevented the transcription by E2F of downstream genes and inhibited cell proliferation. An earlier investigation attributed the growth-inhibitory effect of BRCA1 to the upregulation of p21WAF1/CIP1, a cyclin-dependent kinase inhibitor (Somasundaram *et al*., 1997), in a p53-independent way. Fabbro *et al*. (Fabbro *et al.,* 2004) demonstrated that the BRCA1/BARD1 complex is required for ATM/ATR (ataxia-telangiectasia-mutated and Rad3-related)-mediated phosphorylation of p53 at Ser-15 following IR or UV radiation-induced DNA damage and subsequent transcription of p21 by p53. In summary, BRCA1 induced G1/S arrest may occur through a number of distinct pathways that involve many important BRCA1 interacting proteins, including ATM, ATR, BARD1, RB1, p53 and p21 and their downstream effectors.

- S phase cell cycle checkpoint: S phase checkpoint primarily represents an inhibition of replication initiation upon DNA damage, for

instance, after ionizing radiation exposure. A lack of an IR-induced S-phase checkpoint results in persistent DNA synthesis. Xu *et al.* (Xu *et al.,* 2002) found that HCC1937 cells (a BRCA1-deficient breast cancer cell line) were defective in the S phase checkpoint. Besides, they found that this process is controlled by BRCA1 phosphorylation. ATR phosphorylates BRCA1 and activates the S phase checkpoint in response to stalled replication forks, while its phosphorylation by ATM induced this checkpoint after exposure to ionizing radiation.

BRCA1 interacts with several other proteins that play an essential role in the S phase checkpoint. These include the mediator of DNA damage checkpoint protein 1 (MDC1), H2AX, p53 binding protein 1 (53BP1) and MRE11/RAD50/NBS1.

- G2/M cell cycle checkpoint: G2/M cell cycle checkpoint delays movement of G2 cells into the mitosis (M) phase. Loss of this checkpoint allows cells with damaged DNA to proceed into the M phase, increasing the likelihood of abnormal chromosomes being passed to the daughter cells. Xu *et al.* (Xu *et al*., 1999) demonstrated that elimination of full-length BRCA1 abolishes this checkpoint upon certain types of DNA damage. BRCA1 regulates the expression, phosphorylation and cellular localization of CHEK1, a known regulator of the G2/M cell cycle checkpoint (Yarden *et al*., 2002). Their data also indicated that BRCA1 affects the expression of the Wee1 kinase, an inhibitor of Cdc2/cyclin B kinase, and the 14-3-3 family of proteins that sequesters phosphorylated Cdc25C and Cdc2/cyclin B kinase in the cytoplasm.

Phosphorylation of BRCA1 by ATM is also required for activation of the G2/M checkpoint (Xu *et al*., 2001). CHEK2-mediated phosphorylation of S971 of BRCA1 is involved in BRCA1 function in modulating DNA damage response, G2/M cell cycle checkpoint upon IR radiation and repressing tumour formation (Lee *et al.,* 2000). Aurora-A, one of the kinases known to regulate mitotic progression in various organisms, was shown to bind with and phosphorylate BRCA1 at S308. This phosphorylation is correlated with impaired function of BRCA1 in regulating G2/M transition (Ouchi *et al.,* 2004).

- Spindle checkpoint: during the mitotic phase, duplicated DNA is first condensed and packed to form sister chromatids, which are then equally separated into newly formed daughter cells. In the metaphase, the sister chromatids attach to the mitotic spindle at kinetochores that consist of protein complexes associated with centromeric DNA. Then, a large ubiquitin ligase called the anaphase-promoting complex (APC) and its co-factor, Cdc20, are activated. The activated APC-Cdc20 promotes the cleavage of the cohesion complex between the sister chromatids and triggers the onset of the anaphase. The spindle checkpoint ensures the accuracy of chromosome segregation by preventing cells with un-aligned chromosomes from exiting mitosis. Any premature or missegregation of sister chromatids will lead to the loss or gain of chromosomes in daughter cells, leading to aneuploidy, which is a prevalent form of genetic instability of human cancers. Molecular components of the spindle checkpoint include two evolutionarily conserved protein families MAD and BUB, as well as other components. Wang *et al*. (Wang *et al*., 2004) observed that in BRCA1 deficient cells the spindle

checkpoint is defective and there was a decreased expression of a number of genes involved in the spindle checkpoint, like MAD2, Polo-like-Kinase, BUB1, BubR1 and ZW-10. BRCA1 knockdown also caused the accumulation of multinucleated cells, suggesting a defect in the coordination of cytokinesis and karyokinesis. These findings suggest that BRCA1 transcriptionally regulates gene expression for orderly mitotic progression (Bae *et al.,* 2005).

I.3.4 BRCA1-related breast cancer

Germline mutations in BRCA1 confer an estimated 56-85% lifetime risk of developing breast and/or ovary cancer. Inherited mutations of the BRCA1 gene account for 40-45% of hereditary cancers (Ford *et al.,* 1994, Easton *et al*., 1995), while BRCA2 for about a 35-40% of the cases (Fig. V) (Wooster *et al.,* 1995). However, BRCA1 mutations comprise about 80% of families whose members have a high incidence of both breast and ovarian cancers. Hereditary breast cancers represent a 5-10% of all breast cancers and around 12% of women in the world will develop breast cancer in their lifetimes. Only BRCA2 mutations are associated with male breast cancer.
BRCA1 mutation carriers have also a significantly increased risk of pancreatic, endometrial, and cervical cancers and of prostatic cancers in men younger than age 65 (Thompson *et al.,* 2002).

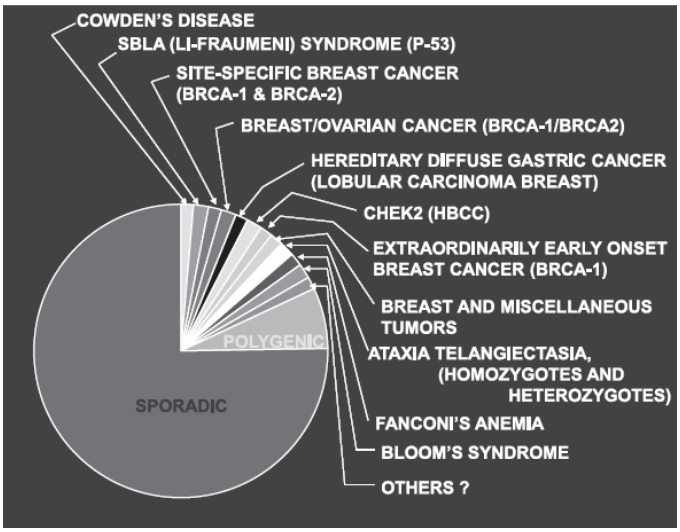


Fig V: ***Circle graph showing relative frequencies of sporadic and hereditary breast cancers*** *(From Breast Cancer, 2nd edn. Winchester et al., 2006, pp.61-82)*

The *BRCA1* gene fits the profile of a classical "tumour suppressor gene". A tumour suppressor is a protein which, when working properly, prevents the development of one or more types of cancer (Knudson, 1993). Loss of function of both alleles of the gene is required for tumourigenesis ("two-hit" hypothesis). In some cases, this results from two somatic mutational events, while in others a mutation in one allele is inherited in the germline and the other occurs somatically (a process called "loss of heterozigosity" (LOH)). Important to this notion is that each wild-type allele is "haplosufficient" for adequate normal function, so that the inherited loss of

one allele has no phenotypic effect (Bignold, 2004). Individuals heterozygous for such a germ-line mutation are at increased risk of developing tumours because of the high probability of a somatic mutation occurring in the remaining normal allele in at least one cell in a susceptible tissue, and this results in the existence of a number of human familial cancer syndromes. Breast and ovarian cancers that develop in carriers of a *BRCA1* gene mutation almost always exhibit loss of the wild-type BRCA1 allele.

Most *BRCA1* mutations are frame-shift mutations resulting in truncated BRCA1 proteins, although point mutations in the C-terminal transcriptional activation domain or N-terminal RING domain are known (Gayther *et al*.,1995). Large chromosomal rearrangements affecting BRCA1 gene have been found to be major founder mutations in some ethnic groups, and it could be due to the unusually high frequency of *Alu* sequences (41,5%) present at the *BRCA1* locus (Welcsh *et al.,* 2001). These are polymorphic short interspersed elements of approximately 300 bp, frequent in primate genomes and prone to recombination (Szabo *et al.,* 2004).

In contrast, *BRCA1* gene mutations are rare in sporadic breast cancer cases and relatively uncommon in sporadic ovarian cancers (Futreal *et al*., 1994). However, it has been demonstrated that BRCA1 expression levels are reduced in sporadic tumours (Thompson *et al.,* 1995; Taylor *et al.,* 1998), generally correlating with high grade (poorly differentiated cells) and higher proliferation rates. The data generated from the clinical studies cited above strongly suggest that decreased BRCA1 expression contributes to sporadic breast tumour progression.

Several potential mechanisms can lead to a permanent decrease in BRCA1 levels, and several of them have been reported to account for the downregulation of BRCA1 in sporadic breast cancers. One mechanism is the hypermethylation of the *BRCA1* promoter. Methylation is associated with greater chromatin compaction and lack of accessibility (Rice *et al*., 2000). Examination of methylation patterns in the *BRCA1* promoter region indicated that preferential methylation of some sites occurs (see Fig. Q), apparently, only in tumours (Rice *et al.,* 1998). Another mechanism is allelic loss of heterozygosity (LOH) in the absence of mutations. If one allele were subject to inactivation by methylation, then LOH would lead to the presence of two methylated alleles and loss of expression. Currently, however, no studies have clearly demonstrated a functional link between LOH and promoter methylation. A third possible mechanism responsible for low BRCA1 protein levels might be the inhibition of transcription and translation of the *BRCA1* gene. Overexpression of HMGA1 proteins (which are proteins that enhance or inhibit gene transcription by acting as architectural proteins) (Baldassarre *et al.,* 2003) or ID4 (Turner *et al*., 2007) results in the downregulation of the *BRCA1* promoter activity.

In an attempt to recapitulate the effects of BRCA1 function deficiency, different mouse models have been engineered. The murine homolog of *BRCA1* (*Brca1*) encodes a 1,812 aa protein with 57% sequence identity to human BRCA1 (Abel *et al.,* 1995). Targeted deletion of exons 5–6 of *Brca1* (which encode the RING domain) revealed that *Brca1* is essential for embryonic cell proliferation (Hakem *et al.,* 1996) since *Brca1*$(5–6)^{-/-}$ mice died

by embryo day 7,5–8,5 primarily due to elevated cell death and growth retardation. Similar findings were reported by other investigators (Liu *et al.,* 1996; Gowen *et al*.,1996). *Brca1*$(5–6)^{-/-}$ embryos exhibited decreased expression of the p53 inhibitor Mdm-2, normal p53 levels, and increased p21 levels, suggesting that impaired growth might be due to the transcriptional activation of p53. Accordingly, *p53*$^{-/-}$*Brca1*$^{-/-}$ and *p21*$^{-/-}$*Brca1*$^{-/-}$ embryos showed increased survival time and died by embryo day 10 (Hakem *et al.,* 1997). Weaver *et al* developed an animal model that featured mammary targeted mutation of *Brca1* gene via an MMTV-Cre transgene (Weaver *et al.,* 2002). Animals homozygous for the mutation developed breast cancers after a long latent period that resembled pretty much the BRCA1 mutant cancers. The latent period for mammary tumourigenesis was significantly decreased in mice heterozygous for p53 ($p53^{+/-}$). This data indicated that inactivation of *p53* and *Brca1* deficiency synergistically induce mammary tumour formation in mice, and it might be the case also in human tumours since in the great majority of BRCA1 tumours there is inactivation of p53. However, experimental data also indicated that the inactivation of p53 (and its associated DNA damage response) is not sufficient for *Brca1* deficient cells to undergo malignant transformation since mutant mice developed tumours in a stochastic fashion, suggesting additional factor(s) are needed for tumourigenesis to occur (overexpression of oncogenes, inactivation of other tumour suppressor genes, etc.).

The breast cancer syndrome associated with BRCA1 mutations is characterized by the following features:

- At least four or more affected members of a kindred
- An increased frequency of early onset breast cancer cases (i.e., before age 40)
- Lifetime (cumulative) risk of female breast cancer of over 50% (about 85-90% by age 70)
- Frequent association with ovarian cancer (60% lifetime risk by age 70)
- Cancers are frequently multicentric and bilateral
- Often exhibit poor nuclear grade (how closely they resemble normal breast cells)
- Very high frequency of p53 mutations (84% as compared with 20-25% in sporadic cancers)
- Are frequently estrogen and progesterone receptor negative (Breast Cancer Linkage Consortium, 1997). Only 10 to 36% of tumours in these patients are estrogen receptor positive (Lakhani *et al*., 2002)
- They rarely exhibit amplification of the *HER2/neu* or *Cyclin D1* genes (quite frequent in sporadic cancers) but have a higher frequency of *MYB* amplification (29%) (Kauraniemi *et al*.,2000)

According to their characteristics, most BRCA1 tumours are to be classified as “basal-like” phenotype breast cancers (ER- and PR-negative, low level of luminal cytokeratins, BCL2, P27, ERBB2 and high expression of basal cytokeratins 5/6 and 17). DNA microarray analyses of human breast

cancers suggest a characteristic pattern of gene expression alterations in BRCA1 vs. BRCA2 cancers (van't Veer *et al*., 2002).
BRCA1 is becoming a potential biomarker in the treatment of breast cancer. In the future, the classification into the BRCA1-related breast cancer might have a significant impact on the clinical management of the disease because the absence of BRCA1 results in increased sensitivity to DNA damage-based chemotherapy, whereas the presence of BRCA1 promotes an increase in sensitivity to antimicrotubule agents (James et al., 2007).

OBJECTIVES

Considering the connection between mutations in the BRCA1 gene and familial breast cancer, and the involvement of ovarian hormones, specially progesterone, in the normal and pathological development of this tissue, we searched for a functional relationship between BRCA1 protein and the progesterone receptor (PR) in breast cancer cells.

Many different hypothesis have been proposed to explain the cancer tissue specificity displayed by BRCA1-mutation carriers, mostly targeted to breast and ovary tissue. We work on the hypothesis that the regulation by BRCA1 of ovarian hormone receptors' activity may explain, in part, the specific mammary carcinogenesis associated to mutations in the BRCA1 gene.

Then, our objectives are:

-to study the effect of the overexpression/knockdown of BRCA1 in the transcriptional activity of PR in our breast cancer cell model, T47D cell line. Effect on our PR-regulated promoter model, the MMTV promoter, and on other endogenous PR-target genes.

-to get further insight into the mechanism by which BRCA1 might affect PR transcriptional activity. This includes:

-study the possible physical interaction between BRCA1 and PR

-study the effect that the enzymatic activity of BRCA1 as an E3 ubiquitin ligase might have on PR

-study of the possible interaction of BRCA1 in the processes ocurring at the promoters of PR-target genes: effect on recruitment of PR, cofactors, chromatin structure.

-to study the effect of the overexpression/knockdown of BRCA1 on the cellular processes regulated by progesterone in cells *in vitro:* short-term proliferation, long-term proliferation and cell survival.

RESULTS

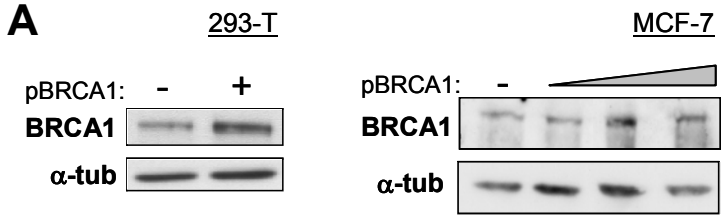
R.1 BRCA1 alters progesterone receptor transcriptional activity

R.1.1 Exogenously overexpressed BRCA1 inhibits the transcriptional activity of exogenous and endogenous progesterone receptor

In previous reports, BRCA1 had been shown to inhibit both ligand-independent (Zheng *et al*, 2001) and dependent (Fan *et al*, 1999, Fan *et al*, 2001) transcription mediated by the estrogen receptor-α (ERα). We wanted to investigate if it would be also the case for another member of the steroid receptor family, the progesterone receptor (PR).

As a first approach, we tested the effect of transiently overexpressed BRCA1 (which is expressed, in a variable amount, in all human-derived cell lines) on exogenously expressed PR by transient transfection of a luciferase reporter construct. Overexpression of the protein may amplify and give insight into possible functions and effects.

Before doing that, we tested the expression level and the functionality of the BRCA1 protein that was produced from our transfected plasmid. The overexpression was assessed by Western blot analysis in the cell lines used for the reporter induction in later experiments, 293T and MCF7 cells (Fig. 1A). We took advantage of the already reported coactivation of p53 transcription by BRCA1 (Zhang *et al*, 1998) to test for the functionality of the protein. MCF7 breast cancer cells, which are p53 wt, were co-transfected with a luciferase reporter gene under the control of a synthetic promoter containing 13 p53-response elements (p53RE-Luc), +/- p53 and +/- BRCA1 plasmids, and induction was detected by luciferase expression as indicated in Materials and Methods (Fig. 1B). As expected, the reporter construct was induced to a basal level by the endogenous wt-p53, while in the breast cancer cell line T47D, which expresses a mutated form of p53, the activation was null (data not shown). Exogenous expression of wt-p53 and BRCA1, though not very much overexpressed (see Fig. 1A), further induced the reporter construct around three-fold, which demonstrates that the transfected pBRCA1 is functional, at least, to an extent enough for the p53 coactivation action.



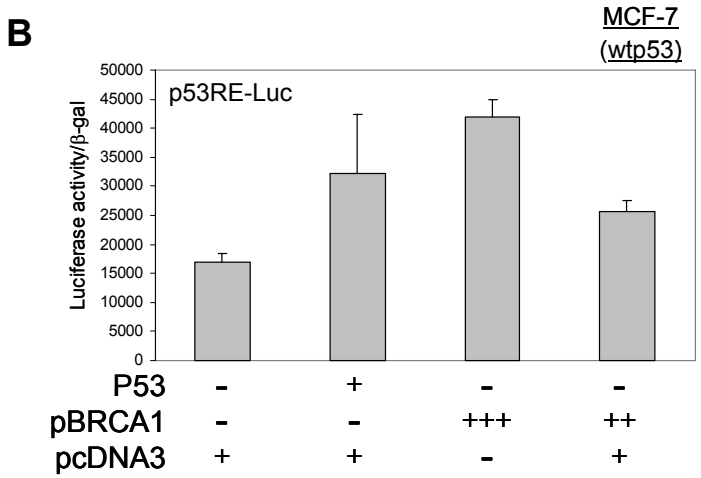


Figure 1: ***Assessment of overexpression and functionality of pBRCA1***
(A) To check the overexpression of BRCA1, 293T and MCF7 cells were transfected with different amounts (from 0,8 to 2 ug) of a plasmid encoding human BRCA1 (pBRCA1). After 48h, cell extracts were collected and BRCA1 overexpression was checked by Western blot analysis. α-Tubulin is shown as loading control.
(B) MCF7 cells were co-transfected with a reporter for p53 activity, p53RE-Luc and a p53 expression plasmid (exogenous p53, P53) or pBRCA1 in two plasmid doses (+++ 1.6, ++ 0.8). Between 36-48 h after transfection, cells were harvested for luciferase activity determination. The values represent the mean luciferase activity units normalized by β-gal ± SD of a representative experiment performed in triplicate.

Secondly, we analyzed the effect of the transfected pBRCA1 on the luciferase reporter for the transcriptional activity of ERα. 293T (ER negative) and MCF7 (ER positive) cells were co-transfected with the reporter plasmid containing estrogen-responsive elements, ERE-Luc, and with the indicated plasmids encoding for BRCA1 and +/- ERα, as shown in Fig. 2A and 2B. Cells were treated with vehicle (ethanol) or with estrogen hormone (estradiol, E2) for 24 hours and assayed for luciferase activity. Although the differences were not statistically significant, we could observe a repetitive and reproducible tendency of BRCA1 to decrease the induction of the reporter by estradiol.

To examine the effect of BRCA1 on the transcriptional activity of exogenous PR we used an MMTV-Luc reporter. The mouse mammary tumour virus (MMTV) promoter is a model promoter for the regulation by several steroid hormones (i.e. progesterone, androgens and glucocorticoids, but not estrogens (Cato *et al*, 1986)).
293T and MCF7 cells (both PR negative) were co-transfected with the reporter, pBRCA1 and a plasmid encoding for PR-B (i.e. the isoform described to be more transcriptionally active (Richer *et al*, 2002)) and luciferase expression was measured upon treatment with the synthetic progestin R5020 at a physiological concentration (10nM) (Fig. 2C). The effect of BRCA1 over the PR activity was more consistent, it reduced by 50% the basal and hormone-induced expression of the reporter when BRCA1 was overexpressed.

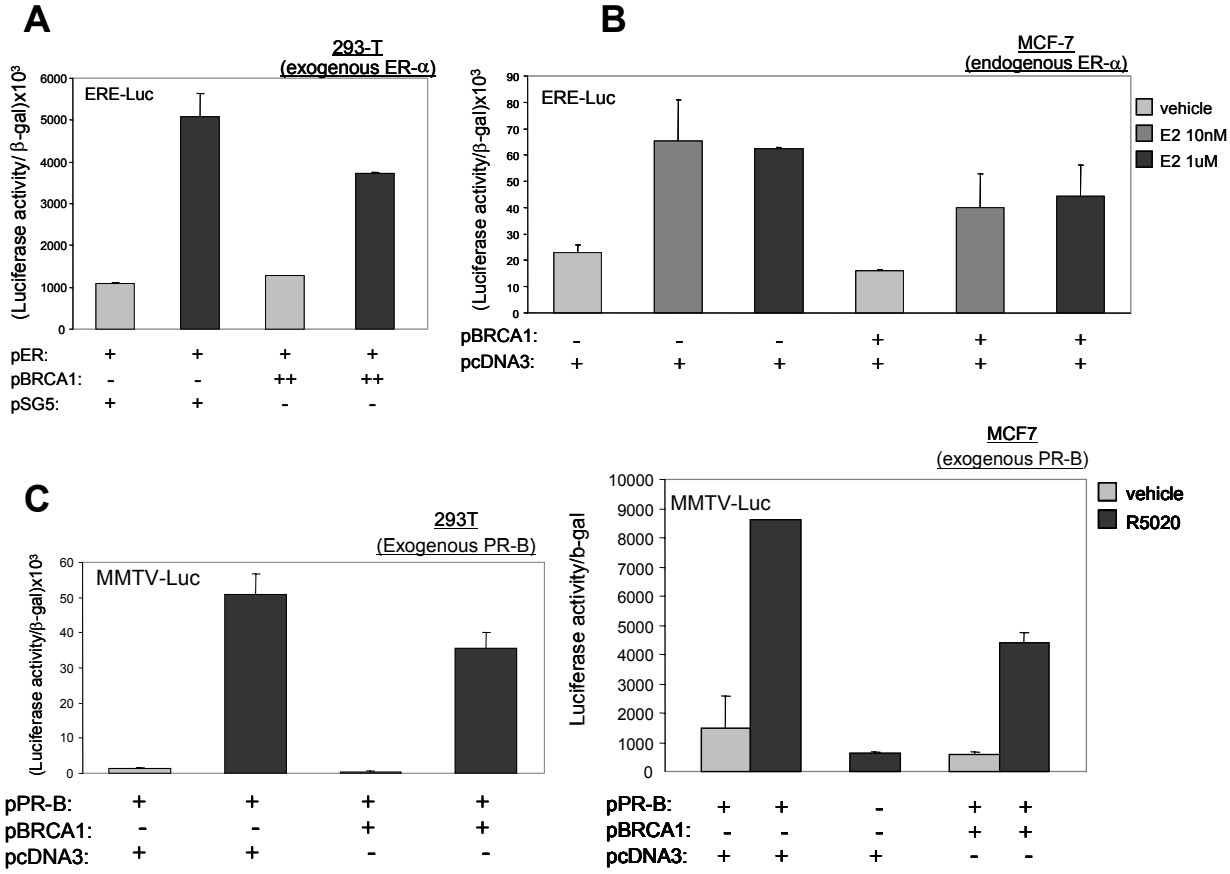


Figure 2: ***Effect of overexpressed BRCA1 on exogenous ER and PR transcriptional activity***

(A) 293T cells were co-transfected with an ERα-luciferase reporter, ERE-Luc, with ERα and pBRCA1 (++ 0.4, + 0.2 ug). After treatment (E2, 10 nM for 24 h), luciferase activity was measured and normalized by β-gal expression. Values are expressed as luciferase arbitrary units, ± SD of a representative experiment performed in triplicate.

(B) MCF7 cells were co-transfected with ERE-Luc and pBRCA1, and treated with vehicle (ethanol) or E2 at a 10 nM or 1 uM concentration for 24 h when cell extracts were collected. Luciferase activity was then assayed and normalized by β-gal. Values are expressed as luciferase activity arbitrary units, ± SD of a representative experiment performed in triplicate.

(C) 293T and MCF7 cells were transfected with the PR-luciferase reporter MMTV-Luc, pPR-B and pBRCA1, and treated with vehicle (ethanol) or R5020 10nM. After 24 h of treatment, luciferase activity was measured and normalized by β-gal expression. Values are expressed as luciferase arbritary units ± SD of a representative experiment performed in triplicate.

Next, we examined if we could reproduce these observations on the transcription driven by the endogenous progesterone receptor on chromatin-embedded promoters. For these experiments, we used the breast cancer cell line T47D-MMTVL, which expresses both PR and ER and has a stably integrated single copy of the MMTV-Luc reporter in the genome (Truss *et al,* 1995). Since we wanted to check the expression of not only the reporter but also physiological and mechanistically important endogenous genes, it was necessary to establish a bona fide set of genes regulated by progesterone in

GENE SYMBOL Average fold-change

R5020/etOH 6h				R5020/etOH 2h		R5020/etOH 1h		R5020/etOH ½ h	
TGFA	4,50	AP2B1	-3,06	IL6ST	2,57	IL6ST	1,85	TGFB3	1,63
CAV1	4,33	GATA3	-3,01	**EGF**	2,26	GADD45A	1,53	KAI1	1,62
DUSP1	4,07	**CCNG2**	-2,53	**DUSP1**	1,96	H1F0	1,49	AP2B1	1,60
ELL2	3,21	HIST1H2AC	-2,47	GADD45A	1,95	**EGF**	1,46	ERBB2	1,52
HMGB3	2,96	OAS2	-2,45	OAS2	1,90	ELL2	1,45	NOTCH3	1,50
RPS6KA5	2,81	ERBB3	-2,40	**CCND1**	1,79	CHD1L	1,44	ELL2	1,49
JUN	2,65	KRT6B	-2,40	PLAU	1,75	STAT5A	1,40	AR	1,43
CCND1	2,54	ITGA2	-2,28	TGFA	1,74			H1F0	1,43
SNAI1	2,36	IGFBP5	-2,25	RPS6KA5	1,70			JUN	1,43
EGF	2,25	SCGB2A1	-2,24	TP53BP2	1,69	CDKN2B	-1,48	MUC1	1,43
GRB2	2,21	ERBB2	-2,19	JUN	1,69	TGFB3	-1,46		
IL6ST	2,17	TGFB3	-2,13	CAV1	1,62	IGFBP5	-1,46		
ETV5	2,09	AR	-1,95	ELL2	1,58	KRT17	-1,46	TIMP3	-1,53
TP53BP2	2,07	SP1	-1,92	FN1	1,55	GATA3	-1,46	ETV5	-1,49
XLHSRF1	2,06	RASD2	-1,87	CHD1L	1,53	CDKN2B	-1,45	TIMP3	-1,46
E2F3	2,04	KRT14	-1,86	SAP30	1,50	GATA3	-1,42		
GADD45A	2,02	KRT18	-1,86	HMGB3	1,47				
CCNE2	2,02	ALPP	-1,79	BCAR1	1,46				
SAP30	1,94	KRT19	-1,78	CHD3	1,46				
KPNA3	1,93	FGFR2	-1,77	CDC42BPA	1,45				
RAMP	1,92	SPRR2C	-1,76	GRB2	1,45				
CHD1L	1,91	CACMKIINa	-1,75	XLHSRF1	1,42				
PCAF	1,90	PIK3CA	-1,74	TGFB3	1,42				
CXCL12	1,88	BTN2A2	-1,72	**HSD11B2**	1,42				
NMU	1,82	NOTCH3	-1,72						
TCL6	1,77	H1F0	-1,71						
HSD11B2	1,77	WIG1	-1,71	TIMP3	-2,40				
BIRC5	1,75	KAI1	-1,71	ANTXR1	-2,25				
MCM6	1,75	CDC7L1	-1,70	ETV5	-2,08				
BTG1	1,72	28SrRNA	-1,67	GATA3	-1,79				
AKAP13	1,72	HSPB1	-1,67	**CCNG2**	-1,71				
DCK	1,72	CDC42BPA	-1,65	CDKN2B	-1,69				
TIMP3	1,72	NCOA2	-1,64	KRT18	-1,66				
CDK8	1,71	NFYB	-1,63	IGFBP5	-1,59				
CDC2	1,69	NCOA3	-1,63	CDKL2	-1,54				
CDC14B	1,69	CHD3	-1,62	CENPA	-1,53				
BNIP3	1,68	KRT17	-1,62	NFYB	-1,51				
MAD2L1	1,68	NF1B	-1,61	CDC14B	-1,51				
PRC1	1,67	MUC1	-1,61	FGF7	-1,49				
TGS	1,66	KIAA0349	-1,60	ERBB3	-1,43				
SOS1	1,65	UNG2	-1,60	H2AFJ	-1,42				
CENPA	1,65	CDC2L5	-1,59	FGFR2	-1,40				
BCAR1	1,65	ESR1	-1,59						
SURB7	1,64	LMNA	-1,59						
STAT5A	1,64	H2AFJ	-1,59						
MAP3K3	1,62	CHEK2	-1,58						
CDK9_f	1,61	KRT5	-1,58						
CDC6	1,61	FN1	-1,57						
GTF2F2	1,59	CDKN2B	-1,56						
STRBP	1,59	FGF7	-1,55						
AREG	1,59	CDKN1B	-1,55						
RFC3_d	1,58	CCNF	-1,55						
BRCA2	1,58	POLR2J2	-1,55						
RBBP7	1,58	NCOR2	-1,55						
DDX5	1,58	PLAU	-1,54						
BCCIP	1,56	TP53BP1	-1,54						
CDKN3	1,56	SMARCD2	-1,53						
DKFZp686F2198	1,56	NCOR1	-1,52						
PECI	1,56	CDKL2	-1,52						
chr_6q25_2_26	1,56	S100A6	-1,51						
KPNA4	1,56	KPNB1	-1,50						
SMARCC2	1,55								
EXT1	1,54								
TOP1	1,54								
ANKT	1,54								
DC13	1,53								
CCNB1	1,53								
RPS6KA1	1,52								
FLJ32001	1,51								
SCN9A	1,51								
ANTXR1	1,51								

Figure 3: ***Gene expresión profile of T47D-MMTVL cells in response to R5020.*** *T47D-MMTVL cells were serum-starved and treated with R5020 10 nM for a period of ½, 1, 2 or 6h, when RNA was extracted for microarray hybridization. Data was analyzed by applying the Significance Analyses of Microarrays (SAM) method. Genes showing an statistically significant change in expression over ethanol (etOH) treatment are listed (fold-change ≥1,4, p<0,05). In red, genes showing upregulation and in blue, genes showing downregulation.*

our model cell line, the T47D-MMTVL. For this purpose, we collaborated in the design of a customized human cDNA microarray platform containing more than 800 genes, all of them expressed in human mammary tissue (Miñana B., Sumoy L., Beato M., Jordan A., Ballare C., Melia M.). In this platform there are different interlinked biological pathways represented: cell cycle, DNA repair, DNA damage, apoptosis, chromatin remodeling, BRCA1 and breast cancer-related genes, PR and ER-related genes, among others. T47D-MMTVL cells were plated in medium without phenol red and supplemented with 10% charcolized-serum, and 24 h later medium was replaced by fresh medium without serum. After two days in serum-free conditions, cells were treated with R5020 10 nM for 30 min, 1, 2 and 6 h or ethanol (as reference). Cells were then harvested, and RNA extracted for microarray hybridization. Upon analysis of the data with the SAM method (see Materials and Methods), we obtained a list of genes reproducibly regulated (activated or repressed, fold change ≥1,4, p<0,05) by R5020 in T47D cells (Fig. 3). As expected, the number of regulated (induced and repressed) genes increases with longer hormone treatments, being the 6 h time point a compromise between rapid and long-term effects of the hormone on gene expression.

All these genes are surely regulated by R5020/PR by means of a variety of mechanisms. Some of them have been dissected and described in more detail in the literature (Quiles *et al*., 2009; Richer *et al*., 2002; Tung *et al.,* 2006; Vicent *et al*., 2006). In further experiments, we monitored the expression of genes selected from the data obtained from the microarray analysis (*DUSP1, EGF, 11b-HSD, CCND1, CCNG2, CHD4*) and incorporated the gene *HEF1* (*NEDD9*) as a previously reported PR-A-regulated gene (Richer *et al*., 2002).

Thus, we studied the effect of BRCA1 overexpression on the expression of these selected genes. Since the T47D-MMTVL cell line is particularly difficult to transfect, we used transduction by an adenoviral vector to overexpress BRCA1 (*AdBRCA1*) (see Material and Methods for more details). Fig. 4A shows the level of overexpression obtained in T47D-MMTVL cells using different amounts of the adenovirus, and a one-hour versus overnight infection procedure. For the following experiments we used an overnight infection with 4000 viral particles/cell, which caused a good overexpression of the protein and minimum cytopathic effect. In these cells, we checked the transcriptional activity of the endogenous PR by quantitative Real Time PCR (qRT-PCR), upon overexpression of BRCA1 (Fig. 4B). In accordance with the previous results, the overexpression of BRCA1 inhibited the progestin-induced expression of genes like *DUSP-1*, *EGF* or *11b-HSD* (Fig. 4B). At longer times of induction (6 h), BRCA1 overexpression also inhibits the basal level of expression of some of the genes, like *DUSP-1* and *EGF* (right panel).

Regarding the effect of the overexpression of BRCA1 on those genes repressed by the progestin treatment, BRCA1 decreased more than two-fold the basal expression and the expression in the presence of hormone of genes such as *Cyclin G2* (*CCNG2),* or decreased the basal level to the level

observed upon hormone addition in the case of *CHD4*, indicating that the overexpression of BRCA1 is helping in the inhibition of these genes.
The level of expression of the housekeeping gene *GAPDH* indicates that the BRCA1 overexpression is not having an indiscriminate effect over the transcription of active genes.

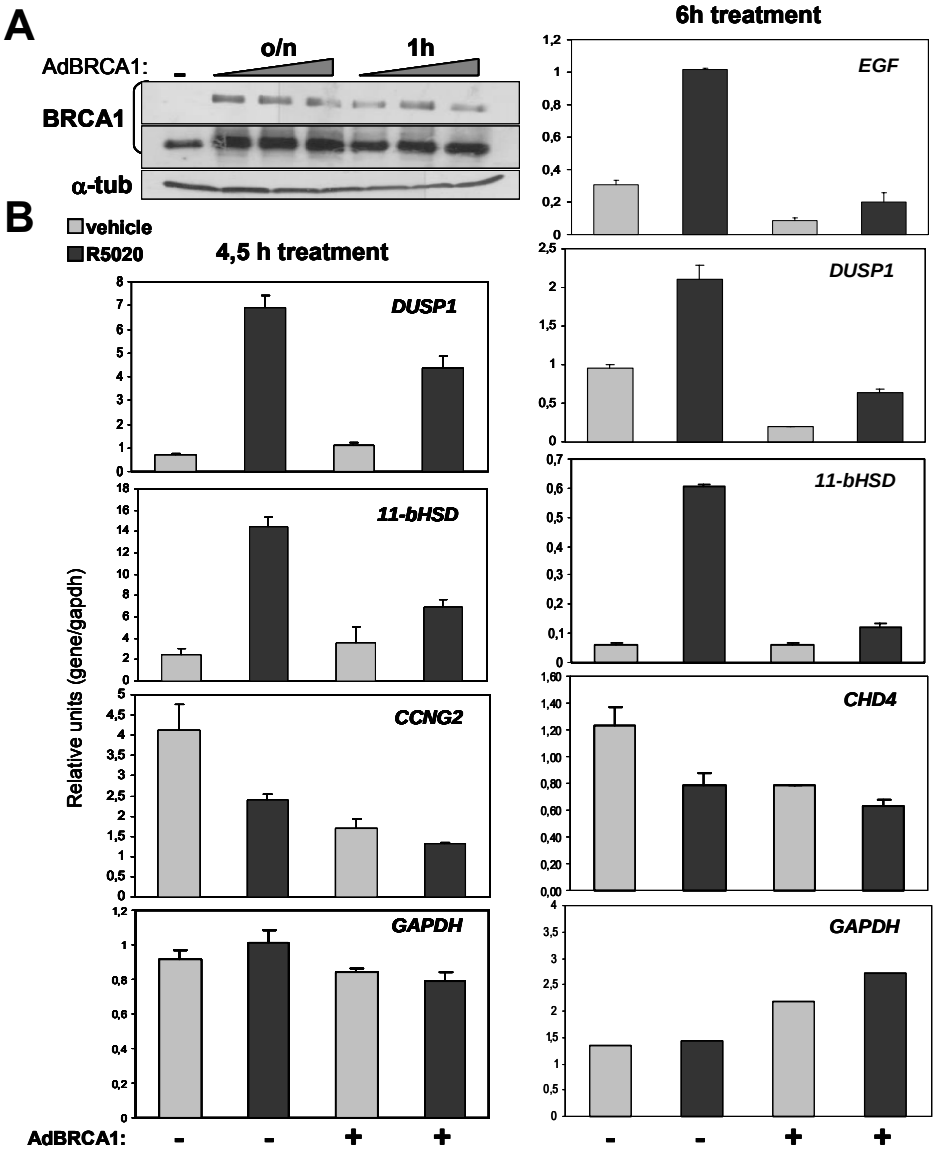
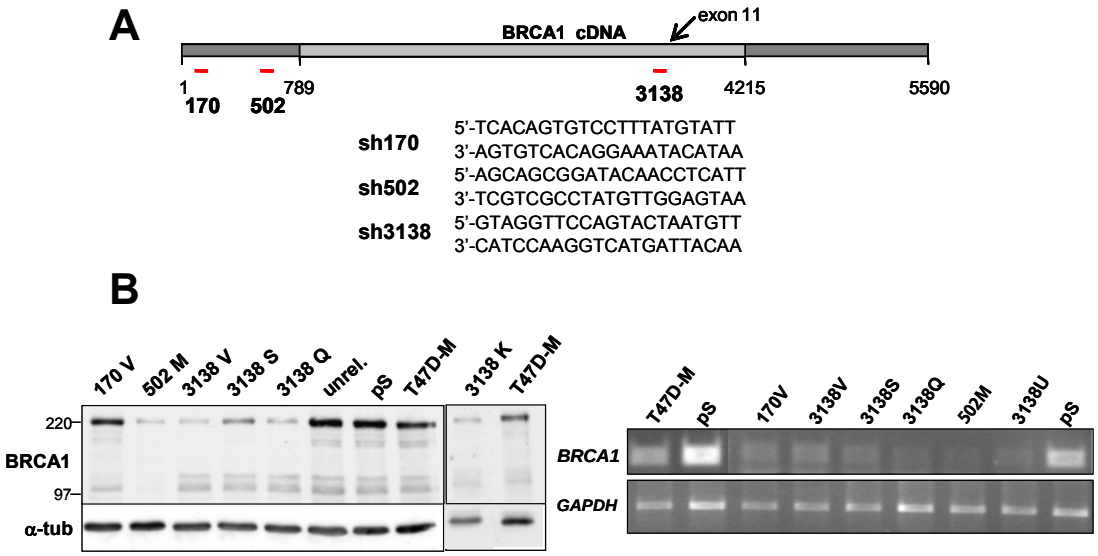


Figure 4: ***Effect of overexpressed BRCA1 on endogenous PR transcriptional activity***
(A) Overexpression of BRCA1 by adenoviral transduction. T47D-MMTVL cells were infected with different amounts of viral particles (vp) per cell (4000, 8000 and 10000), following two procedures, infection overnight or for 1h. After that period, the level of BRCA1 overexpression was assessed by Western blot analysis. Middle pannel shows a longer exposure of the upper membrane. In following experiments, it was used an o/n infection with 4000 vp/cell.

