Circadian Modulation of the Estrogen Receptor Alpha Transcription

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Abstract

The circadian clock is a molecular mechanism that synchronizes physiological changes with environmental variations. Disruption of the circadian clock has been linked to increased risk in diseases and a number of disorders (e.g. jet lag, insomnia, and cancer). Period 2 (Per2), a circadian protein, is at the center of the clock's function. The loss or deregulation of *per2* has been shown to be common in several types of cancer including breast and ovarian [1, 2]. Epidemiological studies established a correlation between circadian disruption and the development of estrogen dependent tumors. The expression of estrogen receptor alpha (ERα) mRNA oscillates in a 24-hour period and, unlike Per2, ERα peaks during the light phase of the day. Because up regulation of ERα relates to tumor development, defining the mechanisms of ERα expression will contribute to our comprehension of cellular proliferation and regulation of normal developmental processes. The overall goal of this project is to investigate the molecular basis for circadian control of ERα transcription. Transcriptional activation of ERα was measured using a reporter system in Chinese hamster ovary (CHO) cell lines. Data show that Per2 influences ERα transcription through a non-canonical mechanism independent of its circadian counterparts. Breast cancer susceptibility protein 1 (BRCA1) was confirmed to be an interactor of Per2 via bacterial two-hybrid assays, in accordance with previous studies [2]. BRCA1 is a transcriptional activator of ERα promoter in the presence of octamer transcription factor-1 (OCT-1) [3]. Our results indicate that the DNA binding

domain of OCT-1, POU, to directly interact with Per2 and BRCA1, *in vitro*. Pull-down assays were used to map direct interaction of various Per2 and BRCA1 recombinant proteins and POU. Chromatin immunoprecipitation assays confirmed the recruitment of PER2 and BRCA1 to the estrogen promoter by OCT-1 and the recruitment of Per2 to the ERα promoter decreases ERα mRNA expression levels in MCF-7 cells. Our work supports a circadian regulation of ERα through the repression of *esr1* by Per2 in MCF-7 cells.

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List of Abbreviations

AF-1	Activation-Function Domain D-domain
BARD	BRCA Associated Ring Domain protein
BIC	Breast Cancer Information Core
BRCA1	Breast Cancer Associated 1
BRCT	BRCA C-terminus
CHO	Chinese hamster ovary-K1
CKIε	Casein Kinase1ε
CREB	cAMP-Response Element-Binding Protein
Cry	Cryptochromes
DBD	DNA Binding Domain
DMEM	Dulbecco's modified Eagle medium
EGFR	Epidermal Growth Factor receptor
ER	Estrogen Receptors
ERE	Estrogen Response Element
ERα	Estrogen Receptor Alpha
Esr1	Estrogen Receptor Alpha gene
EV	Empty Vector
FBS	Fetal Bovine Serum
FPLC	Fast Protein Liquid Chromatography
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HER2	Human Epidermal Growth Factor Receptor 2
HSP-90	Heat Shock Protein 90
IFN-γ	Interferon-γ
IGF-1	Insulin Like Growth Factor-1
IPTG	Isopropyl β-Thiogalactopyranoside
LBD	Ligand Binding Domain
NES	Nuclear Export Signal
NES	Nuclear Export Signals
NHR	Nuclear Hormone Receptors
NLS	Nuclear Localization Signals
NLSs	Nuclear Localization Signals
NR	Nuclear Receptors
OCA-B	OCT-1 Associated Coactivator
ONPG	o-nitrophenyl- beta-D-galactopyranoside
ONR	Orphan Nuclear Receptors
PAS	Per, ARNT, Sim domain
per	Period Genes
PolII	RNA polymerase II
PPAR	Proliferator-activated Receptor α, γ and δ
PR	Progesterone Receptor
RING	Really Interesting New Gene domain
SCN	Suprachiasmatic Nucleus
SERDs	Selective Estrogen Receptor Down-Regulators ()
SERM	Selective estrogen receptor modulators

SMRT	Silencing Mediator for Retinoid and Thyroid Hormone Receptor
UTR	Untranslated Region

Attributions

Linda Villa performed the experiments in Figs. 4.2.B, 4.3.B (right two panels), 4.4.B-C, 4.5, 4.7, 4.8, 4.9.B, and collaborate in 4.9.C. Kevin Kim and Shane McTighe developed the bacterial two-hybrid screening and analyzed the sequencing data. Complete results of their studies were included in Kim's MS thesis and are summarized in Fig. 4.1.A. Xiao Yi performed the experiments in Figs. 4.1.B, 4.2.A, 4.3.A, 4.3.B (left two panels), 4.6, and 4.9.A while a PhD student in Dr. Finkielstein's lab. Mr. Yi's results were previously summarized in his prospectus and in various progress reports to his committee members throughout his stay in the lab. Carlo Santos identified the BRCA1/hPer2 interaction and Sarah Cousins found the BRCA-responsive Oct-1 site in *esr1* (Fig. 4.4.A). Xiangping Fu performed the RNA extraction and qRT-PCR and Marian Vila Caballer statistical analysis in Fig. 4.9.C. Linda Villa wrote her thesis. Dr. Finkielstein formulated the hypothesis, proposed the model, and provided assistance and direction when needed.

Chapter 1

Introduction

Estrogen

The sex hormone, estrogen, is a key steroid derivative in the estrous cycle and also responsible for secondary sexual characteristics such as breasts in women. Estrogen is not only important in female reproduction and development, but also necessary for maturation of sperm in males. Estrogens have function outside of reproduction in both females and males. These hormones have also been found to have an influence in cardiovascular and bone health as well as cognition and behavior [4-9]. There are three types of estrogen; estrone, 17β-estradiol, and estriol. These steroids derive from cholesterol. Main sites for estrogen synthesis are the ovaries for 17β-estradiol, whereas estrone and estriol are primarily made in the liver. The precursors of estrogen, androstenedione and testosterone, are aromatized in the final synthesis step of cholesterol to estrone and 17β -estradiol, respectively [7].

The ovaries of premenopausal women are the main sites of estrogen synthesis [10]; in contrast, estrogen originates from extragonadal sites in men and postmenopausal women. The most potent and second most abundant estrogen, 17β-estradiol, is the main ligand for estrogen receptors (ERs). The two metabolites of 17β-estradiol, estrone and estriol bind to ERs, albeit with a much lower affinity [11].

Aromatase, the key enzyme for estrogen synthesis, can also be found in the breast, nervous tissue, fat, muscles and cells in the testes, suggesting a role for estrogen function in these sites [7, 12-15]. The initiation of puberty, in young girls, commences upon the increase of estrogen serum concentration: signaled by gonadotropins [6]. Estradiol serum concentration varies during the menstrual period; it is at its highest concentration in the preovulatory phase of the cycle [5, 16]. The concentration of estradiol is at its lowest in premenstrual girls and postmenopausal women. Curiously, estrogen also oscillates in a day/night rhythm. Along with testosterone, estrogen is at its highest concentration at the late of night, suggesting a circadian regulation of hormones [17-19].

Estrogen's canonical pathway involves its diffusion through cell membranes seeking out and binding to the estrogen receptor (ER). A detailed explanation of the signaling pathway involving ligand-receptor interactions is presented in the estrogen receptor alpha signaling section.

Estrogen Receptors

Estrogen receptors belong to a large family of steroid nuclear receptors (NRs). They are either nuclear hormone receptors (NHR) or orphan nuclear receptors (ONR) (the ligand has yet to be determined for ONR) [20]. These receptors are typically ligand activated transcription factors with similar structural features: a DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) [21].

ERα, was first identified and characterized by Jensen in 1962 [9], and first cloned in 1986 a subtype, ERβ was cloned 9 years later [9]. These receptors have various isoforms and splice variants [22]. ERα and ERβ are synthesized from different

chromosomes and have distinct functions in cells, with some overlapping functions [23-25]. The subtypes, ERα and ERβ, share a similar DBD, however the sequence homology between the two is only around 55% [26]. The difference in sequence homology results in different binding affinities for certain ligands, for example ERα has a higher affinity for 17β-estradiol than ERβ [11]. Because ERα and ERβ bind 17β-estradiol, albeit with different affinities, the determining factor for estrogen signaling in tissues is the relative abundance of the two receptors at the target tissue.

Although both subtypes are widely expressed, there are notable differences in specific tissues. ERα RNA is expressed in endometrium cells and hypothalamus; ERβ RNA is found in prostrate, lung, brain, heart and endothelial cells [25, 27]. ERβ is typically found in breast tissue; however, ERα is upregulated in breast cancer tissue [7, 25]. The upregulation of ERα in breast cancer tissue has yet to be understood, but it is this receptor that is one of the main targets for the treatment of breast tumors[28-31].

Structure of ERα

The synthesis of ERα is encoded by *esr1* located on the 6q25.1 chromosome [32]. Unlike the majority of nuclear receptors, *esr1* consists of seven distinct promoters encoding ERα. Seven estrogen promoters spanning 450 kb of chromosome 6 generate various isoforms of ERα [33]. The isoforms transcribed from the seven promoters typically vary at the 5' untranslated region (UTR) and yet, still result in the expression of a 66 kDa ERα protein [22]. Interestingly, the promoter used for the transcription of ERα is dependent on the tissue and cell line being examined [22]. The transcriptional regulation of ERα has not been fully examined, however the first ERα promoter

identified was the A promoter (~163bp). It is this promoter that has been found to be the major promoter used when ERα is upregulated [33]. The gene transcripts consist of eight exons and seven introns (Figure 1.1). The eight exons encode for six regions named A-F. Exon 1 encodes for the A/B region largely involved in protein-protein interactions and transcriptional activation of target gene expression. This region contains the activation-function domain (AF-1) primarily involved in binding directly or through co-activators and co-repressors to transcription machinery. ERα AF-1 region is variable compared to ERβ and highly unstructured. The A/B region contains several phosphorylation and sumoylation sites. Phosphorylation sites in the A/B region are involved in non-ligand activation of ERα for downstream signaling. This region may be unstructured, however upon binding to co-activators/co-repressors the unstructured takes form. The AF-1 domain is able to bind to ERE sequences and activate transcription with the minimum presence of the DNA binding domain.

The end of exon 2 and exon 3 encode for region C. Region C is the highest conserved region among NRs. This region comprises the DNA binding domain (DBD). The DBD is critical in receptor dimerization and sequence specific binding to DNA. The globular domain contains a hydrophobic base consisting of two α-helices. The base is involved in the recognition of DNA, and required in the DNA dependent DBD dimerization of the ER [22].

The N terminal end of exon 4 encodes for region D. Region D is known as the hinge region (D-domain). The D-domain is a highly variable region that has very little structural information. The region contains part of the nuclear localization signals (NLS)

and sites for posttranslational modifications, specifically sumoylation and acetylation [34, 35].

Regions E and F encoded by exons 4-8 contain the second most highly conserved domain among ERs, the ligand-binding domain (LBD). This region also contains the domain necessary for homo- and hetero-dimerization of the receptor. In the absence of a ligand the ERα LBD is bound to heat shock protein 90 (HSP-90). The heat shock protein holds ERα in a structural conformation ready for estradiol binding [36].

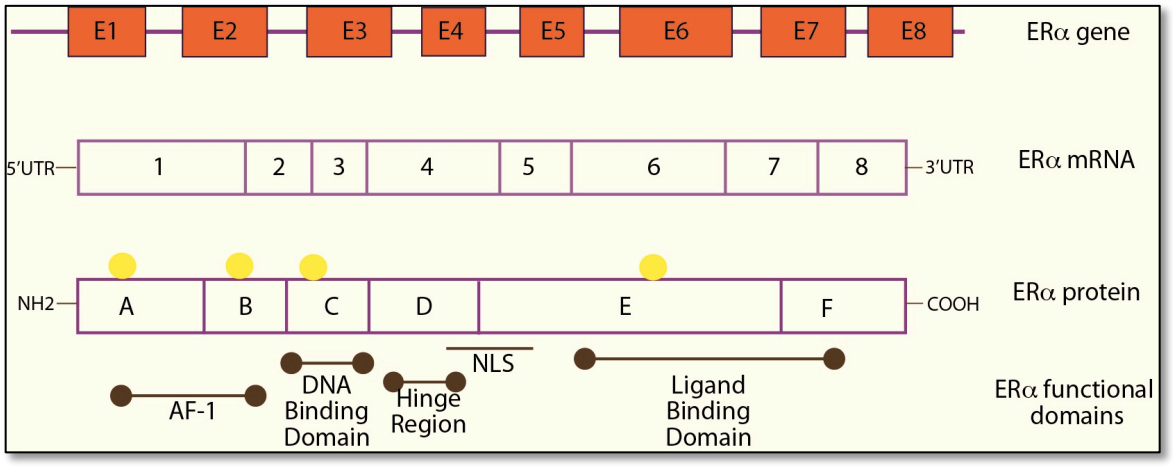


Fig. 1.1 *Organization, post-translational modifications and functional domains of ERα.* E1 represents exons that make-up the mRNA of ERα. Yellow dots on protein schematic represent phosphorylation sites for ERα.

Estrogen receptor α signaling

There are several known pathways of estrogen receptor signaling including ligand dependent (canonical and non-canonical signaling), ligand independent, and non-nuclear activated signaling [7]. The classical pathway of estrogen receptor signaling is ligand dependent. Upon binding to estradiol, the estrogen receptor disassociates from HSP-90, undergoes conformational change and moves to the nucleus [7, 37]. In a non-classical signaling event, ERα still binds estrogen, however, the complex does not bind directly to DNA. Instead the ERα-estrogen complex binds to transcription factor(s) that in turn binds to DNA. Such is the transcriptional activation of collagenase and insulin like growth factor-1 (IGF-1) when ERα is recruited by the transcription factor, Jun/Fos to the AP-1 site of the gene, respectively (Figure 1.2). These genes do not contain the common estrogen response element (ERE) site for ERα binding but are still ERα regulated via protein-protein interactions [8].

ERα and G-proteins can be found in plasma membrane invaginations. ERα and G-proteins interact with each other as well as recruit signaling molecules necessary for ERα non-nuclear signaling. Epidermal growth factor receptor (EGFR) and ERα interaction and activation will recruit kinases such as Src, Shc and a subunit of P13K (p85α) [38]. The recruitment of these kinases lead to phosphorylation of ERα and activation of a multiple kinase signaling cascades [39]. In addition, the recruitment of these kinases to the caveolae will activate secondary signaling cascades such as the MAPK, P13K and PKC to prompt proliferation, metastasis and survival of the cell [40].