(B) T47D-MMTVL cells were infected with +/- AdBRCA1 at 4000 vp/cell and treated with R5020 (10 nM) for 4,5 and 6 h. Gene expression of selected PR-target genes was assessed by quantitative Real Time PCR (qRT-PCR) in triplicate experiments (± SD). The expression of the housekeeping gene GAPDH is shown as a control.

R.1.2 Knockdown of BRCA1 enhances the transcriptional activity of the progesterone receptor

Another strategy to assess the influence of BRCA1 on the PR transcriptional activity would be to knockdown the expression of BRCA1 instead of overexpressing it. This would resemble more accurately the *in vivo* situation of a BRCA1 mutant carrier. For that purpose, we firstly created four T47D-MMTVL-derived cell lines carrying a constitutively expressed vector-based RNAi system (Brummelkamp *et al*, 2002). We designed two shRNAs, *sh170* and *sh502*, directed against all the BRCA1 isoforms, and the *sh3138* directed against all the isoforms excepting the 97 kDa form that lacks exon 11 (Fig. 5A). Exon 11 is the longest exon of BRCA1 and so it is an obvious target for the design of siRNAs against BRCA1. Although this isoform might remain in the cells, it is believed to be a mutant and inactive form of BRCA1 (McEachern *et al*, 2003, Weaver *et al*, 2002). The T47D-MMTVL cell line expresses all the isoforms of BRCA1 (i.e. the 220 and 97 kDa isoforms, mainly) and we also verified by genomic sequencing that the BRCA1 gene presents no mutations. As control cell lines (*control*) we used a cell line expressing the empty vector (*pSuper*, *pS*) and a cell line expressing an unrelated siRNA (*unrel.*). From the pool of cells containing the shRNA-cassettes, we selected several clones (designated with a letter) by serial dilution, in order to have a more homogeneous population of cells. The levels of BRCA1 protein and mRNA expression in several of the clones are illustrated in Fig. 5B. The level of BRCA1 downregulation in various clones was around or more than 75%. As expected, in the sh3138 clones still remains the 97kDa isoform of BRCA1. For later experiments, we chose one of the best clones for each of the shRNA sequences, sh170v, sh502m and sh3138q.



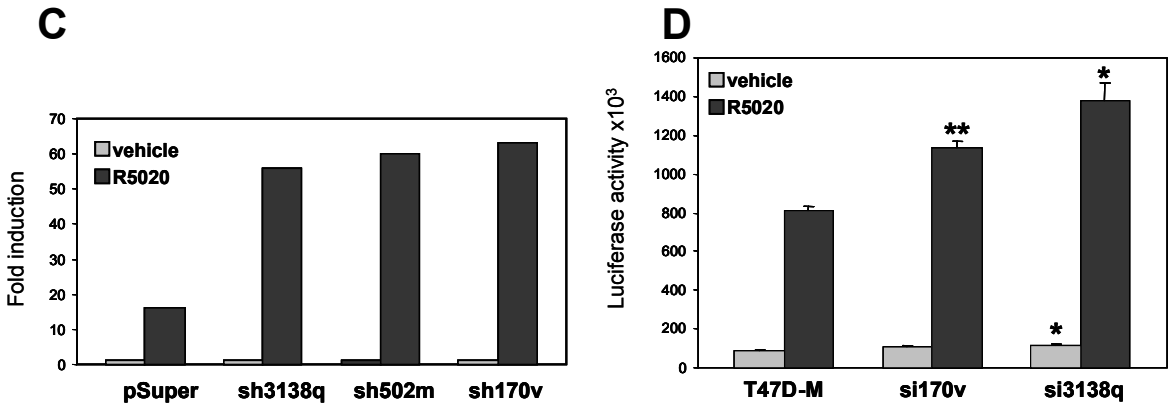
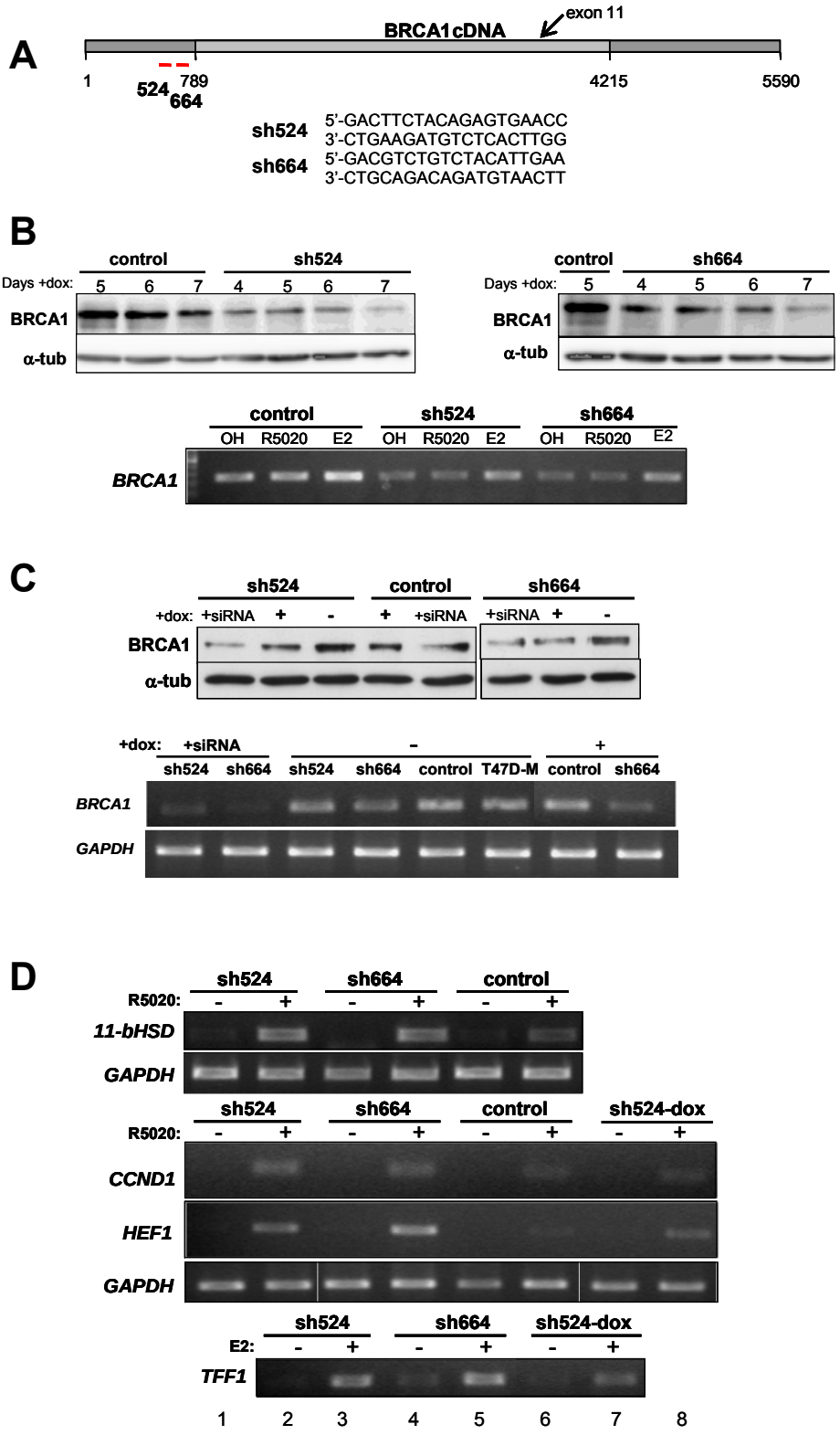


Figure 5: ***Effect of BRCA1 knockdown on endogenous PR transcriptional activity (constitutive RNAi system)***
(A) Sequences and position on the BRCA1 cDNA of the three shRNAs used in the vector-based constitutive RNAi system to knockdown BRCA1 expression.
(B) Western blot and RT-PCR showing the level of BRCA1 downregulation in several clonal cell lines derived from T47D-MMTVL cells. As loading controls, α-tubulin and GAPDH are shown, respectively. Sh170v, sh502m and sh3138q cell lines were chosen for subsequent experiments.
*(C) and (D) Sh170v, sh502m, sh3138q and pS or T47D-MMTVL control cell lines were treated with vehicle (ethanol) or R5020 (10 nM) for 24 h. Cell extracts were collected and luciferase activity derived from the endogenous MMTV-Luc reporter was determined. Values are expressed relative to vehicle (ethanol) as fold induction (C) or are expressed as luciferase arbitrary units ± SD (D). Brackets * indicate statistically significant differences (**, p<0,01 and *, p<0,05, two-tailed t-test).*

Sh170v, sh502m, sh3138q and pS control cells were treated with R5020 (10 nM) or ethanol, and assayed for luciferase activity of the endogenous MMTV-Luc reporter. The result is expressed as fold of induction relative to ethanol treatment in Fig. 5C. The hormone induction of the reporter gene in the three clones assayed was significantly (more than three-fold) higher than the induction in the control cells.

The shRNAs clones were maintained in culture and we observed that along passages we started loosing the silencing and so the effect of the knockdown of BRCA1 on PR activity (although still statistically significative, a representative experiment is shown in Fig. 5D). This led us to change to an inducible RNAi system instead of a constitutively expressed one in order to avoid a possible process of adaptation in the cell to overcome the RNA interference. We designed two shRNAs, sh524 and sh664, against BRCA1 (Fig. 6A) for this new system (described with more detail at Materials and Methods). In this case, the siRNAs are not produced in the cells until they are treated with doxycycline. Fig. 6B shows the downregulation of the BRCA1 protein and mRNA achieved along a maximum of seven days of doxycycline treatment. In order to get even a better silencing we finally adopted the strategy of inducing the endogenous siRNA with doxycycline for six days and transfecting an exogenous siRNA as well. The level of silencing achieved this

way is shown in Fig. 6C. In the last experiments, we have also used a single transfected Stealth siRNA (from Invitrogen, siBRCA1sth) that performed well transfected alone (Fig. 6E).



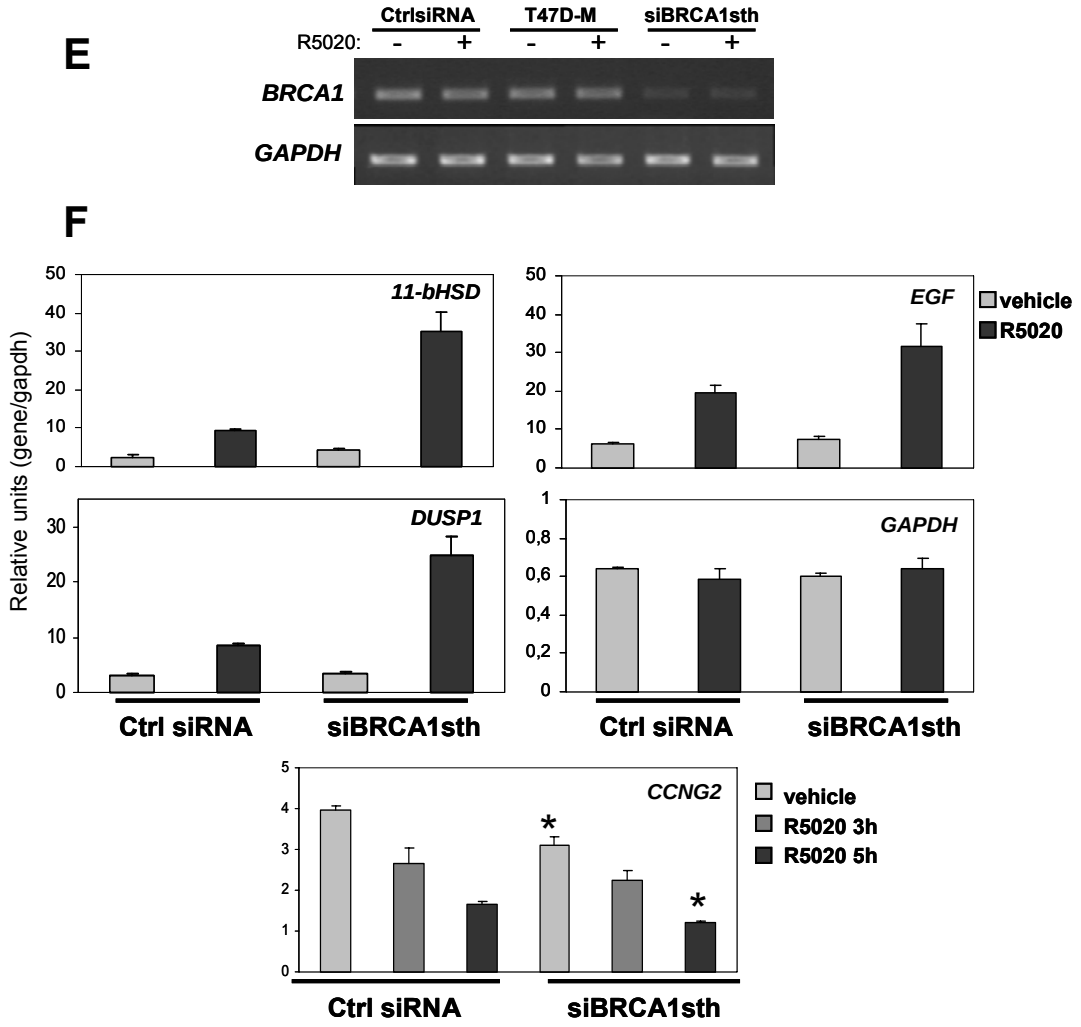


Figure 6: ***Effect of BRCA1 knockdown on endogenous PR transcriptional activity (inducible RNAi system and transfected siRNAsth)***

(A) Sequences and position on the BRCA1 cDNA of the two shRNAs used in the vector-based inducible RNAi system to knockdown BRCA1 expression.

(B) Western blots showing the level of BRCA1 downregulation upon induction of the RNAi system with doxycycline during 4 to 7 days. Below, RT-PCR showing the level of BRCA1 expression in cells induced with doxycycline for 6 days and treated with R5020 or E2 (10 nM) for 6h.

(C) Western blot and RT-PCR showing the level of BRCA1 expression in cells induced with doxycycline for 6 days and transfected with an exogenous siRNA against BRCA1 (100 nM). In the last experiments, we have also used a single transfected Stealth siRNA against BRCA1 (siBRCAsth) and control siRNA (Invitrogen) that performed well by itself (E).

(D) T47D-MMTVL cells carrying the inducible RNAi system were induced for six days with doxycycline and transfected with exogenous siRNA or control siRNA. After 72 hours of transfection, cells were treated with vehicle, R5020 (10 nM) or E2 (10 nM) for 6 h and RNA was extracted. The expression of PR and ERα target genes was checked by RT-PCR. As a control, the gene expression in these cell lines was compared with a control cell line or one of the cells carrying the cassette of the RNAi system without being induced with doxycycline.

*(E) T47D-MMTVL cells were transfected with Stealth siRNA against BRCA1 (siBRCA1sth, Invitrogen) and, after 72 h, they were treated with R5020 (10 nM, 6h or 3 and 5 h). Gene expression was analized by qRT-PCR in triplicate (± SD). Brackets * indicate statistically significant differences (*, p<0,05, two-tailed t-test).*

Cells carrying the inducible RNAi system were treated for six days with doxycycline and were transfected with exogenous siRNA or control siRNA. After 72 hours of transfection, cells were induced with vehicle or hormone for six hours and RNA was extracted. cDNA was generated by RT-PCR and we checked the expression of progestin-induced genes like *11b-HSD*, *cyclinD1 (CCND1)* and *HEF1* by PCR (Fig. 6D). We observed a perceptible increase in the expression of the progestin-regulated genes, and also in the estrogen-regulated gene *pS2 (TFF1)*, in the cells with the BRCA1 knockdown compared to the control cell line and compared with the very same cell line without being induced with doxycycline neither transfected (lanes 7 and 8).

We further corroborated this observation analyzing the induction of some PR-target genes by qRT-PCR in cells transfected with the Stealth siRNA. After six hours of hormone treatment, the expression of hormone-regulated genes (*DUSP-1, EGF, 11b-HSD*) was analyzed (Fig. 6F). We detected 2 to 3 fold overinduction of the genes induced by R5020.

As regards the expression of genes downregulated by the hormone, like *CCNG2*, the knockdown of BRCA1 produced a slight decrease (statistically significant) in the basal expression as well as in the expression after hormone at three and five hours of progestin treatment (lowest panel). In conclusion, the knockdown of BRCA1 increases the transcription of progestin-regulated genes and seems to enhance the inhibition of progestin-repressed genes.

R. 2 Mechanism by which BRCA1 affects PR transcriptional activity

R.2.1 BRCA1 physically interacts with the progesterone receptor

Given that BRCA1 interacts directly with two other steroid hormone receptors (i.e. ERα and androgen receptor (AR) (Park *et al*, 2000)), we wanted to determine whether BRCA1 could also interact with the progesterone receptor.

Initially, we used a GAL4 transcription activator yeast two-hybrid system (Matchmaker Gal4 Two-hybrid System 3; Clontech) to investigate the interaction. In this system, the yeast GAL4 transcription activator has been divided into the following two separate functional domains: the transcription activation domain (AD) present on plasmid pGADT7 (pAD), which encodes for the *LEU2* gene as a selectable marker; and the DNA binding domain (BD) present on the plasmid pGBKT7 (pBD), which encodes for the *TRP1* gene as a selectable marker. A bait protein is expressed as a fusion to the BD and another protein is expressed as a prey annexed to the AD. If the two fusion proteins interact in this system, they will bring in close proximity the GAL4-AD and the GAL4-BD to the GAL4/GAL1 promoters, which in turn will initiate the transcription of the *HIS3* and *ADE2* reporter genes. Protein-protein interactions are then detected by the ability of co-transformed yeast cells to grow in selective medium lacking Leu, Trp, His and Ade (SD-Leu,-Trp,-His,-Ade).

The sequences encoding BRCA1 and PR-B were cloned into the pBD and pAD plasmids and they were co-transformed into the yeast reporter strain. Owing to the inherent capability of both pBD-wtBRCA1 and pBD-PR to transactivate the reporter system by themselves (which is what usually occurs with transcription factors), we were obliged to use the pAD-PR plasmid in combination with transcriptionally inactive forms of BRCA1 fused to the pBD plasmid in order to detect only the yeast growth due to protein interaction. These “transcriptionally inactive” forms of BRCA1 consist in mutant forms of BRCA1, found in BRCA1-related breast tumours, which present point mutations at the transactivating domain (in C-terminus) that truncate or inactivate its transcriptional activity (Lu *et al*, 2000). We used four different mutants (pBD-Gln1756insC 5382, Tyr1853insA 5677, Pro1749Arg 5365, Ala1708Glu 5242), whose expression was confirmed by Western blot (Fig.7A). From these four, the Ala1708Glu 5242 and the Pro1749Arg 5365 were still able to transactivate the yeast system by themselves. The reason for this must be that the Ala1708Glu and the Pro1749Arg mutations are located outside the minimal TAD region and so partial transcriptional activity is retained (see Introduction).

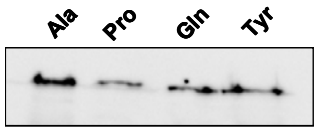
The other two constructs were used for the co-transformation with the AD-PR and AD-ER, and we also checked the interaction between PR and ER, previously described, as a positive control (Ballare *et al*, 2003). We assayed the interaction in selective medium lacking Leu, Trp, His and Ade, and +/- R5020 or E2. Table 1 summarizes the results obtained with all the combination of plasmids in three experiments. Yeast co-transformed with

plasmids encoding ER in combination with the two mutant BRCA1 forms grew in selective medium with or without E2 indicating that we were detecting the already described interaction BRCA1-ER. In contrast, yeast co-transformed with plasmids encoding the mutant forms of BRCA1 in combination with PR showed very faint growth in selective medium.
In some cases, the pBD or AD fusion moiety may occlude the normal site of interaction, or may impair the proper folding of the hybrid protein, and thus interfere with the ability of the proteins to interact (van Aelst *et al*, 1993). We can neither rule out the possibility that the point mutations may affect the conformation or even the interacting faces of the BRCA1 protein disturbing the interaction between BRCA1 and PR, and even between BRCA1 and ER since the interaction detected in terms of growth was very weak.

Therefore, we continued the study of the interaction between BRCA1 and PR in mammalian cells by using *in vivo* co-immunoprecipitation.
T47D-MMTVL cells growing in white medium + 10% CSS were replaced in white medium with 0% serum for 24 h before preparation of nuclear extract. Nuclear extract was immunoprecipitated against BRCA1 (since BRCA1 is much less abundant than PR in these cells) or using a mouse monoclonal control antibody. As shown in Fig. 7C, the BRCA1 antibody co-precipitated BRCA1 along with both PR-B (130 kDa) and PR-A (85 kDa) and also ERα, while the control antibody precipitated neither BRCA1 nor any PR or ER. This was observed when using a mild washing buffer (120 mM NaCl and 0,1% NP-40 concentration) and not when using a disruptive washing buffer (420 mM NaCl concentration).

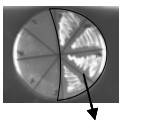
A

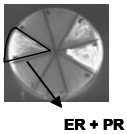
TAD-inactivating BRCA1 point mutations:
Ala1708Glu 5242
Pro1749Arg 5365
Gln1756insC 5382
Tyr1853insA 5677



1

***BD*-bait + *AD*-prey**	-	+ E2	+ R5020
ER + PR		✓	
BRCA1wt	✓	✓	✓
Tyr1853insA 5677			✓
Tyr1853insA 5677 + PR			
Tyr1853insA 5677 + ER	✓		
Gln1756insC 5382	✓	✓	✓
Gln1756insC 5382 + PR		✓	
Gln1756insC 5382 + ER		✓	
Ala1708Glu 5242	✓	✓	✓
Ala1708Glu 5242 + PR	✓	✓	✓
Pro1749Arg 5365	✓	✓	✓
Pro1749Arg 5365 + PR	✓	✓	✓





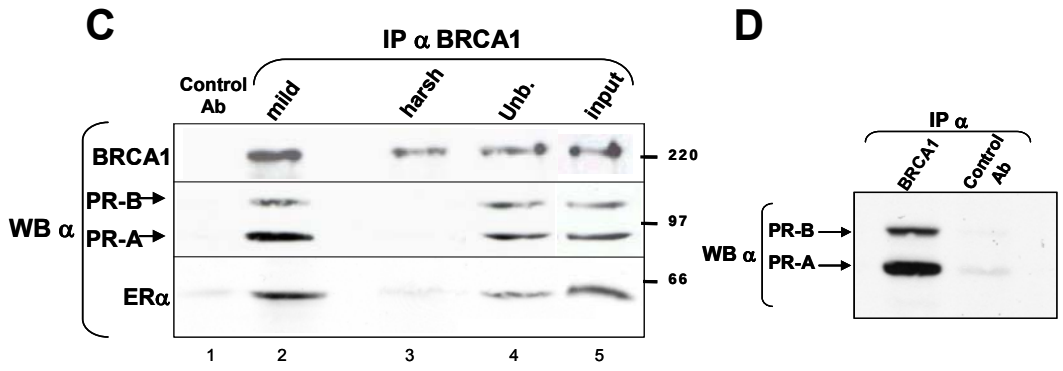


Figure 7 and Table 1: ***BRCA1 physically interacts with endogenous PR in human breast cancer cell lines***

(A) Expression in yeast of the TAD-inactivating point mutants of BRCA1 used as baits in the yeast two-hybrid system experiments (Matchmaker Gal4 Two-hybrid System 3, Clontech).

(Table 1) Summary table of the results obtained in triplicate yeast two-hybrid experiments. The first column shows the combination of BD-bait and AD-prey proteins tested. The signs √ represent growth in selective medium and its size accounts for the intensity of growth.

(C) In vivo co-immunoprecipitation of BRCA1 and PR and ERα in breast cancer cell lines. Nuclear extracts were prepared from serum-starved T47D-MMTVL cells and immunoprecipitated with an antibody against BRCA1. Co-immunoprecipitation was detected using a mild washing buffer (120 mM NaCl concentration) and not with harsh conditions (420 mM NaCl concentration), by Western blot analysis.

(D) Nuclear extracts were prepared from R5020-treated (10 nM, for 15h) T47D-MMTVL cells and immunoprecipitated using an antibody against BRCA1. Co-immunoprecipitation of PR was detected by Western blot analysis.

To examine if the interaction detected in untreated cells was maintained in the presence of hormone, T47D-MMTVL cells growing in white medium and 10% CSS were replaced with white medium without serum + R5020 10nM for 15 h prior harvesting for preparing nuclear extract. As shown in Fig. 7D, BRCA1 is still able to interact and co-immunoprecipitate with the two isoforms of PR.

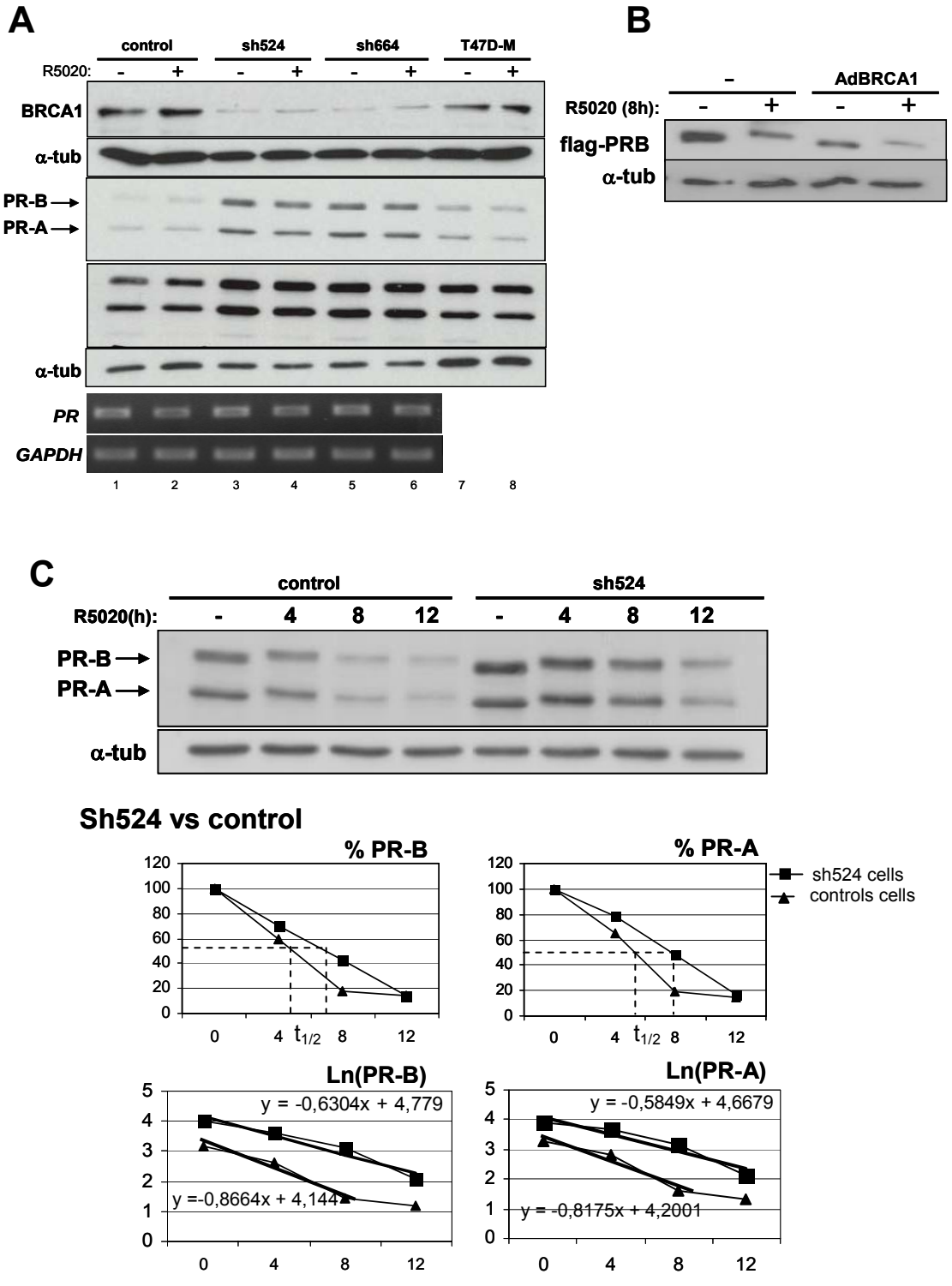
In conclusion, BRCA1 interacts *in vivo* in breast cancer cells with the two isoforms of PR in a ligand-independent manner.

R.2.2 BRCA1 regulates the degradation of the progesterone receptor by the ubiquitin-proteasome system

R.2.2.1 BRCA1 affects the level of PR protein and its rate of degradation upon hormone addition while not affecting mRNA synthesis

We checked in the shRNA-expressing cell lines the level of expression of the progesterone receptor. We observed that there was a noteworthy increase in the amount of the two isoforms of PR correlating with the knockdown of BRCA1 (Fig. 8A). This increase in the protein level was not due to a concomitant increase of PR mRNA synthesis (Fig. 8A lowest panel).

Then, we checked the effect of the overexpression of BRCA1 on PR protein level (Fig. 8B). In this experiment, we used a derivative cell line from T47D-YV (a PR-negative clonal derivative cell line from T-47D, Sartorius *et al*, 1994) engineered to express a flag-tagged form of PR-B protein (T47D-YV-flagPRB, Quiles *et al*., 2009). According to their observations, this cell line behaves similar to T47Dwt cells. In concordance with the previous data, the overexpression of BRCA1 downregulated the level of PR protein, in presence and absence of hormone.



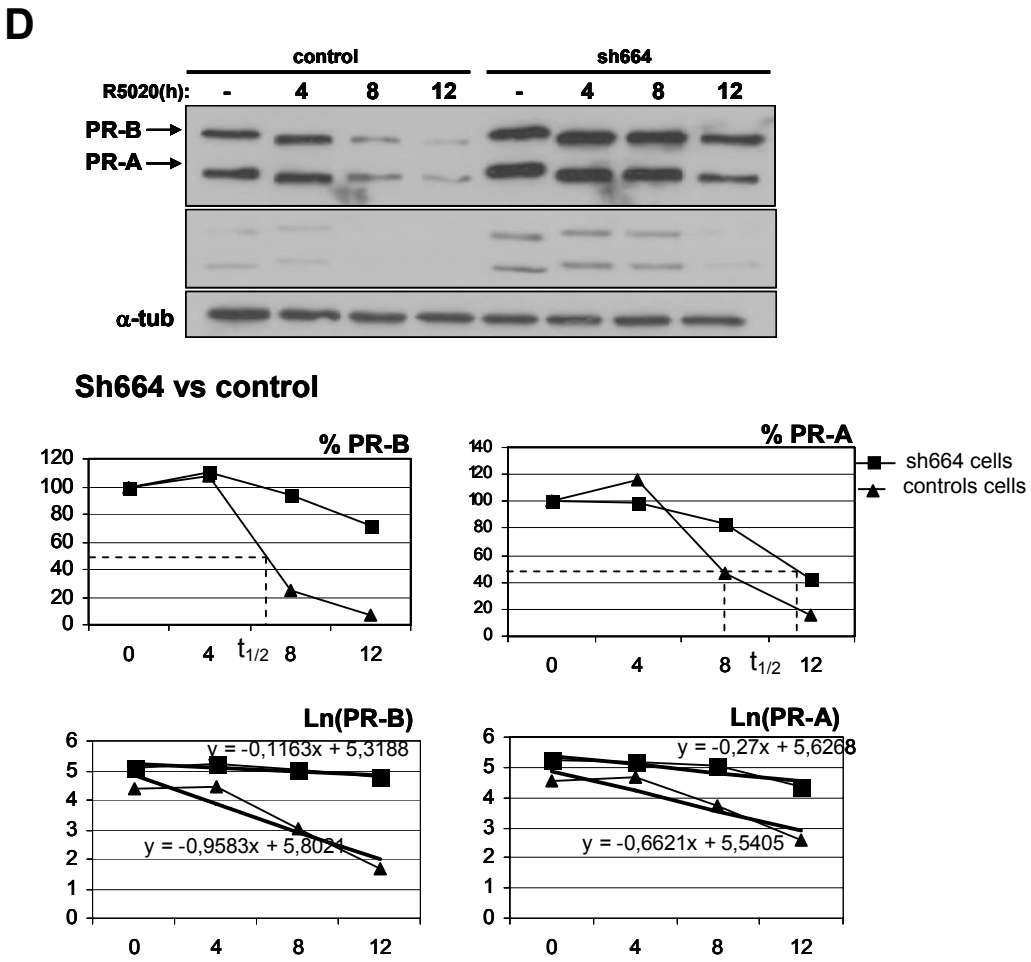


Figure 8: ***BRCA1 affects PR protein level and its rate of degradation through a mechanism independent of mRNA synthesis control***
(A) Western blot showing the level of PR protein expression in the shRNA-expressing cell lines compared to control cell lines. Lower panel, RT-PCR showing the level of PR mRNA expression in the shRNA-expressing cell lines.
(B) T47D-YV-flagPRB cells were infected with +/- AdBRCA1 to overexpress BRCA1. Cell extracts were prepared and the level of PR protein expression was checked by Western blot.
(C) and (D) Control and shRNA-expressing cell lines were treated with R5020 (10 nM) for 0 to 12 h. PR protein level was detected at the different treatment time points by immunoblot and quantified by densitometry. Graphs a) represent the percentage of remaining PR-B and -A protein, taking the time 0 as the 100% level. Graphs b) represent the values of the densitometry as Ln(PR). A trendline was extrapoled from the data and its formula is shown beside each line.

PR protein level is maximally regulated upon hormone binding, when it is extensively and rapidly downregulated (Nardulli *et al*, 1988). We wanted to study if the rate of protein degradation upon hormone addition was also affected by the lack of BRCA1. Control and shRNA-expressing cell lines were treated with progestin 10 nM for 0 to 12 hours. PR protein level was detected at different time points by immunoblot and quantified by densitometry (Fig. 8C for sh524 and Fig.8D for sh664 cell line).

Again, at the 0 time point of progestin treatment, we observe a higher level of PR protein in the two BRCA1-knockdown cell lines. Another observation is that, still in the absence or with very low levels of functional BRCA1 protein, progestin-induced PR degradation is taking place in the shRNA-expressing cell lines. The upper graphs in Fig.8C and 8D (representing the percentage of remaining PR-B and A (compared to time 0)) show that, in addition to starting with a higher level of receptor, the half-life of both PR-B and PR-A (indicated as $t_{1/2}$) is higher in the BRCA1-knockdown cells than in control cell lines (in sh524 cells 7h for PR-B versus 4,5 h in control cells, 8h for PR-A vs 5h in control cells; in sh664 cells >12h for PR-B vs 6,5h in control cells and 11,5h for PR-A vs 8h in control cells).
An approximate protein degradation rate can be determined from the slope of the lower graphs in Fig. 8C and 8D (representing the Ln(band intensity) (corresponding to PR immunoblotting) versus time). We observe that the slope smoothes out for the two isoforms of PR in the two BRCA1-knockdown cell lines, more significantly at the sh664 cell line (in the sh524 cell line, -0,63 versus -0,87 for PR-B and -0,58 versus -0,82 for PR-A; in the sh664 cell line, -0,11 versus -0,95 for PR-B and -0,27 versus -0,66 for PR-A). In all, this would indicate that BRCA1 is affecting the basal level of PR content in the cell and also the progestin-induced degradation rate of the receptor, even though degradation is still occurring in the absence of BRCA1.