The presence of ERα is critical in the diagnosis of breast cancer. If ERα is upregulated in breast carcinomas, there are options for anti-estrogen therapy. This is

especially true for the majority cases of sporadic breast cancer. Unfortunately, for those that are ERα negative, anti-estrogen therapy is not an option. That also goes for cases where ERα is mutated and unable to recognize the drugs targeting the receptor [8]. Antiestrogens (i.e. Tamoxifen) are meant to bind to ERα and prevent the activation of the signaling pathway [41]. The problem with anti-estrogens is the ability for ERα to circumvent the drug and become resistant after years of treatment [8].

Whereas ERα is a key player in mammary carcinogenesis, its definitive role is still under investigation. It is essential to understand the molecular mechanism behind ERα transcriptional regulation to elucidate novel and effective therapies.

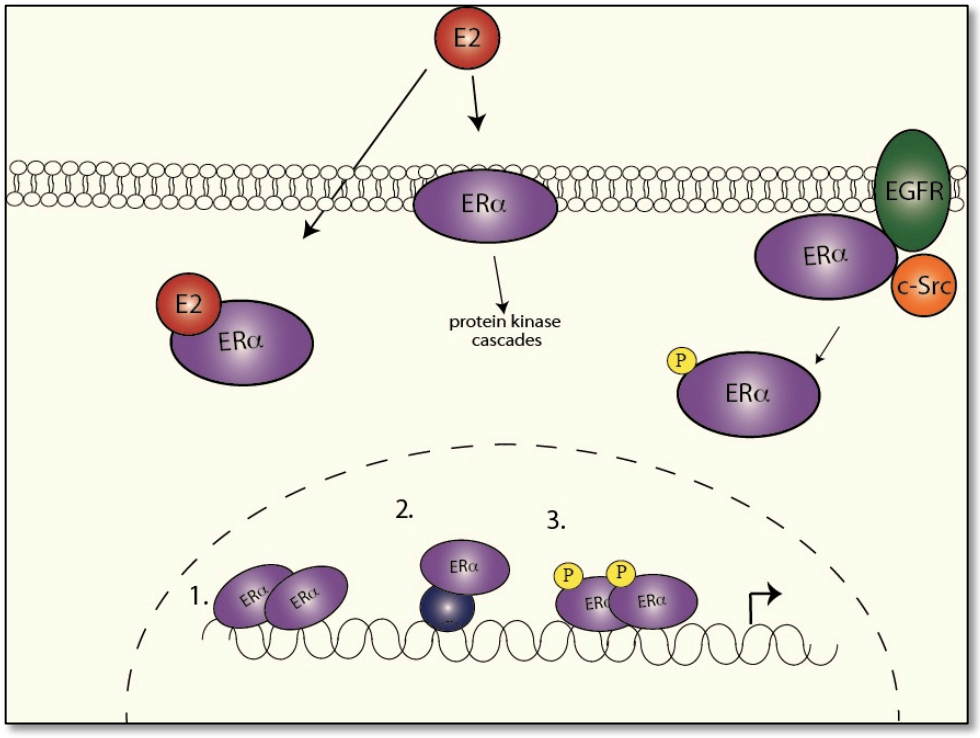


Fig. 1.2. *Estrogen receptor α signaling*. (1) Estradiol binds to the ER α , ER α translocates to the nucleus and directly binds to estrogen response elements (ERE). (2) ER α binds estradiol and indirectly activates downstream signaling via DNA bound transcription factors. (3) Phosphorylation of ER α via G-proteins and recruited kinases activate ER α , ER α translocates to the nucleus and binds to EREs.

Estrogen dependent breast cancers

Breast cancer is the most common form of cancer in women wide world [42]. Approximately 5-10% of breast cancers can be attributed to *breast cancer associated 1* (*brca1*) and *brca2* germline mutations [43]. However, 90% of all breast cancer cases are sporadic and do not have mutated *brca1* or *brca2* genes. Although sporadic breast cancer accounts for 90% of all cases, it is the less studied cancer of the two. Non-familial breast cancers are categorized according to prognostic markers. Three markers, ERα, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) have been identified [44, 45]. Of the three markers, ERα is upregulated in 75-90% of sporadic

breast cancers [29, 46]. This type of breast cancer relies on the availability of estrogen for growth, hence the name estrogen dependent breast cancer. Having even the slightest number of cells expressing ERα results in a significantly positive response to hormone therapy [47].

The importance of steroids in sporadic tumor formation was first shown when Beatson removed ovaries from a premenopausal woman in 1896 [48]. This resulted in a better prognosis of the patient and even regression of breast cancer. However the positive effect lasted for a short period of time and only helped 1 of 3 women [31]. Unbeknownst to Beatson, his work created a link between the ovaries and some forms of breast cancer. Studies following Beatson's clinical work confirmed the link between the ovaries and breast cancer, however it was not until 1923 when Allen *et. al* identified ovarian hormones possibly responsible for the development of breast cancer [4]. Allen and Doisy described the follicles as the site of release for the ovarian hormones and their relevance in the estrus cycle and mating instincts [4]. The hormone of interest was later characterized to be estrogen.

Current treatments for estrogen dependent breast cancers

Selective estrogen receptor modulators (SERM) are a group of drugs able to have an antagonistic effect in target tissues and have an estrogen like effect in non-targeted tissue [31]. The advantage of SERMs in treatment is the ability to maintain ERα function in non-breast cancer tissues. The abrogation of ERα signaling in non-targeted tissues may cause bone deterioration and increase cholesterol levels in the body [49-51]. SERMs directly bind to ERα on the AF-2 ligand-binding domain. The binding of these drugs

triggers a different conformational change from what would have been triggered by estrogen bound to ERα [52]. The SERM-ERα complex fails to recruit necessary co-activators to initiate routine downstream signaling of the receptor [53]. Tamoxifen, the first SERM drug approved by the FDA in 1977, is currently administered to pre- and post- menopausal women with advanced ERα positive breast cancer [31]. This drug is administered as an adjuvant therapy to reduce the reoccurrence of breast cancer incidence. If prescribed for a longer length of time (~5 years), tamoxifen will reduce the recurrence of breast cancer in premenopausal women [28]. Tamoxifen will also reduce the risk of contralateral (breast cancer in the other breast) breast cancer [28]. Although the benefits of tamoxifen are strong, tamoxifen has been found to increase the incidence of endometrial cancer [28, 54]. Another drug in the SERM group, raloxifene, has been found to not increase the incidence of endometrial cancer. In addition, this drug is anti-estrogenic in the uterus and breast and antagonist in bone and lipids. This drug is also used as a treatment for osteoporosis in post-menopausal women [55].

Some drugs have been found to have full anti-estrogenic effects, meaning the drugs bind to ERα to prevent downstream signaling in all tissues [52]. These drugs are known as selective estrogen receptor down-regulators (SERDs). Fulvestrant™ has 100-fold higher binding affinity to ERα than does tamoxifen. The downside of Fulvestrant and other SERDs is the propensity for bone density loss and high cholesterol because of anti-estrogenic effect in all tissues [56].

Drugs such as exemestane™, anastrazole™, and letozole™ block the critical enzyme, aromatase [57]. The enzyme is key in the biosynthesis of cholesterol into estrogen [42]. The initial studies with earlier aromatase inhibitors prevented the synthesis

of estrogen in all tissues, however recently made inhibitors are exhibiting selectivity for breast and uterus tissue [52]. New inhibitors have a higher potency and less toxicity rendering them the better option for treating estrogen dependent breast cancer [52].

Estrogen receptor positive breast cancer can be hormonal therapy resistant. Studies have shown estrogen dependent breast cancer can go from an ERα positive to ERα negative phenotype. It has been suggested, when the tumors begin to proliferate, the tumor cells down-regulate the transcription of ER [58, 59]. Studies have also found the importance of cyclin D1 expression, a key cell cycle regulatory protein, and whether hormonal breast cancer will positively respond to hormonal therapy [60]. The high expression level of cyclin D1 shortens the length of time tamoxifen has an effect, and in addition the high expression of cyclin D1 can also induce acute anti-estrogen resistance [60].

Circadian Rhythm and Physiology

The light/dark cycle seen among eukaryotes and prokaryotes is an outward manifestation of various internal oscillators known as circadian rhythm. Circadian rhythms are internally driven by a hierarchy of oscillators to maintain a rhythm of approximately 24 hours [61]. At the top of the hierarchy is the master clock, the suprachiasmatic nucleus (SCN) located within the anterior hypothalamus of the brain (Fig. 1.3) [62]. The SCN receives neural signals from the retina synchronizing our physiology to different environmental cues. External signals propagate the brain oscillators and from there to peripheral organs [63]. Although rhythms are influenced by external cues, such as light/dark pulses or changes in temperature, they persist in non-

cyclical conditions (*i.e.*, complete darkness) [64]. The biological importance of rhythms is apparent in all cellular levels of the organism from the oscillation levels of enzymes and hormones to physiological oscillations of body temperature and immune responses [65]. An important factor about circadian rhythms is their ability to be reset by external cues. This allows organisms to anticipate environmental changes and organize their physiology and behavior during the day. Disruption of circadian rhythm has been linked to myriad diseases and physiological conditions (*i.e.*, jet lag, insomnia, coronary heart disease, major depression and the increase risk of cancer) [64, 66].

Circadian disruption and breast cancer

Epidemiological studies indicate a higher incidence of breast cancer in Western industrialized societies than in developing countries [67, 68]. It has been proposed that changes in environmental factors, *i.e.*, light/dark cycles due to altered shift work, could be one of the reasons for a higher incidence of breast cancer [67, 68]

Different studies found that the increase risk of breast cancer may be due to exposure to light during the period of night. This includes women working graveyard and rotating shifts [67]. Other factors such as the number of times the graveyard shift was worked and the years of working night shifts also were associated 1.5 fold increase in the risk for breast cancer [67, 68]. The risk of breast cancer was increased by 23% when the group worked in rotating shifts after 1-14 years [67]. Although still controversial, bedroom lighting is thought to contribute to an increased risk; the brighter the room, the higher the risk for breast cancer [67].

Although more studies have supported the idea that disruption of circadian rhythm leads to an increase in tumor development, the mechanism is not fully understood. Therefore, it is necessary to further investigate the molecular pathways between the circadian disruption and the increase in tumor development to generate novel therapeutic solutions.

The first connection between circadian disruption and the increase of mammary tumor development was reported in the 1960's [69, 70]. These studies suggested that the increase in light exposure accelerated breast epithelial stem cell proliferation leading to an increase of mammary gland development and ultimately spontaneous mammary tumors [69, 70].

An increase in the risk of breast cancer is thought to be a product of circadian disruption. The "Circadian Disruption Hypothesis" puts forth the theory that environmental factors (*i.e.,* light at night time) may disrupt endogenous circadian rhythms and increase the risk of breast cancer [71].

The pineal gland, signaled by the SCN, is responsible for the release of the hormone melatonin. Melatonin is specifically secreted during the night and its release is dose dependent on the amount of light present; the brighter the light the less secreted melatonin [62, 71, 72]. Support for the former hypothesis comes from experiments where ablation of the pineal gland results in tumor growth, but the administration of melatonin may inhibit or suppress the formation of tumors [69]. Shah et al. were able to show the suppression of melatonin (through the presence of constant light or surgically removing the pineal gland) lead to an increase in tumor development [70]. Mice were then administered melatonin, for about a week, to see if there were any anti-tumor effects. The

mice in constant light and the "pinealectomized" mice responded to the melatonin and showed tumor suppression [70].

The administration of melatonin as a hormone replacement therapy is already in practice; however, melatonin may only inhibit a single pathway of estrogen signaling. Because hormone therapies are dependent on estrogen being upregulated it is important to understand the relationship between melatonin, estrogen receptor and circadian physiology.

Mammalian Circadian clockwork

Circadian rhythms are controlled by 8 core gene products that sustain oscillations. The feedback loops, both positive and negative are as follows (Fig. 1.3): Clock/Bmal1 heterodimer binds to a response element known as an E-box within the promoter region of *period genes 1*, *2* and *3*, (*per*), *cryptochromes* (*cry*) and *clock controlled genes* (ccg) to initiate transcription at the beginning of the day [62, 65, 66, 73]. The Per proteins accumulate during the day in the cytoplasm where they are phosphorylated by Casein Kinase1ε (CKIε) making them unstable and degraded by the ubiquitin-proteasome pathway. Later in the day, Cry begins to accumulate in the cytosol leading to the formation of the Per2/Cry/CKIε complex [66]. This complex translocates into the nucleus. After shuttling, Cry is thought to disrupt the Clock/Bmal1 heterodimer (the exact mechanism is still under debate). The Clock/Bmal1 complex is inhibited from initiating the transcription of *per*, *cry*, and *rev-erbα*. The entrance of the complex into the nucleus and inhibition of transcription occurs in the beginning of the night [66]. After accumulation of Rev-Erbα levels throughout the day and early evening, Rev-Erbα returns

to the nucleus and represses *bmal1* [66]. *Bmal1* repression by Rev-Erbα diminishes by the end of the night allowing a high concentration of Bmal1 protein in the nucleus and low concentrations of Per and Cry at the beginning of a new day [61].

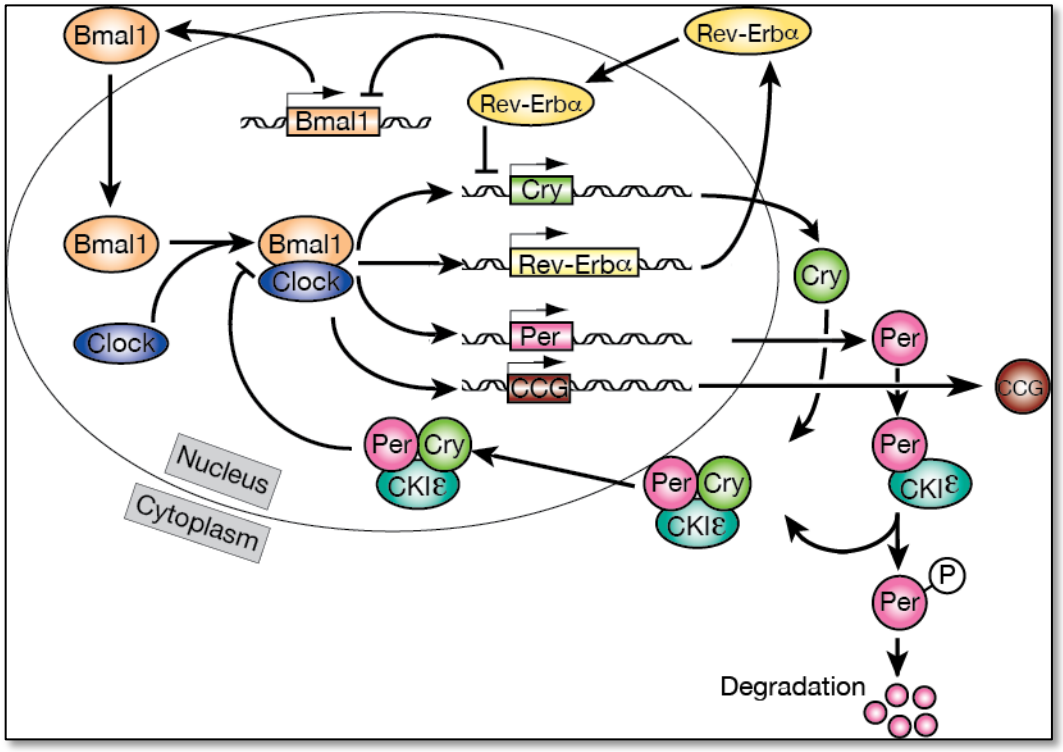


Figure. 1.3. *Mammalian Circadian Clock*. The positive feedback loop involves regulation of bmal1 transcription. Bmal1/Clock selectively binds to E-box enhancers and drives the expression of per, cry and rev-erb α genes. Rev-Erb α protein then represses bmal1 transcription through Rev-Erb α responsee elements in its promoter. The level of bmal1 RNA falls, as per and cry RNA levels peak. When light is present, Per (*i.e.*, Per2) accumulates in the cytoplasm, becomes phosphorylated by CK1 ε , ubiquitylated and degraded. Later in the day, CRY accumulates, associates with Per2/Ck1 ε and this complex translocates to the nucleus where CRY disrupts the Clock/Bmal1-associated transcriptional complex, resulting in the inhibition of *cry*, *per* and *rev-erb* α and de-repression of bmal1 transcription.

Period 2

The *period* (*Per*) genes are core regulators of the circadian clock in mammals whom the ablation or mutation of said genes could lead to disease [1, 74]. The mammalian *per1* gene was first identified in 1997 followed by *per2 and per3* [75]. Of the three *per* products, *per2* studies expose its influence on a widespread of bodily functions such as the homeostasis of metabolism, the immune system and the cell cycle, in brief; the ablation of this gene has ill effects on the organism [76].

Not only is *per2* expressed in a circadian fashion in the SCN, but is also expressed in almost all tissues [76]. One method of regulation of *per2* expression is *via* the non-canonical E box in the promoter of the gene. The Clock/Bmal1 heterodimer binds the site and activates the transcription of *per2,* only to later be disrupted by the gene's product, Per2 [77]. *Per2* gene, along with *Per1,* is directly stimulated by light [78]. The difference between the two genes, with respect to entrainment and induction by light, is that *Per2* is highly sensitive to light in the earlier phase of the night and *Per1* is sensitive in the later phase of the night [78].

Period 2 structure

Per2 contains several structural domains and functional motifs. The N-terminal end of Per2 has a helix-loop-helix motif preceeded by a series of basic residues rendering the motif difficult to bind directly to DNA [78]. The PAS (Per, ARNT, Sim) region, comprises the PAS A, PAS B and PAC domains, is involved in protein-protein interactions and dimerization [79]. The PAS region has also been suggested to be a site for ligand binding [79]. The PAS region is suggested to mediate the response to light,

although it hasn't been directly shown to carry out such response, the ablation of this domain rendered the protein incapable of responding to light stimulation [80]. The PAS region also contains a CoRNR motif seen to be useful for the localization of the protein (ablation of this motif sequesters the protein in the cytoplasm), along with the nuclear localization signals, into the nucleus by Cry1/2 [81]. A proline rich area, towards the carboxyl end, gives flexibility to Per2 to fold-over and help binding a single protein or provides a dock for more than one protein to bind at the same time [76]. The carboxyl end of Per2 is made up of a coiled coil motif capable of dimerization with other helix containing proteins [82].

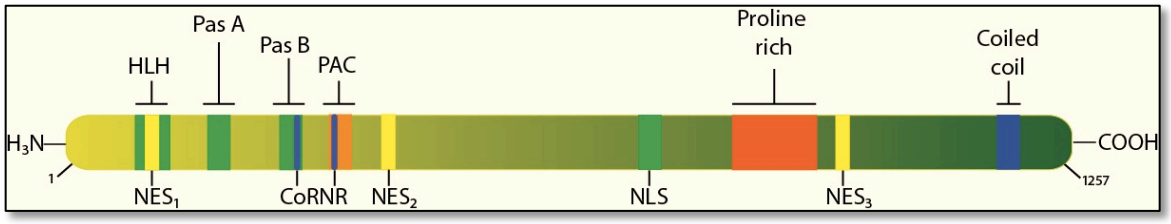


Fig. 1.4 Structural domains and functional motifs of Period 2. The Helix-loop-helix (HLH): green. The PAS region consists of Pas A, Pas B and PAC. Nuclear export signal (yellow). CoRNR motif shared among corepressors for the binding of nuclear receptors (blue). Nuclear localization signal (green). Proline rich sequence (red). Coiled coil domain (blue) for dimerization.

Deregulation of per2 gene expression and disease development

Variants of Per2, including serine to glycine point mutations, are associated with deregulated circadian oscillation. Mutation on *per2* to express glycine instead of serine on residue 662, results in an inability to positively regulate other circadian clock genes, such as *bmal1* [83-85]. The circadian clock is unable to reset itself, via light, with an impaired Per2 [86]. This point mutation changes the amount of Per2 phosphorylation and

in turn changes the clock period length, either shortening or lengthening the period [85, 86]. This change in period length creates an advanced sleep phase syndrome where the person, with such syndrome, experiences sleepiness in earlier phases of the evening (between 6pm-8pm) and experiences an earlier wake up period as well (~1am-3am) [85]. Not only does Per2 deregulation affect the circadian clock, but it is involved in the manifestations of disease in other body functions such as the immune system and metabolism, to name a couple [76].

The immune system is unable to maintain normal levels of proinflamatory cytokines, such as interferon-γ (IFN-γ), in Per2 mutated mice compared to wild type mice [87, 88]. The cytokine IFN-γ is part of a family of cytokines involved in activating macrophages, necessary in the line of defense for the innate and adaptive immunity response. Recent studies have shown Per2 oscillation coincides with the mRNA expression levels of metabolic nuclear receptors such as proliferator-activated receptor α, γ and δ (PPAR) in adipose tissue [89].

Period 2 and breast cancer

These receptors synchronize crosstalk between adipose tissue, muscle and the liver to regulate metabolism [89]. Epidemiological studies have long correlated the disruption of circadian rhythm and the increased risk of breast cancer. However the molecular mechanism and actual key players involved, until recently, have been matters of speculation. The ablation of Per2 in mice increases the risk of tumor formation and sensitivity to γ-radiation [90]. Cell cycle genes, *cyclin D1*, *cyclin A*, *Mdm2* and *Gadd45α* lost an oscillation pattern similar to that of the circadian cycle in mice with mutated *per2*,

suggesting circadian control over cell cycle genes [90]. Such studies led researchers to designate Per2 as a tumor suppressor, further confirmed when a study revealed *per* deregulation in some breast cancers [1]. Although *Per2*'s role in the development of breast cancer is prevalent, it is unknown whether deregulation of Per2 actually is a cause or consequence of breast cancer. Recent studies suggest a regulatory role for Per2 on a key breast cancer protein, ERα [91, 92].

Estrogen receptor alpha expression

Recent studies have shown a strong correlation between nuclear receptor expression and circadian oscillations [89, 93]. One of the first nuclear receptors discovered to exhibit circadian oscillation was estrogen-related receptor α (ERRα) [94]. ERRα expression was found to oscillate in a circadian manner in the uterus and liver [94]. The oscillation of the receptor continued in the absence of light suggesting the oscillation is not driven by light, but driven by the circadian clock [94]. Estrogen treatment in the uterus did not exert influence on the expression of ERRα suggesting it is not regulated by the estrous cycles nor directly by estrogen [94]. Until recently, the regulation of ERα expression was largely defaulted to the estrous cycle, however, studies are showing that ERα is not exclusively regulated by estrogen in all tissues and could be circadian regulated [91, 92, 95, 96].

Period 2 has been found to connect the circadian rhythm and estrogen signaling. In fact, evidence supports an association between ERα and Per2, in the presence of estradiol [92]. Estradiol induces *per2* transcription, furthermore Per2 can directly bind to ERα and enhance degradation of ERα [92]. It has been further suggested that *per2*

expression is transcriptionally mediated by ERα and *per2* is estradiol inducible resulting in a feedback loop [92]. Although studies have shown ERα expression oscillates with the estrous cycle in certain tissues, this does not eliminate the idea that ERα expression could be regulated differently between tissues. In mice, ER transcriptional activity was found to maintain circadian cyclic activity after ovariectomy; in addition, ER activity was asynchronous between organs [95]. Furthermore, recent studies demonstrate ER mRNA oscillates in a circadian fashion in synchronized immortalized cells [96].

Expression of ERα in normal and cancerous breast cells

A recent study found the clock genes exhibiting normal circadian oscillation in immortalized, human mammary epithelium (HME1) cells; in addition, they also measured ERα mRNA and found ERα mRNA oscillated in a circadian fashion [96]. Interestingly, ERα mRNA counter-oscillates to the Per2 mRNA, in other words, at the time Per2 mRNA is at its peak, ERα mRNA is at its lowest levels [96]. Up until these studies it was not known whether this relationship exists in mammary cells or whether the circadian clock genes are present in the mammary cells. The mRNA levels of clock genes were also tested in several breast cancer established cell lines with different ERα expression phenotypes. Non-tumorigenic immortal human cancer cell line, MCF-10A has an ERα negative phenotype. This cell line was entrained and exhibited circadian oscillation of clock genes, however the oscillation was irregular and that peaks were not as pronounced as in the HME1 cell line, *per2* oscillation was difficult to distinguish [96]. It is unknown whether the ablation of ERα in this cell line is the result of irregular circadian function or circadian oscillation is irregular because ERα is not present in this

cell line. Additional cell lines with negative ERα phenotype, HS578T and MDA-MB-231, were also tested to determine whether clock gene oscillations were irregular compared to that of HME1. Results determined that these cell lines, too, had irregular circadian oscillations, for example *Per2* had an initial spike in mRNA concentration, but decreased and remained a low constant thereafter [96]. Breast cancer cell lines MCF-7 and T47D are ERα positive and also revealed deregulated clock genes [96]. Although normal immortalized cells HME1 demonstrated a counter-oscillatory expression of *per2* versus *esr1*, the breast cancer cell lines, MCF-7 and T47D cells did not share the same expression patterns to HME1 [96]. The ERα mRNA expression in T47D slowly decreases over time and in MCF-7 the expression of ERα mRNA slightly peaks at the 12^{th} and 40^{th} hour, but fails to have distinct oscillatory peaks [96].

The expression of ERα is also regulated by BRCA1 in breast cancer cells. Mutations in *brca1* increase the risk for familial breast cancer in women, however *brca1*'s role in sporadic breast cancer has yet to be established. Cells transfected with BRCA siRNA resulted in a lower transcriptional activation of ERα, in other words BRCA1 is a transcriptional activator for ERα in breast cancer cell lines MCF-7 and T47D [3]. Hu et al. reported that BRCA1 is able to transcriptionally inhibit the synthesis of aromatase, the rate-limiting enzyme for the synthesis of estrogen [97].

Furthermore, studies revealed, in a cell line with a negative phenotype of ERα, ERα can be rescued by the transient transfection of *brca1* in the otherwise mutation-carrying breast cancer cell line [3]. The transcriptional activation of ERα by *brca1* is not direct, thus the mediator, Oct-1 is necessary for the recruitment of BRCA1 to the ERα promoter. It is through the protein Oct-1 that BRCA1 transcriptionally activates ERα [3].

Breast Cancer Associated Protein 1 (BRCA1)

BRCA1 was the first gene product identified as a breast and ovarian susceptibility cancer marker [98]. The gene was found through linkage analysis of families with numerous occurrences of breast and ovarian cancer [98].

King *et al*. first identified *brca1* in 1990 in the search for a common gene associated with the predisposition of breast cancer [99]. Mutation of the tumor suppressor gene, *brca1*, can be found in approximately 90% of familial breast cancer incidences [100].

BRCA1 is a transcription factor that recognizes and repairs double stranded breaks in DNA. This protein is key in cell cycle checkpoint arrest. A phosphorylated BRCA1 is necessary for the initiation of G2/M and intra-S checkpoint in the cell cycle [101, 102].

Structure and function of BRCA1

Human BRCA1 is 1863 amino acids and comprises three domains: the N-terminus Really Interesting New Gene (RING) domain, two C-terminus BRCA (BRCT) domains and two Nuclear Localization Signals (NLSs) [98].

The BRCA1 protein shares little homology with other DNA repair proteins with the exception of the RING domain, BRCT domain, Nuclear Export Signals (NES) and the NLS sequences (Fig. 1.4) [43]. The N-terminal RING domain is involved in DNA binding and binding to other RING containing proteins, such as BRCA Associated Ring Domain protein (BARD). The binding of BARD to the ring domain of BRCA1 activates the BRCA1 ubiquitin ligase activity, which is necessary to exert its tumor suppression activity and to regulate cellular responses to radiation [103]. Studies found that the RING

domain is not necessary for ERα binding, but it cannot prevent downstream ERα signaling without a functional RING domain [104]. Two nuclear localization signals are encoded by exon 11 of *brca1* of the protein to the nucleus [43]. Characteristically, transcription factors tend to localize in the nucleus in normal cell function. Breast, ovarian and cervical cancer cell lines were examined to determine the localization of BRCA1 in the nucleus. Two structural tandem repeats are at the C-terminal end of BRCA1 known as BRCA1 C-terminus (BRCT) domains [105]. The domain was first found in BRCA1, however this domain is shared among various DNA damage repair and metabolism proteins [106, 107]. The BRCT domain mediates the recruitment to DNA damage sites and repairs during DNA replication as well as activates the G2/M phase checkpoint of the cell cycle [105, 108, 109]. Truncation and mutations of this domain leads to an increase risk of breast cancer [110, 111].

Fig. 1.5 *Schematic of BRCA1 domain structure and functional motifs*. RING finger domain (green). Nuclear localization signals (NLS) (yellow). Two BRCA1 C Terminus domains (blue).