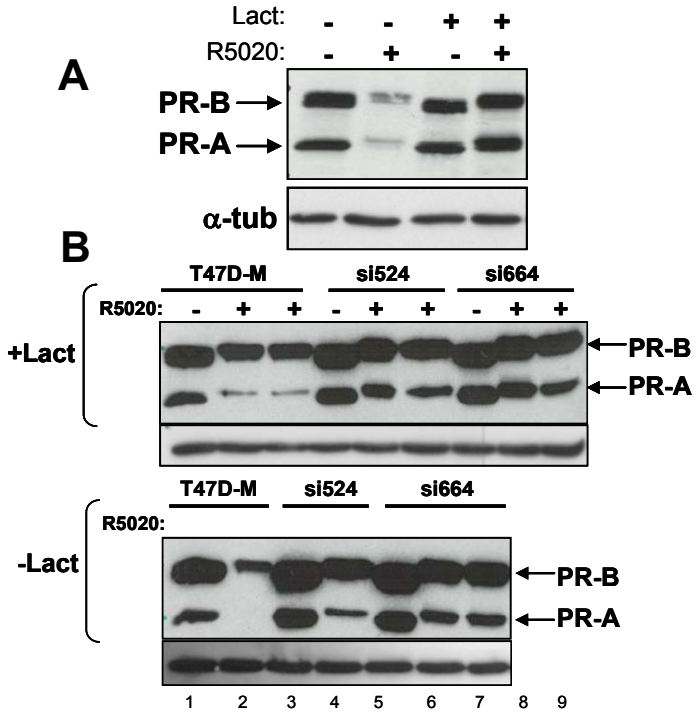


Figure 9: ***PR degradation is mediated by the 26S proteasome***
(A) T47D-MMTVL cells were pre-treated for 4 h with +/- lactacystin (inhibitor of the 26S proteasome, 10 uM) and treated +/- R5020 (10 nM) for 22 h. Cell extracts were prepared and PR protein level was detected by Western blot analysis.
(B) T47D-MMTVL and shRNA-expressing cell lines were pre-treated for 4 h with +/- lactacystin (10 uM) and treated +/- R5020 (10 nM) for 18 h. Cell extracts were prepared and PR protein level was analyzed by Western blot.

Since the basal level and the half-life of PR were increased in the absence of BRCA1 and it was independent of mRNA changes, the effect of BRCA1 on PR stability could be occurring at the post-transcriptional level. Ligand-independent and dependent degradation of PR is mediated by the 26S proteasome (Lange *et al*, 2000; Shen *et al*, 2001). Fig.9A demonstrates how the hormone-induced degradation of the receptor can be blocked by the treatment with lactacystin, a specific inhibitor of the 26S proteasome. The increased apparent molecular weight of lactacystin-stabilized, R5020-occupied PR reflects increased phophorylation at multiple serine residues, related to the activation of the receptor (see Introduction).

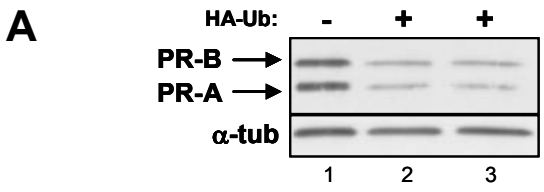
Fig.9B shows the same treatment with the proteasome inhibitor in the control and the BRCA1-knockdown cell lines. In this particular experiment, the treatment with the inhibitor was not as effective as shown in the previous one, but still one can see the accumulation of receptor protein after progestin-treatment in the control cell lines (lane 2 of lower panel compared to lanes 2 and 3 of upper panel). In the case of the BRCA1-knockdown cell lines, it is very evident the increase in the amount of PR protein at the basal and after hormone addition compared to the control cell lines, although still upon treatment with the inhibitor lactacystin it can be noted an increase in the amount of the receptor in cells treated with hormone (lanes 4, 6 and 7 compared to lanes 5 and 6, and 8 and 9). This would indicate that other machines involved in the hormone-driven degradation of the receptor are functional in the BRCA1-defective cell lines.

In conclusion, BRCA1 is involved in the basal progesterone receptor degradation process and also participates in the progestin-induced degradation of the receptor, in which other enzymes (likely, CUEDC2) must also be responsible for the hormone-triggered degradation and are functional in the absence of BRCA1.

R.2.2.2 BRCA1 promotes the ubiquitination of PR *in vivo* and *in vitro*

Although ubiquitin-independent degradation of substrates by the 26S proteasome is known (see Introduction), most proteins are targeted for degradation through the proteasome by ligation to 76-amino acid ubiquitin molecules, assembled as polyubiquitin chains.

The N-terminal 110 amino acid residues of BRCA1, which encode a stable domain containing the RING fingers, displays E3 ubiquitin-ligase activity that is enhanced by the heterodimerization with BARD1 (Wu *et al*, 1996; Hashizume *et al*, 2001; Xia *et al*, 2002).



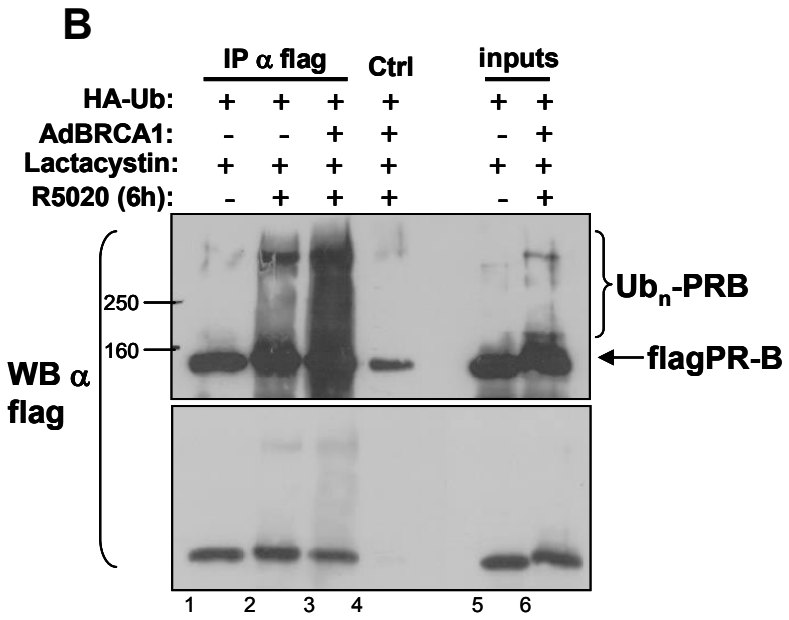


Figure 10: ***BRCA1 enhances the ubiquitination of PR-B in vivo***
(A) T47D-MMTVL cells were transfected +/- a plasmid encoding HA-ubiquitin. Western blot was performed to detect the level of PR protein expression.
(B) T47D-YV-flagPRB cells were transfected and infected to overexpress +/- HA-ubiquitin and BRCA1. They were pre-treated with lactacystin (10 uM) for 1 h and treated with R5020 (10 nM) for 6 h. Nuclear extracts were prepared and immunoprecipitated against flag-tagged PR-B or control antibody, in order to detect ubiquitinated forms of PR-B by Western blot analysis. Lower panel shows a less exposed image of the upper panel.

To determine if BRCA1 might be promoting PR degradation as a consequence of PR ubiquitination we performed an *in vivo* ubiquitination assay. Polyubiquitinated proteins are often difficult to visualize. The proportion of PR that exists as ubiquitin conjugates at a given time point may be very low because the receptor has a half-life of several hours. To visualize ubiquitin-conjugated PR, cells were transfected and infected to overexpress HA-ubiquitin and AdBRCA1. Afterwards, they were pretreated with the proteasome inhibitor lactacystin for one hour and induced with hormone for six hours. Nuclear extracts were prepared cells to perform an immunoprecipitation against flag-tagged PR-B (anti-flag antibody). In this assay we used the derivative cell line T47D-YV-flagPRB in order to facilitate the immunoprecipitation. The overexpression of ubiquitin and the treatment with the proteasome inhibitor and hormone should favour the accumulation of ubiquitinated forms of PR, facilitating the detection by Western blot after immunoprecipitation. One prior observation is that the overexpression of HA-ubiquitin already increases the turnover of PR (Fig. 10A). Overexpression of ubiquitin and the treatment with lactacystin and hormone produced a pronounced accumulation of high molecular weight forms of PR, Ub_n-PR (Fig. 10B, lanes 1 and 2), further increased when BRCA1 is overexpress (lane 3), meaning that BRCA1 induces the ubiquitination of PR *in vivo*.

To test if the E3 ubiquitin-ligase activity of BRCA1-BARD1 heterodimer was directly responsible for the ubiquitination of PR, we performed an *in vitro* ubiquitination assay. PR-B, BRCA1 and BARD1 (forming a heterodimer) were generated and purified from sf9 cells infected with corresponding

baculoviruses. In the presence of ATP, ubiquitin, E1 ubiquitin-activating enzyme and E2 ubiquitin-conjugating enzyme (UbcH5c) in the reaction mix, PR-B was ubiquitinated by the wild type heterodimer BRCA1/BARD1 (Fig. 11A lane 2 and 11C lane 4). As expected, wtBRCA1 is also autoubiquitinated in vitro (Fig. 11B) (Chen *et al*, 2002). Due to its already high molecular weight (220 kDa), the ubiquitinated forms of BRCA1 with the highest molecular weights do not enter the gel and are difficult to visualize.

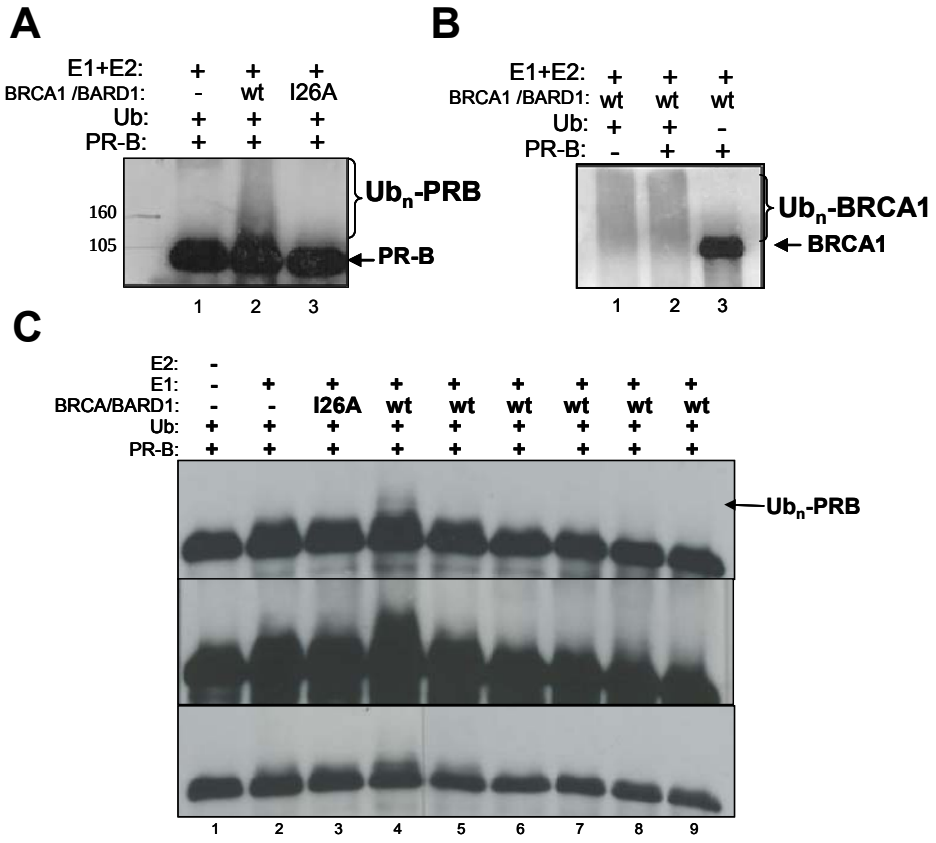


Figure 11: ***BRCA1 directly ubiquitinates PR-B in vitro***

(A) A reaction mix containing enzymes E1, E2 and BRCA1/BARD1wt or I26A mutant heterodimer, ubiquitin and PR-B was incubated, in the presence of ATP, at 37ºC for 1 h. Afterwards, it was run and immunoblotted to detect high molecular weight forms of PR-B.

(B) A reaction mix containing enzymes E1, E2 and BRCA1/BARD1wt and ubiquitin and PR-B was incubated, in the presence of ATP, at 37ºC for 1h. The reaction mix was then run and immunoblotted against BRCA1 to detect high molecular weight forms of BRCA1 due to autoubiquitination.

(C) A reaction mix containing a variety of E2 enzymes (UbcH5c, UbcH6, UbcH10, UbcH1, UbcH8 and UbcH13), E1 and BRCA1/BARD1wt or I26A mutant, ubiquitin and PR-B, was incubated at 37ºC for 1 h. The reaction mix was then run and immunoblotted against PR-B in order to detect high molecular weight forms of PR-B. A more and less exposed image from the same blot are shown.

To exclude the possibility that ubiquitination is due to other contaminating ubiquitin ligases present in the purified proteins, we also included the I26ABRCA1 mutant as a control. The mutation of Ile26 to Ala in the BRCA1 Ring domain does not alter the tertiary structure of the BRCA1 Ring domain but specifically disrupts the contact site of the BRCA1 Ring domain with E2 ubiquitin-conjugase (Brzovic *et al,* 2003). Therefore, the I26ABRCA1 mutant still interacts with its Ring domain-binding partner BARD1 but specifically loses its E3 ligase activity. As shown in lanes 3 of Fig. 11A and 11C, the I26A mutant does not ubiquitinate PR-B in vitro, suggesting that the ubiquitination of PR-B observed in these reactions is specifically dependent on the E3 ligase activity of BRCA1.

Recently, six E2 ubiquitin-conjugating enzymes other than UbcH5c were found to interact and be active with the BRCA1-BARD1 heterodimer (Christensen *et al*, 2007). We tested if any specific conjugating enzyme preferentially drove the ubiquitination of PR. We tested the performance of six different E2 enzymes, -UbcH5c, UbcH6, UbcH10, UbcH1, UbcH8 and UbcH13-, in an *in vitro* ubiquitination assay of PR (Fig. 11C). Of all the E2 ubiquitin-conjugating enzymes tested, only the UbcH5c showed activity towards the ubiquitination of PR by BRCA1-BARD1 (lane 4).

R.2.2.3 Determination of BRCA1-induced PR-B ubiquitination sites

The mechanism and regulation of PR degradation, though extensively studied, is a complex process not yet well understood. In this process, phosphorylation (Lange *et al*, 2000; Shen *et al*, 2001), sumoylation (Abdel-Hafiz *et al*, 2002; Chauchereau *et al*, 2003; Man *et al*, 2006) and ubiquitination (Zhang et al, 2007) take place in the same residues, in a cooperative (phosphorylation and ubiquitination) or competitive way (sumoylation and ubiquitination) (Daniel *et al*, 2007). Among the residues that might be important in the turnover of PR there are some residues that have been characterized in more detail (Fig. 12A). According to the literature, it is likely that more than one residue and region might be important for the turnover of PR, at different levels. Lys388 has been described as the primary site of SUMO attachment, apart from Lys7 and Lys531. This site has also been found to be ubiquitinated and, therefore, important for the degradation of PR. It was speculated the existence of some kind of “destruction box” including this residue. Adjacent to this region, Ser400, a basal phosphorylation site for CDK2 (Pierson-Mullany and Lange, 2004) has been proposed to be a regulator of this “destruction box”, for the degradation of immature or unliganded receptor. However, other point-mutation experiments done over PR sequence have revealed the importance of other residues for the degradation of PR. Importantly, Ser294 phosphorylation seems to be a key regulator of ligand-dependent PR degradation. In the presence of ligand, Ser294 phosphorylation by MAPK leads to rapid PR degradation by the ubiquitin-proteasome pathway. This residue is also supposed to be located within a “destruction box” motif and affect the ubiquitination of neighbouring Lys388, though this is just speculative so far.

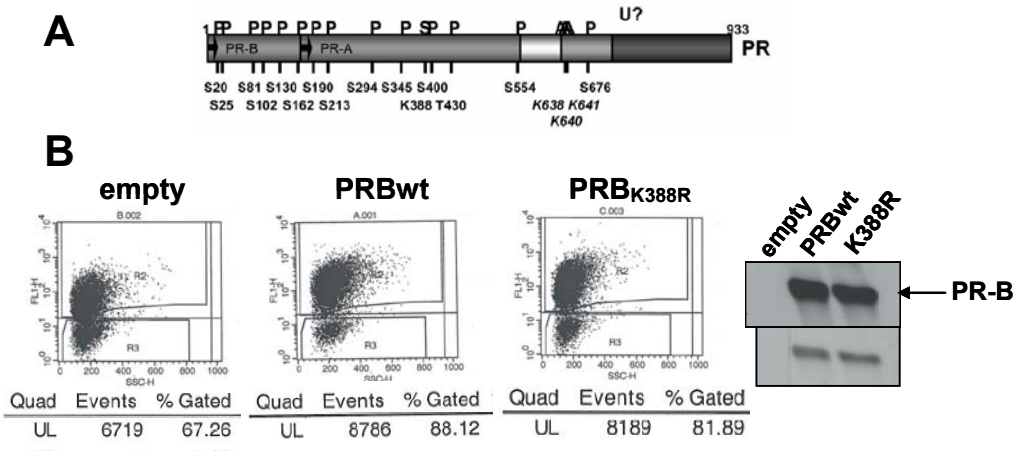


Figure 12: ***Study of the BRCA1-induced PR-B ubiquitination sites: K388***
(A) Schematic drawing of the PR protein indicating the residues described, so far, to be important for the regulation of PR turnover.
(B) Creation of a T47D-YV-derived cell line carrying the expression of the mutant K388R flag-tagged PR-B isoform. Cells stably expressing the mutant plasmid, an empty plasmid or the PR-B wt plasmid were sorted by FACS for the establishment of homogenous cell lines. Right panel: Western blot showing the level of PR-B expression obtained in the three cell lines.

In order to investigate if BRCA1 induces PR degradation through the modification of Lys388, we created a T47D-YV-derived cell line carrying the expression of a mutant K388R flag-tagged PR-B isoform. Fig. 12B shows the expression level of PR-B in the cell lines infected to stably express an empty plasmid, wtPR-B and PR-BK388R mutant, and how these two last cell lines express comparable levels of the receptor.
In future experiments, we want to address if the overexpression of BRCA1 can still cause the degradation of the mutant form PR-BK388R.

Experiments carried out to study the activity of the activation function-3 domain (AF-3, aa1-164, only present in isoform B) in PR-B, revealed the importance of an active AF-3 domain for the ligand-induced degradation of PR-B, pointing to the probable existence of alternate sites of ubiquitin attachment within or close by the N-terminus (Tung *et al*, 2006).
A PEST sequence is a peptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T). This sequence is associated with proteins that have a short half-life. It is hypothesized that PEST sequences act as signals for protein degradation driven by the proteasome or calpain (Rogers *et al*, 1986). An in silico PESTfind analysis (PESTfind Analysis webtool EMBnet Austria) showed the existence of two PEST-like sequences within the first 164 aa of PR-B, encompassing the AF-3 domain and another one in the region of aa 546 to 565, although the best scored sequence was the one between aa 211-238 (Fig. 13A). In order to test if this last region could be important for the BRCA1-driven degradation of PR, we overexpressed BRCA1wt in a T47D-YV derived cell line T47D-YV-PRΔERIDI (Quiles *et al*, 2009) that expresses a form of PR-B lacking aa165-345, and so it lacks the best scored PEST sequence (aa211-238) (Fig. 13A). As shown in Fig. 13B, the overexpression of BRCA1wt still induced the degradation of the

PRΔ165-354, indicating that that missing region of PR does not contain any indispensable residue for the degradation of PR driven by BRCA1.

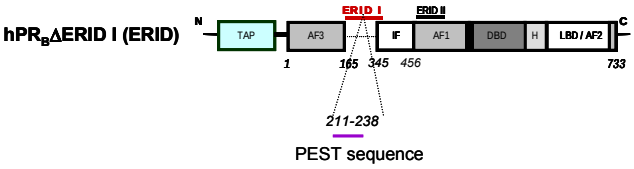
A

```
MTELKAKGPRAPHVAGGPPSPEVGSPLLCRPAAGPFPGSQTSDTLPEVSA 50
IPISLDGLLFPRPCQGQDPSDEKTQDQQSLSDVEGAYSRAEATRGAGGSS 100
SSPPEKDSGLLDSVLDTLLAPSGPGQSQPSPPACEVTSSWCLFGPELPED 150
PPAAPATQRVLSPLMSRSGCKVGDSSGTAAAHKVLPRGLSPARQLLLPAS 200
ESPHWSGAPVKPSPQAAAVEVEEEDGSESEESAGPLLKGKPRALGGAAAG 250
GGAAAVPPGAAAGGVALVPKEDSRFSAPRVALVEQDAPMAPGRSPLATTV 300
MDFIHVPILPLNHALLAARTRQLLEDESYDGGAGAASAFAPPRSSPCASS 350
TPVAVGDFPDCAYPPDAEPKDDAYPLYSDFQPPALKIKEEEEGAEASARS 400
PRSYLVAGANPAAFPDFPLGPPPPLPPRATPSRPGEAAVTAAPASASVSS 450....
```

++++++ potential PEST sequence
------ poor PEST sequence

POTENTIAL PEST SEQUENCES:
211 KPSPQAAAVEVEEEDGSESEESAGPLLK 238
PESTfind score : +10.65
mole fraction of PEDST : 51.02
hydrophobicity index : 34.81

B



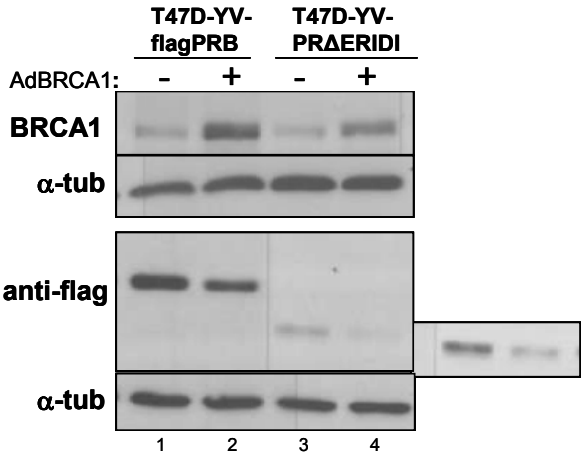


Figure 13: ***Study of the BRCA1-induced PR-B ubiquitination sites: PEST sequences***
(A) PESTfind analysis results over the PR protein sequence, from the EMBnet node Austria web-service. A PESTfind score above +4 indicates a highly potential PEST sequence. At the right, sequence of the best scored PEST sequence found.
(B) Schematic representation of the domains and sequences present and lacking at the flag-PRΔERIDI form. T47D-YV-flagPRB and T47D-YV-PRΔERIDI cells were infected to overexpress BRCA1. Cell extracts were collected and the expression of BRCA1 (upper panel) and flag-PRwt and flag-PRΔERIDI were analyzed by Western blot. The small panel at the right shows a longer exposure of the lanes corresponding to flag-PRΔERIDI expression.

In conclusion, the *in vitro* and *in vivo* data suggest that BRCA1 can directly polyubiquitinate PR, likely through UbcH5c ubiquitin-conjugating enzyme, and in a lysine residue located outside the region encompassing aa 165-345.

R.2.2.4 BRCA1 inhibitory effect over PR transcriptional activity is dependent on its E3 ubiquitin ligase activity

Subsequently, we addressed the question if the E3 ubiquitin ligase activity of BRCA1 was required for the inhibitory effect of BRCA1 over the transcriptional activity of PR. As a first approach, we transfected 293T cells with plasmids for MMTV-Luc reporter, PR-B and a plasmid encoding the wtBRCA1 or the mutant I26ABRCA1, lacking its ability as an E3 ubiquitin ligase enzyme. The cells were treated with hormone for 24 hours, when extracts were collected and luciferase activity determined. Graph in Fig. 14A shows how the cotrans-fection of wtBRCA1 significantly inhibits both the basal and hormone-induced transcription of the reporter, while the co-transfection of the mutant I26ABRCA1 is unable to produce such an inhibition.

We further confirmed these results in T47D cell lines, expressing endogenous PR and on endogenous target genes. We overexpressed wtBRCA1 by infection with adenovirus and I26ABRCA1 by transfection of the corresponding plasmid. Cells were then starved and treated with R5020 (10 nM) for 6 hours and extracts were collected for RNA extraction. Expression of PR-target genes was detected by qRT-PCR. As can be seen in Fig. 14B, the overexpression of the wild type form of BRCA1 caused the total inhibition of progestin-induced expression of the genes tested, and in the case of *DUSP1* and *EGF* genes the basal level of expression was also inhibited to a great extent. However, the overexpression of the mutant I26ABRCA1 did not cause any change in the expression of these genes. This would indicate that the lack of the E3 ubiquitin ligase activity disable BRCA1 for the inhibition of PR transcriptional activity. However, we cannot exclude the possibility that this differences are due to different levels of overexpression of the two proteins (Fig. 14C). This point awaits further confirmation by adenovirus transduction.

Next, we wanted to discern if the inhibitory effect of BRCA1 overexpression was due to the ubiquitin- and proteasome-driven degradation of the receptor or just to ubiquitination of the receptor. For this purpose, we infected T47D cells with adenovirus for wtBRCA1. Cells were pretreated for 30 minutes with or without the specific inhibitor of the 26S proteasome lactacystin (10 uM) and then treated with vehicle or R5020 (10 nM) for 4,5 hours. Expression of target genes was determined by qRT-PCR. Proteasome inhibitors block PR degradation under these conditions, as previously shown (Fig. 9A). The treatment with the inhibitor did not cause any significant change in the expression of the PR-target genes tested, under normal conditions (Fig. 14D). The overexpression of BRCA1 inhibited the expression of these target genes, as expected. However, we observed two different consequences of the treatment with the proteasome inhibitor. In the case of the *DUSP1* gene, the treatment with lactacystin reverted the inhibitory effect of the overexpression of BRCA1 (Fig. 14D, top panel). Yet, in the case of the *11b-HSD* gene, the treatment with lactacystin did not revert the inhibition by BRCA1 overexpression. This would indicate that, probably, these genes are differently regulated by BRCA1 overexpression (Fig. 14D, bottom panel). In one case, the regulation is dependent on the proteasome-driven degradation

of proteins and, in the other case, the regulation is not dependent on degradation but just ubiquitination.

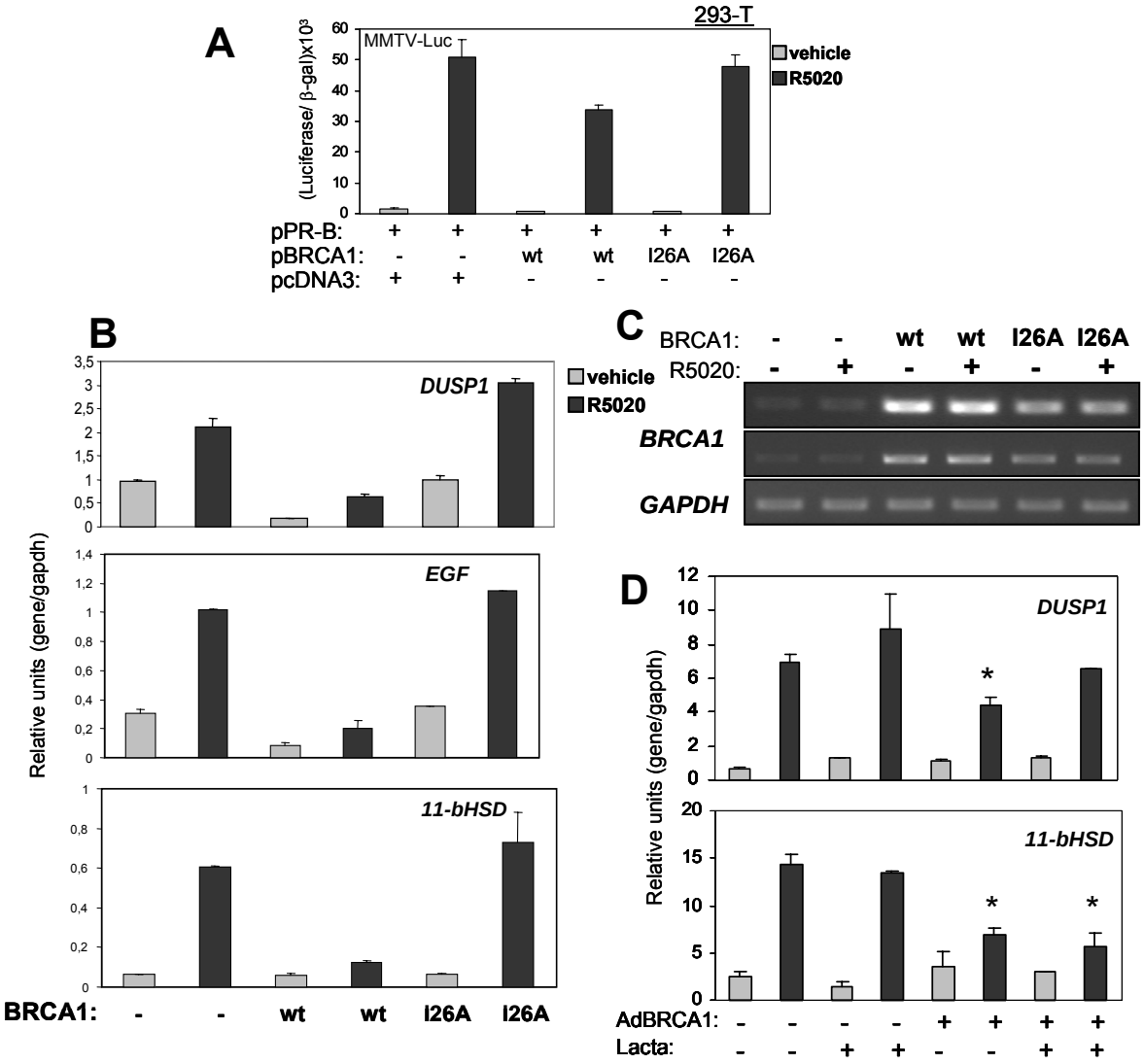


Figure 14: ***BRCA1 inhibitory effect over PR is dependent on its E3 ubiquitin ligase activity***

(A) 293-T cells were co-transfected with the reporter MMTV-Luc, pPR-B and pBRCA1wt or I26A mutant BRCA1. Cells were treated with vehicle (ethanol) or R5020 (10 nM) for 24 h. Luciferase activity was then determined and normalized by β-gal expression. Values are expressed as luciferase arbitrary units ± SD of a representative experiment performed in triplicate.

(B) T47D-MMTVL cells were infected with AdBRCA or transfected with pI26ABRCA1, and treated with R5020 for 6h. RNA was extracted and expression of PR target genes (DUSP1, EGF, 11-bHSD) was determined by qRT-PCR.

(C) RT-PCR showing the level of BRCA1 mRNA expression in cells infected with AdBRCA1 or transfected with the pI26ABRCA1. Middle panel shows a less exposed image of the upper panel.

(D) T47D-MMTVL cells were infected with +/- AdBRCA1, pre-treated with +/- lactacystin (proteasome inhibitor, 10 uM) and then treated with vehicle or R5020 (10 nM) for 4,5 h. The expression of PR-target genes (DUSP1 and 11-bHSD) was assessed by qRT-PCR, and expressed as ± SD of triplicate experiments.

R.2.3 BRCA1 effects at the promoter of PR-regulated genes

R.2.3.1 BRCA1 presence at the hormone-responsive regions of promoters regulated by PR

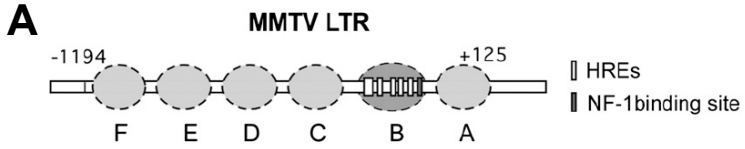
We next wondered if BRCA1 levels would affect the events occurring at the promoter of the PR-regulated genes.

First, we examined if BRCA1 could be detected at the hormone-responsive regions (HREs) of the promoter of some of the PR-regulated genes that are transcriptionally affected by the presence/absence of BRCA1. For this purpose, we have employed the chromatin immunoprecipitation (chIP) technique. For these series of chIP experiments, we mainly focus our analysis on the chromatin structure of the MMTV promoter, in which the first HREs were identified (Beato *et al*., 1995). In T47D-MMTVL cells, the MMTV promoter is covered by positioned nucleosomes of which the structure and dynamics have been intensively studied (Vicent *et al*., 2004 and 2006; Truss *et al*., 1995). The positioned nucleosome B (Nuc B) covers the region containing a cluster of five degenerated HREs and a binding site for the ubiquitous transcription factor nuclear factor 1 (NF1) (Richard-Foy and Hager, 1987; Piña *et al.,* 1990) (Fig. 15A).

We observed a rapid recruitment of PR to the nucleosome B region (at 10 minutes), that is maintained along time of treatment, for hours (Vicent *et al.,* 2004). As for the presence and timing of recruitment of BRCA1, we repeatedly observed the behaviour illustrated in Fig. 15B. We detect a quite prominent amount of BRCA1 present at the nucleosome B region at the basal level, in the absence of hormone, which is partially diminished after 5 to 10 minutes of hormone addition. The signal is recovered to a level slightly higher than the basal level or similar at 30 min-1h. Although this profile of recruitment is found consistently, we have observed variations the amount of BRCA1 protein present in the basal level (as can be seen in Fig. 16C).

As control, chromatin material was immunoprecipitated with a control normal IgG rabbit antibody (lane 9). Additionally, chromatin prepared from BRCA1-knockdown cell lines treated with R5020 10nM for 30 minutes was immunoprecipitated with the specific antibody against BRCA1, and the signal was insignificant (lane 13).

We also detected the presence of the BRCA1-protein partner, BARD1, in the nucleosome B region, and in this case BARD1 was recruited only after treatment with hormone and its permanence at the promoter was much more stable (fourth panel).



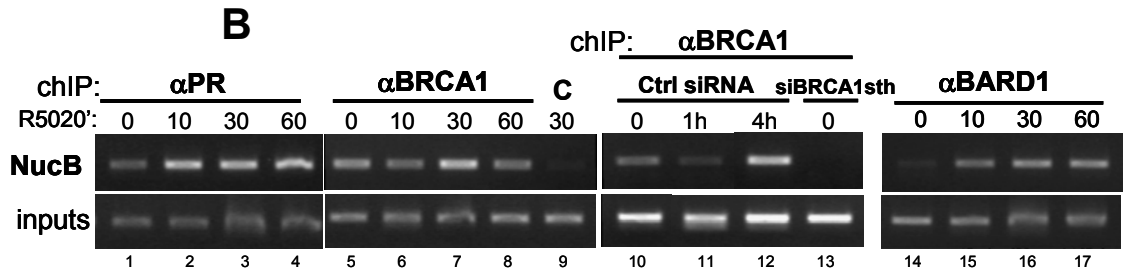


Figure 15: ***BRCA1 and BARD1 presence at the progesterone responsive element of the MMTV promoter***

(A) Structure of the MMTV promoter at T47D-MMTVL cells. The positioned nucleosome B (NucB) covers the region containing a cluster of five degenerated HREs.

*(B) T47D-MMTVL cells were treated +/- R5020 10 nM for 10 min to 4 hours. Chromatin was prepared and immunoprecipitated with antibodies against PR, BRCA1, BARD1 or control antibody (**C**), as indicated. The precipitated DNA was subjected to PCR analysis with specific primers flanking the region of the nucleosome B (**NucB**) of the MMTV promoter. A sample of the chromatin used in each immunoprecipitation was used directly for PCR analysis (inputs).*

In the third panel, T47D-MMTVL cells were transfected with control siRNA or siRNA against BRCA1 (siBRCAsth) for 72 hours, prior to chromatin immunoprecipitation.

While it has not been demonstrated that BRCA1 can interact directly with a specific sequence within undamaged DNA, it has been established that BRCA1 can bind to various sequence-specific DNA binding transcription factors to stimulate or inhibit transcription (reviewed at the Introduction and at Rosen *et al*., 2006). Our next question was if PR could be the protein that brings BRCA1 to the promoter of the target genes, since we have previously shown that the two proteins are able to interact *in vivo.*

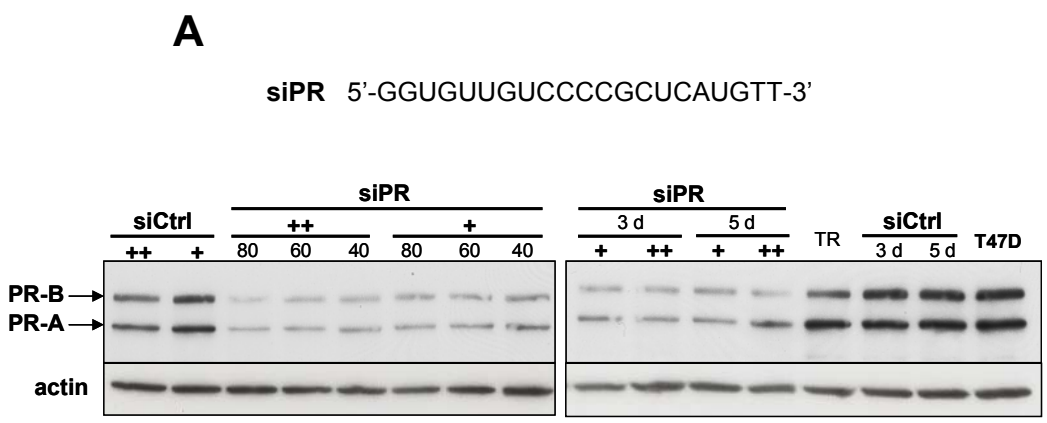
In order to answer this question, we envisaged the possibility to knockdown the expression of PR and then check if the recruitment of BRCA1 was affected. As a first approach, we contemplated the option of using an siRNA directed against PR to knockdown the expression of PR protein in T47D cells. Fig. 16A shows in a Western blot the level of downregulation of the PR protein obtained transfecting the siRNA (which was previously reported in the literature, Hardy *et al*., 2006) at different concentrations and cell confluency. Finally, we transfected the siRNA at a 100 nM concentration, 60% confluency and for 72 hours, obtaining a considerable knockdown of the protein (around 75 % knockdown). We performed chIP assays in cells transfected with the siRNA against PR or control siRNA, upon 30 minutes of hormone treatment. In this case, we checked the presence of BRCA1 and PR in the hormone-responsive region of an endogenous PR-target gene, 11β-HSD, because this cell line lacked the exogenous MMTV construct. PR binds to the region -1778 to -1596 of the 11β-HSD promoter, regulating its expression (Subtil-Rodríguez *et al*., 2008).