Mutations in brca1 associated to breast cancer

Although mutations found in *brca1* are indicators for early onset of breast cancer, *brca1* does not contain germline mutations in sporadic breast cancer [3]. The presence of a specific germline mutation on *brca1* will increase the risk for breast cancer to approximately 65% by the age of 70 years [112]. This is not to dismiss the importance of

this gene in the development of sporadic breast cancer. Studies indicate when breast cancer is attributed to the mutation on the BRCA1 gene, breast epithelial cells are ERα-negative and in sporadic tumors, where the BRCA1 gene is not mutated, the cells are ERα-positive. Approximately 90% of mutated *brca1* breast tumors have ERα negative phenotype with the remaining 10% of mutated *brca1* breast tumors expressing ERα [113, 114]. The deficiency of ERα in mutated *brca1* tumors renders the tumor unresponsive to hormone therapy targeted for the receptor, such as tamoxifen. However, not all germline mutations on *brca1* increase the risk for early onset of breast cancer [115].

The Breast Cancer Information Core (BIC) database (http://research.nhgri.nih-.gov/projects/bic/) was created by the National Human Genome Research Institute to identify mutations among men and women that have a high penetrance and occur often in cases of breast cancer. The database has compiled information on mutations that have been found on *brca1* and *brca2,* including deletions, insertions, and non-sense mutations directly related to breast cancer prevalence [116]. The mutations recorded on the website are categorized based on the frequency of occurrence and their clinical importance [117]. How some high *brca1* mutations abrogate function has been investigated, resulting in a better molecular understanding for BRCA1 function. For example, a high frequency mutation, C61G is one of the most cited mutations in *brca1 [118].* This missense mutation is found in the conserved ring domain on the N-terminal end of the protein. Mutated BRCA1 is unable to homodimerize and unable to bind to BARD1 [118, 119]. In the absence of BARD1 binding, BRCA1 does not exhibit ubiquitin ligase activity [103]. The frameshift mutations, deletion of nucleotides 185 and 186 (adenine and guanine) and insertion of cytosine at nucleotide 5283 (5283insC), are two more high frequency

mutations linked to breast cancer incidences. Mutation 185delAG causes an early stop codon to result in the truncation of BRCA1. The truncation of the BRCT domain (5283insC) prevents the hydrophobic pocket that stabilized protein-protein interactions [115].

Hypothesis

Even though the presence of ERα in breast cancer prognosis is relevant for the choice of therapy, the spectrum of its molecular regulation has yet to be defined. Abnormal circadian patterns have long been correlated with increasing incidences of sporadic breast cancer in women [62, 67, 68, 71]. *A majority of sporadic cases share an upregulation of the estrogen hormone receptor ERα, this raising the question of whether upregulation of ERα results from circadian deregulation.* In addition, ERα counter-oscillates with Per2 in immortalized cell lines. Thus, does Per2 modulate the expression of ERα? Does Per2 need an intermediary? What is the role of other well-known ERα regulators in circadian-mediated expression?

Studies done in MCF-7 and T47D (*brca1* and ERα positive), *via* siRNA experiments, found that BRCA1 may transcriptionally activate the expression of ERα; furthermore they found that the non-existent expression of ERα in cell line HCC1937 (*brca1* and *p53* mutant and ERα negative) could be reversed when transfected with wt*brca1 [3]*. The transcriptional activation of ERα by BRCA1 is only achieved in the presence of OCT-1 [3]. OCT1 associates with BRCA1 on the ERα promoter. Interestingly, BRCA1 also inhibits downstream ERα signaling creating a feedback loop for ERα regulation (Fig. 1.5). Our data indicate an association between BRCA1 and Per2.

Could this association play a role in the modulation of the expression of ERα? We hypothesize that Per2 in conjunction with BRCA1 modifies the expression of ERα.

Chapter 2

Specific Aims

The overall goal of my work is to investigate the circadian regulation of ERα synthesis and its role in breast cancer incidence. Defining the mechanism of ERα expression will contribute to our comprehension of cellular proliferation, regulation of normal developmental processes and to our understanding of how circadian disruption acts on disease initiation and progression.

Recent studies have linked the circadian protein Per2 to and estrogen signaling through direct interaction at the protein level [92]. Are the incidences of some sporadic breast cancer occurrences a consequence of an imbalanced circadian homeostasis? In order to answer this question, we first need to define the role of Per2 in the transcriptional regulation of ERα.

ERα is regulated by BRCA1 and through that association, we ask whether Per2 interacts with BRCA1 to transcriptionally modulate ERα expression. Therefore, we aim to:

Aim 1: Determine the molecular basis for Per2 control of ERα transcription.

Our preliminary data indicates Per2 directly associates to BRCA1 and the Oct-1 transcriptional regulator. Thus, we propose that the interplay among these molecules controls the expression of the receptor in a circadian fashion. We aim to define the

response element that mediates the interaction, the protein domains involved in their regulation, and the effect on ERα expression on unscheduled circadian protein levels.

By unveiling this mechanism, we will provide a platform for understanding the effect of altering our body physiology on disease development. We expect this work will set the foundation for further screening purposes for long-term exposure to circadian disruptors.

Aim 2: Demonstrate the functional regulation of Per2 of ERα expression. The literature supports a correlation between the expression of Per2 and ERα, however it is not known whether Per2 has a direct influence on the transcriptional expression of ERα. Our preliminary results indicate Per2 to be a repressor of ERα. Thus we propose Per2 has a repressive regulatory role for the expression of ERα. These studies will demonstrate the functional regulation of Per2 for ERα.

Chapter 3

Materials and Methods

Plasmid constructs

Full length *per2*, constructs of *per2*, *pou*, *brca1* and constructs (cDNA) were cloned into SalI and NotI sites of pGEX-4T-3. Fragments of Per2 protein: residues 1 to 172, 173 to 355, 356 to 574, 575 to 682, 683 to 872, 873 to 1120, 1121 to 1255, and 822 to 1255. Constructs of BRCA1 protein: 1 to 178, 1 to 400, 1646 to 1859, and 1670 to 1863.

The *per2*, *per2* constructs, *brca1*, *brca1* fragments and *pou* were cloned into pCS2+*myc*-tag and pCS2+FLAG-tag vectors modified for ligation independent cloning (Novagen). Full length *per2*, *per2* (575-1255), *per2* (683-872) cDNAs were cloned into NotI and XhoI sites of pBT. *Brca1* cDNA fragments, 1 to 400 and 1670 to 1863, were cloned into NotI and XhoI sites of pTRG. Stratagene's QuickChange kit was used to mutate pCS2+*myc-brca1*, pCS2+FLAG-*brca1* and pGEX-4T-3 *brca1* as follows: BRCA1 stop codon at residue 1835, insertion of cysteine at residue 5382, stop codon at residue 143, stop codon at residue 321, cysteine to glycine at residue 61, and deletion of two threonines at residue 185. QuickChange was also used to mutate pGEX-4T-3 *pou*: Q27A, R20A, and V47A.

Brca1, *per2*, and *pou* were cloned into pcs2+EGFP-tag vector modified for ligation independent cloning (Novagen). Constructs of the *esr1* (ERα promoter), +139 to +294, +76 to +294, and -124 to +294 are referred to as 100NT, 200NT and 400NT, respectively.

ERα promoter and constructs were cloned into KpnI and NheI sites of the pGL2 luciferase reporter vector (Promega).

Cell culture and transient transfections

Human breast cancer cell line MCF-7 and Chinese hamster ovary-K1 (CHO) were purchased from the American Type Culture Collection (ATCC). MCF-7 was propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin/streptomycin and 0.01 mg/mL bovine insulin (Sigma). CHO was propagated in ATCC formulated F-12K medium, supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL penicillin and streptomycin. Cell lines were all maintained at 37ºC/5%CO_2. Transfections for CHO were performed using Lipofectamine (Invitrogen) according to manufacturer's instructions. Unless stated otherwise, cells were seeded into 6- and 12-well plates for qRT-PCR, protein expression and luciferase transient transfections. MCF-7 transfections were carried out in Opti-MEM antibiotic-free medium (Invitrogen) in a 1:4.4 ratio of Lipofectamine LTX to DNA. Cells in DMEM containing 10% fetal bovine serum and 0.01 mg/mL bovine insulin were incubated with Lipofectamine LTX complexes for 12 hours before changing to medium with antibiotics. MCF-7 cells were collected 48 hours after incubation for protein expression tests and qRT-PCR. In the event of estradiol stimulation, transfections in MCF-7 cells were carried out with Lipofectamine LTX in phenol-red free DMEM (Cellgro). Cells in phenol-red free DMEM containing 10% charcoal treated fetal bovine serum (Atlanta Biologicals) and 0.01 mg/mL bovine insulin were incubated with Lipofectamine LTX complexes for 12 hours before changing to medium with antibiotics. Cells were kept in hormone free

media for a total of 48 hours before 17β-estradiol stimulation. One hour before collection, cells were stimulated with either 10nM 17β-estradiol (Sigma) or DMSO vehicle. Cells were collected for qRT-PCR.

Bacterial two-hybrid screening

BacterioMatch II system (Stratagene) was used to screen for two-hybrid interactions between a specific bait (pBT-Per2) and target plasmid pair from a library (pTRG cDNA library). Both the bait vector (200 ng) and pTRG (200 ng) vector were co-transformed into BaterioMatch II validation reporter competent cells. Aliquots of pBT-Per2 and pTRG were plated on nonselective and selective screening (5 mM 3-AT) medium. After 24 hours incubation, strong interactors were isolated from the selective media. Positive clones were maintained in LB tetracycline/chloramphenicol (tet/cam) agar plates. Specificity between bait and target proteins were validated through activation of, *aadA* gene encoding streptomycin resistance. Positive colonies from selective screening medium were patched on a dual selective screening medium plate containing both streptomycin and 3-AT. Transformed pBT-LGF2/pTRG-Gal11P, from selective screening media, was used as a positive control. Negative controls were pBT-Per2 co-transformed with either empty pTRG or pTRG-Gal11P vectors. All cDNA was sequenced to identify and confirm clone identities.

Immunoprecipitation Assays

Cells pellets collected after transfecting with pCS2+*myc-brca1* (and/or constructs), pCS2+*myc-per2* (and/or constructs), and pCS2+*myc-pou* were resuspended in lysis buffer

(10 mM Tris-HCL pH 7.5, 137 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 80 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF and protease inhibitors). Extracts for immunoprecipitation of endogenous and transfected proteins, (1 mg and 100 μg, respectively) were incubated with either α-FLAG M2 agarose beads (Sigma) or α-myc (3 μg) (9E10) beads (Santa Cruz) overnight at 4ºC. Beads were washed four times with lysis buffer, followed by immunoblotting using specific primary antibodies. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG immunoblotting was used for all immunoprecipitation assays. Chemiluminescence reactions were performed using the SuperSignal West Pico substrate (Pierce).

GST-fused protein purification

GST-fused proteins were expressed by inducing 1 L of E. coli cultures with 0.1 mM isopropyl β-thiogalactopyranoside (IPTG) followed by purification via manufacturer's protocol. Proteins were cleaved from the GST tag with thrombin (10U/uL). Proteins were separated (HiLoad 16/60 Superdex 75 prep grade column) to obtain single monomeric state of each protein in buffer A (20 mM Tris-HCL pH 7.4, 100 mM NaCl, and 5 mM EDTA). Protein concentration was determined by Bradford protein assay (Bio-Rad). The Bradford assay is a method used to determine total protein concentration of my samples. It is based on the proportionally binding of proteins to the Coomasie dye and absorbance is measured with the spectrophotometer at 595 nm.

Protein pull-down assays

Approximately 20 μg of GST bound protein was incubated with 3 μl of in vitro transcribed and translated [^{35}S]-POU, Per2 or BRCA1 in binding buffer (20 mM Tris-HCL pH 7.4, 100 mM NaCl, 5 mM EDTA and 0.1% Triton X-100) at 4ºC for 1 h. Beads were washed with low and high salt binding buffer (100 mM and 1 M NaCl, respectively), resolved by SDS-page and analyzed by autoradiography.

Luciferase transient transfections

CHO and MCF-7 cells were seeded into 12-well plates for luciferase assays. Cells were co-transfected with 200 ng pGL2-*esr1* and increasing amounts (50 ng - 200 ng) of pCS2+*myc*-POU, pCS2+*myc*-BRCA1, and pCS2+*myc*-Per2. pCMV-β-Gal (100 ng) was also included for transfection normalization. Total DNA (1 μg) was kept constant with empty vector, pCS2+*myc*. Cells were collected 24 and 48 hours after transfection (CHO and MCF-7 cells, respectively), lysed in 1X cell lysis buffer (Promega), and reporter activity was measured using Bright-Glo Luciferase Assay System (Promega) according to manufacturer's protocol. Luciferase activity was obtained using a Glomax Luminometer (Promega). Luciferase activity was normalized with the β-galatosidase activity. The β-galatosidase activity was determined by o-nitrophenyl- beta-D-galactopyranoside (ONPG) assay.

Electrophoretic mobility shift assays

Complementary oligonucleotides were purchased from Integrated DNA technologies comprising residues +76-+87 of ERα promoter. Oligonucleotides (forward primer, 5'-

GCTATGGCCTATGCATATGAAGCCTTTATT-3' and complementary oligonucleotide 5'-AATAAAGGCTTCATATGCATAGGCCATAGC-3') were annealed by heating to 95°C for 5 min and were allowed to cool to room temperature overnight. The end labeling of DNA with [γ-^{32}P] was done with T4 polynucleotide kinase (NEB) followed by purification with NucAway spin columns (Ambion). Recombinant proteins and [γ-^{32}P] – double stranded DNA were incubated for 20 min at room temperature in binding buffer ((20 mM HEPES, 2 mM $MgCl_2$, 10% glycerol and 2 mM DTT, pH 7.9, 36 ng poly(dI·dC)). Complexes were analyzed on non-denaturing polyacrylamide electrophoresis via 5% gels in a 1X Tris borate-EDTA buffer. Gels were visualized by autoradiography.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were done as previously described [3]. Briefly, samples collected in 1 mL of cell lysis buffer were sonicated for ten seconds three times with one-minute intervals at an output of 1 on Branson Sonifier 450. The samples were precleared with 40 µl of A/G sepharose slurry for 45 min at room temperature. After preclear, 1% of the supernatant was aliquoted out for DNA input and the rest was equally divided for IgG internal control (1 µg), RNA polymerase II (1 µg) and α-FLAG M2 agarose beads (10 µl of 50:50 slurry) or α-myc (9E10) beads (3 µg). Samples were incubated overnight at 4°C. Samples with antibodies were incubated for an additional 2 hours at 4°C with protein A slurry. After serial washes, bound complexes were extracted with cell extraction buffer (1% SDS, 0.1 M $NaHCO_3$). Complexes were obtained from two 15-minute washes with cell extraction buffer.