In this region, we detected the recruitment of PR at 30 minutes of hormone induction that was significantly diminished after PR-knockdown (Fig. 16B). We also detected the presence of BRCA1, which was further enhanced by the treatment with progestin for 30 minutes (graph in Fig.16B). Although the

knockdown of PR did not affect the basal level recruitment of BRCA1, we did not see the enhancement in BRCA1 recruitment upon progestin treatment in cells with PR-knockdown.

Given that the siRNA transfection was not completely abolishing the expression of PR, we wanted to confirm our findings in another system that could allow us to study the recruitment of BRCA1 in the same breast cancer cell line background with the possibility to switch on and off the expression of PR. For this purpose, we used the already mentioned T47D-YV cell line (a PR-negative clonal derivative from the T47D cell line) and its derivative engineered to express a flag-tagged form of PR-B protein (Quiles *et al*., 2009). In this way, we would have the same cell line background in two different versions, with undetectable levels of endogenous PR protein expression or with expression of an exogenous PR protein to an extent similar to the one found in T47D wt cells.

We treated the cells with progestin for 30 minutes and performed chIP analysis against PR and BRCA1 at the nucleosome B of the MMTV, at the hormone-responsive region of the *11β-HSD* gene and, additionally, analyzed the presence of PR and BRCA1 in a hormone-responsive region found at the *EGF* gene promoter (Cecilia Ballaré personal communication). We observe the expected recruitment of PR at the HREs of these three promoters and detect the presence of BRCA1, with a very low basal level, that is enhanced by the addition of progestin, in cells expressing the flag-tagged PR-B (lanes 8 and D, Fig.16C). We did not detect any noticeable recruitment of PR in the PR-null T47D-YV cell line and no signal was detected for BRCA1, either. Since the amount of BRCA1 protein detected at the basal level in the PR-expressing cell line is very low, it would mean that this basal level is unchanged in the presence or absence of PR, in accordance with the experiments of the siRNA transfection.



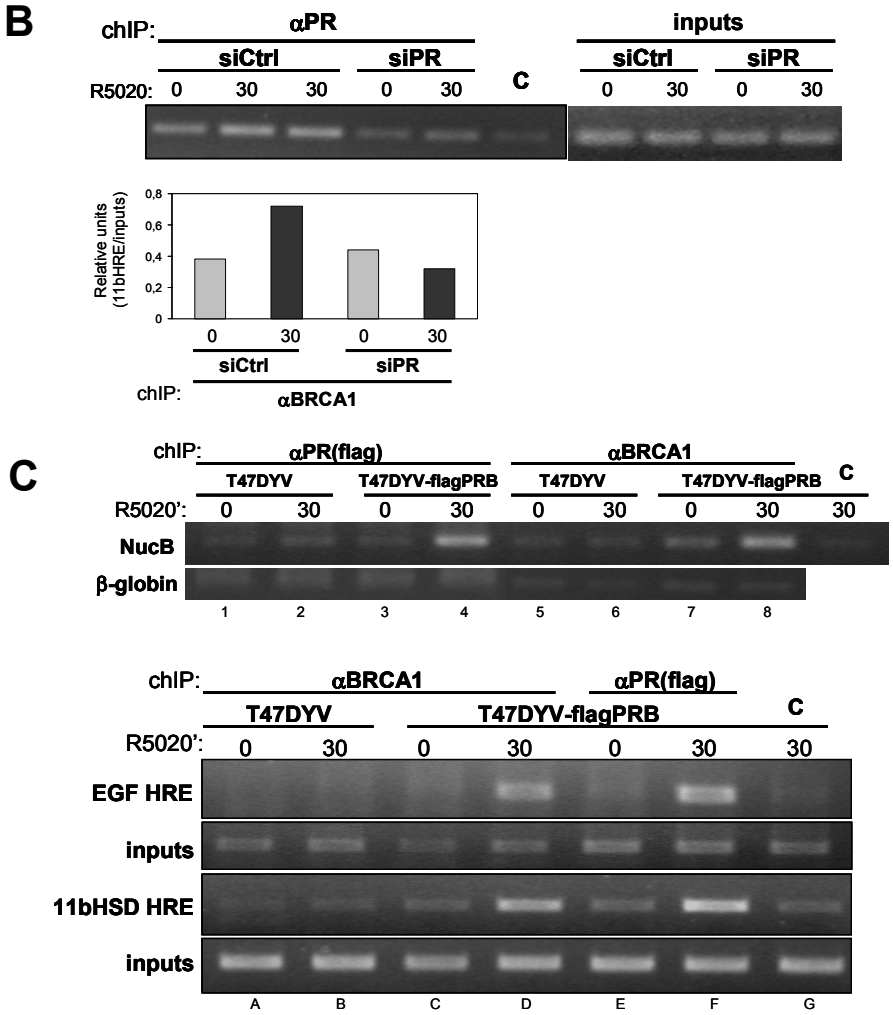


Figure 16: ***The absence/presence of PR protein affects the recruitment of BRCA1 to the hormone-responsive elements of PR-target genes***

*(A) Sequence of the siRNA used against PR (both isoforms). Western blots showing the level of PR protein knockdown obtained in T47D cells transfected with the siRNA (**siPR**) or control siRNA (**siCtrl**) at two different concentrations (+, 100 nM, ++, 200 nM), at different percentages of cell confluency (40, 60, 80 %) and for 3 or 5 days before cell extract preparation. In the first panel, cells were harvested after 3 days of siRNA exposure. In the second panel, cells were transfected at 40 % cell confluency. "TR" stands for cells just exposed to the transfection reagent. Actin is shown as loading control.*

*(B) T47D cells were transfected with the siRNA against PR (**siPR**) or control siRNA (**siCtrl**) for 72 h before being treated +/- R5020 10 nM for 30 min. Chromatin was prepared and immunoprecipitated with antibodies against PR, BRCA1 or control antibody (**C**), as indicated. The precipitated DNA was subjected to PCR analysis with primers flanking the HRE located at the distal region of the promoter of the 11β-HSD gene. A sample of the chromatin used in each immunoprecipitation was used directly for PCR analysis (inputs). PCR analysis of the precipitated DNA with BRCA1 antibody was quantified and normalized (by inputs) and is represented graphycally.*

*(C) T47D-YV (PR null) and T47D-YV-flagPRB cells were treated with +/- R5020 10 nM for 30 min prior to chromatin preparation. Chromatin was immunoprecipitated with antibodies against the flag-tag (for PR), BRCA1 or control antibody (**C**). Precipitated DNA was PCR-analyzed with primers flanking the nucleosome B (**NucB**) of the MMTV promoter, the HRE found at the EGF gene promoter (Cecilia Ballaré personal communication), the distal HRE of the 11bHSD promoter or against the β-globin gene as loading control. A sample of the chromatin used in each immunoprecipitation was used directly for PCR analysis (**inputs**).*

R.2.3.2 Effect of BRCA1 on the recruitment of PR and/or its coregulators

We next determined if BRCA1 knockdown affects the level or timing of PR recruitment to the promoter of regulated genes, since BRCA1 affects the amount of PR protein in the cell.

Control and BRCA1 knockdown T47D-MMTVL cells were exposed to 10 nM R5020 for 0 to 10 minutes (Fig. 17A) and 0 to one hour (Fig. 17B). PR protein association with the nucleosome B of the MMTV promoter was analyzed by chIP. Overall, progestin-bound PR recruitment was not significantly changed by decreased BRCA1 expression.

We performed a complementary experiment, this time overexpressing BRCA1 in T47D-MMTVL cells. Control and cells overexpressing BRCA1 were exposed to 10 nM R5020 for 0 to 30 minutes and chIP experiments were performed to detect PR and BRCA1 at the nucleosome B. Fig. 17C shows how the overexpression of BRCA1 increases its recruitment on the promoter at 10 and 30 minutes of hormone treatment. Concomitantly, we detect a slight though consistent decrease in the amount of PR recruited at all time points tested. In all, it would mean that it is possible that overexpression of BRCA1 actually affects the amount of PR recruited to the promoter of regulated genes.

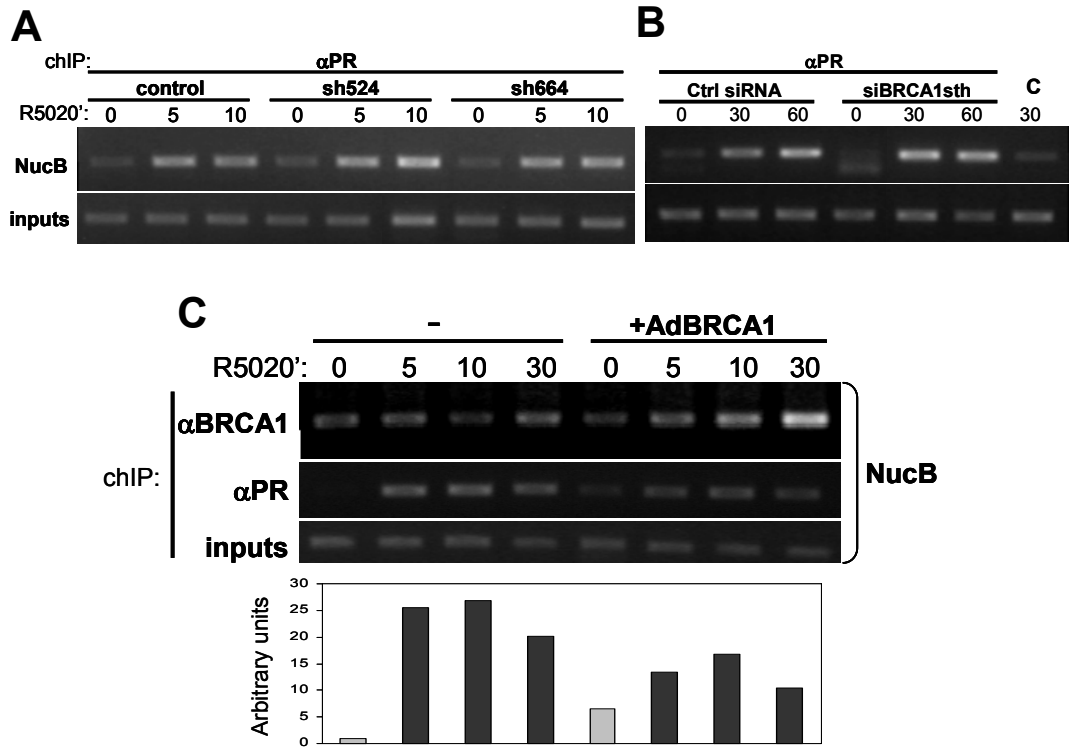


Figure 17: ***BRCA1 expression level might affect the amount of PR recruited to the HRE region of the MMTV promoter***

*(A) Control and shRNA-expressing T47D-MMTVL cells were treated with +/- R5020 10 nM for 5 or 10 min. Chromatin was prepared and immunoprecipitated against PR. Precipitated DNA was analyzed by PCR with primers flanking the nucleosome B (**NucB**) of the MMTV promoter. A sample of the chromatin used in each immunoprecipitation was used directly for PCR analysis (**inputs**).*

*(B) T47D-MMTVL cells were transfected with control siRNA (**Ctrl siRNA**) or siRNA against BRCA1 (**siBRCA1sth**) for 72 h before being treated +/- R5020 10 nM for 30 or 60 min. Chromatin was prepared and immunoprecipitated against PR or control antibody (**C**), as indicated. The precipitated DNA was subjected to PCR analysis with specific primers flanking the nucleosome B (**NucB**) of the MMTV promoter. A sample of the chromatin used in each immunoprecipitation was used directly for PCR analysis (**inputs**).*
*(C) T47D-MMTVL cells were infected +/- AdBRCA1 to overexpress BRCA1. Cells were treated with +/- R5020 10 nM for 0 to 30 min and chromatin was prepared. Chromatin was immunoprecipitated against BRCA1 and PR. Precipitated DNA was PCR analyzed with primers flanking the nucleosome B (**NucB**) of the MMTV promoter. PCR analysis of the precipitated DNA with the PR antibody was quantified and represented graphycally.*

Ligand-bound steroid receptors recruit coactivators such as members of the p160 family of coactivators (steroid receptor coactivator-1, 2 and 3), cAMP response element binding protein (CREB)-binding protein (CBP), p300 as well as ATP-dependent chromatin remodeling complexes, like SWI/SNF, to modifiy the promoter chromatin architecture and allow subsequent recruitment of additional transcription factors as well as the members of the basal transcriptional machinery for transcription activation (Li *et al*., 2004).
The progesterone receptor interacts with both SRC-1 and 3 although it preferentially binds to SRC-1, which recruits CBP and significantly enhances acetylation of K5 of histone H4 (Li *et al*., 2003; Vicent *et al*., 2004). We wanted to determine if BRCA1 could be affecting the recruitment of PR coactivators such as SRC-1 or SRC-3. Control and BRCA1-knockdown cells were subjected to chIP assays with specific antibodies against SRC-1 and SRC-3. No significant changes were observed in the recruitment of SRC-3 between the two cell lines (Fig. 18A) while a slight delay was observed in the case of SRC-1 in the BRCA1 knockdown cells (right panel).

Our own previous observation that overexpression of BRCA1 leaded to the inhibition of progestin-induced genes but also to the inhibition of progestin-repressed genes made us envision what scenario could correspond to this outcome. Following the reasoning of other known corepressor complexes, we thought that, rather than hindering the recruitment of coactivators, BRCA1 could enable the action of repressive activities such as histone deacetylation.
Many of the transcription coactivators contain intrinsic histone acetyltransferase (HAT) activity, whereas many of the transcription corepressors complexes contain subunits with histone deacetylase (HDAC) activities. Therefore, mainly, histone acetylation has been correlated with gene activation and deacetylation with repression (Spencer *et al*., 1997; Schubeler *et al*., 2004; Bartsch *et al*., 1996; Sakai *et al*., 2003). BRCA1 has been found to interact with HDAC1 and 2 (Yarden *et al*., 1999), and this interaction was suggested to be involved in the mechanism of the ligand-independent repression of the ERα receptor by BRCA1 (Zheng *et al*., 2001). We checked if BRCA1 levels affect HDAC recruitment at the promoter of progestin-regulated genes. HDAC1 and HDAC3 are the deacetylases commonly associated with nuclear receptor activities (Kinyamu *et al*., 2004).
We performed a time-course chIP assay in control and BRCA1-knockdown cells to monitor the presence of HDAC1 at the nucleosome B of the MMTV promoter. We observed a complex profile of displacement and recruitment of

HDAC1 in the promoter, with peaks at 5 and 60 minutes, similar to previous observations previously reported (Aoyagi and Archer, 2007). In BRCA1-knockdown cells the levels of HDAC1 are lower and peak later, at 30 minutes, meaning that the promoter is less exposed to the deacetylase activity of HDAC1 (Fig. 18B), compatible with an enhancement in the transcription of the gene.

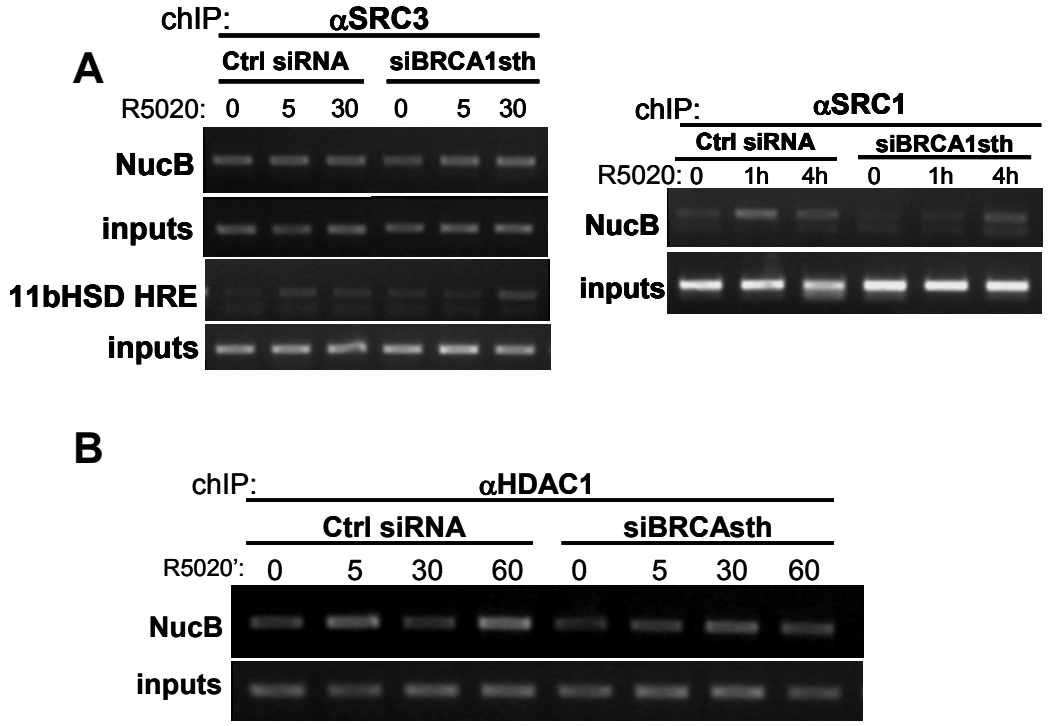


Figure 18: ***BRCA1 expression level has little effect on the presence of coactivators like SRC1 and 3 but it affects the presence of corepressing activities such as HDAC at the HREs of the MMTV promoter***

*(A) Control and siBRCA1sth transfected T47D-MMTVL cells were treated with +/- R5020 10 nM for 5, 30 min, 1 or 4 h. Chromatin was prepared and immunoprecipitated against SRC-3 and SRC-1. Precipitated DNA was PCR analyzed with primers flanking the nucleosome B (**NucB**) of the MMTV promoter and the HRE region of the 11βHSD gene promoter. A sample of the chromatin used in each immunoprecipitation was used directly for PCR analysis (**inputs**).*

*(B) T47D-MMTVL cells were transfected with control siRNA or siBRCA1sth for 72 h before being treated +/- R5020 10 nM for 5, 30 or 60 min. Chromatin was prepared and immunoprecipitated against HDAC1. The precipitated DNA was subjected to PCR analysis with primers flanking the nucleosome B (**NucB**) of the MMTV promoter. A sample of the chromatin used in each immunoprecipitation was used directly for PCR analysis (**inputs**).*

R.2.3.3 Effect of BRCA1 on the histone code: histone H2A ubiquitination

Apart from the relationship that BRCA1 could have in transcription at the level of the basal machinery of transcription, on specific transcription factors or on coregulators, to which there are many links described in the literature and in this work, we wondered if BRCA1 could also be directly related to another level of transcription regulation of the utmost importance like it is the histone code. Chromatin is subjected to a diverse array of post-translational modifications that largely impinge on histone amino termini, thereby regulating access to the underlying DNA. Distinct histone amino-terminal modifications (acetylation, phorphorylation, methylation, ADP ribosylation and ubiquitination) can generate synergistic or antagonistic interaction affinities for chromatin-associated proteins, which in turn dictate dynamic transitions between transcriptionally active or silent chromatin state. The combinatorial nature of histone amino-terminal modifications is known as the “histone code” (Jenuwein and Allis, 2001).

Monoubiquitination is the least understood of the histone modifications, although recent publications are starting to clarifiy its role in transcription regulation. In general, histone H2A monoubiquitination has been linked to gene repression while H2B monoubiquitination has been linked to gene activation and transcription elongation (reviewed at Weake and Workman, 2008). In humans, H2A ubiquitination is mediated by at least two E3 ubiquitin ligases, Ring1B and 2A-HUB, both of which are associated with transcriptional silencing (Cao *et al.,* 2005; Wang *et al*., 2004; Zhou *et al.,* 2008). However, there are candidates for additional E3 enzymes mediating histone ubiquitination in human cells. One of these candidates is BRCA1 itself, who can catalyze ubiquitination of both H2A and H2B *in vitro*, although its *in vivo* relevance has not been explored (Mallery *et al*., 2002).

We focused our attention on the possible effect that BRCA1 could have on the monoubiquitination of histone H2A. First, we checked if we were able to detect any change in the total levels of histone H2A monoubiquitination in T47D breast cancer cells depending on the level of BRCA1 expression. Total histones were acid-extracted from control and BRCA1-knockdown T47D total extracts and probed with an antibody against total H2A and an specific antibody recognizing the monoubiquitinated form of histone H2A (uH2A) (Fig. 19A). We did not see any significant change in general H2A monoubiquitination after BRCA1 depletion. Nevertheless, the fact that the depletion of the E3 ubiquitin ligase 2A-HUB neither caused a change in the total level of H2A ubiquitination (Zhou *et al*., 2008) prompted us to go on in the study of the modification of histone H2A by BRCA1 but at specifically localized targets on the chromatin template. We were considering the possibility that BRCA1 might have a local effect rather than a global effect on histone H2A ubiquitination.

For this purpose, we performed chIP assays directed to the detection of uH2A at the hormone-responsive region of a promoter regulated by PR. Remarkably, another nuclear receptor, AR, counts among its coactivators with a histone deubiquitinase enzyme, 2A-DUB (Zhu *et al*., 2007). This deubiquitinase is recruited to the promoter of PSA, an AR-target gene, in

response to ligand, removes the repressive uH2A mark from the acetylated nucleosomes, and dissociates linker histones in a stepwise manner.
We were able to detect the presence of uH2A at the hormone-responsive region of the MMTV promoter before hormone induction and how this repressive mark gradually disappears from the promoter upon hormone addition, and it comes back at 60 minutes, coinciding with the recruitment of BRCA1 (Fig. 19B, first panel). We repeatedly observed that the presence of uH2A mark was significantly decreased from the hormone-responsive region following knockdown of BRCA1 (Fig. 19B, second panel). Overall, these data would indicate that BRCA1 is affecting the levels of monoubiquitinated H2A at the promoter of PR-regulated genes, probably by directly ubiquitinating the histone protein.

As regards the amount of total H2A present at the nucleosome B region, we detect a slight decrease in the signal upon 30 minutes of hormone addition in the control cell line; while at the BRCA1-knockdown cells there is a greater decrease upon 60 minutes of hormone addition (fig. 19B, first panel). This observation correlates with the data by Vicent *et al* (Vicent *et al.,* 2004). They report the displacement of histones H2A/H2B at the NucB region upon 30 minutes of R5020 treatment in this same cell line. However, in order to detect the displacement occurring at the NucB with more sensitivity it is recommended to shear chromatin at the mononucleosome level (Vicent *et al.*, 2004) instead of the 200-400 bp fragments of chromatin used in previous experiments. We sheared chromatin at around the mononucleosome level (~200 bp) and performed a chIP analysis of control and siBRCA1sth cells to check the presence of total H2A at the NucB region (Fig. 19B, third panel). We observed the expected displacement of H2A in control cells and a significant decrease in the signal at the BRCA1-knockdown cells at time 0 and upon hormone addition, indicating that, concomitant with the decrease in the amount of uH2A at the NucB region there is a minor presence of localized total histone H2A.

In parallel, we analyzed the presence of the uH2A mark in a promoter previously described to be regulated by ubiquitination of H2A, to serve as a control for the detection of this histone mark. The *HOXC5* gene, like other members of the Homeobox (HOX) family, has been described to be regulated by ubiquitination of H2A in human cell lines (Wei *et al*., 2006). Ubiquitinated H2A was localized on a 5' regulatory region of the gene promoter and it was dependent on the activity of the E3 ligase Ring 1B.
We detected the presence of the uH2A mark on the 5'regulatory region of this gene by chIP assay (Fig. 19C) and, unexpectedly, we also observed the decrease in the signal in the BRCA1-knockdown cell lines, indicating that the influence of BRCA1 might be more general than expected or that HOX genes might be also targets of the action of BRCA1 transcriptional control.

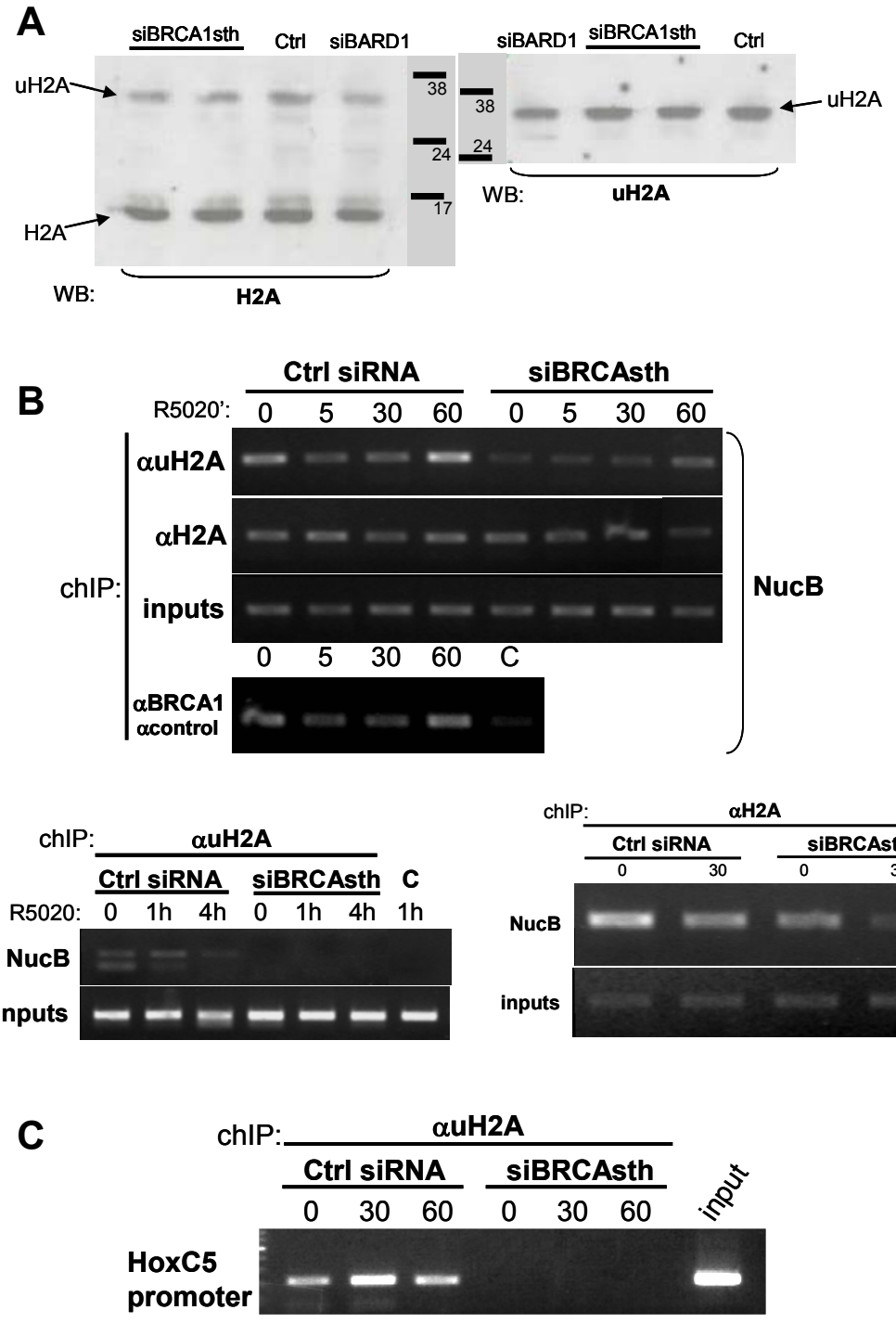


Figure 19: ***BRCA1 affects the level of monoubiquitinated histone H2A present at the HREs of a promoter regulated by PR***

(A) Total histones were acid-extracted from control and siBRCA1sth transfected T47D-MMTVL cells total extracts. 4-6 ug of extracted histones were run in a high percentage gel, transferred and probed with an antibody against total histone H2A (H2A) or an specific antibody recognizing monoubiquitinated histone H2A (uH2A). Total histone H2A antibody also recognizes, to some extent, monoubiquitinated H2A protein.

*(B) T47D-MMTVL cells were transfected with control siRNA (**Ctrl siRNA**) or siBRCA1sth for 72 h before being treated +/- R5020 10 nM for 5 to 60 min, or for 1 and 4 hours. Chromatin*

*was prepared and immunoprecipitated against total histone H2A, monoubiquitinated histone H2A (**uH2A**), BRCA1 or a control antibody (**C**), as indicated. The precipitated DNA was subjected to PCR analysis with primers flanking the nucleosome B (**NucB**) of the MMTV promoter. A sample of the chromatin used in each immunoprecipitation was used directly for PCR analysis (**inputs**).*
*(C) T47D-MMTVL cells were transfected with control siRNA (**Ctrl siRNA**) or siBRCA1sth. After 72 h, cells were treated +/- R5020 10 nM for 0, 30 or 60 min. Chromatin was prepared and immunoprecipitated against monoubiquitinated H2A (**uH2A**). Precipitated DNA was analyzed by PCR with primers flanking a 5' regulatory region of the HOXC5 gene promoter previously reported to present this post-translational modification in histone H2A. A sample of the chromatin used in one immunoprecipitation was used directly for PCR analysis (**input**).*

R. 3 Effect of BRCA1 on cell biology processes induced by progestins

R. 3.1 Short-term progestin-induced proliferation

Progesterone plays a prominent biological role in the normal breast development and in breast tumourigenesis. Proliferating breast tumour cells often express ERα and PR (Clarke *et al*., 1997; Russo *et al*., 1999). While estrogens have a pronounced proliferative effect, progesterone has been shown to have biphasics effects on breast cancer cell growth *in vitro*, with an initial peak of proliferation at the 18-24 hours, which may last from one (Groshong *et al*., 1997; Musgrove *et al*., 1991) to multiple rounds of cell division followed by cell growth inhibition (Fig. 20A).

To assess the effect of BRCA1 on short-term progestin-induced cell proliferation we performed a double-staining with bromodeoxiuridine (BrdU) and propidium iodide. BrdU is a synthetic nucleoside analogue of thymidine, which is incorporated into newly synthesized DNA of replicating cells (mostly marks cells in S-phase and a small fraction of G2/M phase cells).
BRCA1 knockdown cells sh170v and sh502m, and pS and T47D-MMTVL control cells were grown in white medium + 10% charcoal-stripped serum (CSS) for 48 hours and then were replaced with 0% CSS for 24 hours. Cells were treated with ethanol (vehicle), R5020 10 nM or estradiol (E2) 10 nM. Sets of cells were harvested at 24 hours of E2 treatment and at 15 and 24 hours or 8, 18 and 40 hours of progestin treatment (Fig. 20B). After BrdU addition and immunodetection, the cells were stained with propidium iodide and the percentage of BrdU incorporation determined by flow cytometry.
Fig. 20B shows a representative experiment. We obtained the expected profile of progestin induced proliferation in control and shRNA-expressing cell lines. We detected no significant differences in the R5020-induced proliferation between the two cell lines, while a significant ($p<0.05$ for the upper graph and $p<0,01$ two-tailed t-test for the lower graph) increase in BrdU incorporation was observed in the BRCA1-downregulated cell line upon estradiol treatment. It would indicate that the decrease in BRCA1 expression is only affecting the short-term proliferation induced by E2 and not by the progestin R5020.

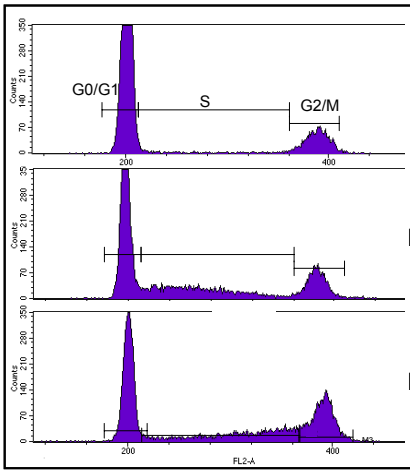
It is believed that the growth-promoting effects of progestins are due to PR's function as an activator of cytoplasmic kinase cascades rather than its direct activation of transcription, at least for the first round of cell proliferation (Skildum *et al*., 2005). Progestin treatment of breast cancer T47D cells

activates the signal-transducing c-Src/Ras/Erk pathway, which is essential for the induction of cell proliferation (Migliaccio *et al*., 1998; Skildum *et al*., 2005). One of the targets of the kinase pathway is the PR itself, which is phosphorylated at Ser294 as fast as five minutes after hormone induction (Vicent *et al.,* 2006). Besides, Ser294 phosphorylation greatly increases transcriptional activity of liganded PR at PRE-containing promoters (Shen *et al.,* 2001).

Similarly, agonist-occupied ERα stimulates the c-Src/Ras/Erk pathway in MCF-7 cells (Migliaccio *et al*., 1996). Under proliferative conditions, estradiol activation of this pathway leads to stimulation of G1-S transition of MCF-7 cells (Castoria *et al*., 2001). Besides, the MAPK activation mediated by growth factors or estrogen signals are known to augment the ligand-induced transactivation function of nuclear ERα through phosphorylation of Ser118 within the ERα N-terminal AF-1 domain (Thomas et al., 2008; Weitsman et al., 2006).

We wanted to check the level of activation of the c-Src/Ras/Erk pathway in the BRCA1-knockdown cell lines. In order to do so, we determined the level of phosphoryation of the receptors, at Ser294 for PR-B and Ser118 for ERα, with specific phospho-Ser294 and Ser118 antibodies, by Western blot. ShRNA-expressing cell lines were serum-starved and treated with E2 10nM for 45 minutes or R5020 10 nM for 10 minutes. Fig. 20C shows that there are no significant differences in the level of phospho-Ser118 ERα and total ERα between the control and the BRCA1-knockdown cell lines. As for phospho-Ser294 (Fig. 20D), we observe a clear increase in the level of phosphorylation of the receptor upon hormone addition in the shRNA-expressing cell lines, which correlates with the already observed increase in the total PR-B level. Therefore, we do not detect an over-activation of the pathway that leads to the activating phosphorylation at Ser118 of ERα while the increase in Ser294-phosphorylation observed is concomitant with the increase in total PR-B protein level.

A



vehicle

	Events	% Gated	% Total	Mean	CV
All	9897	100.00	64.82	239.78	31.02
G0/G1	7740	78.21	50.69	201.92	2.41
S	262	2.65	1.72	290.68	15.22
G2/M	1867	18.86	12.23	389.01	2.89

R5020 18h

	Events	% Gated	% Total	Mean	CV
All	9933	100.00	59.28	253.71	28.62
G0/G1	5173	52.08	30.87	198.49	2.36
S	2927	29.47	17.47	269.19	13.76
G2/M	1811	18.23	10.81	383.54	2.33

R5020 24h

	Events	% Gated	% Total	Mean	CV
All	10112	100.00	48.88	288.50	29.35
G0/G1	4375	43.27	21.15	201.58	2.59
S	2748	27.18	13.28	311.79	13.92
G2/M	3110	30.76	15.03	390.53	2.76

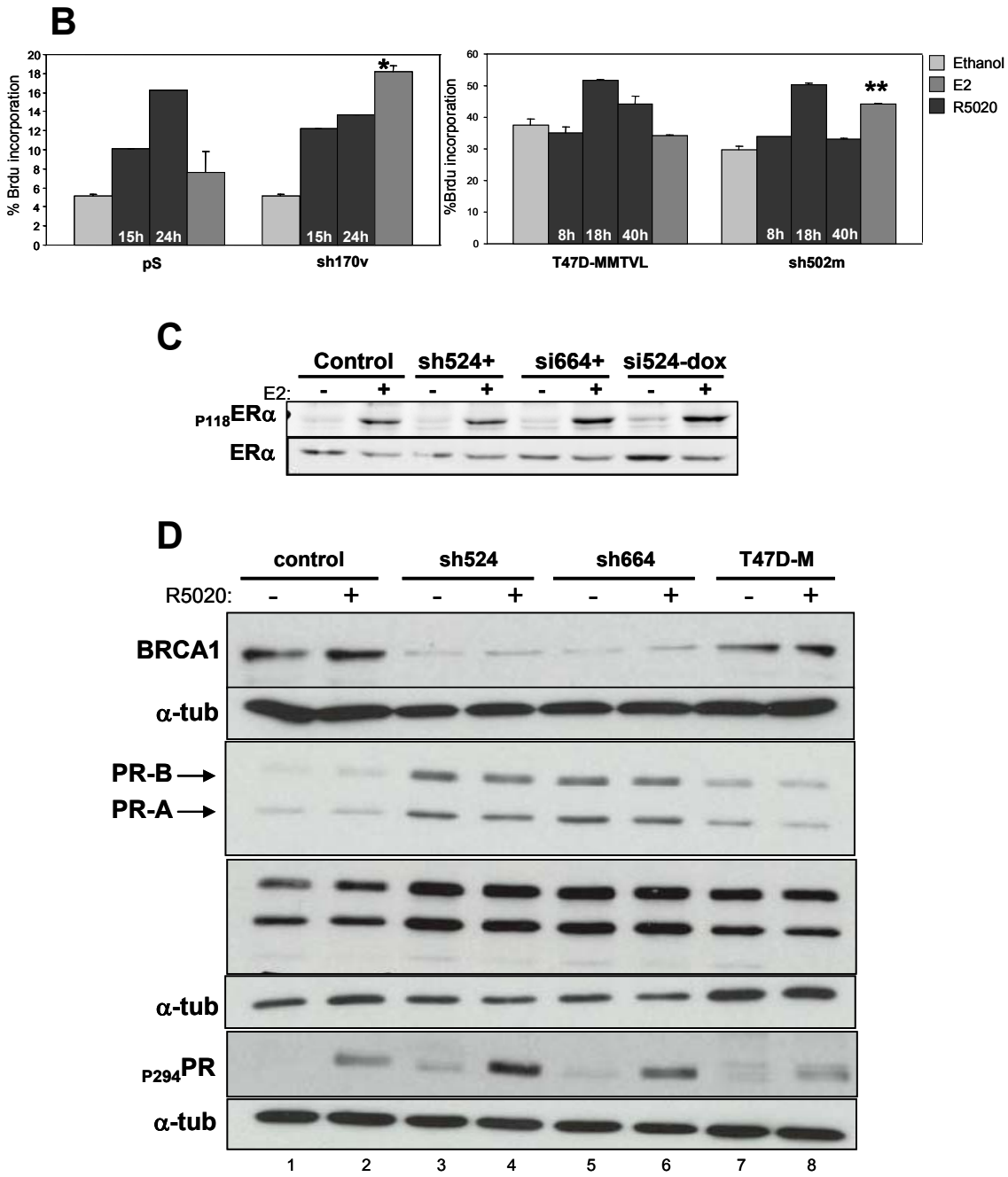


Figure 20: ***BRCA1 does not affect the short-term progestin-induced cell proliferation***

(A) Graph showing the progression into the cell cycle of serum-starved T47D cells upon progestin treatment (10 nM) for 24 h. A CV<3 was taken as a requisite for subsequent cell cycle analysis.

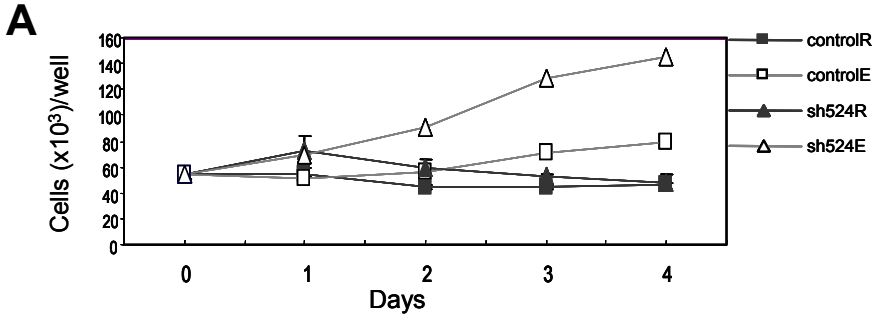
(B) Control pS and T47D-MMTVL cells and shRNA-expressing cell lines sh170v and sh502m growing in white medium + 10% charcoal-stripped serum (CSS) were replaced with 0% CSS medium for 24 h and treated with ethanol, E2 10 nM or R5020 10 nM. Sets of cells were harvested at 15 h of ethanol treatment, 24 h of E2 treatment and 15 or 24 h of R5020 treatment (left) or 8, 18 or 40 h of R5020 treatment (right). During the last 2-4 h of treatment, cells were incubated with BrdU and harvested for BrdU immunodetection and IP staining.

*Percentage of BrdU incorporation was measured by flow cytometry. Brackets * indicate statistically significant differences (**, p<0.01 and *, p<0.05, two-tailed t-tests).*
(C) Control, shRNA-expressing cell lines and one of the shRNA-expressing cell lines not induced with doxycycline were serum-starved and treated with +/- E2 10 nM for 45 minutes, when cell extracts were prepared. Western blot was performed with specific antibodies against phosphorylated Ser118-ERα ($_{P118}$ERα) or against total ERα.
(D) Control, T47D-MMTVL (T47D-M) and shRNA-expressing cell lines were serum-starved and treated with +/- R5020 10 nM for 10 minutes, when cell extracts were prepared. Western blot was performed with antibodies against BRCA1, PR, a specific antibody against phosphorylated Ser294-PRB ($_{P294}$PR) and α-tubulin as loading control.

R. 3.2 Long-term progestin-induced proliferation and survival

To address the responsiveness of the T47D breast cancer cells expressing normal or low amounts of BRCA1 under conditions of long-term hormone exposure, cells were induced to express the sh524 or empty vector (control), plated in triplicate and treated with R5020 10nM or E2 10 nM after have been serum-starved overnight. Cells were collected and counted with a Coulter counter from one through four days of treatment. Media was replenished every 48 hours (Fig. 21A). We observed a very similar behaviour between the two cell lines upon progestin stimulation, while there was a significant difference between the two cell lines in the number of cells after two to four days of estrogen treatment, being higher in the BRCA1-knockdown cell line, in accordance with the previous experiments of short-term proliferation. This would indicate, again, that the lack of BRCA1 does not affect either the short-term proliferation induced by progestins nor the long-term profile of growth of T47D cells.

Additionally, long-term exposure to progestins may increase cell survival (Moore *et al.,* 2006). To test this effect in the BRCA1-knockdown cell lines, control and shRNA-expressing cell lines were grown to confluency in quadruplicate for each treatment. The cells were then changed to serum-free white medium and incubated in this medium for six days with 10nM R5020 or vehicle (ethanol). Fresh hormone or ethanol was added every 48 hours. After six days, cells in the supernatant and still attached to the flask were counted and assayed for viability with trypan blue staining (Fig. 21B). We observed a significant protection from cell death in control cells treated with progestin. As for the BRCA1-knockdown cell lines, we observe a remarkable resistance to the cell death induced by serum withdrawal, which is not further rescued by progestin treatment since the cell death induced is barely discernible.



B

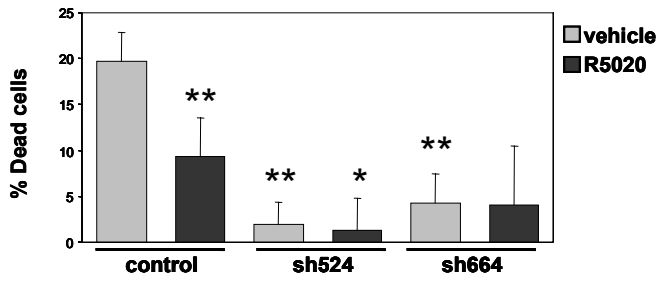


Figure 21: ***BRCA1 effect on long-term progestin-induced proliferation and cell survival***

(A) Control and BRCA1-knockdown cells were plated in triplicate and treated with R5020 10 nM or E2 10 nM after been serum-starved o/n. Cells were collected and Coulter-counted at one to four days of treatment.

*(B) Control and shRNA-expressing cell lines were grown to confluency and changed to serum-free white medium plus R5020 10 nM or vehicle (ethanol), in quadruplicate. Fresh hormone or ethanol was added every 48 h, during 6 days. Cells still attached and in the supernatant were then collected, trypan blue-stained and counted to assess viability (**, $p<0.01$ and *, $p<0.05$, two-tailed t-test).*

R. 3.3 Effect of irradiation and progesterone treatment on the cell cycle of BRCA1 wt/ko cell lines

Progesterone has been described to protect cells against γ-irradiation-induced apoptosis in a p53 and cell cycle distribution independent manner (Vares *et al*., 2004). Progesterone also increases the rate of cell proliferation after irradiation, cells showing a higher number of chromosome aberrations than cells treated with radiation alone. We wanted to test which would be the response of cells lacking functional BRCA1 in the setting of γ-irradiation-induced DNA damage and progesterone treatment.

Ionizing radiation, which produces DNA strand breaks and various types of oxidized bases, is often used to induce p53 and activate the G1 checkpoint response. The T47D cell line expresses mutant p53 protein and so displays absence of wtTP53-specific characteristics, such as radiation-induced cell cycle arrest at the G1/S boundary. It presents instead an apparent accumulation of G2/M phase cells at 16 hours after irradiation, in contrast with the behaviour of a wtTP53-expressing cell line like MCF-7, which displays the typical G1 arrest after γ-irradiation (Fig. 22A). The G2 checkpoint will arrest damaged cells in G2, delaying entry into mitosis until the damage has been repaired. Efficient cell cycle arrest at the G2/M boundary following ionizing radiation exposure requires intact function of BRCA1 (Scully and Livingston, 2000; Yarden *et al*., 2002).

T47D control and shRNA-expressing cell lines were plated in white medium and serum-starved overnight. They were treated with ethanol, R5020 10 nM or E2 10 nM and left for two hours before being irradiated with a single dose of 10 Gy radiation. The cells were collected and stained with propidium iodide for cell cycle analysis 18 hours for R5020-treated and 24

hours for E2-treated cells after γ-irradiation. After irradiation, control cells double the number of cells in the G2/M fraction compared to unirradiated cells, independently of the treatment applied (Fig. 21B).
A smaller fraction of irradiated BRCA1-knockdown cells was arrested in the G2/M phase after irradiation compared to the control cell line. Although the BRCA1-knockdown cell line showed radiation-induced accumulation in G2/M phase, the accumulation of cells in the G2/M phase compared with the same treatment without irradiation never got higher of 1,3 fold enrichment, while in the control cell line the enrichment was of 2 fold, no matter the treatment. This behaviour is indicative of a deficient G2/M checkpoint, as expected in a BRCA1-mutant background (Ree *et al*., 2003) (Fig. 21B). However, no further effect is observed in the presence of progestin hormone, as observed in control cells.

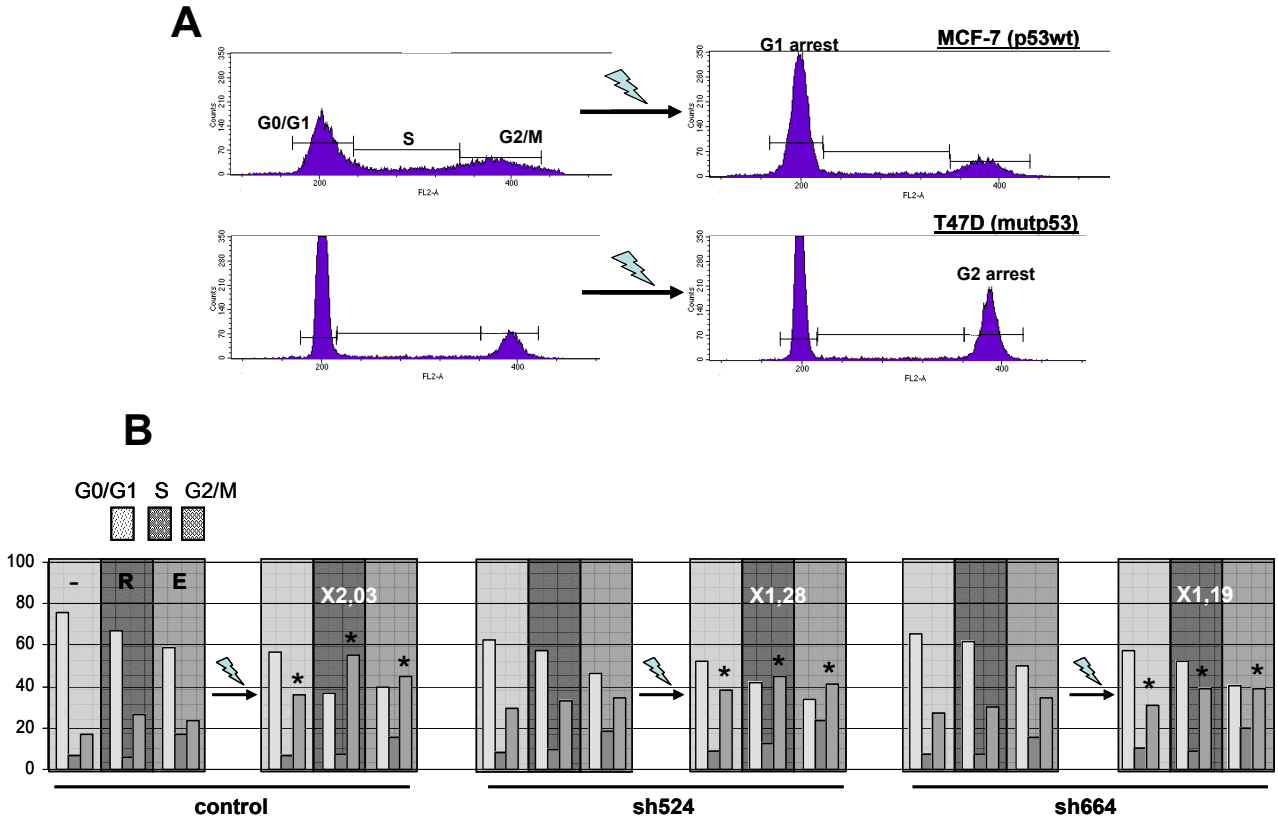


Figure 21: ***Effect of irradiation and hormone treatment in the cell cycle of BRCA1 knockdown cells***
(A) Ionizing radiation (10Gy) produces a pronounced G1 arrest in cells with intact p53 activity (MCF-7 cells) while in p53 mutant cells like T47D produces an apparent accumulation of cells at G2/M.
*(B) Control and shRNA-expressing cell lines were plated in white medium and serum-starved o/n. They were treated with vehicle (ethanol, **-**), R5020 10 nM (**R**) or E2 10 nM (**E**) for two hours before being irradiated with a single dose of 10 Gy. Cells were collected and cell cycle was analyzed after 18 h and 24 h of R5020 and E2 treatment, respectively. The G2/M fraction is marked with * and the number indicates the fold enrichment in the G2/M population of cells after irradiation.*

DISCUSSION

In the present work we have tried to demonstrate a functional relationship between the breast cancer 1 protein (BRCA1) and the progesterone receptor (PR) in breast cancer cell lines.

We are mainly interested in a better understanding of the biology of the progesterone receptor in mammary cells and its role in breast cancer malignancy. Therefore, the study of the relationship between BRCA1 and PR would widen our knowledge about the mechanism of action and control of the receptor. At the same time, it turned out to be very appealing the possibility to give answer or, at least, bring some light on the long-run enigma that surrounds the tissue specificity of BRCA1 mutations. Despite being expressed ubiquitously and having such a general function in the cell (see Introduction), mutations in BRCA1 predispose individuals to, mainly, breast and ovary cancers. This enigma has generated several speculative and no mutually exclusive ideas (Monteiro, 2003):

-*Differential tissue requirement*. An idea that is often invoked to explain tissue specificity in cancer predisposition is that functional BRCA1 protein is required to maintain the normal phenotype in breast and ovary epithelia, but not in other tissues. Thus, loss of BRCA1 would allow cancer development in breast and ovary but would not be expected to affect other tissues.
In unaffected tissues there might be functional redundancy whereby other proteins perform the same function as BRCA1. However, it has been argued that the severe embryonic lethality in *Brca1*-/- mice is inconsistent with this hypothesis (Elledge *et al*., 2002), although it is possible, if unlikely, that cells only establish the redundant pathways after embryonic development.

-*Specific tissue requirement due to particular forms of DNA damage*. Particular tissues might be more exposed to a certain type of damage that requires BRCA1 for its repair. And so, although the DNA repair machinery (in this case BRCA1) is present in several tissues, only the tissues exposed to that particular type of damage are affected by the lack of the specific machinery. Interestingly, mammary gland tissue does have increased levels of carcinogenic estrogen metabolites that can adduct DNA (Fishman *et al*., 1995). In addition, it could be that a major determinant for the generation of mutations is the high rate of proliferation in the mammary gland. This could account for the generation of single and eventually double-strand breaks due to stalled replication forks (replication stress), which would be at the origin of breast tumours.

-*Delayed apoptotic response*. Elledge *et al* proposed the existence of a delayed apoptotic response in breast and ovary (Elledge *et al*., 2002). It is based on the idea that loss of BRCA1 function would lead to apoptosis or severe proliferation defects in tissues other than breast and ovary, therefore preventing the accumulation of additional mutations required for tumour formation. Indeed, evidence derived from mouse models indicates that *Brca1* is an essential gene and its loss is incompatible with embryonic development. According to this idea, cells undergoing loss of the wild-type allele of *BRCA1* (LOH) would be eliminated rapidly unless tissue-specific factors could suppress

or delay lethality. In this case, temporary suppression of lethality in breast and ovary cells lacking BRCA1 would allow sufficient time to accumulate additional mutations required for oncogenesis. The balance between proliferation and apoptosis is tightly maintained in the mammary gland, and cells undergo apoptosis after each estrogen cycle, indicating that the apoptotic response might be controlled by tissue-specific factors (Anderson *et al.,* 2004).

-Differential loss of heterozygosity (LOH). Tissue specificity for BRCA1 tumours might reflect higher rates of loss of the wild-type allele in breast and ovary than in other tissues. Mechanisms of LOH are tumour- and chromosome specific (Lasko *et al.,* 1991) but still very little is known about the mechanisms of LOH in epithelial tumours in general. Non-disjunction with chromosome loss or with reduplication, interstitial and terminal deletion, gene conversion, mitotic recombination between homologous chromosomes and translocations are several mechanisms by which a cell might undergo LOH.
Among the different mechanisms, mitotic recombination has a key role in tumourigenesis and seems to be major cause of LOH in mice (Shao *et al*., 1999).

As mentioned in the Introduction, the human *BRCA1* gene is unusual in its high content of *Alu* sequences. It has been proposed that these sequences mediate recombination during meiosis promoting the deletion of large portions of the *BRCA1* gene and are at the origin of several germline mutations (Welcsh *et al*., 2001). One speculative idea would be that mitotic recombination between *Alu* sequences in somatic cells might account for the loss of the wild-type allele and that the recombination rates would vary in a tissue-specific manner. Some experimental evidences point to the possibility that mitotic recombination would indeed vary in a tissue-specific fashion, like in mice (Shao *et al.,* 2001). In fact, in human lymphocytes there is interindividual variation in mitotic recombination rates leading some authors to propose that it could be the basis for differences in lifetime risk of cancer (Holt *et al*., 1999). It is conceivable, although still speculative, that a basis for differences in mitotic recombination rates could be the tissue-specific chromatin environment at a particular locus.

One possible explanation for a tissue-specific chromatin environment might derive from differential transcription rates at the *BRCA1* locus. *BRCA1* transcripts are most abundant in testis and thymus, breast and ovary and at maximal levels in phases of proliferation. Moreover, *Brca1* expression is modulated during postnatal development of the mammary gland. Hormones acting on the mammary gland like estrogen and prolactin (Favy *et al.,* 1999) have been shown to upregulate *BRCA1* expression. Nevertheless, the structure of the *BRCA1* gene promoter does not fit the description of a highly regulated gene since it does not contain a TATA box but “initiator elements”, which are frequently involved in the expression of “housekeeping” genes (Yang *et al*., 2007).

Against this hypothesis some recent evidences suggest that phenotypic effects may result from BRCA1 haploinsufficiency, without the need to loose the remaining wild-type allele (King *et al*., 2007; Cousineau *et al*., 2007). A concept already suggested for the function of the tumour suppressor p53 in 1998 (Venkatachalam *et al.,* 1998).

-Modulation of steroid hormones' action on their target tissues. *In vitro* and *in vivo* findings, until present, do not point to a unique hypothesis. Nevertheless, one of the most argued models is the one that searches for a role of BRCA1 in regulating the activity of ovarian hormones on its target tissues. Absence of functional BRCA1 might result in unregulated activity of ovarian hormone receptors on their target tissues, such as breast and ovary, and so it could explain tissue specificity. In addition, several observations underline the existence and importance of this relationship.

We have previously reviewed the importance of ovarian hormones for the development, proliferation and differentiation of the normal human mammary gland and its implication in breast cancer. Both estrogen and progesterone act in the mammary gland through the activation of signaling cascades (endocrine or paracrine) and the induction of transcriptional programs so that they induce cyclical and profound changes in the mammary gland that, by all means, need to be strictly regulated and kept in pace. Taking this into account, is not surprising to observe that many known breast cancer risk factors have to do with exposure and action of ovarian hormones on this tissue or, even, on breast cancer stem/progenitor cells. Early age at menarche, regular ovulation, late age at menopause, alcohol intake and use of oral contraceptives and postmenopausal hormones, all increase exposure to ovarian hormones and are associated with increased breast cancer risk. Breastfeeding, increased parity and an earlier age at first pregnancy are associated with reduced risk in the majority of breast cancers.

Although they share most of the risk factors associated to breast cancer in the general population, the results of several population-based studies suggest that the usual protective effect of early age at first birth and increasing parity is not observed in BRCA1-mutation carriers (Hartge *et al.,* 2002; Jernstrom *et al.,* 2004). In fact, BRCA1-mutation carriers are particularly susceptible to develop breast cancer because of pregnancy (Jernstrom *et al.,* 1999). BRCA1 has been particularly implicated in the normal morphogenesis of the mammary gland during pregnancy (Hoshino *et al*., 2007) and, what is more, *Brca1* is induced in waves corresponding to the periods in which intense proliferation and morphogenesis take place in the mammary gland.

During pregnancy, progesterone and prolactin activate the "*alveolar switch*", a genetic program that coordinates changes in mammary epithelial cell proliferation, migration, differentiation and apoptosis (Oakes *et al*., 2006). It consists of a first proliferative phase, in which epithelial cells proliferate within the ductal branches and developing alveoli; a second differentiative phase and a final involution phase, in which surplus cells are removed by apoptosis. It is clear from the role of progesterone in the massive tissue remodeling that takes place in the mammary gland that pregnancy might constitute a real risk factor in a scenario where progesterone might act in an uncontrolled way, like, as it is proposed, in the absence of functional BRCA1.

There are other observations that allude to a close functional relationship between BRCA1 implication in tumourigenesis and progesterone and estrogen action in the breast. For instance, there is a low incidence of breast cancer among BRCA1-mutant carriers women after 49 years and among men, in both

cases with very low ovarian hormones level (Liede *et al.,* 2004). Studies on the effect of prophylactic oophorectomy have provided some of the most convincing evidence that hormone-related factors influence the risk of breast cancer in BRCA1-mutation carriers. This procedure removes the major source of the two hormones, estrogen and progesterone. Prophylactic oophorectomy was associated with a 59% reduction in risk of contralateral breast cancer in BRCA1- mutant carriers (Metcalfe *et al.,* 2004).
BRCA1 was shown to inhibit the transcriptional activity of the estrogen receptor (Zheng et al., 2001; Fan *et al.,* 1999 and 2001; Xu *et al*., 2005) and cancer-associated BRCA1 mutants failed or were unable to repress ERα activity.

There are, also, some evidences that argue against this hypothesis. One of them is that analyses of BRCA1 tumours have revealed that they are mostly ER and PR-negative. Evidence from a breast cancer prevention trial (King *et al.,* 2001) indicates that cancer risk was not significantly reduced by the use of tamoxifen, an anti-estrogen, in women carrying mutations in BRCA1. This result would suggest that either the tumours initiate from ERα-negative cells, or that loss of ERα is an early event in tumour development.

Nevertheless, some recent publications propose that ERα-negativity is likely to occur after loss of the wild-type allele of BRCA1. In one of them (Li *et al.,* 2007), they observe that, in mouse models lacking the full-length form of BRCA1, ER-α is highly expressed in the premalignant mammary gland and initiation stages of tumourigenesis, although its expression is gradually diminished during mammary tumour progression. The absence of BRCA1 turns the proliferation of ERα-positive cells from a paracrine to an autocrine or endocrine fashion. Consequently, BRCA1-mutant cells are sensitized to estrogen-induced cell proliferation *in vitro* and mammary tumourigenesis *in vivo*. Similarly, King et al (King et al., 2002) observed that progesterone receptor expression was significantly higher in benign mammary epithelial cells adjacent to a BRCA1 mutant breast cancer than in sporadic cancers. Another study (Hosey *et al*., 2007) proposes that the lack of BRCA1 activity has a direct effect on the ER-α gene transactivation, resulting in the loss of ER-α mRNA and protein expression.

Therefore, so far, it remains unclear whether these breast tumours originate from ERα-negative (and hence PR-negative) cells or whether receptor expression is lost during tumour development. In the next section, this aspect is commented from the point of view of the cancer stem cell hypothesis, as well.

In this work, we wanted to explore the existence of a relationship between BRCA1 and the action of the progesterone receptor in mammary cell lines, at the cellular and molecular level. For this, we have mainly used breast cancer cell line models and *in vitro* experiments and have obtained evidence for a relationship BRCA1/PR at the mechanistic level. The data presented here in combination with existing data in the literature will be discussed from the point of view of their physiological significance and we will propose an integrative model.

R.1 BRCA1 alters progesterone receptor transcriptional activity

In 1999 BRCA1 was first described to affect the transcriptional activity of ERα in breast and prostate cancer cell lines (Fan *et al*., 1999). BRCA1 was shown to inhibit the ligand-independent activity (Zheng *et al*., 2001) and the ligand-dependent activity of ERα (Fan *et al.,* 2001; Xu *et al*., 2005). At the beginning of this thesis project, the relationship between BRCA1 and estrogen signaling had been centring the attention in the search of a liaison BRCA1 function-ovarian hormones activity in the mammary gland. This is due to the general belief that estrogen is the main circulating hormone responsible for the normal development of the mammary gland and a major stimulus for proliferation once the onset of breast cancer is declared. However, to date, the biology of progesterone in the mammary gland and in tumour cells is better understood and, therefore, its implication in breast cancer tumourigenesis is gaining interest.

First, we examined if BRCA1 levels had any *effect on the transcriptional activity of PR*. As a first approach, we tested the effect of overexpressed BRCA1 on the transcriptional activity of the progesterone receptor in an exogenous system. One problem was that previous studies indicated that BRCA1 overexpression can result in induction of high levels of spontaneous apoptosis (Thangaraju *et al.,* 2000; Yan *et al*., 2002) or prolonged cell cycle arrest incompatible with life (MacLachlan *et al*., 2000). Another concern was the correct overexpression of the protein, since it is a large protein (around 220 kDa) and it may be difficult to obtain enough amounts of full-length transcripts and protein. In consequence, we checked that the protein was correctly overexpressed at the correct size by Western blot analysis. To test its functionality, we analized its ability to coactivate p53-dependent transcription (Zhang *et al,* 1998). We verified that the overexpressed full-length protein did not induce apoptosis and could coactivate p53-dependent transcription.

We proved that the transfection of BRCA1 inhibited, to a certain extent, the transcriptional activity of transiently transfected ER and PR in 293T and MCF7 cells. Although the effect on ER was not so evident, in the case of the PR reporter, the inhibition of the PRE-luciferase construct (MMTV-Luc) was of around 50%. However, the eukaryotic genome is structurally organized into nucleosomes to form chromatin. During gene expression, there is a need for distinct multiprotein complexes to modulate higher-order chromatin structure (Eissenberg *et al*., 2001), modify nucleosomal structures (Narlikar *et al.,* 2002), and bind to regulatory sequences to initiate transcription. The organization of DNA in chromatin influences the ability of transcription factors like steroid hormone receptors to interact with their cognate recognition sites (Wolffe *et al*., 1993) and, at the same time, they can interact with the repressive chromatin structure and remodel the chromatin to allow other transcription factors to bind (Hager *et al.,* 2000). Transiently transfected DNA does not assume the highly organized chromatin structure characteristic of stable, replicating templates (Archer *et al.,* 1992) and so may not fully replicate the steroid receptor transactivation process as it occurs on promoters within chromosomes (Deroo *et al*., 2001).

To determine whether this weak effect might be due to the artificial nature of the transient transfection assay, we examined the effect of BRCA1 in a chromatin environment on a stably-integrated MMTV-Luc reporter and endogenous genes, in the breast cancer cell line T47D, that expresses endogenous PR. In addition, we overexpressed BRCA1 using an adenoviral vector instead of transient transfection. These recombinant adenoviruses are capable of transducing high-level transgene expression to a wide variety of cell lines with a high yield of infection (Campbell *et al.,* 2001; Le Page *et al*., 2000).
The overexpression of BRCA1 clearly inhibited the expression of the tested genes. At shorter times of hormone treatment (4,5h) BRCA1 overexpression inhibits the progestin-induced expression of the upregulated genes. However, at longer times of hormone treatment (6h) it is noteworthy that BRCA1 inhibited as well the basal level of expression (that is, in the absence of hormone induction) of some of the tested genes. Oddly, we also observed that BRCA1 overexpression had the same inhibitory effect on genes selectively downregulated by hormone addition, like *CCNG2* and *CHD4*.

To corroborate these data, we examined the transcriptional activity of PR in an endogenous system in the absence of functional BRCA1, by RNA interference. It is important to note that, as other tumour suppressor genes, BRCA1 follows the "two-hit hypothesis" (Knudson, 1971), which implies that both alleles that code for BRCA1 must be affected before an effect is manifested (a process called *loss of heterozigosity* (LOH)). This is due to the fact that if only one allele is damaged, the second can still produce sufficient correct protein. That is the reason why we combined different strategies in order to obtain the greatest downregulation of the protein possible.
After BRCA1 knockdown, we detected a clear upregulation of progestin-induced genes. The knockdown of BRCA1 produced also a decrease in the basal expression and enhanced the repression of progestin-downregulated genes.

Examined PR-target genes are regulated through a variety of mechanisms. Quiles *et al* (Quiles *et al*., 2009) dissected some of these mechanisms of gene expression through the establishment of breast cancer cell lines expressing engineered mutant forms of PR-B. They describe *CCND1* as a gene that depends on the transcriptional genomic action of PR and on the activation of signaling pathways initiated by the interaction of PR with ER at the cytoplasm. Transcript accumulation is maximal at 6 h. They observed that the majority of progestin-induced genes fall into this mechanism of regulation by PR, as seems to be also the case for *EGF* and *DUSP1* (Vicent *et al*., 2006). *11β-HSD* have been described to be regulated by PR through the STAT5A-mediated recruitment of PR to a distal promoter region and through the activation of the JAK/STAT signaling pathway (Subtil-Rodríguez *et al*., 2008). *HEF-1* (*NEDD9*) is believed to be induced mostly by the PR-A isoform (Richer *et al*., 2002).

It should be emphasized that the effect of overexpressed or BRCA1 knockdown on PR transcriptional activity is not caused by non-specific transcriptional repression of the RNAPII since other genes like *GAPDH,* or even other progestin-regulated genes like *c-FOS* and *NCOA3* (data not shown), are not affected by the BRCA1 overexpression or knockdown. Although BRCA1 can associate with the RNAPII (Horwitz *et al*., 2006), other studies have shown that

BRCA1 modulates transcription of only a subset of genes, likely through interaction with specific transcription factors. Thus, BRCA1 has little or no effect on transcription controlled by transcription factors like Jun, Fos, Gal4, USF, E2F-1 or Sp1 (Fan *et al*., 2002).

In conclusion, BRCA1 affects the transcriptional activity of PR on a wide variety of target genes. Surprisingly, while the knockdown of BRCA1 enhances the transcriptional induction and repression of PR-target genes (positively or negatively regulated respectively), the overexpression of BRCA1 provoked the inhibition on both types of PR-regulated genes. During the course of this thesis, another group also published that BRCA1 affected PR transcriptional activity (Ma *et al*., 2006) though the mechanism was not resolved.

R. 2 Mechanism by which BRCA1 affects PR transcriptional activity

Even though the relationship of BRCA1 and the estrogen receptor was described about ten years ago, there is just a few scattered and even contradictory data concerning the mechanism by which BRCA1 affects the transcriptional activity of this receptor. Similarly, although recently described, the relationship between BRCA1 and PR is starting to be described in more detail and it has captured our interest.

Physical interaction between BRCA1 and the progesterone receptor

One of the most robust data implying a direct relationship between ERα and BRCA1 was the finding that the two proteins interact *in vivo* in breast cancer cell lines (Fan *et al.,* 2001). The BRCA1:ERα interaction was ligand independent and was mapped to the N-terminus of BRCA1 (amino acids 67–100 and 101–134) and AF-2 domain of ERα (amino acids 338–379 and 420–595) hormone (Fan *et al.,* 2001; Ma *et al*., 2005). Two tumour-associated BRCA1 mutations at the interacting surface between BRCA1 and ERα (L63F and I89T) were found to impair the ability of BRCA1 to repress ERα activity. Besides, BRCA1 contains a conserved helical motif (amino acids 86–95) resembling a previously identified nuclear corepressor motif (Lxx(I/H)Ixxx(I/L), where x=any amino acid), the mutation of which disrupted the ability of BRCA1 to bind and repress ERα (Ma *et al*., 2005). These evidences support the idea that the physical interaction between BRCA1 and ERα is functionally important.

This observation prompted us to investigate if BRCA1 would also interact with PR. Although, due to its inherent transcriptional activity, we were incapable of using the yeast two-hybrid for this purpose, we studied the interaction by *in vivo* co-immunoprecipitation. We detected a *ligand-independent interaction between BRCA1 and the two isoforms of PR*, A and B, as well as with ERα.
Since Ma *et al* (Ma *et al*., 2006) mapped the interacting regions to the amino acids 1-324 and the C-terminus aa 1314-1863 in BRCA1 and 166-456 and 457-687 in PR, we have not attempted to repeat this work. Surprisingly, these regions do not overlap with the interacting regions between BRCA1 and ERα. This might also explain why we were unable to detect the interaction between the BRCA1 mutant constructs and PR-B at the yeast two-hybrid

system, since the two point mutants used had the mutations localized right at the C-terminus of BRCA1 (aa 1756 and 1853).

BRCA1 regulates the degradation of the progesterone receptor by the ubiquitin-proteasome system

The level of expression of transcription factors that act in conjunction with RNAPII to effectively synthesize mRNA are critical to transcriptional regulation. Consequently, the level and activity of transcriptional activators and repressors are tightly controlled. Accumulating evidences indicate that ubiquitin plays an important role in transcriptional regulation through proteasome-dependent and –independent mechanisms (Muratani and Tansey, 2003). The ubiquitin-proteasome pathway tightly regulates a large number of key transcriptional regulators including p53, c-Fos, c-Jun and also nuclear receptors (Nawaz and O'Malley, 2004).

PR protein is not an exception. Its sequence indicates that the protein is expected to be quite unstable, with an instability index of 64,32 (above 40 is considered to be unstable, from ProtParam at ExPASy server) and an estimated half-life of around 30 hours (in mammalian reticulocytes *in vitro*), which was calculated *in vivo* to be around 20 hours (Nardulli *et al.,* 1988). In addition to its inherent instability, PR protein level is extensively and rapidly downregulated in response to ligand binding (Nardulli *et al*, 1988), changing the half-life of the liganded receptor to around 6-8 h.

Protein molecules are continuously synthesised and degraded in all living organisms. The concentration of individual cellular proteins is determined by a balance between the rates of synthesis and degradation, which, in turn, are strictly regulated processes. Protein degradation exhibits first-order kinetics unlike protein synthesis, which is zero-order. Protein degradation is energy dependent and is limited by the concentration of the reactants, whereas protein synthesis cannot be completed in the absence of any one of the necessary reactants. Usually, degradation rates are measured in chase experiments with cycloheximide treatment (a protein synthesis inhibitor). We calculated an approximate degradation rate without cycloheximide treatment because the cycloheximide addition at 20 ug/ml caused the almost total inhibition of the progestin-induced degradation of the receptor. This behaviour has also been observed in ERα degradation (Nonclercq *et al*., 2004).

One of our more remarkable observations regarding the relationship between BRCA1 and PR was that there is an *increase in the basal amount of PR protein* in the BRCA1-knockdown cells compared to control cell lines, which, importantly, did not correlate with an increase in the PR mRNA level. In concordance, the overexpression of BRCA1 decreased the amount of PR protein.

As regards the amount of PR protein expressed in the presence of hormone, we observed that *BRCA1 also participates in the hormone-triggered degradation of the receptor* since the absence of functional BRCA1 affects the half-life and protein degradation rate of the protein in the presence of progestin. However, BRCA1 is not indispensable for this hormone-driven degradation of the receptor and other mechanisms must be working in the absence of BRCA1.

Ligand-independent and dependent degradation of PR is mediated by the ubiquitin and 26S proteasome system (Lange *et al*, 2000; Shen *et al*, 2001). This process can be blocked by specific inhibitors of the 26S proteasome leading to the accumulation of ubiquitinated PR species (Lange *et al*., 2000). To date, only one enzyme, CUEDC2, has been described very recently to be directly implicated in the ubiquitination of the receptor, by promoting PR ubiquitination at Lys388 (Zhang *et al*., 2007).

Of all the interacting protein partners of BRCA1, the association with BARD1 to form a very stable and predominant heterodimer in the cell has been gaining more interest and significance. The heterodimer displays E3 ubiquitin ligase activity and this is thought to contribute to many of the biological functions of BRCA1, as well as its tumour suppressor activity (Baer and Ludwig, 2002).

Following with the attempt to parallel the BRCA1-ERα relationship, recently, ERα was described as a putative substrate for the BRCA1/BARD1 ubiquitin ligase activity (Eakin *et al.,* 2007). The ligand-binding domain (at K302) of ERα is monoubiquitinated by BRCA1/BARD1 *in vitro* but whether this happens also *in vivo* and its biological implications are not known.

We wanted to explore the possibility that BRCA1 might be altering PR content and degradation by means of its E3 ubiquitin ligase activity. Our *in vivo* and *in vitro* experiments confirm that the *BRCA1/BARD1 heterodimer*, with the UbcH5c E2 enzyme, *specifically ubiquitinates PR-B*. In these particular experiments, we tested only the PR-B isoform, which is flag-tagged in the T47D-YV-PRflag cell line and it is also the isoform generated and purified for *in vitro* experiments. Besides, in the two cases the receptor was activated by the presence of hormone in the preparation of the extract or in the procedure of purification. We would expect to obtain the same results of ubiquitination *in vivo* and *in vitro* for the PR-A isoform, and specially on unliganded receptors, according to our previous results showing that BRCA1 affects the amount of both isoforms of the receptor present in the whole cell and independently of hormone binding.