Quantitative reverse-transcription polymerase chain reaction

Transfected MCF-7 cells were collected and pellets washed two times with PBS and resuspended in 50 μl of PBS. Cells were DNAse treated (BioRad) and incubated at 37ºC for 30 min. Samples were sorted via fluorescence-activated cell sorting (FACS) analysis to obtain all EGFP fluorescing cells (transfected) from the non-EGFP fluorescing cells. Total RNA was extracted (Ambion) and reverse transcribed into cDNA. Primers were specifically designed to measure ERα mRNA expression and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). qRT-PCR was done with iScript RT-qPCR.

Far-UV circular dichroism

For acquisition of far-UV CD spectra, proteins (10 μM) were in 5 mM Tris-HCl (pH8.0), 100 mM KF, and 0.1 mM DTT. Experiments were carried out in a 1-mm path length quartz cell at 23ºC using a Jasco J-815 spectropolarimeter. Spectra were obtained from five accumulated scans from 260 to 195 nm using a bandwidth of 1-nm and a response time of 1 s at a scan speed of 20 nm/min. Buffer spectra were subtracted from the protein spectra to account for any background. Spectra were deconvoluted to estimate secondary structure content with the online server DICHROWEBB. NRMSD is the normalized root mean square difference of the experimental and calculated spectra.

Chapter 4

Results

Per2 is a novel interactor of the breast cancer type 1 susceptibility protein, BRCA1.

Potential Per2 binding partners were initially identified using a human liver cDNA library *via* the two-hybrid system (BacterioMatch II). Full length *per2* and three constructs *per2* (575-1255), *per2* (683-872) in pBT plasmid were used to screen the cDNA library. Constructs were designed based on the inclusion of functionally relevant domains in protein-protein interactions. Tentative positive interactors (67 strong and 53 weak) from the original $4x10^6$ clones were identified from the non-selective antibiotic containing media. Positive clones were selectively screened on 5 mM of 3-AT (Fig. 4.1A). Selectively screened clones were further verified via dual selective screening medium. The positive interactors of Per2 were validated through direct co-transformation of pBT-Per2 and pTRG targets. Negative controls included the co-transformation of pTRG with empty pBT plasmid (Fig. 4.1B). Interestingly, BRCA1 fragments were among the positive identified clones to interact with Per2. Several fragments were identified to associate to pBT-Per2, including the N- and C-terminal ends of BRCA1 suggesting BRCA1 is a novel interactor of Per2 with multiple binding sites.

BRCA1 and Per2 associate in multiple cellular setting cells.

To determine whether novel interactors BRCA1 and Per2 form a complex in cells, we transfected CHO and MCF-7 cells and analyzed binding of BRCA1 to endogenous Per2 *via* reciprocal immunoprecipitation. CHO cells express both BRCA1 and Per2 as opposed to MCF-7 cells, which express BRCA1, and have undetectable protein levels of Per2 [120]. CHO cells were transfected with *myc*-BRCA1 (1-178), *myc*-BRCA1 (1-400), *myc*-BRCT BRCA1 and empty vector (EV) to determine whether the constructs bind to mPer2 (Fig. 4.2.A). The N- and C- terminal ends of BRCA1 bound and immunoprecipitated endogenous mPer2 confirming their interaction. In addition, MCF-7 cells were transfected to determine whether this interaction occurs in a breast cancer cell line. Due to the low endogenous levels of Per2 in MCF-7 cells, we transfected *myc*-Per2, and immunoprecipitated endogenous BRCA1 (Fig. 4.2.B). As shown in Fig. 4.2.B, binding of myc-Per2 to BRCA1 occurs in breast cancer cell line MCF-7.

Period 2 binds to the N- and C- terminal ends of BRCA1.

To characterize the novel interaction between the circadian protein, Per2 and BRCA1, we mapped the area of binding of BRCA1 to Per2 *in vitro* (Fig. 4.3.A). Five recombinant proteins comprising the N- and C-terminal ends of BRCA1; BRCA1 full-length, BRCA1 (1-178), BRCA1 (1-333), BRCA1 (1-400), and BRCT (1646-1859), were incubated with [^{35}S]-labeled Per2. Results show Per2 binds to the N-terminal end of BRCA1, specifically residues 1-333 (Fig. 4.3.A). This region contains a zinc finger domain (1 to 109) and a nuclear export signal (NES) (81-99). Ubiquitin ligase activity is mediated through the RING finger domain of BRCA1; furthermore this activity is

increased when bound to BARD1 [103]. Interestingly, Per2 also binds to the carboxyl terminus end of BRCA1, named BRCT. The BRCT domain typically recognizes and binds phosphorylated proteins [106, 107] and is involved in transcriptional regulation *via* the recruitment and binding of proteins such as RNA polymerase II. In short, Per2 can bind BRCA1 *in vitro* and in mammalian cells (CHO and MCF-7).

BRCA1 directly binds residues 356-574 and 683-872 of Per2.

To fully understand the functional purpose for the BRCA1/Per2 interaction, we must first distinguish the possible functional domains involved in the binding of each protein. Not only did we want to determine what residues of Per2 binds to BRCA1, but we also mapped the regions where the N- and C- terminal ends of BRCA1 bind to Per2 (Fig.4.3.B). Seven recombinant constructs spanning Per2 and five constructs of BRCA1 were selected based on secondary structure prediction, sequence homology and molecular modeling. Per2 GST-fused recombinant proteins Per2 (1 to 172), Per2 (173 to 355), Per2 (356 to 574), Per2 (575 to 682), Per2 (683 to 1120), Per2 (1121 to 1255) were incubated with [^{35}S]-labeled BRCA1 full-length, BRCA1 (1 to 178), BRCA1 (1 to 400), or BRCT (1646 to 1859) to determine binding of Per2 to BRCA1 through GST pull down assays (Fig. 4.3.A and fig. 4.3.B). Results show the N- and C- terminal GST-fused constructs of BRCA1 bind to two distinct regions of Per2 (356 to 574 and 683 to 872). Residues 356 to 574 include the N-terminal end of the PAS domain, the entire PAC domain and the NES signal [79]. Period 2 (356 to 574) includes the C-terminus end of the PAS domain implicated in dimerization and protein-protein interactions [79]. Residues 683 to 872 contain a NLS signal and the N-terminal end of the proline rich area of Per2. This region

is largely unstructured and is proline rich, suggesting a flexible and readily available binding area within Per2. Interestingly, the binding of BRCA1 to Per2 spans not only a NLS, but also an NES, both signals important for efficient localization of Per2 suggesting BRCA1 may deter Per2 movement out of the nucleus because BRCA1 is typically localized in the nucleus.

Estrogen Receptor alpha (ERα) promoter has a response element for OCT-1.

The relationship between mutations in BRCA1 in hereditary breast tumors and the loss of ERα expression resulted in the identification of an ubiquitous transcription factor, OCT1 that mediates BRCA1's transactivation of ERα [3]. To identify whether Per2 is involved in transcriptional regulation of ERα, we first characterized the binding of the POU, to the estrogen promoter (Fig. 4.4). Although the ERα promoter (spanning promoter A, B and C) has 26 response element sites for OCT-1, we focused on the response elements spanning promoter A. As previously stated, ERα promoter A is the promoter involved in the overexpression of ERα. We found a single OCT-1 response element within promoter A (Fig. 4.4.A). Chromatin immunoprecipitation assay of pCS2+*myc*-POU transfected MCF7 cells resulted in POU and RNA polymerase II (Pol II) binding to the ERα promoter (Fig. 4.4.B). Furthermore, when increasing concentrations of pCS2+*myc*-POU (50-200 ng) were co-transfected with the reporter pGL2-*esr1* (200 ng), luciferase activity was evaluated in transient transfections (Fig. 4.4.C). The transcription activity of ERα has a linear relationship with increasing concentrations of transfected POU in CHO cells. In summary, the OCT-1 site of interest for ERα transcriptional regulation is 78 bp downstream of the transcription start site.

ChIP assay confirmed POU association to its response element in promoter A in MCF-7 cells and, the dose-dependent association between POU transfection and ERα activity in CHO cells.

Single amino acid mutations on the highly conserved DNA binding domain of OCT-1 abrogate binding to the estrogen promoter.

To further our understanding of POU's binding to the estrogen promoter, we mutated critical residues and determined those necessary for binding to the promoter. Critical residues for DNA binding have been identified in OCT-1 orthologs and were found conserved in human Oct-1 POU (Fig. 6B). Residues arginine-20 (R20), glutamine-27 (Q27), glutamic acid-51 (E51) and valine-47 (V47) were mutated to alanine on the POU domain. Single mutations, along with double and triple mutations were also generated to test the binding ability of the various recombinant proteins to esr1. POU mutants' binding was tested by electrophoretic mobility shift assays (Fig. 4.5.C). Our results show single point mutations within POU key residues were enough to abrogate direct binding to *esr1* response element. Only one of the mutants POU-Q27A was used for further studies. Far-UV CD analysis was used to verify that POU-Q27A overall secondary structure content was similar to that of the wild type protein (Fig. 4.5.B). Use of the online server DICHROWEB resulted in identification of approximately 31% alpha helix, 15% beta sheets and 49% random coil for POU and POU-Q27A using the CDSSTR algorithm (Fig. 4.5.B) [121, 122]. Finding what residues are critical for POU will help in determining faults in the mechanism that can ultimately lead to an upregulated ERα expression.

The OCT-1 DNA binding domain, POU, binds the N- and C-terminal ends of BRCA1.

BRCA1 and OCT1 association has been found in other transcription regulating mechanisms, such as the transcriptional regulation of DNA damage response gene, *gadd45* and the transcriptional regulation of spindle checkpoint gene, *mad2* [123, 124]. However, studies have yet to examine the direct binding of BRCA1 to OCT1 DNA binding domain, POU. We transfected *myc*-POU in MCF-7 cells to determine whether POU and BRCA1 form a complex in cells. We found POU directly binds BRCA1 in MCF-7 cells (Fig. 4.2.B, lane 2). We performed GST-pulldown assays to map the POU binding region of BRCA1 (Fig. 7A). POU directly binds to GST-BRCA1 (1-333), GST-BRCA1 (1-400), GST-BRCA1 (852-1379) and GST-BRCA1 (1670-1863) (Fig. 7A, lane 3, 4, 5 and 6, respectively). However, POU did not bind to GST-BRCA (1-178) (Fig. 7A). The DNA binding domain, POU does in fact bind to BRCA1 in MCF-7 cells, but more specifically this binding occurs on the N- and C- terminal ends of BRCA1.

The OCT-1 DNA binding domain, POU binds Per2.

Because OCT-1 is a transcriptional mediator for BRCA1 and BRCA1 directly binds Per2, we also asked whether OCT-1 and Per2 directly interact with each other. Due to the low protein concentration of Per2 in MCF-7 cells, we used CHO cells to immunoprecipitate endogenous Per2 with myc-tagged POU. Interestingly, POU is able to immunoprecipitate Per2 in CHO cells (Fig. 4.2.A, lane 4). To further investigate the association between Per2 and POU we mapped the Per2 residues that bind POU. Pull-down assays were used to map the direct interaction of GST-Per2 constructs and [^{35}S]-

labeled POU (Fig. 4.6.A). Radiolabeled POU was found to bind to an unstructured region of Per2 (Fig. 4.6.A). Residues 683 to 872 of Per2 contain a NLS and the N-terminal end of a proline rich area suggested to give Per2 flexibility to possibly bind two proteins at once.

Period 2, residues 683 to 872 competes POU off the estrogen promoter, in vitro.

Earlier electrophoretic mobility shift assays (EMSAs) confirmed POU binding to the estrogen promoter in Fig. 4.4.D. It was after we determined the binding of POU and Per2 that we asked whether Per2 influences POU's binding to the estrogen promoter resulting in the modulation of ERα transcriptional activity. EMSAs were carried out essentially as previously described in Materials and Methods; however in this experiment we asked whether, once bound, can Per2 compete POU off the double stranded DNA and, if incubated together before DNA addition, does Per2 alter POU association to the estrogen promoter (Fig. 4.7.A and B, top panels). Increasing concentrations of GST-fused proteins, Per2 (683 to 872 and 822 to 1255) were added to the POU/*esr1* (see oligonucleotides in electrophoretic mobility shift assays section) complex. As expected for the non-binding region of POU to Per2 (822 to 1255), there was no change in POU binding to the estrogen promoter (Fig. 4.7., C and D bottom panels). However Per2 (683 to 872) did decrease the binding of POU to the promoter (Fig. 4.7, C and D middle panels.). Following the schematic depicted in figure 4.7.B, top panel, Per2 and POU were first incubated followed by the addition of the estrogen promoter. Per2 (683 to 872) is able to sequester POU from binding to the estrogen promoter. In result, Per2 can compete off POU from the estrogen promoter once bound as well as prevent POU to readily bind

to the promoter. The result suggests Per2 can modulate the transcriptional activation of ERα by competing POU off the estrogen promoter.

BRCA1 does not modulate POU's binding to the estrogen promoter in vitro.

BRCA1 transcriptionally modulates ERα, however the mechanism to regulate ERα promoter is not known. To determine whether BRCA1 alters POU binding to the estrogen promoter, we introduced increasing concentrations of known binding fragments of BRCA1 (fragments 1 to 333 or fragment 1646 to 1859) (Fig. 4.8.A). This resulted in no change with either the N- or C-terminal ends of BRCA1. Furthermore, incubating the N- or C-terminal ends of BRCA1 with POU before the addition of esr1 also resulted in no change (Fig 4.8.D, top and bottom panel). Although POU directly binds to BRCA1, BRCA1 does not change the binding of POU to the promoter.

POU recruits BRCA1 and Per2 to the ERα promoter and Per2 transcriptionally represses the expression of ERα.