We wanted to further characterize the ubiquitination of PR-B by BRCA1 and looked for the residue that was being ubiquitinated and subsequently was leading to the degradation of the protein. A few residues have been related to the ubiquitination and stability of PR protein in the literature. Lys388 is the best characterized one, as the primary site of SUMO attachment and it was also found to be a ubiquitin target site (Man *et al.,* 2006; Zhang *et al*., 2007). The region encompassing aa 1-162, corresponding to the PR-B-specific AF-3 domain, was also found to be important for the ligand-induced degradation of the receptor (Tung *et al*., 2006). Ser400, was proposed to be a regulator for the degradation of immature or unliganded receptor (Pierson-Mullany and Lange, 2004), while Ser294 phosphorylation seems to be a key regulator of ligand-dependent PR degradation (Lange *et al*, 2000; Shen *et al*, 2001).

A few PEST sequences are found along the sequence of the receptor, being the one at region 211-238 the highest scored one in an in silico PESTfind analysis.

Overexpression of BRCA1 affected both the full length PR-B as well as the PRΔ165-354, which lacks the 211-238 PEST sequence, indicating that this region do not include residues crucial for the BRCA1-induced degradation of

PR. It remains to be explored if mutation of Lys388 disrupts the ability of BRCA1 to regulate PR stability and if phosphorylation at Ser400 has some role in the degradation of unliganded receptor.

Another aspect that remains to be studied is the type of Ub chain catalyzed by BRCA1 on PR protein. BRCA1 was shown to catalyze, primarily, Lys 6 ubiquitin chains. However, this type of linkage does not seem to signal for degradation of the substrate, at least as observed on the autoubiquitination of the BRCA1/BARD1 heterodimer (Wu-Baer *et al.,* 2003). BRCA1 can also catalyze the formation of Lys 48 ubiquitin chains, which typically send target proteins to degradation via the 26S proteasome. Among the few described substrates of the BRCA1/BARD1 E3 ubiquitin ligase activity RNAPII is another example of protein targeted for ubiquitination and degradation (Starita *et al*., 2005; Horwitz *et al*., 2006).

Although more experiments are needed to confirm the exact implication of **BRCA1 on ligand-independent and –dependent PR degradation**, we could envision the following **model** (Fig. I) taking into account the data obtained experimentally and already published information.

In the absence of hormone, BRCA1 might be the E3 ubiquitin ligase in charge of the degradation of unstable intermediate PR-chaperone complexes. According to existing data, this process might be regulated by CDK2 activity at several levels. Active cyclin A/CDK2 complex can phosphorylate BRCA1 on tyrosine residues (Wang *et al*., 1997) and inhibit the ubiquitin ligase activity of the heterodimer BRCA1/BARD1 (Hayami *et al.,* 2005), thus preventing the ubiquitin ligase activity of the heterodimer on PR. This would occur, principally, during the S phase of the cell cycle, when the cyclin complex is active. This phase also coincides with the peak of PR transcriptional activity (Moore *et al.,* 2007). Moreover, CDK2-dependent Ser400 phosphorylation might enhance the basal transcriptional activity of PR and is required for increased PR nuclear localization (Pierson-Mullany and Lange, 2004; Moore *et al*., 2007). On the other hand, BRCA1 induces p21, a potent inhibitor of CDK2 (MacLachlan *et al*., 2000). Likewise, DNA damage-induced ATM activation signals through a comprehensive network of proteins leading to CDK2 inhibition thus preventing DNA synthesis (Woon *et al.,* 2003) and favouring BRCA1 action. Therefore, CDK2 and BRCA1 mutually control their activity and they must be strictly regulated since the lack of functional BRCA1 and abnormal activation of CDK2 both contribute to the formation of basal-like breast cancers (Corsino *et al*., 2008).

Consequently, the lack of BRCA1 would result in the accumulation of basal PR protein to a level higher than the one found in homeostasis. It is likely that part of this excess of PR protein is not folded in the proper hormone-binding conformation and so might be not functional, but certain amount of this excess could increase the supply of activated PR.

In the presence of hormone, other machineries, likely CUEDC2, might also operate in PR degradation. It is not unprecedented that a single protein is targeted by multiple E3 ligases. For example, p53 can be targeted by several E3s (Dornan *et al.,* 2004).

Although mainly regulated through MAPK pathway activation and Ser294 phosphorylation, CDK2 also favours ligand-induced PR degradation, through Ser400 phosphorylation (Lange, 2004). It also alters PR function indirectly by increasing the recruitment and activity of the coactivator SRC-1 (Moore *et al.,* 2007). Progestins activate CDK2, perhaps allowing for the coordinate regulation of PR action during cell cycle progression.

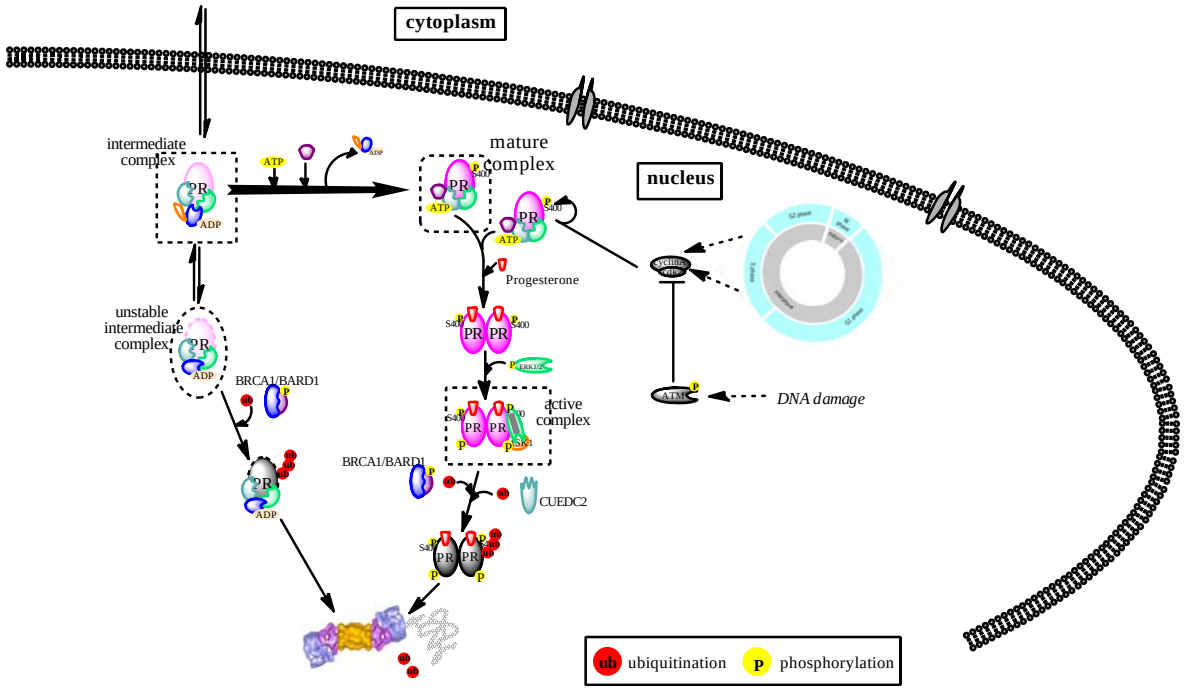


Figure I: Model for the role of BRCA1 in ligand-independent and dependent PR degradation

Finally, in order to directly relate the E3 ubiquitin ligase activity of BRCA1 with the inhibitory effect of BRCA1 over PR transcriptional activity, we performed a series of experiments with the mutant I26A-BRCA1. This mutant displays a disabled E3 ubiquitin ligase activity due to its inability to effectively interact with the E2 ubiquitin-conjugating enzyme (Brzovic *et al,* 2003). Our preliminary observations indicate that the *E3 ubiquitin ligase activity of BRCA1 is indispensable for the inhibition of PR transcriptional activity*. Similarly, it was found that mutants that disrupt the RING domain structure of BRCA1 (C61G and C64G) failed to inhibit ERα transcriptional activity (Fan *et al.,* 200a). These mutants lack the E3 ubiquitin ligase activity (Ruffner *et al.,* 2001).
Indeed, the other E3 ubiquitin ligase described for PR ubiquitination, CUEDC2, is also a regulator of PR activity. CUEDC2 interacts with PR, repressing the transcriptional activity of PR and blocking progesterone signaling. For the repression of PR activity it was essential the ubiquitinable Lys 388 of PR, since,

otherwise, CUEDC2 was unable to induce the degradation of PR and inhibit its activity (Zhang *et al.,* 2007).

The last experiment of this section gave us one more clue about the mechanism of action of BRCA1 over PR activity. We overexpressed BRCA1 in T47D cells and checked by qRT-PCR the expression of PR-target genes upon the addition of hormone and co-treatment with the proteasome inhibitor lactacystin. Although the analysis of a more exhaustive list of genes is pending, we observed two different behaviours. On the one hand, the treatment with the inhibitor reverted the inhibition of *DUSP1* transcription by BRCA1 overexpression. On the other hand, the inhibitor had no effect over the inhibition of *11β-HSD* expression. These two different outcomes would be telling us about *two different mechanisms of action*. In the case of *DUSP1*, the inhibitory effect of BRCA1 is reverted by the use of lactacystin and it might indicate that BRCA1 is necessary working by means of the proteasome system, probably through PR degradation. In the case of *11β-HSD*, the effect of BRCA1 is not reverted by the drug even though the ubiquitin ligase activity of BRCA1 is essential to drive this inhibition, according to the prior result (still, preliminary). This would indicate that the inhibitory effect of BRCA1 is acting through ubiquitination but not necessarily leading to the proteasome action and degradation.

The inhibitor of the proteasome, lactacystin, did not cause any significant change in the expression of PR-target genes, in accordance with previous reports (Shen *et al.,* 2001). The facts suggest that, under select conditions, multiple nuclear receptors are degraded concomitantly with transcriptional activation induced by these same receptors. In general, the addition of the 26S proteasome inhibitors MG132 and lactacystin were inhibitory to nuclear receptor function, indicating that the prevention of degradation may be deleterious to regulation of transcription by certain receptors.

In the case of the *androgen receptor*, inhibition of the proteasome suppresses AR transactivation, AR nuclear translocation, and interaction between AR and AR coregulators (Lin et al., 2002).

In contrast, inhibiting proteolysis of the *glucocorticoid receptor* by the inhibitor MG132 results in an increase in GR-mediated transcriptional activation of the MMTV promoter, in an open or closed chromatin structure (Deroo *et al*., 2002). Inhibition of the proteasome results in an increase in the global and promoter levels of trimethyl histone H3K4 and phospho-RNAPII but not in more GR being recruited (Kinyamu and Archer, 2007). In the same line, Stavreva *et al* (Stavreva *et al*., 2004) propose that longer GR residence at the MMTV may enhance initiation complex formation and that proteasome inhibition favours GR occupancy, while chaperone inhibition favours GR loss. The equilibrium between these two components helps to set the transcriptional level.

Proteasome inhibition abolished *estrogen receptor* transcriptional activity. It is likely that ubiquitinated ER is transcriptionally incompetent, as on inhibition of proteasome activity transcription does not occur and RNAPII is never recruited to the pS2 promoter. When proteasome activity is blocked, the pS2 promoter remains associated with unidentified ubiquitinated proteins. These proteins may be directly responsible for preventing transcription either by masking the ERE or through epigenetic closure of the promoter (Reid *et al*., 2003). MG132 partially blocked the basal transcription of an ERE-dependent reporter and modified the

ability of E2 to induce its expression (Laïos *et al.,* 2005). However, Fan *et al* (Fan *et al*., 2004) described that proteasomal degradation is not essential for transcriptional activity of ER and that the ubiquitin-proteasome system functions to limit E2-induced transcriptional output at longer time points. At early time points the use of the inhibitor decreases E2-induced pS2-reporter expression. They propose that proteasome inhibition can have promoter-specific effects on gene transcription, maybe dependent on the type of ERE sequence.

In a genomic approach designed to examine the impact of proteasome inhibition on GR and ER-mediated gene expression in MCF-7 cells, transcript profiling revealed that inhibiting proteasome activity modulates gene expression by GR and ER in a similar manner in that several GR and ER target genes are upregulated and others downregulated after proteasome inhibition (Kinyamu *et al*., 2008).

In the case of the *progesterone receptor*, Qiu *et al* (Qiu *et al*., 2003) reported that PR in lactacystin-treated cells appears to be stabilized because it fails to be exported from the nucleus and degraded. This stabilized PR that is trapped within the nucleus is transcriptionally impaired, suggesting that ubiquitinated PR is transcriptionally inactive. In a similar study, Dennis *et al* (Dennis *et al.,* 2005) attributed the suppression of PR-mediated gene expression in lactacystin-treated cells to a decrease in RNAPII recruitment, although this was not specific to PR but generalized to a subset of RNAPII-regulated genes.

Nonetheless, Shen *et al* (Shen *et al.,* 2001) showed that, upon a similar lactacystin treatment procedure as used by us, the inhibitor of the proteasome inhibited the hyperactivation of PR when it was enhanced by hyper-phosphorylation, but did not affect the normal level of activation, in accordance with our results.

Our observations do not contradict the belief that rapid degradation and efficient active transcription by PR are linked, since BRCA1 seems to be more involved in the unliganded PR degradation than on the progestin-induced turnover. In any case, the ubiquitin-proteasome implication on transcriptional activation is considered to be a mechanism of fine-tuning for limiting ER or PR-dependent gene transcription and imparting a continuous response to hormone signaling (Reid *et al*., 2003).

Besides, it is necessary to differenciate between the effects of ubiquitination and degradation, which, in the case of steroid hormone receptors, are normally sequential events in the cell. If separated one from the other the effect is expected to be different. While the inhibition of degradation may inhibit transcription driven by ER or PR, as mentioned before, in these conditions, ubiquitinated forms of the receptors do accumulate in the cell, and it is likely that ubiquitinated receptors are transcriptionally incompetent (Reid *et al.,* 2003). On the other hand, if the succession of events is stopped before ubiquitination of the target protein, it would lead to accumulate non-ubiquitinated forms of the receptor, that might be available for activation by the hormone, and then, maybe, an increase in the recruitment of the receptor at the promoter of regulated genes would be observed.

To sum up, we found that BRCA1 affects the content of PR protein in the cell in a basal situation and also affects the ligand-triggered degradation of the

receptor. This effect is probably related to the capability of BRCA1 to ubiquitinate PR-B *in vivo* and *in vitro*.

BRCA1 effects at the promoter of PR-regulated genes

We have demonstrated in our experiments that *BRCA1 is present at the promoter*, specifically at the hormone-responsive region*, of PR target genes* like the MMTV promoter, *11β-HSD* or *EGF* promoters, which get affected by the lack or presence of functional BRCA1 in our transcription assays. BRCA1 has previously been found associated to the promoter of regulated genes and of particular interest is the case of ERα-regulated genes.

There are opposite reports regarding the presence of BRCA1 at the promoter of ERα-regulated genes. BRCA1 has been described to be present at the promoter of the ERα-target gene pS2 at the basal state and then leave the promoter upon hormone addition (Zheng *et al.,* 2001), although a recovery of the signal was detected at 12 hours of hormone addition (earliest time point tested) in another publication (Wang *et al*., 2005). This recovery might reflect an even earlier recovery of the protein, that would be in agreement with our observations (if we consider to exist some parallelism between the BRCA1-ERα and BRCA1-PR relationship) since in our experiments the signal is recovered between 30 min or 1h after hormone addition.

However, in a recent publication BRCA1 was found to follow a cyclical pattern of recruitment, induced by the treatment with hormone, at the HRE of the ER-regulated gene *CyclinD1* (Wen *et al.,* 2008). These differences might reflect a different regulatory behaviour of BRCA1 in these two genes, even though been both ER-regulated genes, or simply different experimental conditions or particular differences between cell lines, T47D and MCF7 cells. We have also experienced some fluctuations in the levels of BRCA1 detected at the promoter of PR-target genes (specially the basal level) that might be attributed to the same factors. Our finding that BRCA1 is also able to affect the basal level of expression of some target genes would point to the prior presence of BRCA1 at the promoter of those genes.

The next question regarding the presence of BRCA1 at these promoters was if PR could be the sequence-specific DNA binding protein tethering BRCA1 to DNA. Our chIP experiments +/- PR would indicate that the *progestin-enhanced recruitment of BRCA1 is PR-dependent*. Our immunoprecipitation assays showing the physical interaction of the two proteins would further support this idea. However, our results do not rule out the possibility that BRCA1 enhanced recruitment upon progestin treatment could be due to an incorporation of the protein to actively transcribed genes that, in this case, would only occur in the presence of PR protein and upon progestin treatment.

On the other hand, these experiments do not give an answer to the *presence of BRCA1 at time 0* of progestin treatment. We envision diverse explanations for this. The first one is that BRCA1 could be tethered to the promoter of regulated genes by the interaction with the RNAPII holoenzyme (in concrete, BRCA1 interacts with the polymerase II and the basal transcription factors TFIIF, TFIIE and TFIIH (Scully *et al.,* 1997)). This would imply the presence of certain amount of RNAPII at the promoter of these genes. Previous

characterization of the MMTV promoter revealed that, although the promoter is hormone inducible, there is some basal transcriptional activity along with constitutive binding of factors such as NF-1, as determined by footprinting assays (Mymryk *et al*., 1995).

Recent global analyses have determined that many human and Drosophila genes have engaged polymerases molecules trapped at a promoter-proximal site (Price, 2008; Nechaev and Adelman, 2008; Wade *et al*., 2008; Core and Lis, 2008). Notably, this behaviour is prevalent at genes involved in development and response to stimuli, suggesting that this mechanism of action plays an important role in rapid and precise control of gene expression. At these genes, RNAPII begins transcription but stalls after synthesizing a short RNA, and it is the release of this engaged polymerase from the promoter-proximal region that is rate limiting for transcription. In fact, it is possible that BRCA1 presence could even serve to stabilize the preinitiation complex on the core promoter, as previously published (Horwitz *et al*., 2006).

A second possibility would implicate the existence of unliganded PR bound to the promoter of regulated genes, which would be tethering BRCA1 to the promoter in absence of hormone. There are some reports implying that PR is capable of binding to HREs in the living cell in the absence of added ligand, whereas hormone agonists further increase this interaction (Gass *et al*., 1998). Nevertheless, important aspects for the binding of the receptor to its target sequence like dimerization, disassembly from hsp proteins, phosphorylation and conformational changes are quite strictly dependent on ligand binding (Truss and Beato, 1993; Dilworth and Chambon, 2001). A last possibility is that BRCA1 might be recruited through the interaction with other transcription factors like STAT1, Oct-1, NF-κB, c-Myc, ELK-1 or ZBRK1 (reviewed at the Introduction). More experiments are needed to give an answer to this open question.

Keeping in mind the fact of the presence and recruitment of BRCA1 at the hormone-responsive region of PR-target genes, we had necessarily to focus our attention on the effect that BRCA1 might have on this scenario, on the main actors enroled in the transcription function: transcription factors, cofactors, basal machinery and chromatin status. Moreover, we had to take into consideration the evidence that the E3 ubiquitin ligase activity of BRCA1 might be essential for its interference with PR-driven transcription.

We firstly checked how the *profile of PR recruitment* was affected by the lack of BRCA1. According to our chIP assays, BRCA1 overexpression reduces the amount of PR recruited, although the differences detected were not that striking to suppose it would explain the strong transcriptional effect. Similarly, the changes in PR function observed along cell cycle are not attributable to changes in the level of PR recruitment but of coactivators like SRC-1 and -3 and CBP (Narayanan *et al*., 2005). Besides, the effect of BRCA1 on PR is not attributable either to an effect of ligand affinity of the receptor (P.Katiyar personal communication).

It has also been investigated if the BRCA1 inhibitory effect on ERα could be explained by a change in the recruitment of ERα, and again the results were contradictory. While the effect of BRCA1 on the transcription of the pS2 gene by ERα was suggested to be due to reduction of the recruitment of the receptor to the promoter (Wang *et al.,* 2005), the effect on the transcription of the *CyclinD1*

gene was not due to a change in ERα recruitment but of cofactors (Wen *et al.,* 2008).
In conclusion, the effect of BRCA1 on the total amount of PR, before and after hormone addition, might represent a secondary mechanism of control of PR transcriptional activity, according to our observations.

This led us to another important actor in the plot; namely, *coregulators*.
It has long been proposed that the diverse functions of BRCA1 manifest through its ability to interact with many different proteins. Among these proteins, there are proteins involved in transcriptional repression like CtIP (Yu *et al.,* 2000), RB1, RbAp46 and 48 and histone deacetylases HDAC1 and 2 (Yarden *et al*., 1999). We reasoned that if the overexpression of BRCA1 enhances its recruitment along with PR upon hormone addition (Fig. 16C) and this is causing the inhibition of progestin-induced genes but also on progestin-repressed genes, it is likely that BRCA1 associates with a repressive function. To date, we have checked the recruitment of the deacetylase HDAC1, and we observed a lower level of HDAC1 presence at the promoter of PR-regulated genes in the absence of BRCA1 protein. This observation is in agreement with the article by Zheng *et al* (Zheng *et al*., 2001) that implicated HDAC1 in the mechanism by which BRCA1 affected ERα transcriptional activity.

We did not observe a significant change in the recruitment of SRC-3 depending on the presence/absence of BRCA1 and detected a delay in the recruitment of SRC-1 in absence of BRCA1, although the changes were not very pronounced. As for its relationship with other cofactors, the overexpression of p300, the CBP homolog, did not rescue the inhibition of PR activity by overexpressed BRCA1 (Ma *et al.,* 2005).

In the future, it would be interesting to extend the study to other corepressors and coactivators that could be implicated in steroid receptor-driven transcription. Although the repression of PR transcriptional activity by CUEDC2, the other PR E3 ubiquitin ligase, appeared not to involve the interference with coactivators (SRC-1 and JDP-2) or corepressors (NcoR and SMRT), this mechanism of action have been previously described for BRCA1. Wen *et al* (Wen *et al*., 2008) reported that BRCA1 affected the recruitment of the SMRT repressor, besides interfering in the recruitment of coactivators like SRC-3 and CBP, as a part of the mechanism of inhibition of ERα activity. Fan *et al* (Fan *et al.,* 2001 and 2002) also described the downregulation of p300 by BRCA1 and the competition for binding to the AF-2 domain of ERα as a possible explanation for the interference on ERα activity.

Similar to the receptor, the turnover of PR cofactors could be regulated by BRCA1. It was previously shown that the degradation of nuclear receptor cofactors is tightly regulated by the proteasome and it might even be ligand-induced (Lonard *et al*., 2004).
Another aspect that awaits further study is the involvement of the relationship between the RNAPII and BRCA1 (see Introduction) in the effect on PR transcriptional activity.

Finally, *the histone code and chromatin structure*. In this respect, we have found another link between the E3 ubiquitin ligase activity of BRCA1 and transcriptional control. While ubiquitination of other components of the transcriptional machinery (RNAPII, PR, cofactors) by BRCA1 is contemplated,

we suggest that transcriptional control is due, at least in part, to enhanced histone H2A ubiquitination in the presence of BRCA1.

Although total levels of monoubiquitinated histone H2A (uH2A) are not affected, we observe that uH2A correlates, at the promoter of PR-target genes, with the presence of BRCA1 (Fig. 19B). Lack of BRCA1 recruitment significantly decreases the level of H2A ubiquitination present at the promoter. This would explain, to some extent, the modest effect we observe of BRCA1 overexpression on the transcription of reporter plasmids transiently transfected. In the case of reporter plasmids transiently transfected and non structured in chromatin, the overexpression of BRCA1 would not exert its inhibitory effect by affecting chromatin structure and so other mechanisms, probably less forceful, are taking place. Likewise, this mechanism of action could be the responsible for the repressive effect of BRCA1 overexpression we observed on the expression of PR-repressed genes.

To date, there are no other reports showing a direct relationship between BRCA1 and histone H2A ubiquitination *in vivo* since it was firstly described to ubiquitinate H2A and H2B *in vitro* (Mallery *et al.,* 2002). Maybe its local influence on particular promoters shown in this work and not a general effect over global H2A ubiquitination discouraged further research on its possible implications *in vivo*. Or maybe it was just a matter of lack of appropriate specific antibodies, at that time. It remains to be explored the possible implication of BRCA1 on histone H2B monoubiquitination *in vivo*, as well.

Histone ubiquitination has gained interest and importance during the last few years, and it is likely that various H2A ubiquitinases/deubiquitinases enzymes will be discovered to regulate distinct transcriptional programs. The data by Zhou *et al* (Zhou *et al.,* 2008) suggested that regulation of H2A ubiquitination occurs in a gene- and enzyme-specific fashion.

The mechanism of how H2A ubiquitination may lead to gene silencing or repression is largely unknown. It has been proposed that uH2A may directly or indirectly block recruitment of the basal transcriptional machinery (Cao *et al*., 2005) or, alternatively, interfere on the activity of RNAPII at events downstream of recruitment (Dellino *et al*., 2004).

Two recent studies (Zhu *et al*., 2007 and Zhou *et al*., 2008) propose that histone H2A monoubiquitination serves to pause RNAPII at the promoter-proximal region, constituting an important step in the regulation of RNAPII transcriptional elongation. Histone H2A monoubiquitination acts to prevent the recruitment of FACT (a positive factor por elongation that acts as a histone chaperone to exert displacement of H2A/H2B dimers (Belotserkovskaya *et al.,* 2003)) at the transcriptional promoter region, blocking RNAPII release at the early stage of elongation.

Some of the roles of uH2A in repression of transcription might relate to the finding that uH2A enhances the binding of the linker histone H1 to reconstituted nucleosome *in vitro* and this mechanism seems to work also *in vivo*. Actually, 2A-DUB is recruited to the promoter of the PSA gene in response to ligand probably through the androgen receptor, removing the repressive uH2A mark from the acetylated nucleosomes and dissociating linker histones in a stepwise manner (Zhu *et al*., 2007). This idea is consistent with the structure of the

nucleosome in which the C-terminus of H2A appears to interact with linker histones (Luger *et al.,* 1997).
Crosstalk between uH2A and H3K4 methylation was recently observed in studies on the H2A deubiquitinase USP21 (Nakagawa *et al.,* 2008). Specifically, uH2A inhibits MLL3-mediated di- and tri-methylation of H3K4, repressing transcription initiation, *in vitro*. In addition, uH2A and Ser-10 H3 phosphorylation (associated with transcription activation) inversely correlate during cell cycle progression (Joo *et al*., 2007).Thus, it is possible that uH2A contributes directly to transcriptional repression by regulating higher-order chromatin structure, in addition to inhibiting H3K4 methylation.
Indeed, we have detected a decrease in the amount of total histone H2A at the NucB region of the MMTV promoter that might correlate with a facilitated displacement of H2A/H2B dimers, maybe through FACT interaction. These aspects await further research.

Apart from the involvement of histone H2A in transcriptional repression, this histone modification has also been shown to be physiologically relevant. The levels of uH2A are significantly diminished in prostate cancer compared to benign prostate tissues, suggesting that the activation of a cohort of AR target genes in such cancer is likely to refer to, in part, low uH2A levels (Zhu *et al.,* 2007). Alterations in histone H2A and H2B monoubiquitination have even been related to transcriptional dysregulation caused by mutant huntingtin protein (Kim *et al.,* 2008).

Unexpectedly, lack of BRCA1 also abrogated the presence of uH2A at the 5'-regulatory region of the HOX family gene *HOXC5.* Ubiquitination of H2A at this site was shown to be triggered by Ring1B (Wang *et al*., 2004). We do not believe that BRCA1 could be affecting the levels of this enzyme in the cell, since the lack of Ring1B was demonstrated to largely reduce the total levels of uH2A and we do not see a change in total uH2A.
It is possible that this gene forms part of the set of genes that BRCA1 could be regulating by means of H2A ubiquitination. Homeobox genes are, indeed, crucial regulators of cell growth and differentiation, and absent or aberrant expression of HOX genes has been implicated in cancer development, including invasive breast carcinomas (Makiyama *et al.,* 2005) and prostate cancer (Miller *et al*., 2003). BRCA1 could even regulate the expression of this gene through PR itself since estradiol, progesterone, testosterone, retinoic acid, and vitamin D have been shown to regulate HOX gene expression (Daftary *et al.,* 2006) in the embryo and in the adult organism. In the adult, endocrine regulation of HOX gene expression is necessary to enable such diverse functions as hematopoiesis and reproduction.

Taking into consideration the results presented in this work and data existing at the literature, we propose a coherent, but speculative, view **model** to explain the **influence of BRCA1 on transcriptional control over the progesterone receptor.**

First step: before any addition of hormone and activation of the PR. (Fig. II).

In the absence of hormone, BRCA1 regulates the levels of PR protein, as commented before. Meaning that, in the absence of functional BRCA1, the cell accumulates higher amounts of PR protein in a basal state.

At the promoter level, we find BRCA1 recruited to the promoter, near to the hormone responsive regions, of PR target genes. We speculate with two possible theories to explain this recruitment. One possibility is that the specific transcription factor regulated by BRCA1, in this case PR, would be tethering BRCA1 to the promoter of the target gene, implying that a certain amount of the receptor is bound to the HRE in the absence of hormone.

Another possibility is that BRCA1 could be targeted to a wider set of genes by means of its interaction with the RNAPII and RNA helicase A (Anderson *et al.,* 1998), which are recruited for the basal transcription of these genes. Once the specific induction of some of these genes is elicited by some stimulus (like progesterone induction), BRCA1 would transiently leave the promoter, to be specifically recruited by the specific transcription factor, in this case PR.

In this context and according to our observations, BRCA1 is already responsible for the monoubiquitination of histone H2A, likely serving as a repressive mark to maintain silent the promoter. Intriguinly, although the mechanism of repression by histone H2A ubiquitination is largely unknown, it was proposed to pause RNAPII at the proximal-promoter region, constituting an important step in the regulation of RNAPII transcriptional elongation (Zhu *et al*., 2007; Zhou *et al.,* 2008).

Besides, through the interaction with HDAC1, it is possible that BRCA1 might bring this histone deacetylase to the promoter. In the same way, the H2A ubiquitin ligase 2A-HUB (Zhou *et al*., 2008) associates with N-CoR/HDAC1/3 complex at the promoter of repressed genes and inhibits RNAPII elongation by blocking FACT recruitment.

(figure on next page)

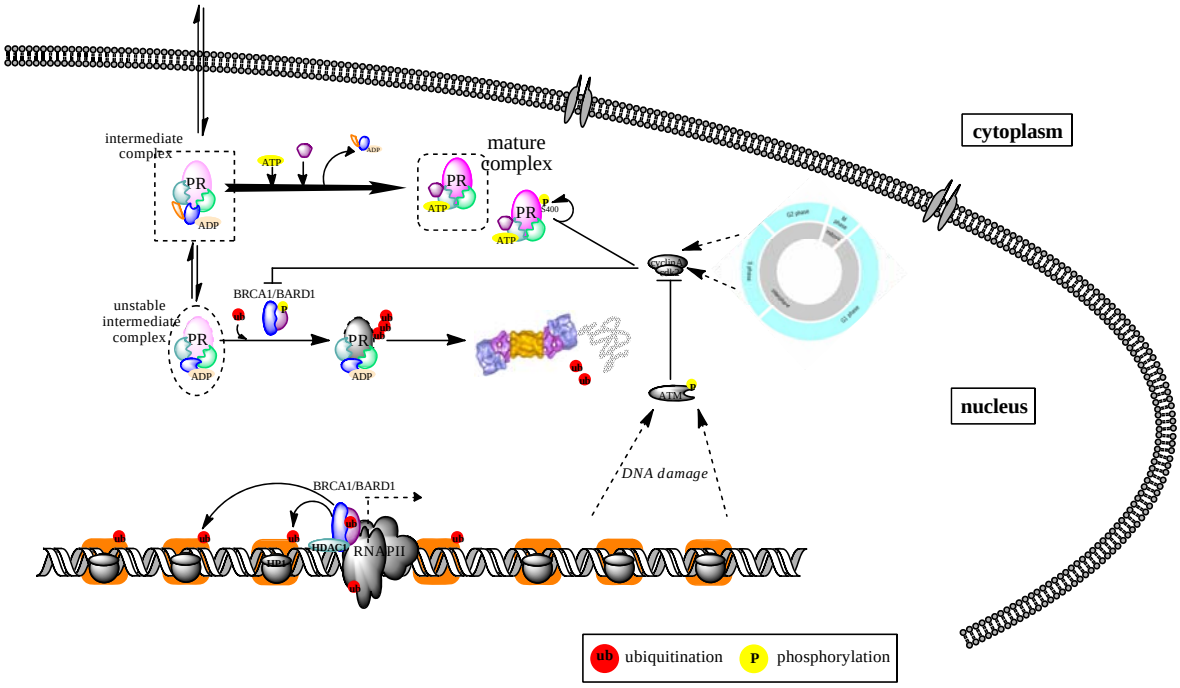


Figure II: Model for the influence of BRCA1 on transcriptional control over the progesterone receptor: in the absence of hormone

Second step: upon hormone addition and PR activation (Fig. III).

Upon hormone binding, the complex of chaperones is disassembled from the receptor so that it can dimerize and bind to the HRE. The events occurring at the promoter in this step of activation have been explored in more detail in the model promoter MMTV (Vicent *et al*., 2006 and 2004). Accordingly, PR can lead to the displacement of a repressive complex containing HP1γ and recruits an ATP-dependent chromatin-remodeling complex that uses the energy of ATP hydrolysis to remodel local chromatin (implying the displacement of histones H2A/H2B dimers) and allow the binding of further PR molecules, NF1, coactivators and the basal transcriptional machinery.

In line with the hypothesis previously proposed, if the promoter is already "poised" for transcription, the arrival of the specific transcriptional activator would be responsible for the subsequent promoter clearance, RNAPII phosphorylation (Ser5) and transcription elongation (Spilianakis *et al*., 2003).
During the first minutes of PR recruitment and initiation of transcription, BRCA1 partially leaves the promoter. Concomitantly, the repressive uH2A mark also diminishes at the hormone responsive region. This temporary leave of BRCA1 from the promoter might be necessary for loosening the repressive state of the promoter and allowing the proper commence of elongation.

Shortly after, BRCA1 is recovered and further recruited to the promoter. Our observations would indicate that BRCA1 is recruited specifically by ligand-bound PR. Upon BRCA1 recruitment, uH2A is recovered. Besides, the fluctuating binding of HDAC1 seems to be significantly lost in the absence of BRCA1, meaning that BRCA1 might also serve as a docking platform for corepressors like histone deacetylases. Of note, several nuclear receptor

coregulators have been shown to regulate both ligand-dependent and independent transcription, like ERα corepressor SAFB1 (Townson *et al*., 2004), coactivator CoCoA (Kim *et al.,* 2003), or BRCA1 itself (Zheng *et al.,* 2001; Fan *et al*., 2001).

In this moment, when BRCA1, PR and active polymerase coincide at the promoter, it is possible that BRCA1 could exert its ubiquitin ligase activity on other substrates, apart from histones. Although we believe BRCA1 is mainly involved in the ubiquitination and degradation of unliganded PR (and so regulates the amounts of available PR for activation), BRCA1 also regulates ligand-bound PR turnover as another mechanism of control of PR transcriptional activity. In all, in both cases, the outcome would be the control of the amount of active PR protein. According to our observations using proteasome inhibitors, we believe this mechanism of control must be more relevant for some PR target genes (like *DUSP1*) than for others. This might relate to differences in regulation of progesterone-responsive promoters, which suggests diversity in how individual promoters achieve transcription. Additional binding sites for transcription factors and the architecture of the core promoter must increase specificity of regulation.

The proteolytic strategy to couple the activity of transcription factors to their destruction allows tight control over transcription, by ensuring that the activation of any gene is linked to the ongoing degradation of its transcriptional regulator. One way this coupling could be achieved is by the coordinated action of the ubiquitination and transcription machineries. It has been proposed a “kamikaze” model for activation, in which simply activating transcription is the signal for activator turnover (Thomas *et al.,* 2000).

Ubiquitination can also influence transcription by regulating the association of transcription factors with partner proteins necessary for proper activation.

BRCA1 can ubiquitinate the elongating form of RNAPII, phosphorylated on Ser5, and target it for degradation (Starita *et al*., 2005). In addition, the ubiquitination of RNAPII can result in the destabilitzation of TFIIE and TFIIH in the PIC and the concomitant inactivation of transcription, without the action of the proteasome (Horwitz *et al.,* 2007). Indeed, the ubiquitin-proteasome system appears to function in the switch from hypo- to hyper-phosphorylated state of the RNAPII, allowing the elongating polymerase to recruit a diverse collection of elongation factors (Shilatifard *et al.,* 2003). This mechanism was also suggested to function in nuclear receptor-mediated transcriptional regulation (Kinyamu *et al.,* 2005).

Ubiquitination of the locus then functions to recruit components of the proteasome. At least five 19S subunits were found recruited to transcriptionally active genes in yeast (Gonzalez *et al*., 2002). Components of the 26S proteasome are recruited to ER and AR responsive endogenous gene promoters (Kang *et al.,* 2002; Reid *et al*., 2003). One 20S subunit was found to interact with the SRC coactivator and recruit the proteasome to the entire sequence of ERα target genes, implicating a role for the proteasome in both transcription initiation and elongation (Zhang *et al*., 2006). Moreover, the chaperone-like ATPase function of proteins of the 19S complex are ideally suited for orchestrating the rearrangements that are required for transcription,

disengaging inhibitor molecules, stimulating chromatin remodeling, or do all of the above.
Recruitment of the 26S proteasome then has a dual role; destroying the activator, -preventing reinitiation of transcription-, and converting RNAPII from an initiation to an elongation-competent form that can transcribe the entire gene. As transcription elongates, the BRCA1-proteasome complex would move with polymerase, reconfiguring chromatin structure and allowing the disengagement of RNAPII at either the end of the gene, or when a damaged DNA segment is detected.

Finally, following gene transcription, the changes in phosphorylation of the CTD make RNAPII and the proteasome to assume its initiation-competent form.
(figure on next page).

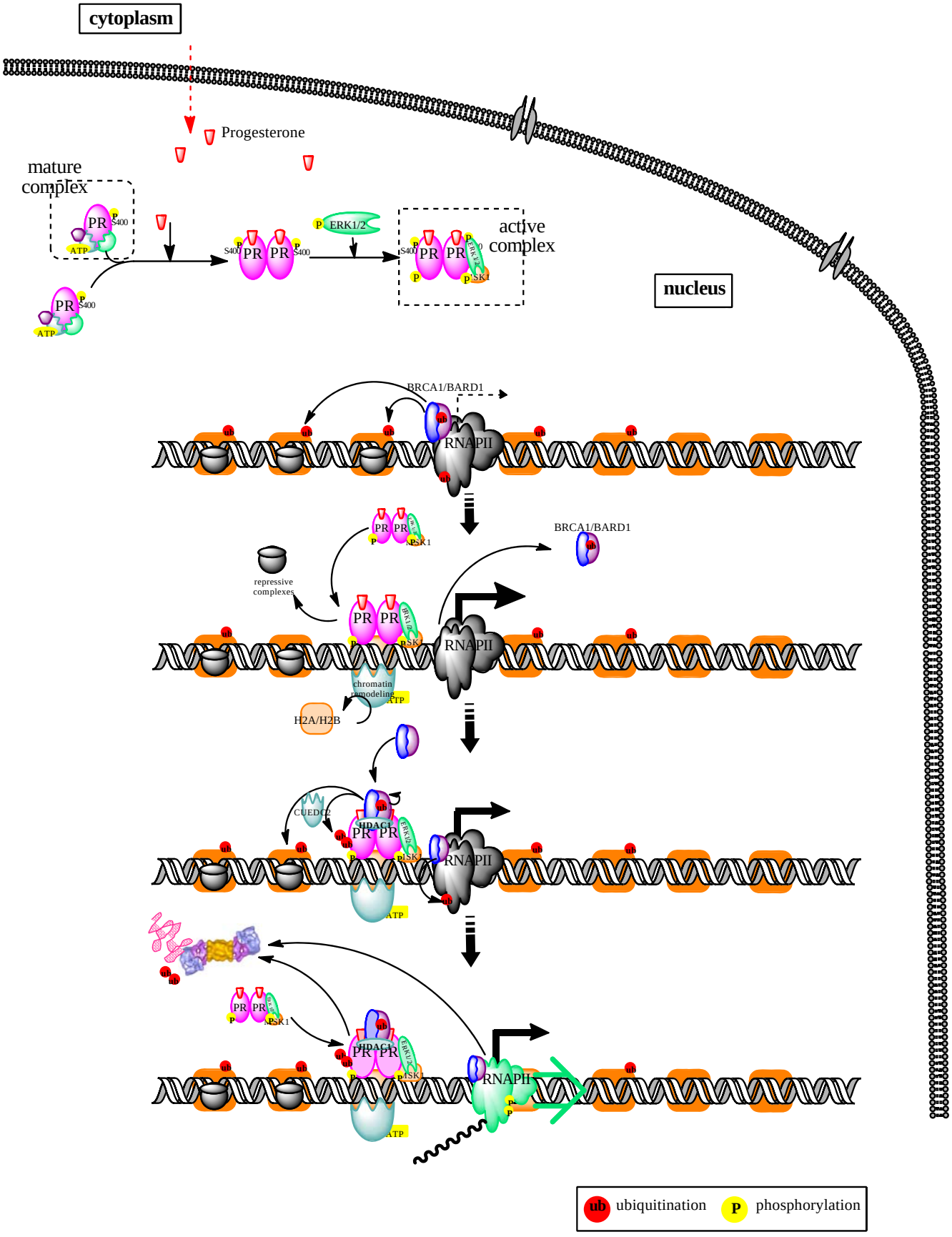


Figure III: Model for the influence of BRCA1 on transcriptional control over the progesterone receptor: upon hormone addition and PR activation

In the absence of functional BRCA1 (Fig. IV), this highly regulated model of transcriptional activation would be disturbed at several different points. To begin with, there would be a certain excess of basal PR protein available for activation and also after hormone addition (even though CUEDC2 or other E3 ubiquitin ligases would still be active). The lack of BRCA1 at the promoter of regulated genes in the basal state would imply the decrease in uH2A and this might lead to an increase in the basal level of transcription of some genes.
Once the receptor is activated, it would find the promoter of BRCA1-regulated genes much more accessible due to the decrease in ubiquitinated histone H2A. In those cases in which an overload is possible, the availability of more ligand-bound PR than usual would result in an increase in the number of PR molecules recruited to the promoter of these target genes. Also, the lack of ubiquitin marks on histone H2A would promote the binding of FACT, or other histone-interacting proteins and chromatin remodellers, which would facilitate transcription by the displacement of histone H2A/H2B dimers. Besides, the RNAPII would loose the regulation by BRCA1 mentioned before.
(figure on next page).

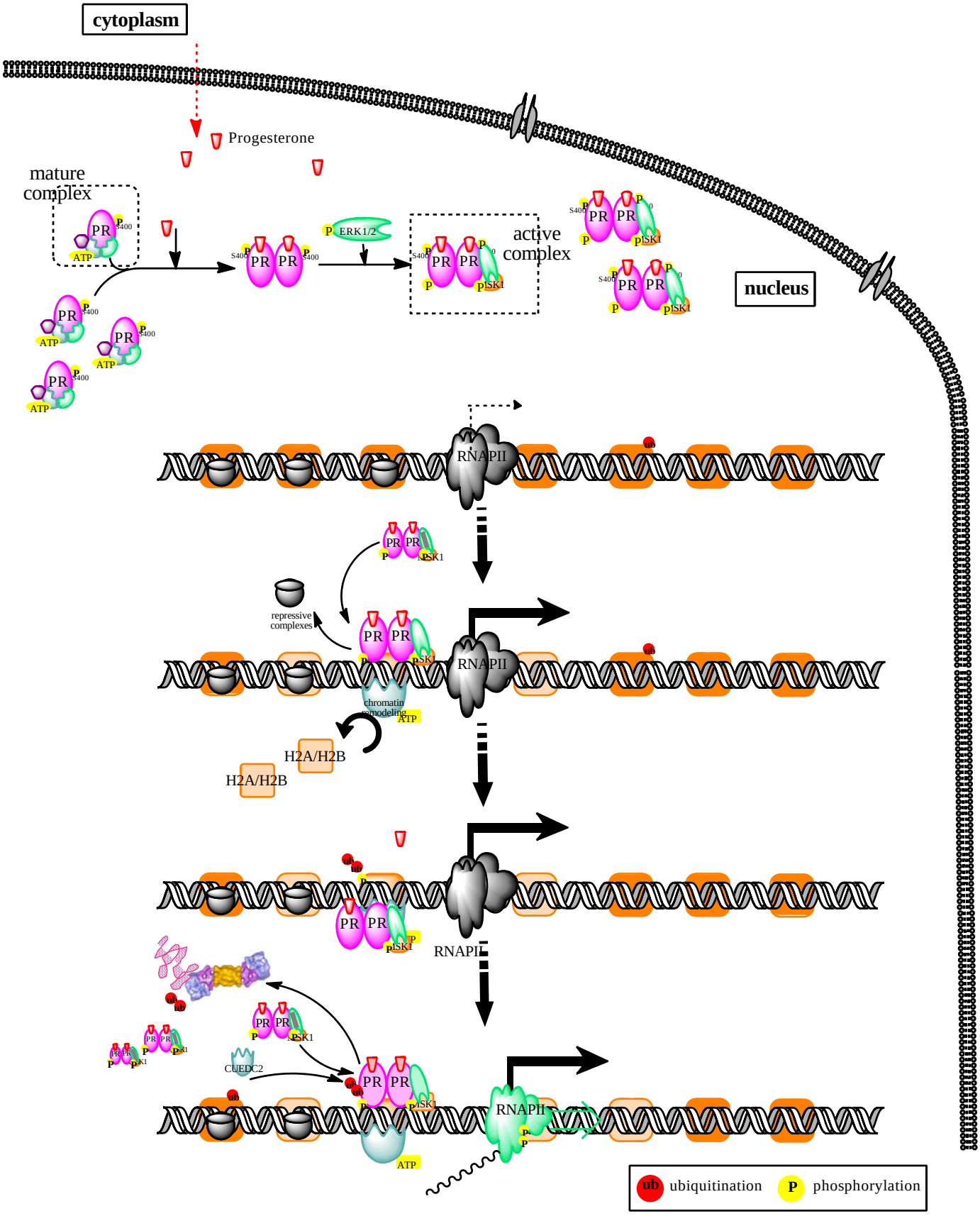


Figure IV: Model for the influence of BRCA1 on transcriptional control over the progesterone receptor: upon hormone addition and PR activation, in the absence of functional BRCA1

At surface value, it may seem counter-intuitive that the transcriptionally active, agonist-bound PR would recruit a corepressor that in turn attenuates gene expression. However, it is firmly established that hormone-responsive gene expression is a readily reversible process, the duration and magnitude of which have to be tightly controlled by combinatorial actions of functionally distinct coregulators in response to physiological fluctuations in signaling.
Furthermore, emerging evidence indicates that several distinct mechanisms exist to attenuate the ligand-dependent response. For example, ligand-dependent corepressors may recruit HDACs to antagonize the actions of HATs and thus reduce chromatin accessibility to the transcriptional machinery (Fernandes *et al*., 2003).

In this way, the transient interaction between BRCA1, PR, RNAPII and the proteasome is a self-limiting mechanism that resets the regulatory clock for another round of transcription. This model is probably a gross simplification but similar models have been proposed as mechanism of action of other nuclear receptors (Metivier *et al.,* 2003; Stavreva *et al*., 2004; Nawaz and O'Malley, 2004; Kinyamu and Archer, 2007; Reid *et al.,* 2003) or transcription factors in general (Muratani and Tansey, 2003).

R. 3 Effect of BRCA1 on cell biology processes induced by progestins

Although we have not moved into the use of *in vivo* models for the study of BRCA1 influence on the biological processes regulated by progestins, we have taken advantage of some of the already described effects of progestins on the cell biology of breast cancer cells in assays *in vitro*.

First, we tested the *cell cycle profile* of cells lacking functional BRCA1 during the first 40 hours of progesterone treatment, which would include the *first round of proliferation induced by progestins* in breast cancer cell lines.
Numerous data suggested that a crosstalk between PR and multiple signaling pathways (Lange *et al.,* 1998) could explain the apparently paradoxical dual proliferative and antiproliferative action of progesterone. These studies, using T47D cells, described a biphasic effect of the hormone. It consisted of a transient acceleration of the cell cycle followed by a long-term growth inhibition and arrest in G1 phase (Groshong *et al*., 1997; Musgrove *et al.,* 1991; Lange *et al.*, 1998), accompanied by cellular changes that permit other factors to influence the final proliferative or steady differentiative state.
The data suggest that progestins act to increase transiently the rate of progression of actively cycling cells rather than to accelerate the entry of quiescent cells into the cell cycle (Musgrove *et al*., 1991). Therefore, the ultimate physiological effect of progesterone, proliferative or antiproliferative, is a result of composite signal pathways, depending on growth factors/cytokine-signaling pathways, which are specific to and different in each cell line or even may depend on the cell culture conditions and cell state. For instance, a population of cells constantly growing exponentially and with low proportion of quiescent cells may preclude the detection of any stimulatory response or, conversely, cell populations with a high proportion of quiescent cells and a low proportion of S-phase cells may blunt responses.

On the contrary, estrogen stimulates the entry of cells into S phase but this is not followed by later inhibition (Musgrove et al., 1991). E2 exerts its mitogenic action inducing first a rapid activation of the ERK and PI3K pathway in breast cancer cells (Castoria *et al*., 2001; Stoica *et al.,* 2003), and, secondly, inducing the expression of several genes with important functions in the cell cycle like *Cyclin D1* (Sabbah *et al.,* 1999). The activation of the ERK and PI3K cascades are related with cell cycle progression (Robinson *et al.,* 1997). Progesterone also stimulates intracellular phosphorylation cascades, like ERK (Ballare *et al.,* 2003), which are essential for the proliferative response (Skildum *et al.,* 2005).

We detected a significant increase in the BrdU incorporation and, so, DNA synthesis, in BRCA1-knockdown cells compared with control cell lines at 24 hours of estrogen treatment, while no remarkable differences were detected in the time-course assay with R5020 treatment (Fig. 20B). The effect of BRCA1 on E2-induced proliferation is in accordance with data reported by Razandi *et al* (Razandi *et al*., 2004) showing that overexpression of BRCA1 prevented E2-induced proliferation in MCF-7 cells.

As for the activation of signaling pathways, we have not analyzed in detail the activation of the pathways but just two of the described most important outcomes of the cascade of activating phosphorylations, that is the phosphorylation of Ser294 and Ser118 of PR and ERα, respectively (Vicent *et al.,* 2006; Weitsman *et al*., 2006). These two residues are major sites of phosphorylation by ERK and become phosphorylated in a matter of minutes.
We have not detected any noteworthy change in the level of Ser118 ERα phosphorylation in the presence or absence of BRCA1. We observed an up-regulation of Ser294 phosphorylation in the BRCA1-knockdown cell lines, which correlated with the increase in the level of total PR (Fig. 20 C and D). Our observations do not discard the possibility of an enhanced activation of the ERK pathway in cells lacking functional BRCA1 expression since we have just explored the phosphorylation of one of the multiple substrates and not directly the activity of the kinases. Other assays would be necessary to assess definitively this aspect, as well as the activation of other pathways involved in the proliferative phenotype induced by ovarian hormones.

Contradictory information exists about the influence of BRCA1 on steroid hormone-activated signaling pathways. Razandi *et al* postulated in their article that overexpressed BRCA1 upregulates ERK-directed phosphatase (MKP-1 or DUSP-1) activity and protein expression upon E2 treatment, then downregulating ERK pathway activation, while not affecting PI3K/AKT signaling. However, Yan *et al* (Yan *et al.,* 2002 and 2008) showed the activation of ERK1/2 upon BRCA1 overexpression in MCF-7 cells. Recently, Ma *et al* (Ma *et al.,* 2007) described a mechanism to explain the influence of BRCA1 on ERα activity by stating that lack of functional BRCA1 enhances ERα activating phosphorylation on Ser167 (and not Ser118) through, in part, the inhibition of a phosphatase, PP2A, that regulates the activation of c-Akt, main kinase involved in the phosphorylation of Ser167 of ERα. They further state that the PI3K/AKT pathway is the main pathway regulated by BRCA1 with regard to its effect on ERα activity and not ERK.

Wen *et al* (Wen *et al*., 2008) very recently reported that BRCA1 knockdown enhanced E2-stimulated ERK1/2 and AKT activation, though the level of Ser118 ERα phosphorylation was not affected and neither the E2-induced short-term proliferation.

In any case, the influence of BRCA1 on the short-term induced proliferation by E2 would implicate BRCA1 in the signaling pathways activated by the hormone, since it is well recognized that the rapid proliferative effect triggered by E2 treatment during the first hours of hormone treatment are mainly due to the activation of membrane-initiated signaling cascades, followed, in a second term, by the transcriptional induction of genes, which may take a few more hours. Either ERK or PI3K/AKT, BRCA1 effect on the signaling pathways must be affecting differently the outcome of such activation given that, although both pathways are relevant for the E2 and R5020-induced breast cancer cell proliferation (Pedram *et al*., 2002; Carnevale *et al.,* 2007), we only observe a detectable effect over the short-term induced proliferation by E2.

We also assayed the *proliferation induced by long-term treatment* with E2 and R5020 in cells expressing or not wild-type levels of BRCA1.

While estrogen has a persistent proliferative effect, repeated exposure to progesterone or R5020 every 48 hours produces permanent growth arrest (Groshong *et al*., 1997). We wanted to see if the lack of BRCA1 would make any difference in the behaviour of the cells upon long hormone treatment in terms of proliferation.

Again, we observed a significant difference in the number of cells between controls cells and BRCA1-knockdown cells at four days, and even before, of E2 treatment, while no differences are noticed with the R5020 treatment.

In the publication by Ma *et al* (Ma *et al*., 2005) that came out during the course of this thesis, they perform a similar assay treating with 100 nM progesteroneT47D cells transfected to overexpress BRCA1. They observe that BRCA1 overexpression inhibits the progesterone-stimulated proliferation of cells at four days of treatment. It seems there must be differences in the cell culture conditions or even differences in the characteristics of the T47D subline, since they observe long-term stimulation of proliferation by progestins in the control T47D cell line, which is rarely observed (Musgrove *et al*., 1991; Skildum *et al*., 2005). Where progestin stimulation has previously been described, relative increases in cell number in progestin-treated cultures are rarely more than two-fold even after extended treatment (Hissom *et al.,* 1989).

Lastly, we performed some experiments aimed at analyzing a possible connection between the *cell survival properties* assigned to progestins (Moore *et al.,* 2006) and to loss of BRCA1 function. Lack of functional BRCA1 suppresses spontaneous and induced (by withdrawal of serum-derived survival factors, exposure to ionizing radiation or treatment with the chemotherapeutic agent paclitaxel) apoptosis in breast and ovarian cancer cell lines (Thangaraju *et al.,* 2000). The BRCA1-dependent apoptosis occurring after serum withdrawal proceeds through an H-Ras/MEKK4/JNK signaling pathway followed by increased expression of Fas and FasL and by activation of caspase-8, in a p53-independent fashion.

We explored the cell death induced by serum withdrawal in control and BRCA1 knockdown cells upon progestin treatment. Although the T47D breast cancer cell line is quite resistant to serum withdrawal-induced cell death, even at six days of serum lack, we detected a progestin-induced protection against cell death. We were unable to see any change in this progestin-induced protection in the BRCA1 knockdown cells given that the lack of functional BRCA1 already enhanced their resistance to cell death in a basal situation, being the level of cell death at six days of starvation almost negligible.

We also examined the pattern of cell cycle distribution in progestin-treated control and BRCA1 knockdown cell lines upon a DNA-damaging stimulus like ionizing radiation.

Progesterone increases the rate of cell proliferation and inhibits the apoptotic process after irradiation (Vares *et al.,* 2004). BRCA1 is important for the G2/M checkpoint following ionizing radiation (Yarden *et al.,* 2002). The cell cycle analysis we performed gave us little information about the relationship between progestin treatment, BRCA1 function and cell survival, and it would be necessary to perform more specific experiments to resolve this question. By just analysing the cell cycle we could only detect the deficient G2/M checkpoint of the BRCA1 knockdown cells, which was already expected (Ree *et al*., 2003). We could have measured cell death or apoptosis at 5-7 days of irradiation exposure, when modulation of radiation sensitiviy is better sensed. Then, we would be able to see if the confluence of BRCA1 lack and progestin treatment, upon DNA damage, implies a benefit for the survival of the cells. We could also measure the capacity of cells to divide in these conditions, after irradiation, by treating the cells with cytochalasin-B that blocks cytokinesis and leads to accumulation of multinucleated cells, which then serves as an index of proliferation (Vares *et al*., 2004). We could have also measure more specifically the G2/M checkpoint by detecting phospho-H3 by flow cytometry, which serves as an actual marker of mitotic cells.

Aside from the uncertainties surrounding the influence of BRCA1 on the biological effects of progestin on breast cancer cell lines *in vitro*, Ma *et al* and Poole *et al* (Poole *et al*., 2006) found that a deficiency of Brca1 confers an exaggerated progesterone-induced growth response in the mammary glands of intact and ovariectomized adult female mice. This would point to a factual effect of BRCA1 in the progesterone-induced growth of the mammary gland *in vivo*. And, more importantly, this effect is observed over normal mammary cells instead of transformed breast cancer cell lines, in which it could be easier to observe phenotypes related to increased tumourigenesis and proliferation. Furuta et al (Furuta et al., 2005) also demonstrated that loss of functional Brca1 causes a failure in mammary acinus formation and enhances the proliferation of mammary epithelial cells using a in vitro 3D culture system.

As hypothesized later, it is possible that the biological effects driven by the BRCA1 lack might be blunted in the breast cancer cell lines we have used in our studies (T47D and MCF7 cells), not only for being already transformed cell lines but also because they resemble the luminal-epithelial-like phenotype of breast cancer (ER and PR-positive, Lacroix *et al.,* 2006) instead of the basal/myoepithelial-like phenotype that is characteristic of BRCA1-related tumours. It might be a matter of target cell, since, as proposed in the model

below, the ER/PR-positive cells that might be the target of tumourigenic events in luminal epithelial breast cancers might not be the same target cells (stem ER/PR-negative cells) that in the case of basal breast cancers. However, as stated after, though ER/PR-positive cell lines might not be the target for the overgrowth promoted by BRCA1 defficiency, important events might also be induced by the lack of the protein in these cells (as exposed in this thesis) that, at the end, would be the responsible for the development of the tumour.

-BRCA1 as a stem cell regulator in the mammary gland promoting the transition from basal epithelial phenotype to glandular phenotype: the last of the hypothesis proposed to explain the BRCA1-related tumourigenesis and tissue specificity has to do with the cancer stem cell hypothesis. Accumulating data has provided support for the cancer stem cell hypothesis, which holds that cancers originate in tissue stem and/or progenitor cells through the dysregulation of self-renewal processes (Wicha *et al.,* 2006). Evidence for the existence of a "cancer stem cell" component in human breast cancer has been generated (Al-Hajj *et al*., 2003).

The phenotype of these "cancer stem cells" ($CD44^+/CD24^{-/low}$ and ESA^+ expression) has been associated to basal-like and BRCA1 hereditary breast cancers (Honeth *et al.,* 2008). Foulkes (Foulkes, 2004) proposed that the clinical, molecular, and pathological features of breast cancer in BRCA1 mutation carriers fit a model in which BRCA1 functions as a stem cell regulator.
In another study (Wicha *et al.,* 2006), *in vitro* and mouse models suggest an important role for BRCA1 in regulating the differentiation of ALDH1+/ER- primitive stem cells into ER+ epithelial cells. The loss of BRCA1 would cause the persistence of the "primitive" basal phenotype and it would also increase the rate of damage in the DNA of these stem cells, which need, in fact, very few mutational hits to become frank cancers (Liu *et al*., 2008).
Vassilopoulos *et al* (Vassilopoulos *et al*., 2008) also provides evidence for the cancer stem cell origin of BRCA1 tumours and propose that BRCA1 plays a role in the differentiation of normal breast stem cells.

Finally, we propose an integrative **model to explain the BRCA1-related breast tumourigenesis** having into consideration diverse ideas proposed in the previously reviewed hypothesis to explain BRCA1 breast cancer (Fig. V).

The cancer stem cell hypothesis would point to the idea that BRCA1 tumours arise from ER-negative (and PR-negative) cells. They suggest that the BRCA1 loss of function occurs in primitive ER-negative stem/progenitor cells. The carcinogenetic event would be the loss of BRCA1 expression by LOH of the second allele in BRCA1 mutation carriers, or the downregulation, by other mechanisms (hypermethylation of the *BRCA1* promoter or of the two alleles, inhibition of transcription or translation) in non-mutation carriers. It is possible that there could be a higher rate of LOH of the *BRCA1* allele in breast and ovary than in other tissues, probably by mitotic recombination, helped by the mitotic profile of the tissue, the abundant transcription of the gene in the breast and its susceptibility owing to its high content in *Alu* sequences. More than 90% of BRCA1 carcinomas present LOH of the non-mutated allele and, in addition,

LOH has been found in association with *in situ* lesions, non-neoplastic peritumoural tissues and even in other areas in the contralateral breast.
However, it could be possible that phenotypics effects could be observed in heterozigosy for BRCA1 and that the loss of the second allele occurs later.

We would envision a situation in which the lack of BRCA1, possibly starting in heterozigozy and becoming homozigotic, would be sensed in those ER-negative stem cells and also surrounding cell types, including cells expressing ER and PR. Stem cells are subjected to the accumulation of multiple mutations by their long-lived nature. Therefore, the chance to accumulate a second hit in the wild-type allele of the BRCA1 gene could be higher in stem cells than in progenitor cells. This is a possible explanation for the association of BRCA1 cancers with the basal phenotype, “stem cell-like”. Another explanation could be the putative highest aggressiveness and proliferation capacity of cancer cell clones arising from ER-negative stem cells than ER-positive progenitor cells. In this scenario, primitive ER-negative stem cells lacking BRCA1 are not allowed to differentiate into ER-positive progenitor cells, by assymetric division, thus exposing these undifferentiated cells to the effects derived from the lack of BRCA1 expression. This means that they are more exposed to DNA damage and cell cycle desregulation.

In the case of steroid receptor-positive cells, apart from being more exposed to those same risks, these cells would tolerate an exacerbated effect of progesterone and estrogen, including the paracrine signals of proliferation towards neighbouring steroid receptor-negative cells. Similarly to the model proposed by LaMarca and Rosen (LaMarca and Rosen, 2008), we would envision a situation in which basal ER-negative stem cells may divide assymmetrically once to give rise to a luminal ER-positive progenitor cell. This undifferentiated cell would then secrete paracrine factors in response to ovarian hormones stimulation to feedback on ER-negative stem cells and induce their proliferation. Additionally, these same paracrine factors may induce the proliferation and/or differentiation of adjacent ER-negative and ER-positive progenitor cells. This idea was also suggested by the experiments carried out by Mallepell *et al* (Mallepell *et al.,* 2006). In particular, progesterone might be an important proliferative stimulus on these basal stem cells, according to the observations by Sartorius *et al* (Sartorius *et al*., 2005), further remarking the importance of progesterone action on basal-like breast cancers.

As a result, mammary ER-negative stem cells remain in their “primitive” state, much more sensitive to the action of DNA-damaging agents, which might target the p53 tumour suppressor gene or inactivate the ATM kinase, among other events. Besides, paracrine signals coming from ER-positive sorrounding cells would incite them to proliferate, thus becoming precursors or at least best candidates for starting a tumourigenic growth. The protective effect of ovariectomy on breast cancer development in BRCA1 carriers might be due to elimination of paracrine signals from differentiated ER/PR-positive luminal cells to primitive stem cells.

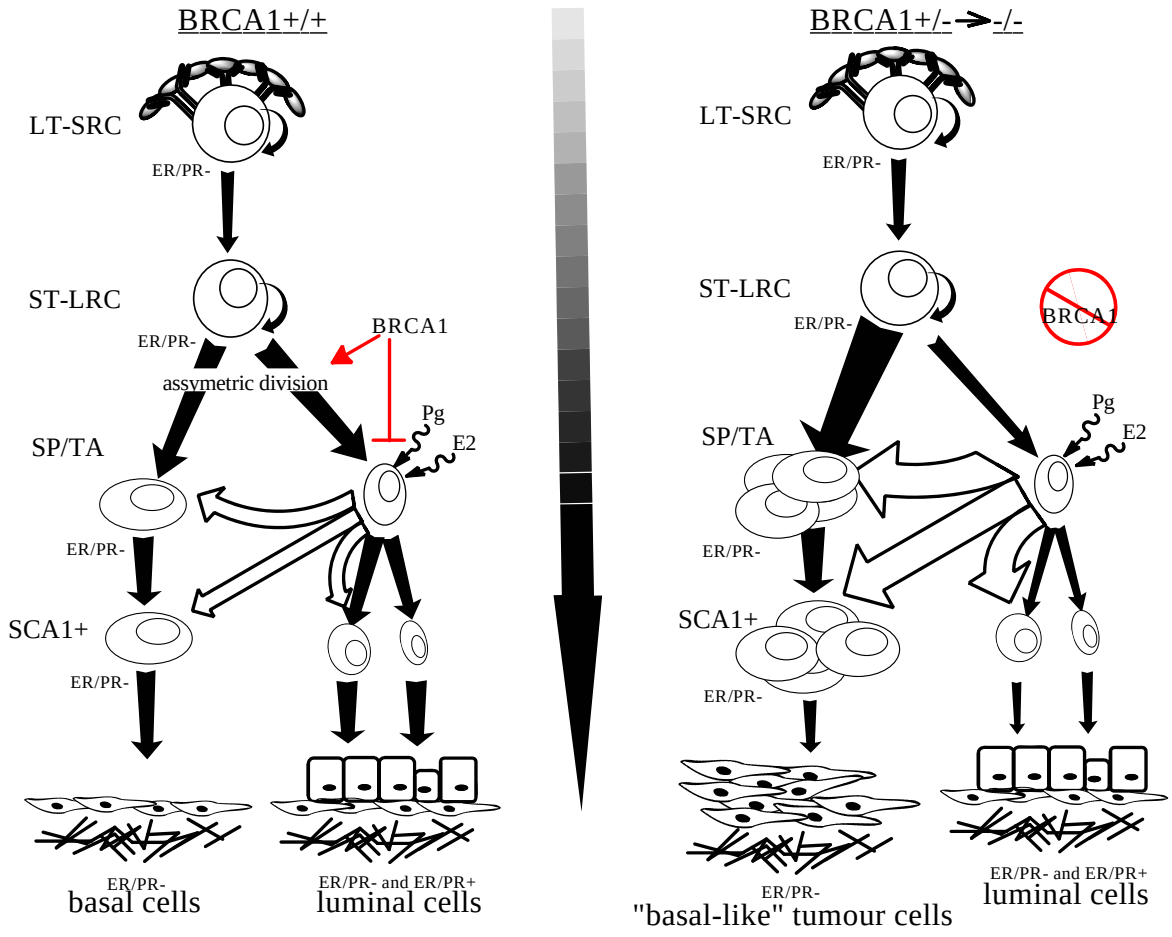


Fig.V: Model for the BRCA1-related breast tumourigenesis

CONCLUSIONS

The conclusions of this thesis can be summed up in:

1. Exogenously overexpressed human BRCA1 inhibits the transcriptional activity of exogenous and endogenous progesterone receptor on the induction of the PR-regulated promoter model MMTV and on the induction of endogenous PR-target genes like DUSP1, 11β-HSD, EGF but also enhances the repression of PR-downregulated genes like CCNG2 and CHD4.
2. Knockdown of BRCA1 by RNA interference enhances the transcriptional activity of PR on the induction of the MMTV promoter and of endogenous PR-target genes like DUSP1, 11β-HSD, EGF, CCND1 or HEF1 while enhancing as well the repression of PR-downregulated genes like CCNG2.
3. BRCA1 interacts *in vivo* with the PR-A and PR-B isoforms in a ligand-independent manner.
4. BRCA1 affects the basal level of PR protein and the protein half-life and rate of degradation upon hormone addition while not affecting mRNA synthesis. The overexpression of BRCA1 decreases the total amounts of PR and the knockdown of BRCA1 increases its stability in the cell. However, still in the absence of BRCA1 expression, other degradation machineries send PR to degradation since the treatment with hormone still induces some degradation along time and the treatment with a proteasome inhibitor accounts for more accumulation of protein.
5. BRCA1 promotes the ubiquitination of PR-B *in vivo* and *in vitro* in combination with BARD1 and the E2 enzyme UbcH5c. The target site for the ubiquitination is located outside the region encompassing aa 165-345 that contains the *in silico* best scored PEST sequence (aa 211-238).
6. According to preliminary results, BRCA1 inhibitory effect over PR transcriptional activity is dependent on its E3 ubiquitin ligase activity since the overexpression of a ubiquitin ligase-deficient BRCA1 mutant fails to inhibit the receptor.
7. The regulation of transcription by BRCA1 based on its E3 ubiquitin ligase activity may operate through two distinct mechanisms: one implying the ubiquitination and degradation of a target protein involved in the regulation of the gene (PR?) and another one implying only the ubiquitination of a certain substrate.
8. BRCA1 is present at the hormone-responsive elements of the MMTV promoter. It is found in a basal condition, leaves the promoter shortly after hormone addition and gets further recruited at 30 min-1h of progestin treatment through the interaction with PR. BARD1 is also detected at the hormone-responsive region.
9. Although BRCA1 knockdown does not seem to affect PR recruitment to the MMTV promoter, the overexpression of BRCA1 diminishes the amount of recruited PR indicating that the changes in PR protein levels may have a reflect on the recruitment of the receptor to target genes.

10. BRCA1 levels do not affect the recruitment of coactivators like SRC-1 and 3 while BRCA1 knockdown diminishes the recruitment of histone deacetylase activity HDAC1, compatible with an enhancement in the transcription of the gene.
11. BRCA1 knockdown greatly reduces the levels of monoubiquitinated histone H2A at the hormone-responsive region of the MMTV promoter and HOXC5 promoter, while the recruitment of BRCA1 coincides with the ubiquitination of H2A, indicating that BRCA1 may exert this function *in vivo*. Besides, the loss of ubiquitin mark on H2A correlates with enhanced displacement of the histone.
12. BRCA1 knockdown does not seem to affect the short-term neither long-term proliferation induced by progestin in T47D cells although it enhances both E2-induced proliferations. It does not either affect the levels of ERα Ser118 phosphorylation and the increase in Ser294 phosphorylation correlates with the increase in total PR protein.

In all, two general final conclusions would be that:

i. BRCA1 regulates the transcriptional activity of PR by a mechanism that implies different aspects. Through the regulation of the basal PR degradation BRCA1 may control the basal level of PR protein. In the presence of hormone, BRCA1 also regulates PR degradation along with other enzymes. This regulation can be reflected in the level of PR recruitment to the promoter of specific target genes. Furthermore, BRCA1 presence at the promoter of PR-target genes can inhibit/control transcription by ubiquitinating histone H2A and serving as a signal for transcription repression. Upon hormone binding to the receptor, BRCA1 may leave the promoter in order to allow for the transcription initiation, but it gets further recruited by PR probably to ensure a controlled transcription by means of the ubiquitination of H2A, attachment of HDAC activity, degradation of PR and ubiquitination of RNAPII.

ii. Although the manipulation of BRCA1 expression levels did not show to have a significant effect on the biological processes tested in breast cancer cells in vitro, further analyses in normal mammary cells and animal models would surely serve to extend the implications of this relationship to physiological aspects.

MATERIALS AND METHODS

Reagents

R5020 and E2 were purchased from Perkin Elmer Life Sciences. Bromodeoxyuridine (BrdU) and propidium iodide (PI) were purchased from Roche. Doxycycline (Dox) and lactacystin (Lact) were from Sigma. Trypan Blue solution was from Sigma. Recombinant proteins UbcH5c, UbcH6, UbcH10, UbcH1, UbcH8 and UbcH13 were from Biomol. Recombinant ubiquitin protein was from Upstate. Recombinant human Ubiquitin-Activating Enzyme E1 was from Calbiochem.

Antibodies

Antibodies to BRCA1 were from Oncogene (Ab1) and from Santa Cruz (C20). PR (Ab4, H190), ER (HC-20), BARD1 (H300) and α-tubulin were from Santa Cruz. Anti-BDGal4 was purchased from Clontech. Anti-flag antibody was purchased from Sigma. Antibody against total H2A was from Cell Signaling and against ubiquityl-H2A (uH2A) was from Upstate. Antibody against phosphorylated Ser294-PRB was from NeoMarkers. SRC-1, SRC-3 and HDAC1 antibodies were from Abcam.

Plasmids

The pAGEMMTVLu (MMTV-Luc) plasmid carrying the mouse mammary tumor virus promoter linked to the firefly luciferase reporter gene was described previously (Klehr *et al*., 1991). pSG5-PRB and pSG5-ERα were a gift from Dr. Pierre Chambon (Kastner *et al.,* 1990; Tora *et al.,* 1989). pcDNA3-wtBRCA1 and pcDNA-Gln1756insC 5382, Tyr1853insA 5677, Pro1749Arg 5365, Ala1708Glu 5242 were a gift from Dr. Barbara Weber. pGADT7 and pGBKT7 were purchased from Clontech. BRCA1wt and BRCA1mutants were subcloned from the pcDNA3 plasmids to the pGADT7 and pGBKT7 plasmids. pGADT7-PRB and pGBKT7-ER plasmids were described previously (Ballaré *et al.,* 2003). pTG13-luciferase (p53RE-Luc) and pCAG3.1/p53 were a kind gift from Dra. Berta Vidal. pSUPER.retro.puro was purchased from Oligoengine. pLVTHM, ptTR-KRAB-Red, pCMC-R8.91 and pMD.G were provided by Dr. Trono (Wiznerowicz *et al.,* 2003). Plasmid pSG5-I26ABRCA1 was provided by Dr. Jeffrey Parvin. Plasmid for the expression of HA-Ubiquitin was a kind gift from Dr. Timothy Thomson. pRAV-Flag-PRB and empty pRAV-Flag were described previously (Quiles *et al*., 2009). pShuttle-CMV vector was provided by Dr. Nieves Calvo.