Although POU binds to both BRCA1 and Per2 it wasn't known whether these interactions occurred at the ERα promoter. Studies have shown BRCA1 transcriptionally activates *esr1*, therefore we aimed to determine whether Per2 transcriptionally represses the ERα promoter [3]. Our results indicate that POU does, in fact, recruit BRCA1 and Per2 to the promoter in CHO cells (Fig. 4.9.A). CHO cells were transfected with pcs2+*myc*, pcs2+*myc-pou*, pcs2+FLAG-*pou* and pcs2+*myc-per2*, pcs2+*myc-brca1* and pcs2+FLAG-*pou,* pcs2+*myc-per2*, pcs2+*myc-brca1*. Figure 4.9.A. lanes 2-3 EV (input, RNA polymerase II and the experimental labeled with the protein immunoprecipitated

with myc-beads), are the results for the experimental negative control. Immunoprecipitation with RNA polymerase antibody resulted in the presence of *esr1* (positive control). Lanes 5-7 the input, RNA polymerase and experimental for pcs2+*myc-pou* transfected CHO cells resulted in the immunoprecipitation of *esr1*. When *pou* and *per2* were co-transfected it resulted in the immunoprecipitation of *esr1*. However, transfected *per2* alone did not result in the immunoprecipitation of *esr1* (lane 16) confirming that Per2 is only present at *esr1* when POU is also found at the promoter (lanes 8-10). As previously shown, BRCA1 is only present at *esr1* when POU is present at *esr1* (lanes 11-13). In addition, luciferase assays show transcription activity of ERα has an inverse relationship with increasing concentrations of transfected Per2 in CHO cells (Fig. 4.9.B). Although we saw that Per2 may repress transcription of ERα in CHO cells, qRT-PCR experiments were carried out to measure the mRNA expression levels of ERα in MCF-7 transfected cells. MCF-7 cells were transfected with pcs2+*egfp-per2*, pcs2+*egfp-pou,* and both plasmids together. The transfected MCF-7 cells were sorted via fluorescence activated cell sorting (FACS) to obtain a group of only transfected MCF-7 cells for RNA extraction. The sorting of transfected MCF-7 cells from untransfected cells circumvents the common problem of low and highly variable transfection efficiency in these cells. The ERα mRNA expression levels did not change when pcs2+*egfp-pou* transfected cells are compared to pcs2+*egfp* transfected cells. Cells transfected with pcs2+*per2* show a decrease compared to cells transfected with pcs2+*egfp* in accordance with the decrease in luciferase activity when CHO cells were transfected with increasing concentrations of pcs2+*per2*. Surprisingly, when cells were co-transfected with

pcs2+*per2* and pcs2+*pou* there is no change in ERα mRNA expression levels suggesting there could be another regulatory factor not taken into consideration.

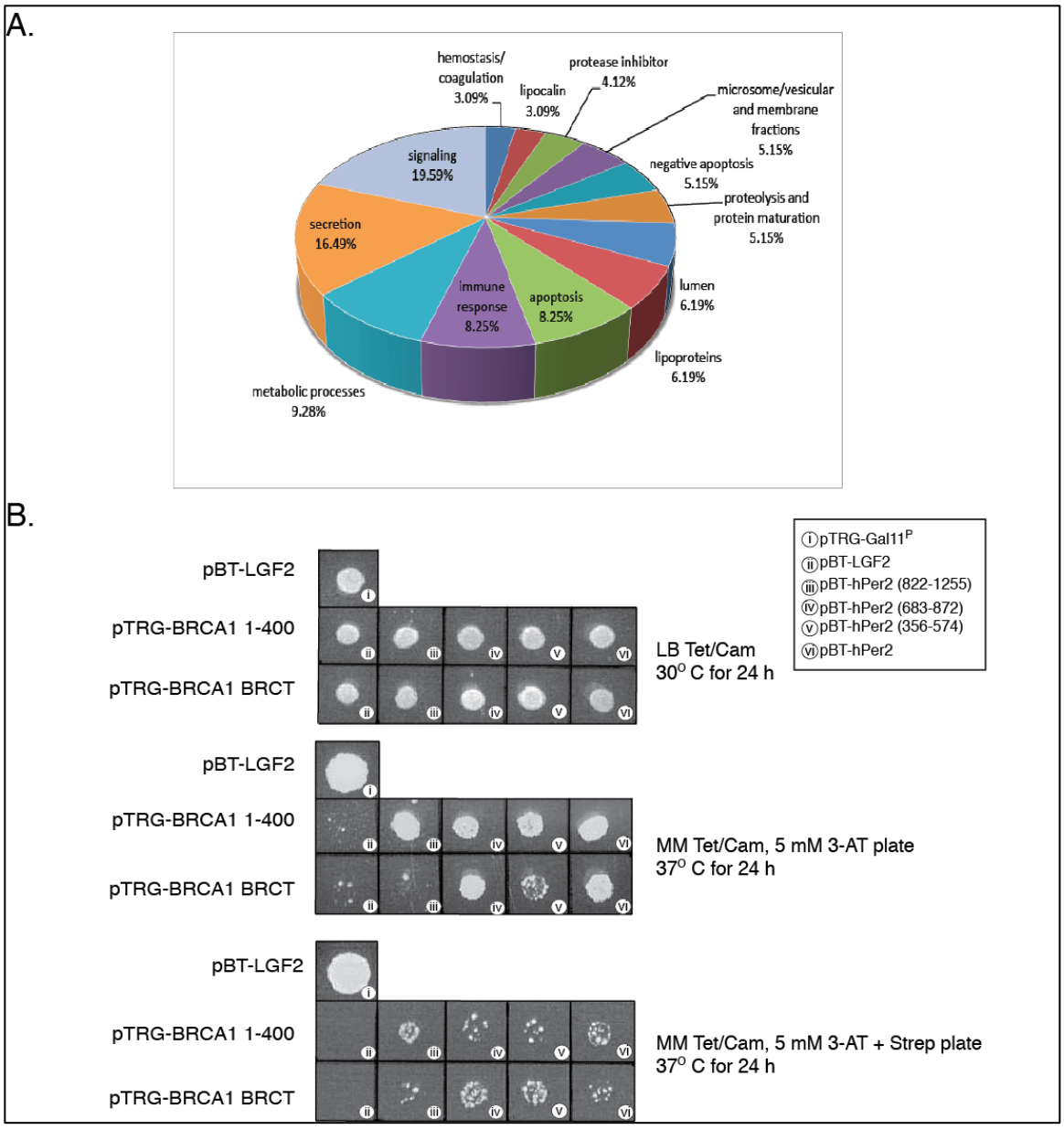


Figure 4.1. *Cluster analysis for found bacterial two hybrid interactors of Period 2 and confirmation of BRCA1 interaction via two hybrid system*. (A) Clones were grouped based on biological function, percentage stated is based on an overall of 120 positive clones identified. (B) Two-hybrid analysis using non-selective and selective plates to confirm Per2 interaction.

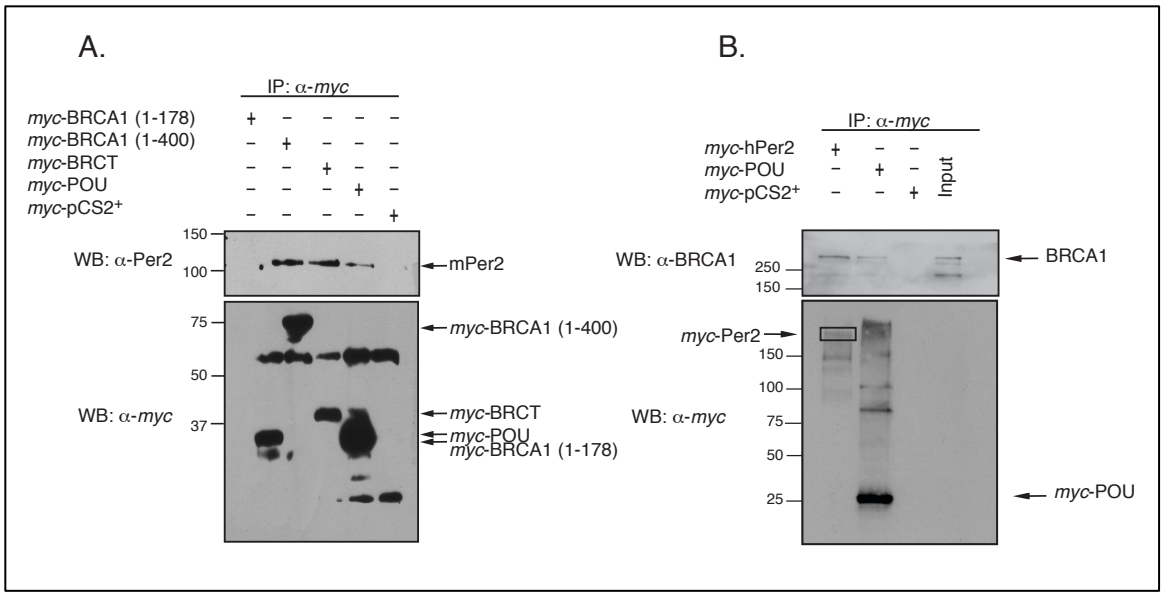


Figure 4.2. *Circadian protein Per2 binds to BRCA1 and DNA binding domain of OCT1, POU in mammalian cells*. (A) CHO cells were transiently transfected (Lipofectamine®) with myc-tagged gene constructs of *brca1*, 1 to 178, 1 to 400 and 1670 to 1863 (BRCT), and *pou*. Endogenous Per2 was immunoprecipitated (IP) using *myc*-beads (3 μg) and resolved by SDS-PAGE and immunoblotting. (B) MCF-7 cells were transfected with pCS2+*myc*-POU and pCS2+*myc*-Per2 (Lipofectamine LTX®) following manufacture's instructions. Cells were collected and proteins of interests were immunoprecipitated using *myc*-beads (3 μg) followed by immunoblotting with antibodies a-BRCA1 and a-*myc*.

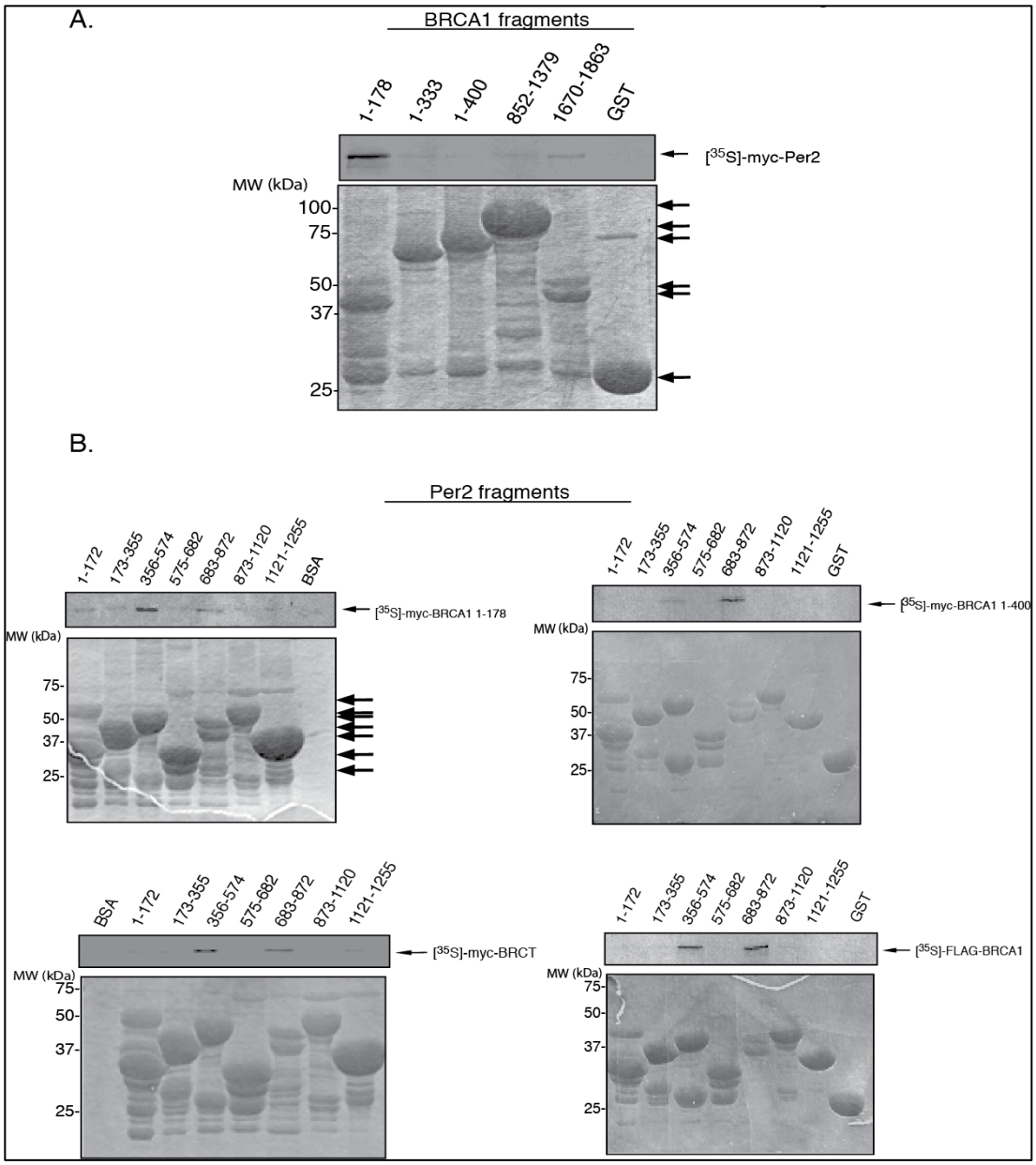


Figure 4.3. *Per2 and BRCA1 bind to distinct regions to one another in vitro*. (A) GST-bound recombinant BRCA1 fragments (1 to 178, 1 to 333, 1 to 400, 852 to 1379, 1670 to 1863) were incubated with [^{35}S]-Per2, pulled-down, and the complexes washed with low and high salt buffers (20 mM Tris-HCL pH 7.4, 100 mM NaCl (1M), 5 mM EDTA and 0.1% Triton X-100). GST was used as negative control. Complexes were resolved by SDS-PAGE and visualize by autoradiography (top panel) and Coomasie staining (bottom panel). Arrows indicate the protein of interest. (B) Seven GST-fused Per2 constructs were pulled down with [^{35}S]-BRCA1 (1-178, 1-400 or 1646-1859) pulled-down, and the complexes washed with low and high salt buffers (20 mM Tris-HCL pH 7.4, 100 mM NaCl (1M), 5 mM EDTA and 0.1% Triton X-100). GST was used as negative control. Samples were monitored by SDS-page followed by autoradiography.