Oligonucleotides

Oligonucleotide		**Sequence 5'-3'**
MMTV Nuc B	up	GGGCTTAAGTAAGTTTTTGGTTACA
	low	TTTACATAAGATTTGGATAAATTCC
β-globin	up	ACACAACTGTGTTCACTAGC
	low	CAACTTCATCCACGTTCACC

11β-HSD HRE region	up	ACCTGAGCGCGGCGGCTTGG
	low	CCTGGCTGCGGGCGGTGCTT
HoxC5 promoter	up	CCCAAAGGGCACATAACGG
	low	TCAGGCCCTAAGGCTCCACTA
11β-HSD	up	ACGCAGGCCACAATGAAGTAG
	low	GCAGCCAGGCTGGATGATG
CCND1	up	CCCTCGGTGTCCTACTTCAA
	low	AGGAAGCGGTCCAGGTAGTT
DUSP1	up	CAGCTGCTGCAGTTTGAGTC
	low	AGAGGTCGTAATGGGGCTCT
GAPDH	up	TTGGTCGTATTGGGCGCCTGG
	low	CAAAGTTGTCATGGAT
EGF	up	TCACCTCAGGGAAGATGACC
	low	CAGTTCCCACCACTTCAGGT
TFF1 (pS2)	up	TTGTGGTTTTCCTGGTGTCA
	low	CCGAGCTCTGGGACTAATCA
BRCA1	up	GGTGGTACATGCACAGTTGC
	low	ACTCTGGGGCTCTGTCTTCA
HEF1 (NEDD9)	up	ACTGTCAGCCTCCCCAGCTCAGGACAA
	low	ATCGTCACACTTGTTCTGGGGCTT

Cell culture and treatments

293T cells (human embryonic kidney transformed cells) and GP2-293 were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 ug/ml) and L-Glutamine (2 mM).
MCF7 cells (human breast adenocarcinoma cells) were grown routinely in Eagle's minimum essential medium (MEM) containing 10% FBS, L-Glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 ug/ml), non-essential amino acids (5mM) and sodium pyruvate (1 mM).
The cell line T47D-MMTVL (Truss *et al.,* 1995) is a derivative of the T47D cell line (human breast ductal carcinoma cell line), containing a single copy of the luciferase gene under the control of the MMTV promoter. These cells were routinely grown in RPMI 1640 medium, with 10% FBS, L-Glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 ug/ml) and 700 ug/ml of G418 (Invitrogen).
The cell line T47D-YV (PR-negative clonal derivative cell line from T47D, Sartorius *et al*, 1994) and its derivatives (T47D-YV-flagPRΔERIDI (Quiles *et al*., 2009), T47D-YV-empty, T47D-YV-flagPRBwt and T47D-YV-flag-PRBK388R) were routinely grown in MEM medium supplemented with 7% FBS, L-Glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 ug/ml).

For luciferase and gene expression experiments, nuclear and total extract preparation, cells were usually plated in phenol red-free medium (since phenol red is a weak estrogen mimic (Berthois *et al.,* 1986)) supplemented with

10% charcoal-stripped serum (CSS) (which is depleted of steroid hormones present in serum) and left for 24 h before being replaced for fresh medium without serum. After 24 h, cells were treated with vehicle (ethanol), R5020 (10 nM) or E2 (10 nM) for the indicated times, in a humidified chamber at 37°C and 5% CO_2.
For chromatin immunoprecipitation and proliferation assays, cells were plated in phenol red-free medium and left for 48h before being serum-starved for 72h and then treated with vehicle, R5020 (10 nM) or E2 (10 nM) for the indicated times. At the proliferation assays, whenever BrdU incorporation is measured, bromodeoxiuridine (BrdU) is added to the culture medium (10 umol/L) during the last 2-4h of hormone treatment. In the case of γ-irradiation, a single dose of 10 Gy of radiation was applied 2 hours after starting the hormone treatment.
At the cell survival experiments, cells are plated and grown to confluency before changing to serum-free conditions. At this time, cells are incubated in this medium with vehicle (ethanol) or R5020 (10 nM) for six days. Fresh ethanol or hormones was added every 48 h.
When indicated, the inhibitor of the proteasome lactacystin (Lact, 10 uM) was added to the cells 1 or 4 h before starting hormone treatment. Doxycycline (Dox, 2,5 mg/ml) was added to the medium when indicated, for four to seven days.

Transient transfection and luciferase assay

Subconfluent proliferating cells were harvested by trypsination and replated at a density of 2,5-3,5x10^5 in 6-well plates in medium without antibiotics. They were transfected with Lipofectamine Plus (Invitrogen) following the manufacturer's instructions. The total transfected DNA was kept constant by addition of appropriate empty vectors. To normalize the efficiency of transfection, a plasmid encoding β-gal (CMV-gal) was co-transfected at a very low concentration. After incubation for 6 h with the adequate amount of transfection mix, medium was replaced for phenol-red free medium with antibiotics. Cells were allowed to recover in this medium for 24 h before being serum-starved and hormone-treated for 24 h.

Cell extracts were collected in 150 ul of lysis buffer (Promega) and were subjected to a single freeze-thaw cycle to ensure complete lysis. Tubes were vortexed and cleared by centrifugation at 13.000 x g for 2 min at 4°C.
Protein was determined by Micro BCA protein assay (Pierce). Equivalent protein amounts, adjusted with lysis buffer, were used for the measurement of luciferase activity and β-gal expression with the Luciferase Assay kit and β-Galactosidase Enzyme Assay kit both from Promega, according to the manufacturer's instructions. Briefly, 20 ul of equivalent amounts of cell lysates (between 1 to 10 ug) are dispensed into individual luminometer tubes. Luciferase assay reagent (100ul) was injected into the tubes by the AutoLumat 953 luminometer (Berthold), which performed a 10-second measurement read for luciferase activity.
For β-gal determination, 50 ul of equivalent amounts of protein cell lysate are pipetted into a 96-well plate. Assay buffer (50 ul) is added to each well and it is

allowed to develop a faint yellow colour, when reaction is stopped by adding 150 ul of 1M sodium carbonate. Absorbance is read at 420 nm in a plate reader.

For the transfection of 293-T cells, we followed the instructions of the BD CalPhos Mammalian Transfection kit (BD Biosciences).

SDS-PAGE and Western blot

Protein extracts were prepared in lysis buffer (Tris 25 mM pH 7.5, SDS 0.5%, EDTA and EGTA 1mM supplemented with protease inhibitors (β-glycerol phosphate 20 mM, $NaVO_4$ 2 mM, PMSF 2 mM and Protein Inhibitor Cocktail (Roche)). Protein concentration was determined with Micro BCA protein assay (Pierce). Equal protein amounts (between 6 to 100 ug, depending on the protein and the antibody) were loaded into 8 to 15 % SDS-polyacrylamide gels, transferred to nitrocellulose membranes and incubated with a dilution (usually in 3% fat-free milk TBS-Tween buffer) of the primary antibody ranging from 1:500 to 1:2000, during 2 h at room temperature or, otherwise, at 4°C overnight. The secondary antibody (horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG, Amersham) was usually diluted 1:10000 in 3 % fat-free milk TBS-Tween buffer . Immunoreactivity was detected using the ECL chemiluminescent system (Amersham), according to manufacturer's instructions.

Histone extraction

For total histone extraction we followed a previously published protocol (Shechter *et al*., 2007). Cells are harvested and washed with PBS. Cell pellet is resuspended in 1 ml hypotonic buffer (10 mM Tris-HCl pH8, 1 mM KCl, 1,5 mM $MgCl_2$ and 1 mM DTT plus protease and phosphatase inhibitors added prior use, see chIP procedure) and incubated for 30 min on a rotator at 4°C to promote hypotonic swelling of cells and lysis by mechanical shearing during rotation. Intact nuclei are pelleted by spinning at 10000g, 10 min, 4°C.

Pelleted nuclei are resuspended in 400 ul of 0,4 N H_2SO_4 and incubated on rotator from 30 min to o/n. Afterwards, nuclear debris are removed by centrifugation at 16000 g, 10 min. Supernatant is further processed by TCA-precipitation. 132 ul of TCA (final concentration 33%) are added drop by drop to the histone solution making sure solutions are mixed well. Solution is incubated on ice for 30 min to o/n. Histones are pelleted by spinning at 16000 g, 10 min, 4°C. Histone pellet is carefully washed twice with ice-cold acetone (acetone is used to remove acid from the solution without dissolving the pellet). Pellet is air-dried for 20 min at room temperature and dissolved in an appropriate volume of water. Protein concentration is determined by Micro BCA protein assay (Pierce) and around 6 ug of histone protein are resolved on a 15 % SDS-PAGE for Western blot analysis.

RNA extraction and reverse transcription (RT)

Cells were harvested and total RNA was purified using Trizol Reagent (Invitrogen) or RNeasy mini kit (Qiagen). Briefly, with the Trizol Reagent, cells

are lysed in a solution of guanidine isothiocyanate and phenol. Addition of chloroform generates a second organic phase into which DNA and proteins are extracted, leaving RNA in the aqueous phase. RNA is then precipitated, washed, dissolved in RNase-free water and stored at -80°C.

For smaller sample volumes, RNA was extracted using the RNeasy mini kit (Qiagen). Samples are first lysed and homogenized. Ethanol is added to the lysate to provide better binding conditions onto the silica-membrane column. In order to remove residual amounts of DNA, an on-column DNase treatment is performed during the procedure. Finally, membrane-bound RNA is eluted with water and stored at -80°C.

For reverse transcription (RT), cDNA was generated from 100 ng of total RNA by using Superscript First Strand Synthesis system (Invitrogen).

PCR and Real Time (RT)-PCR

1ul of RT reaction product was used as the starting material for Polymerase Chain Reactions (PCR). Reactions contained 1,25 U Taq DNA polymerase (Invitrogen), primers (0,2-0,5 uM each), 1x polymerase buffer, 1,5 - 2 mM $MgCl_2$, and 400 uM dNTP mix in a volume of 25 ul. Thermal cycler conditions were adapted to each particular pair of primers. 12,5 ul of the PCR product were analysed by 1-1,2 % agarose gels electrophoresis and visualized by ethidium bromide staining.

When indicated, quantification of gene products was performed by Real Time PCR (qRT-PCR) using the LightCycler 480 SYBR Green Master and Instrument from Roche. The one-component Master Mix for PCR contains a FastStart Taq DNA polymerase and DNA-double-strand-specific SYBR Green dye for product detection. Reactions were prepared in 384-well plates and read in a LightCycler 480 Instrument. Each value was corrected by the expression of the *GAPDH* gene and expressed as relative units.

Customized microarray hybridization and data analysis

A cDNA microarray platform containing 826 cDNA clones was generated at the Microarray Unit of the Centre de Regulació Genòmica (B. Miñana, L. Sumoy, M.Beato, A. Jordan, C. Ballare, M. Melia; http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi; GEO accession number GPL5953). cDNA inserts were PCR-amplified and spotted on Corning UltraGAPS amino-modified glass slides. mRNA samples were processed for first and second strand cDNA synthesis and *in vitro* transcription with T7 RNA polymerase. Universal reference RNA was obtained from Stratagene. RNA was directly labelled with Cy3- or Cy5-dUTP (Amersham) and hybridized to spotted slides. After washing, fluorescent images were obtained using a G2565BA Microarray Scanner System (Agilent) and TIFF images were quantified using GenePix 6.0 (Molecular Devices) software. Raw data was processed using MARGE, an in house developed web implementation of LIMMA, a microarray statistical analysis package of Bioconductor (http://www.bioconductor.org) that is run in the R programming environment. Gene intensities were background subtracted (taking mean of channel intensities and median of background). Spots with intensities <2 times the local

background in either or both dye filter channels (Cy3 or Cy5) as well as controls were excluded from normalization, and were referred as “not reliable”. An intensity dependent normalization algorithm (global lowess) was applied using a smoothing factor f=0.2 for all experiments. Normalized Log_2Ratios (Intensity Cy5/Intensity Cy3) were scaled so that they all had the same median absolute standard deviation across all the arrays, to give the same weight to each gene, and not only due to the magnitude of the expression ratio. The computed B statistic rank value from all replicate hybridizations was used to determine the genes with significant changes. We considered genes that showed a 1,4-fold gene up or downregulation relative to control sample with a B-rank value above the 90^{th} percentile as significant. The value of fold change or copy number relative change was calculated as $2^{Log2Ratio}$, if the value of the ratio was >0, or $2^{-1/Log2Ratio}$, if it was <0.
In order to do the statistical analysis of the data, we have used the opensource, freely available software package for microarray data management and analysis TM4 obtained from TIGR (http://www.tigr.org/software/) that applies the Significance Analyses of Microarrays (SAM) method. This method assigns a score to each gene on the basis of its change in gene expression relative to the standard deviation of the duplicate measurement (Tusher *et al.,* 2001).

RNA interference

For the establishment of cell lines expressing a stable and constitutive RNAi system, we used the pSUPER RNAi system (described at Brummelkamp et al., 2002), which provides a mammalian expression vector that directs intracellular and constitutive synthesis of siRNA-like transcripts (OligoEngine). For this purpose, infectious retrovirus stocks were generated. The GP2-293 packaging cell line stably expresses the viral *gag* and *pol* proteins, but since the VSV-G envelope protein is toxic to cells, it is not integrated into the genome of the cell line. GP2-293 cells were cotransfected by calcium phosphate transfection (BD CalPhos Mammalian Transfection kit (BD Biosciences)) with the plasmid pVSV-G and pSuper-derived plasmids containing the expression cassette for the siRNAs to produce high-titer virus. Target sequences for interference were designed following standard rules (aaN19, GC% 30-70). The sequence and position on cDNA of the siRNAs produced are represented in Figure 5. Viral particles were recovered from the medium after 72 h of transfection and concentrated by sucrose cushion ultracentrifugation (90 min at 26,000 rpm). Viral particles (vectors of the RNAi system) were added to the cells and spinoculated by centrifugation at 1200 x g for 2h at 25 °C. The infected cells were separated in clonal cell lines by serial dilution and were selected for the expression of the siRNA-cassette thanks to the puromycine resistance encoded by the integrated cassette.

For the establishment of cell lines expressing a stable but inducible RNAi system, we used a lentivirus vector-mediated drug-inducible system provided by Dr. Trono (University of Geneva, http://tronolab.epfl.ch/, Wiznerowicz et al., 2003). In short, two different 64-mer oligonucleotides for BRCA1 shRNA (the sequence and position on cDNA are represented in Figure 6) were designed, annealed and cloned into MluI/ClaI-digested pLVTHM

plasmid. Oligonucleotides have the following general structure: 5'-CGCGTCCCC-N19-TTCAAGAGA-rcN19-TTTTTGGAAAT-3' and 5'-AGGGG-N19-AAGTTCTCT-rcN19-AAAAACCTTTAGC-3', being N19 the specific target sequence for BRCA1 and rcN19 its reverse complementary sequence. Target sequences for interference were designed following standard rules (aaN19, GC% 30-70). For the production of the lentiviral particles, $2{,}5x10^6$ 293T cells (Clontech) in 10 cm dishes were transfected with the plasmids ptTR-KRAB-Red, pLVTHM-shBRCA1 (10 ug), pCMV-R8.91 (6,5 ug) and pMD.G (3,5 ug) by CalPhos Mammalian Transfection kit (BD Biosciences). Medium was collected every 24 hours after transfection, for 2 days and concentrated by sucrose cushion ultracentrifugation (90 min at 26,000 rpm). Pellets containing viral particles were dissolved in medium and used for cell infection. Cells were infected by spinoculation, i.e. plates were centrifuged at 1200 x g for 2h at 25°C. Firstly, cells are infected with lentiviral vectors carrying the Doxycycline-responsive KRAB repressor and RedFP (ptTR-KRAB-Red). Afterwards, cells are infected with the corresponding pLVTHM-shRNA-carrying vector. After 3 days of doxycycline (Dox) treatment, the KRAB repressor is expressed and allows for the expression of the shRNAs (and GFP). The induced cell lines are then sorted in a FACSvantageSE (Becton Dickinson) for RedFP-positive and GFP-positive fluorescence. Sorted cells were amplified in the absence of Dox until it was required for the experiment (2,5 ug/ml).

In the case of transient transfection of siRNAs, we followed the instructions for the siRNA transfection with Lipofectamine 2000 (Invitrogen). Briefly, cells were plated at different levels of confluency (40-60%) in medium without antibiotics one day prior transfection. The siRNA oligomer is diluted in Opti-MEM medium (for a 100 nM final concentration) as it is the Lipofectamine 2000 reagent. After 20 min of incubation, both dilutions are combined and incubated for 25 min at room temperature. Mixture is then added to the cells and left overnight before changing to complete medium. After 72 hours, cells are ready to be assayed for gene knockdown.

Yeast two-hybrid system

We followed the Matchmaker Gal4 Two-Hybrid System 3 (Clontech) manufacturer's instructions for the two-hybrid experiments. Briefly, gene sequences encoding for ER, PR, BRCA1 and the different BRCA1 mutants (Gln1756insC 5382, Tyr1853insA 5677, Pro1749Arg 5365, Ala1708Glu 5242) were cloned in-frame at the BD or AD vectors. These vectors were tested separately for protein expression and for the activation of the reporter system in the yeast strain AH109. Yeast competent cells were prepared and transformed with the combinations of plasmids (Table 7B) according to instructions. Cells were plated in selective medium lacking Leu, Trp, His and Ade for detection of protein-protein interactions.

Nuclear extract preparation and co-immunoprecipitation

Nuclear extract from treated or untreated cells was prepared exactly as described by Dignam *et al* (Dignam *et al.,* 1983). Buffers used for the extraction

were: Buffer A (10 mM HEPES (pH 7,9 at 4°C), 1,5 mM $MgCl_2$, 10 mM KCl and 0,5 mM DTT); Buffer C (20 mM HEPES (pH 7,9), 25% (v/v) glycerol, 0,42 M NaCl, 1,5 mM $MgCl_2$, 0,2 mM EDTA, 0,5 mM PMSF and 0,5 mM DTT); Buffer D (0,3 M HEPES (pH 7,9), 1,4 M KCl and 0,03 M $MgCl_2$). DTT and PMSF were added fresh to the buffers just before use.

The procedure for extract preparation was as follows. Cells were harvested and washed with cold PBS. Pelleted cells were suspended in five volumes of buffer A and allowed to stand for 10 min. Cells were collected by centrifugation and suspended in two volumes of buffer A and lysed by 10 strokes of a glass Dounce homogenizer (B type pestle). The homogenate was centrifuged for 10 min at 2000 rpm to pellet nuclei. The supernatant was carefully decanted and the pellet was subjected to a second centrifugation for 20 min at 25000 g to remove residual cytoplasmic material. The pellet was then resuspended in 3 ml of buffer C per 10^9 cells and homogenized with a glass Dounce homogenizer (10 strokes with type B pestle). The resulting suspension was stirred gently with a magnetic stirring bar for 30 min and then centrifuged for 30 min at 25000 g. The resulting supernatant was dialyzed against 50 volumes of buffer D for, at least, 5 hours. The dialysate was centrifuged at 25000 g for 20 min and used for immunoprecipitation.

For the co-immunoprecipitations (co-IP), from 500 to 3000 ug of nuclear extract was incubated with 6 ug of antibody against BRCA1 (Ab1 from Oncogene) for 3 h at 4ºC on a orbital rotator. 60 ul of Protein G beads slurry (Sigma) were washed with washing buffer (50 mM Tris pH 8, 150 mM NaCl and 1 % NP-40) and were pre-absorbed with sonicated salmon sperm (Sigma) and BSA for 3 h at 4ºC with rotation. After extensive washing, the beads were added to the nuclear extract and incubated o/n at 4ºC on a rotator. Beads were afterwards spinned down and washed extensively with washing buffer, before being suspended and boiled in SDS-loading buffer. Supernatant was subjected to SDS-PAGE and Western blot analysis.

Protein degradation rate

Cells were treated with progestin 10 nM for 0 to 12 hours. PR protein level was detected at different time points by Western blot (as described before) and quantified by densitometry (MultiGauge, Fujifilm). The data was represented in two ways. Graphs “a” represent the percentage of remaining PR protein, taking the time 0 as the 100% level. From graphs “b”, representing the Ln of the values, a trendline was extrapoled and from its slope an approximate degradation rate can be estimated.

Ubiquitination assays

In vivo ubiquitination assays were performed as follows. T47D-YV-flagPRB cells were transfected and infected to overexpress HA-ubiquitin and BRCA1. Cells were pre-treated with lactacystin (10 uM) for 1 h and treated with R5020 (10 nM) for 6 h. Subsequently, nuclear extracts (as described before) were prepared and immunoprecipitated with an antibody against the flag tag or control antibody. Precipitated material was run in a SDS-PAGE and

immunodetected by Western blot analysis with an antibody against the flag-tag (Sigma).

In vitro ubiquitination assays were performed as follows (Wu-Baer *et al.,* 2003). Reactions were conducted at 37°C for 1 h in a 30-ul volume containing buffer (50 mM Tris-HCl pH 7,4, 5 mM $MgCl_2$, 2 mM NaF, 10 nM okadaic acid, 2 mM ATP, 0,6 mM DTT) and +/- of the following proteins as indicated: E1 (138 ng), E2 (UbcH5c, UbcH6, UbcH10, UbcH1, UbcH8 or UbcH3; 0,75 ug), ubiquitin (2 ug), E3 (BRCA1/BARD1-purified heterodimer or I26ABRCA1/BARD1 heterodimer, 50 ng) and substrate (purified hormone-activated PR, 200 ng). Reaction is stopped by adding 6x SDS-loading buffer and reaction products are fractionated by SDS-PAGE and detected by Western blot analysis with an antibody against PR (H190, Santa Cruz).

Purification of recombinant proteins from baculovirus systems

The BRCA1/BARD1 and I26ABRCA1/BARD1 heterodimer proteins were produced using the Baculovirus Expression Vector System (Pharmingen) at the laboratory of Dr. Baer (Institute for Cancer Genetics, Columbia University).
To generate recombinant baculoviruses encoding the BRCA1 polypeptide, Sf9 insect cells were transfected with the flag-BRCA1-6His/pVL1392 vector using the BaculoGold transfection kit (Pharmingen). Recombinant viruses encoding BARD1 were obtained by transfecting Sf9 cells with the GST-BARD1/pVL1392 vector. For expression of the BRCA1/BARD1 heterodimer, 500 ml of Sf9 cells were co-infected with BRCA1 and BARD1 recombinant baculoviruses at a MOI of around 2 pfu/cell for each. Cells were harvested 72 h after infection, and the cell pellet was resuspended in lysis buffer (50 mM HEPES pH7,9, 250 mM NaCl, 0,1% NP-40, 10 mM β-mercaptoethanol, 10% glycerol) with protease inhibitors cocktail (Roche). After 15 min incubation on ice, the resuspended cells were sonicated, the cell debris removed by centrifugation at 30000 g for 45 min at 4°C and the supernatant filtered through a 0,45 um filter (Nalgene). To purifiy the BRCA1/BARD1 complex, the filtered lysate was incubated with Ni-NTA agarose resin (Qiagen) for 2 h at 4°C. The resin was then washed twice with lysis buffer, twice with “Ni Wash buffer” (25 mM Tris-HCl pH7,9, 50 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol and protease inhibitors) containing 10 mM imidazole, packed into a column and eluted sequentially with “Ni Wash buffer” containing 30 mM and then 250 mM imidazole. The 250 mM imidazole eluate was pooled, adjusted to 250 mM NaCl and 0,1 % NP-40 and incubated with glutathione-agarose beads (Sigma). After incubation for 3h at 4°C, the beads were washed twice with “GST Wash buffer” (25 mM Tris-HCl pH7,9, 250 mM NaCl, 0,1 % NP-40, 10 mM b-mercaptoethanol, 10% glycerol and protease inhibitors) and twice with “Storage buffer” (25 mM Tris-HCl pH7,9, 50 mM NaCl, 0,02 % NP-40, 1 mM DTT, 20% glycerol and protease inhibitors). The beads were then packed into a column, eluted with “Storage buffer”containing 10 mM glutathione, and the peak protein fractions were pooled and stored in aliquots at -80°C. The same procedure was followed in the case of the I26ABRCA1/BARD1 heterodimer.

For the purification of the progesterone receptor recombinant protein the procedure was as follows.

Recombinant viruses were obtained by transfecting Sf9 insect cells with the plasmid encoding histidine-tagged PRB (His-PRB-pVL1393). After 48 hours of infection, cells are harvested and washed with PBS. Cell pellet is resuspended in 5 ml of ice-cold homogenization buffer (20 mM Tris pH7,8, 0,2 mM EDTA pH8, 2 mM DTT, 10 mM NaCl and protease inhibitors) per ml of cell pellet and is incubated for 10 min. Suspension is homogenized on ice with a glass Dounce (type B pestle) in 4 rounds of 5 strikes and 3 min stand. NaCl 4 M and glycerol are added to take it to 50 mM NaCl and 10% glycerol. The suspension is ultracentrifugated at 4°C at 105000 g. The supernatant (taking out supernatant membranes) contains the protein. 10 ul (approx. 1 uCi) of ^{3}H-Organon2058-solution (a synthetic steroid with progestational activity) are added per ml of protein extract and incubated for 30 min at room temperature.

Activated receptor is purified through a phospho-cellulose (PC) column (Atger *et al.,* 1976). Protein extract is loaded on the PC column and washed with PC-washing buffer (20 mM Tris pH7,8, 1 mM DTT, 10% glycerol, 90 mM NaCl). Activated receptor is eluted in PC-washing buffer with 300 mM NaCl.
Pooled fractions are further purified through a Ni-NTA-Agarose column. First, the eluate is adjusted to 10 mM Imidazole. Afterwards, eluate is loaded on the column, washed (20 mM Tris pH7,8, 1 mM DTT, 10% glycerol, 300 mM NaCl, 20 mM imidazole and a second wash with 90 mM NaCl), and eluted in Ni-washing buffer with 250 mM imidazole. Finally, eluate is desalted by using a NAP-5 column (Amersham).

Site-directed mutagenesis

For the generation of a plasmid encoding the mutant PR-BK388R gene we mutagenized the wt plasmid pRAV-Flag-PRB. We used the QuikChange site-directed mutagenesis kit (Stratagene). The oligonucleotide used for the mutagenesis was designed with the QuikChange Primer Design Program (5'-CCGCCCGCTCTAAAGATAAgGGAGGA-3' and its complementary). It changed a single codon encoding for lysine 388 (AAG) to an amino acid of a similar polarity, arginine (AgG). Briefly, the protocol is as follows. The plasmid and oligonucleotides are denatured and annealed. The oligonucleotide primers are then extended by the Pfu Turbo DNA polymerase. Incorporation of the oligonucleotides generates a mutated plasmid containing staggered nicks. Parental wt DNA template is digested with DpnI endonuclease, which is specific for methylated and hemimethylated DNA (parental DNA). The remaining nicked vector DNA containing the mutation is transformed into XL1-Blue competent cells, which repair the nicked DNA. Mutation was verified by sequencing.

Generation of stable cell lines by retroviral infection

For the establishment of stable cell lines expressing flag-PRB and flag-PRBK388R we used an existing cell line devoid of endogenous PR isoforms A and B expression and stably transduced with a single copy of the MMTV-Luc reporter (Quiles *et al*., 2009). To stably express the PRB forms in these cells we have used a retroviral vector that, at low multiplicity of infection, allows for the controlled integration of a limited number of copies of the gene of interest in the

host genome. First, we generated retroviral particles containing the vectors of interest. $2,5x10^6$ GP2-293 packaging cells (Clontech) were transfected with plasmids pRAV-Flag-PRB wt, pRAV-Flag-PRBK388R or empty pRAV-Flag and pVSV-G (encoding the virus envelope protein) in 10 cm dishes using calcium phosphate transfection (BD Biosciences). Medium was collected every 24 h for two days and centrifuged for 1,5h at 26000 rpm at 4ºC in a 20% sucrose cushion to concentrate viruses. Pellet containing viral particles was dissolved in medium and used for cell infection. Cells ($3,5x10^5$) were infected at different multiplicities in six-well plates using the spinoculation method (plates were centrifuged at 1200 rpm for 2h). The proportion of infected cells was followed by FACS analysis of GFP expression. GFP-positive cells were isolated by two rounds of sorting in a FACSvantageSE (Becton Dickinson).

Adenovirus production

For the generation of recombinant adenoviruses for the expression of BRCA1 (AdBRCA1) and I26ABRCA1 (AdI26A), we used the AdEasy Adenoviral Vector System (Stratagene) following manufacturer's instructions with modifications according to published protocol (Luo *et al.,* 2007). BRCA1 and I26ABRCA1 cDNAs were obtained by restriction enzyme digestion from pCR3-BRCA1 and pSG5-I26ABRCA1 and cloned into the pShuttle-CMV vector. Cloned vector were verified by sequencing. Shuttle plasmid clones were grown and plasmid DNA was purified by conventional alkaline lysis procedure. Plasmid DNA was linearized with PmeI digestion and DNA recovered by phenol/chloroform extraction and ethanol precipitation. Linearized plasmids were electroporated into electrocompetent AdEasier cells (already containing pAdEasy-1 vector). Cells were then plated in two to five LB/kanamycin plates and grown overnight at 37ºC. Several of the smallest colonies were picked and plasmid DNA obtained by alkaline lysis procedure. The presence of recombinant Ad plasmids is checked by PacI digestion. Correct recombinant Ad plasmids are then retransformed into XL10-Gold (a strain not prone to recombination) for amplification. Sufficient amounts of amplified and purified recombinant Ad plasmids are digested with PacI and ethanol-precipitated for the final generation of adenoviruses. HEK-293 packaging cells were plated at a confluency of approximately 50% in two 25 cm^2 dishes for each plasmid. Ad plasmids were transfected into the packaging cells with LipofectAMINE as described before. After transfection, cells are incubated at 37ºC for 14-20 days without any medium change. After this period, adenoviruses generated are released from cells (they are mostly attached to the cells, not in the medium) by performing four freeze-thaw-vortex cycles: cells are freezed in a dry-ice/methanol bath, thaw in a 37ºC water bath and vortex vigorously for 30 sec. For amplification of adenoviruses, the supernatant is used for infecting HEK-293 cells plated at a 80-90% confluency. Cells are collected after 3 to 5 days (when 30-50% of cells are detached) and adenoviruses are released as before. For further amplification, the same procedure is followed increasing the size and the number of plates infected until obtaining high-titer adenoviruses.
Adenovirus encoding wtBRCA1 were also kindly provided by Dr. Didier Marot.

Chromatin immunoprecipitation (chIP)

Chromatin immunoprecipitation was basically performed as described (Strutt and Paro, 1999). After treatment, cells are replaced with fresh medium and proteins are crosslinked to DNA by adding a formaldehyde-containing solution (50 mM HEPES pH8, 0,1 M NaCl, 1 mM EDTA pH8, 0,5 mM EGTA pH 8, 11 % formaldehyde) directly to culture medium to a final concentration of 1% and incubating cells for 10 min at 37°C. Crosslinking is stopped adding Glycine to a final concentration of 0,1 M and incubating for 5 min at room temperature. Afterwards, medium is removed and cells are washed twice with ice-cold PBS containing protease and phosphatase inhibitors (1 mM PMSF, 1 ug/ml aprotinin, 1 ug/ml pepstatin A, 1 uM $NaVO_4$, 20 mM b-glycerophosphate and Protein Inhibitor Cocktail (Roche)). Cells are scrapped in PBS+inhibitors and are pelleted.

Cell pellets are resuspended in 2,5 ml of Cell Lysis buffer (5 mM PIPES pH8, 85 mM KCl, 0,5 % NP-40 + inhibitors) and are incubated for 10 min on ice. After cell lysis, nuclei are pelleted for 5 min at 4000 rpm 4°C and are then resuspended in 1 ml of Nuclei lysis buffer (1 % SDS, 10 mM EDTA pH8, 50 mM Tris-HCl pH 8,1). After 10-min incubation on ice, lysate is sonicated on ice to shear DNA to lengths between 300 and 500 bp. After sonication, the solution is centrifuged 5 min at 4000 rpm 4°C and the supernatant is recovered (chromatin solution). In order to quantify the DNA present at the solution, an aliquot of the chromatin is treated with Proteinase K and the DNA is recovered by phenol/chloroform extraction. DNA can be quantified and also the size of the fragments can be checked in a 1,2 % agarose gel. Chromatin solution is stored on ice at 4°C.

To perform a chromatin immunoprecipitation experiment, 20 to 60 ug of chromatin is used, diluted 10x in chIP buffer (0,01% SDS, 1,1% TritonX-100, 1,2 mM EDTA pH8, 16,7 mM Tris-HCl pH8,1, 167 mM NaCl). To reduce non-specific precipitations, diluted chromatin is pre-cleared with 15 ul of Salmon Sperm DNA/Protein A or G Agarose (Upstate) for 4 h at 4°C with rotation. The pre-cleared chromatin is then collected and an aliquot is reserved for Input information. The corresponding immunoprecipitating antibody is added in a variable amount depending on the antibody (from 1 to 7 ug) and is left o/n at 4°C with rotation. One aliquot is incubated with a control antibody normal rabbit or mouse IgG (Sigma) depending on the specie of the specific antibody used.
After incubation, 30-50 ul of Salmon Sperm DNA/Protein A or G Agarose is added for two more hours of incubation at 4°C with rotation. This way, the protein A/G of the agarose beads serves to precipitate the antibody bound to the specific protein supposedly bound to fragments of DNA. Agarose is pelleted by gentle centrifugation (1 min 3600 rpm) and is extensively washed (5 min at 4°C with rotation) with the following washing buffers: Buffer 1 (0,1% SDS, 1% TritonX-100, 2 mM EDTA pH8, 20 mM Tris-HCl pH8,1, 150 mM NaCl), Buffer 2 (0,1% SDS, 1% TritonX-100, 2 mM EDTA pH8, 20 mM Tris-HCl pH8,1, 500 mM NaCl), Buffer 3 (0,25 M LiCl, 1% NP-40, 1% Sodium deoxicholate, 1 mM EDTA pH8, 10 mM Tris-HCl pH8,1) and two washes with Tris-EDTA buffer.

In order to elute the protein-DNA bound to the agarose beads, elution buffer (1% SDS, 0,1M $NaHCO_3$) is added to the beads, vortexed vigorously for

10 seconds and incubated for 15 min at room temperature, twice. The supernatant is recovered by centrifugation at 3600 rpm for 5 min at room temperature. Crosslinking protein-DNA is reversed by adding 0,2 M NaCl and incubating at 65°C o/n. Afterwards, the sample is treated with Proteinase K and DNA is recovered by phenol/chloroform extraction. DNA is precipitated with ethanol+10% sodium acetate and 0,1 % glycogen, washed and, finally, DNA is dissolved in DNAse-free water (Ambion). Aliquots of this DNA (2 ul) were used for PCR amplification using primers for specific promoter regions.

Short-term proliferation assay

During treatment, cells were pulsed with bromodeoxiuridine (BrdU, 10 umol/L) for 2 to 4 h prior to harvesting. Cells were then harvested in ice-cold PBS and incubated on ice for at least 20 min. Cells were then fixed for a period of 72 h with 70 % ethanol, at 4°C. After that period, DNA is denatured with 2N HCl/0,5% BSA, added in constant mixing and is left for 10 min at room temperature. Pellets are then washed 3 times with ice-cold PBS. Pellets are incubated in a 1/2000 dilution of antibody anti-BrdU (Pharmingen) in PBS 0,5% BSA, for 1 h at room temperature and rotation. Afterwards, pellets are washed twice with PBS and are incubated in a 1/100 dilution of a secondary antibody anti-mouse-FITC in PBS 0,5% BSA, for 45 min at room temperature, dark and rotation. Finally, pellets are washed twice with PBS and processed for Propidium iodide staining (PI).

Pellets are resuspended in 1 ml of Analysis solution (0,3 mg/ml Ribonuclease A (Sigma), 1,14 mM sodium citrate, 15 ug/ml propidium iodide, in PBS) and incubated in this solution o/n at 4°C prior to analysis. Samples were analyzed using a FACS Calibur machine (Becton Dickinson), CellQuest analysis software and ModFit program. A CV<3 was taken as a requisite for good cell cycle population separation.

Long-term proliferation assay

Cells were plated in triplicate and treated accordingly from one through four days, and spent media was replaced every 48 h. At each time point, cells were collected and counted with a Coulter counter (Beckman Coulter). Counter was set to count particles from 8-24 um size.

Cell survival assay

To estimate cell survival to serum withdrawal, cells were plated and grown to confluency, when they were changed to serum-free conditions +/- treatments for a period of 6 days. At that time, cells in the supernatant and still attached were collected and assayed for viability with Trypan blue staining (Sigma) and standard hemacytometer chamber counting. Trypan blue does not react with the cells unless the membrane is damaged, so acting as a dye exclusion method.

In silico PEST motifs analysis

PESTfind tool (http://www.at.embnet.org/toolbox/pestfind/) allows for the identification of PEST motifs in protein target sequences. The PEST hypothesis was based on the observation of protein stabilities and protein primary sequence information (Rogers *et al.,* 1986; Rechsteiner and Rogers, 1996). From these observations, it was concluded that local concentrations of proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST) reduced the half-lives of proteins dramatically and hence, that they target proteins for proteolytic degradation. The quality of "valid" PEST motifs is refined by means of a scoring parameter based on the local enrichment of critical amino acids as well as the motif's hydrophobicity as expressed by the following equation:

PEST score = 0.55 * DEPST - 0.5 * hydrophobicity index.

"Valid" PEST motifs below the threshold score (5.0) are considered as "poor", while PEST scores above the threshold score are of real biological interest. The higher the PEST score, the more likely is degradation of proteins mediated via "potential" PEST motifs in eukaryotic cells.

In silico protein stability analysis

ProtParam (http://www.expasy.ch/tools/protparam-doc.html#ref5) computes various physico-chemical properties that can be deduced from a protein sequence (Gasteiger *et al.,* 2005).

The *half-life* is a prediction of the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell. ProtParam relies on the "N-end rule", which relates the half-life of a protein to the identity of its N-terminal residues (Ciechanover and Schwartz, 1989; Varshavsky, 1997). The N-end rule originated from the observations that the identity of the N-terminal residues of a protein plays an important role in determining its stability *in vivo* (Bachmair *et al.,* 1986).

The *instability index* provides an estimate of the stability of a protein in a test tube. It is based on the method developed by Guruprasad et al (Guruprasad et al., 1990). Statistical analysis of 12 unstable and 32 stable proteins revealed that there are certain dipeptides, the occurrence of which is significantly different in unstable proteins compared with the stable ones. The authors assigned a weight value of instability to each of the 400 different dipeptides and using these values it is possible to compute a instability index. A protein whose instability index is smaller than 40 is predicted as stable and a value above 40 predicts that the protein may be unstable.

Statistical analyses

Results were analyzed by Student's t test. Differences between two means with a $p<0,05$ or $p<0,01$ were regarded as significant.

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