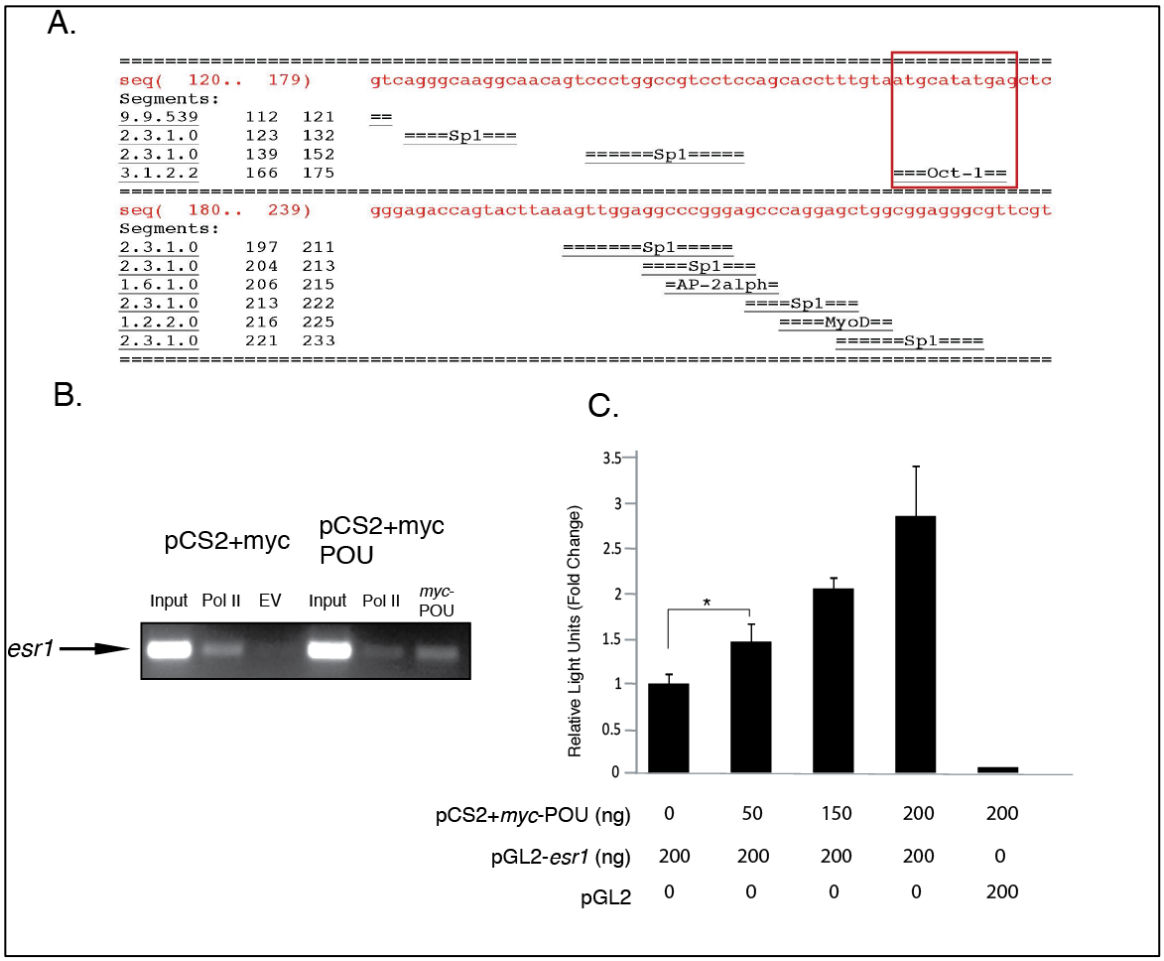
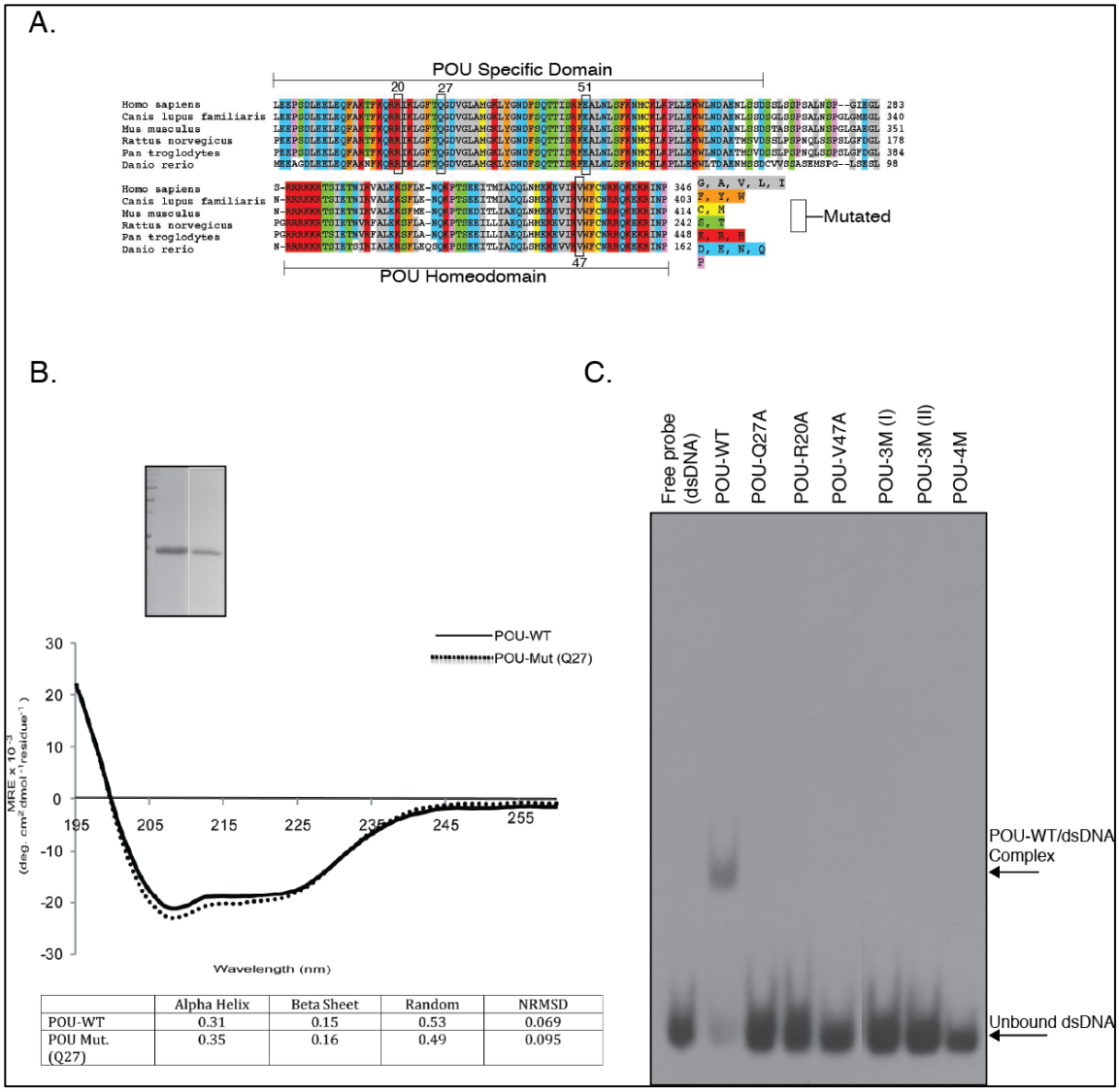


Figure 4.4. *The DNA binding domain of OCT1, POU binds and modulates esr1*. (A) Alibaba 2.0 was used to identify potential response elements for the regions +1 to +163 of the estrogen receptor promoter sequence. The location the Oct-1 binding site is underlined and comprises 78 – 86 bp downstream of the promoter start site. (B) MCF-7 cells were transfected with pCS2+*myc* or pCS2+*myc*-POU and protein-DNA complexes were immunoprecipitated as described in the Materials and Methods section. Oligonucleotides flanking the POU-response element within the estrogen promoter 216 bp were amplified. The RNA polymerase II was used as the positive control. Total input (10%) was used as a loading control. (C) CHO cells were co-transfected with increasing concentrations of pCS2+*myc*-POU (0 to 200 ng), pGL2-*esr1* (200 ng) and β-galactosidase (100 ng). Statistics were performed using the student t-test ($p<0.05$).



	Alpha Helix	Beta Sheet	Random	NRMSD
POU-WT	0.31	0.15	0.53	0.069
POU Mut. (Q27)	0.35	0.16	0.49	0.095

Figure 4.5. *Single nucleotide mutations in POU abrogate binding to the ERα promoter*. (A) Sequence alignment of the POU domain of various species. Conserved residues were mutated from the POU specific and homeodomains. (B) Purifcation of recombinant POU-WT and POU-Q27A was performed essentially as described in the Materials and Methods. Far-UV CD spectra of POU and POU-Q27A (10μM). Dichroweb, online server (http://www.cryst.bbk.ac.uk/cdweb/html/) was used to analyze percent of secondary structure and to validate the goodness of fit data (normalized root mean square deviation < 0.1) Spectra were recorded on a Jasco J-815 spectropolarimeter. (C) Electrophoretic mobility shift analysis (EMSA) was performed using oligonucleotides comprising the +76-+87 region of the ERa promoter. POU and mutant proteins (100 ng) were incubated with the ER promoter DNA in binding buffer (20mM Hepes pH 7.5, 2mM MgCl2, 10% glycerol, 0.1mM DTT and 3 of poly (dI-dC)). Complexes were resolved in a 5% gel and resolved by autoradiography.

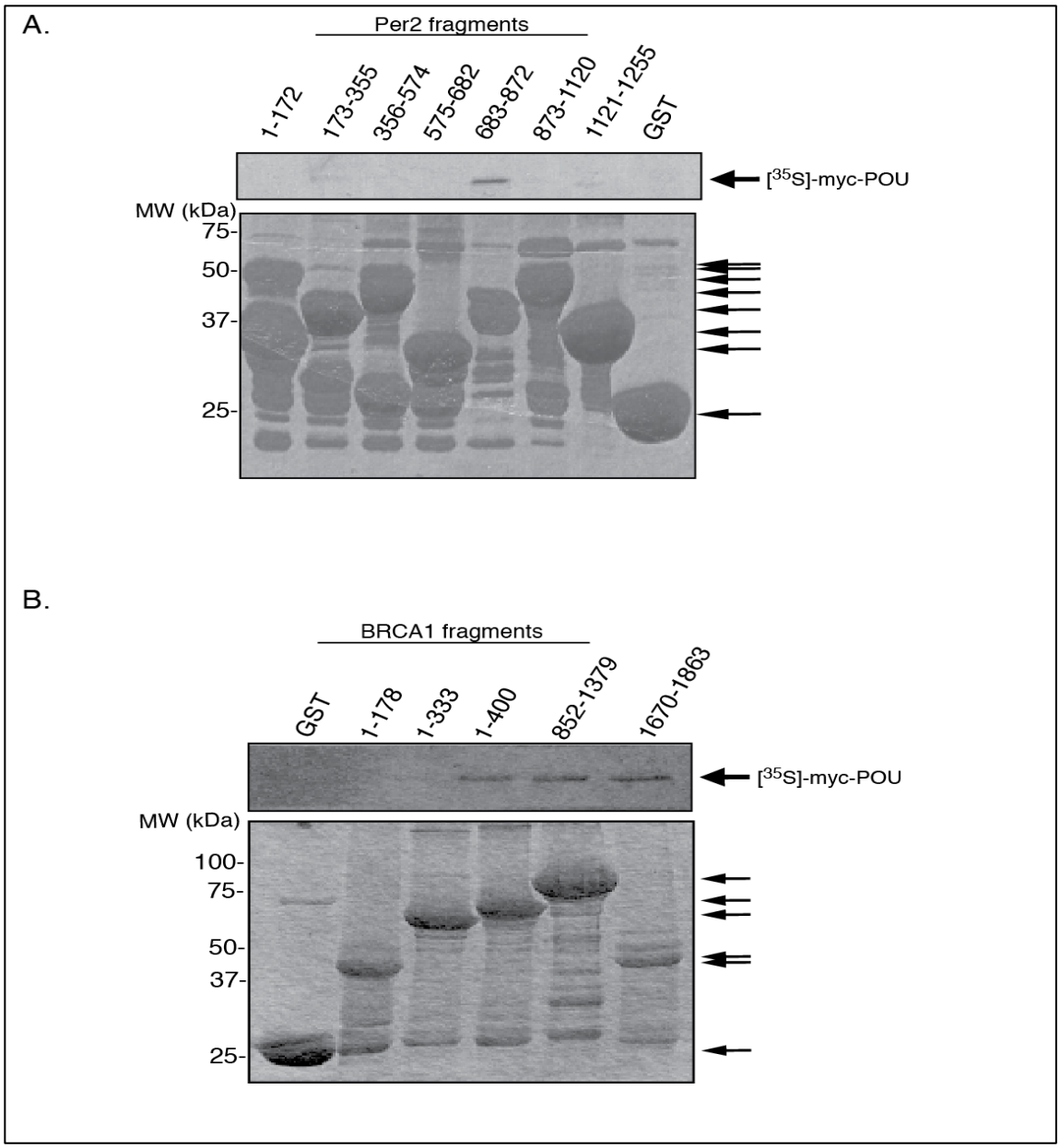


Figure 4.6. *POU binds distinct regions of BRCA1 and Per2*. (A) GST-tagged constructs of Per2 were expressed and purified as described in Materials and Methods, and incubated with [^{35}S] –POU, pulled-down, and the complexes washed with low and high salt buffers (20 mM Tris-HCL pH 7.4, 100 mM NaCl (1M), 5 mM EDTA and 0.1% Triton X-100). GST was used as negative control. Complexes were resolved by SDS-PAGE and visualize by autoradiography. Bound POU to Per2 constructs were determined by autoradiography and Coomasie staining, respectively. Black arrows indicate protein construct of interest. (B) GST-bound BRCA1 constructs (1 to 178, 1 to 333, 1 to 400, 852 to 1379, and 1670 to 1863) were expressed and purified as described in Materials and Methods and incubated with [^{35}S] –POU. Bound complexes were treated essentially as described above.

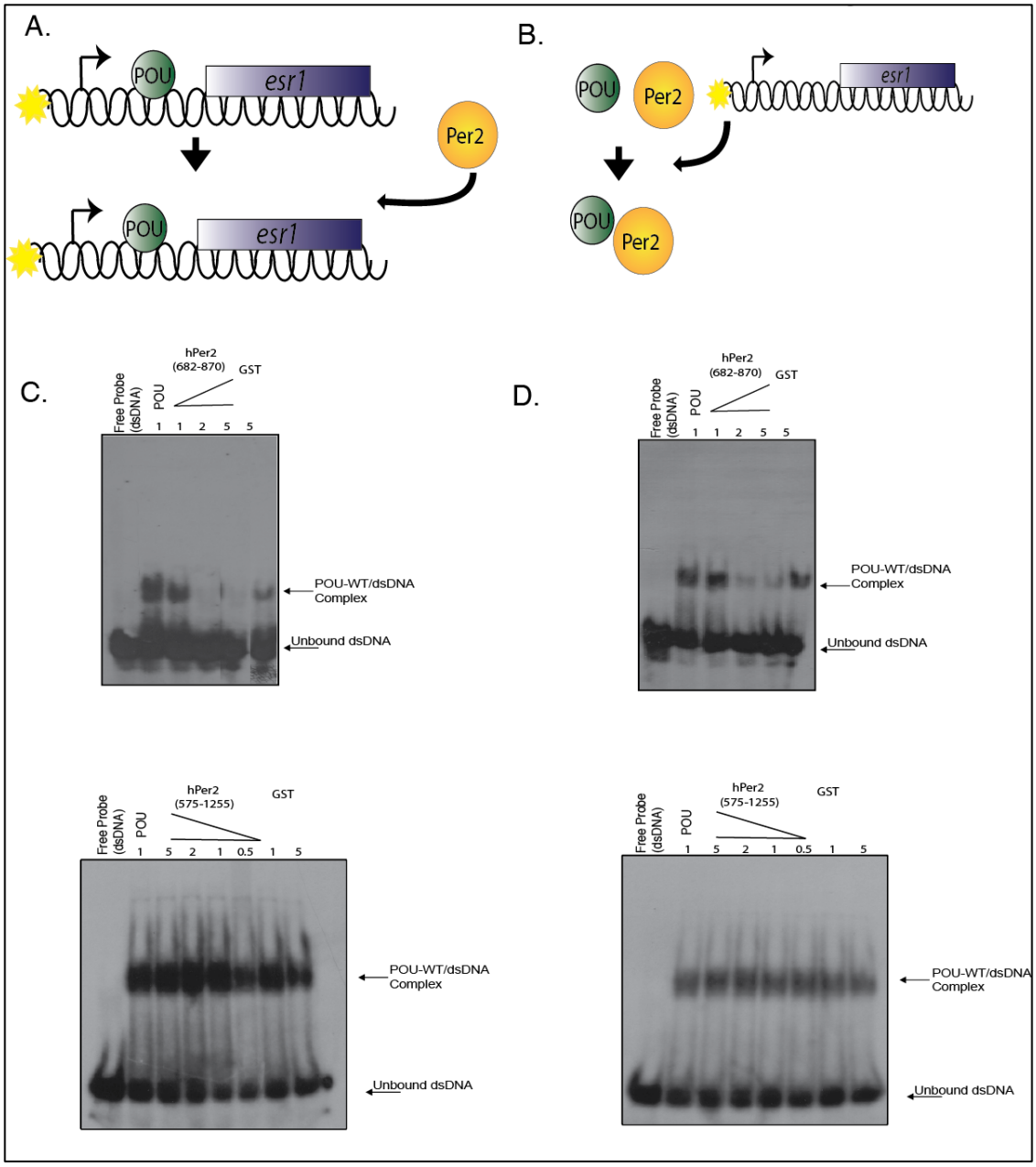


Figure 4.7. *Per2 (682 to 870) competes off POU binding to the ERa* (A) POU is bound to the radiolabeled dsDNA (*esr1)* and then Per2 is introduce. (B) POU and Per2 are incubated first and then radiolabeled dsDNA (*esr1)* is added to the reaction. (C) POU was incubated with radiolabeled dsDNA followed by the addition of GST-fused Per2 constructs, 682 to 870 (top gel) and 575 to 1255 (bottom gel). (D) On the right panel, POU is incubated with Per2 for the initial 20 min. at room temperature then followed by incubation with radiolabeled dsDNA (76 to 87). After the incubation of POU and dsDNA, increasing concentrations of GST-fused Per2 682 to 870 (top gel) and 575 to 1255 (bottom gel), were added to the mixture. Complexes were resolved in a 5% gel and visualized by autoradiography.

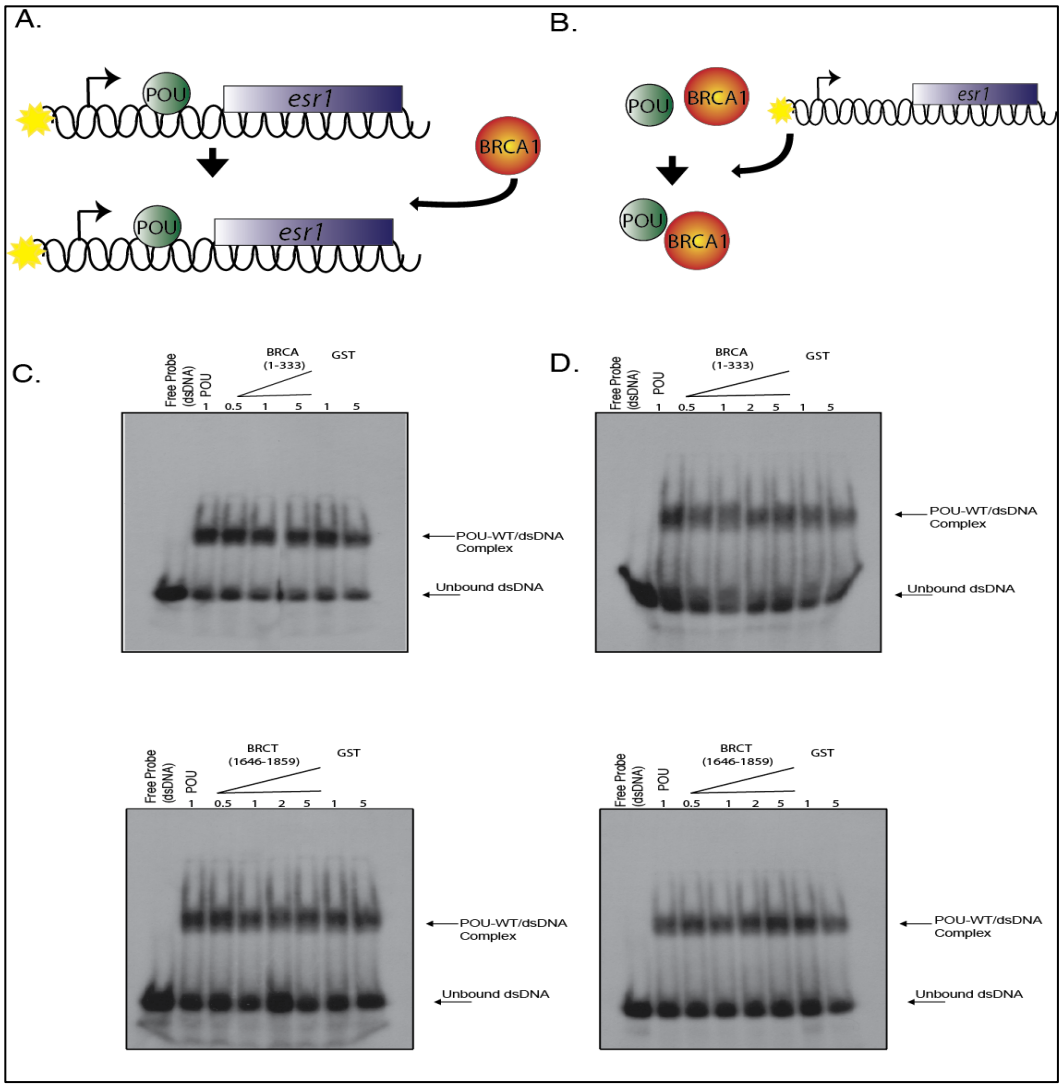


Figure 4.8. *In vitro studies show BRCA1 1 to 333 and 1646 to 1859 do not disrupt POU binding to the ERa.* (A) POU is bound to the radiolabeled dsDNA (*esr1)* and then BRCA1 is introduced to the incubated protein to DNA. (B) POU and Per2 are incubated first and then radiolabeled dsDNA (*esr1)* is added to the reaction. (C) POU and radiolabeled dsDNA were initially incubated for 20 min. at room temperature followed by the addition of increasing concentrations of BRCA1, 1 to 333 (middle panel) and 1646 to 1859 (bottom panel). (D) POU and increasing concentrations of BRCA1, 1 to 333 (middle panel) and 1646 to 1859 (bottom panel) were incubate first followed by the addition of radiolabeled dsDNA. All samples were ran as previously described above. Complexes were resolved in a 5% gel and visualized by autoradiography.

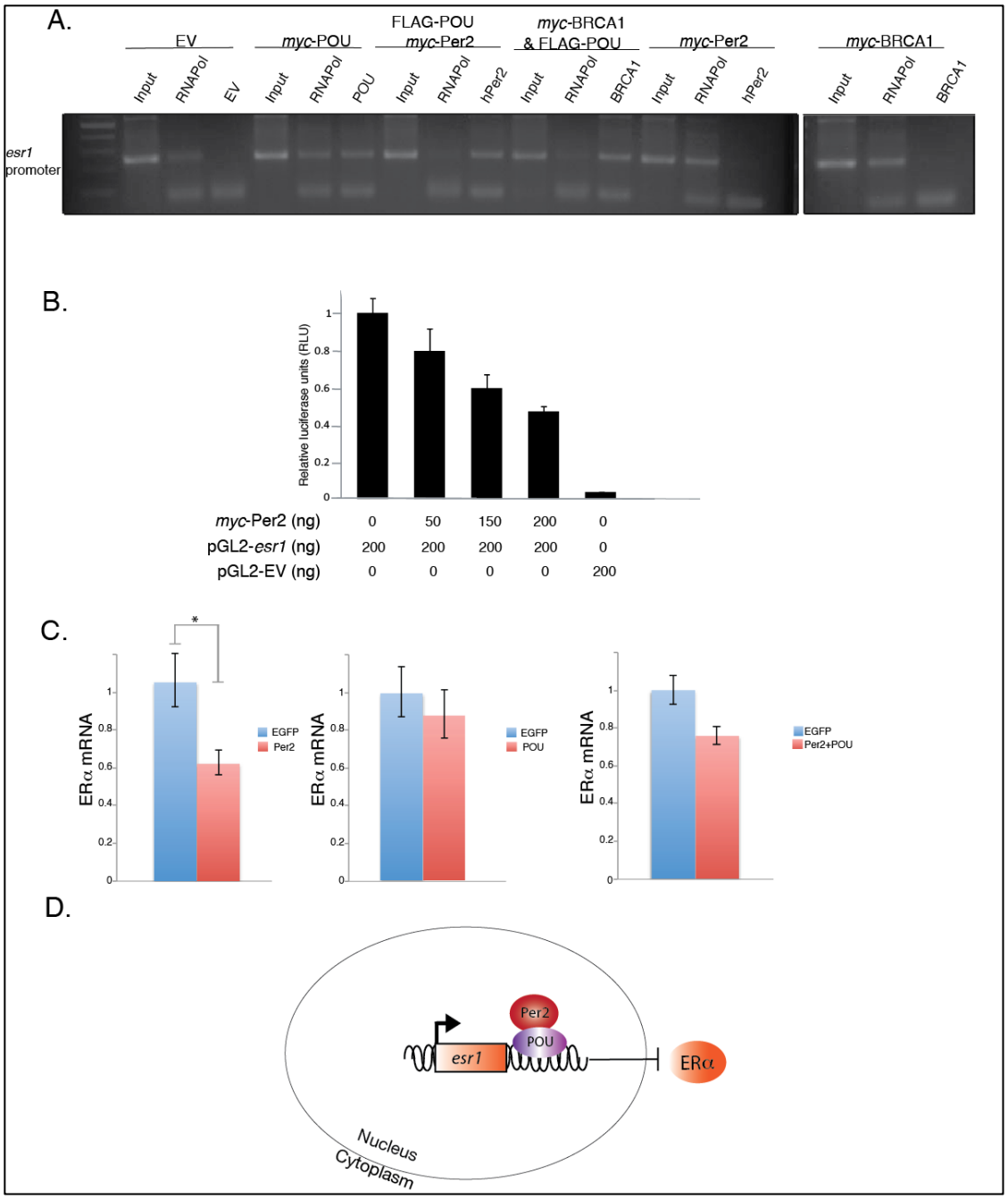


Figure 4.9. *POU recruits BRCA1 AND Per2 to ERα promoter*. (A) CHO cells were transiently transfected with empty vector (EV), *myc*-POU, FLAG-POU and *myc*-Per2, *myc*-BRCA1 and FLAG POU, *myc*-Per2 alone and *myc*-BRCA1 alone using Lipofectamine and following standard procedures. Cells were then fixed with 1.5% formaldehyde in 1X PBS and consecutively washed using Buffer A (10mM Hepes pH 6.5, 10mM EDTA, 0.5mM EGTA and 0.25% Triton X-100) and Buffer B (1mM EDTA). Input DNA was incubated overnight at room temperature with *myc*-beads at 4°C. Supernatants were incubated at 65ºC overnight to reverse protein-DNA cross-links. PCR was performed using primers flanking a region of *esr1* equivalent to 271bp located. (B) CHO cells were co-transfected with increasing concentrations of

pCS2+*myc*-Per2 (0 to 200 ng), pGL2-*esr1* (200 ng) and β-galactosidase (100 ng). (C) MCF-7 cells were transfected with pcs2+*egfp-per2* and pcs2+*egfp- pou*. qRT-PCR was performed. Statistics were performed using the student t-test ($p<0.05$). D. Model depicting *esr1* repression in the presence of Per2.

Chapter 5

Discussion

Epidemiological studies support a relationship between the loss of circadian rhythmicity and the increased risk of sporadic cancer in industrialized societies compared to developing countries [67, 69]. This correlation is exemplified in several studies that link the increased risk of breast cancer to altered shift work [62, 67-69, 71]. Circadian rhythmicity is driven by the transcription/translation system of core clock genes, one of those genes being *per2*. Epigenetic studies show expression of *per2* is deregulated in cell lines derived from breast tumors suggesting a role for *per2* in transformation [1, 2, 120]. Furthermore, in-frame deletion of *per2* in mice results in an animal that is cancer prone and develops spontaneous lymphomas, a phenotype that is exacerbated when the animals are exposed to γ-radiation [90].

In order to determine additional partners for Per2 that might influence its regulation, we screened for interactors using a bacterial two-hybrid system. The two-hybrid system, using the C-terminus of Per2 as bait, rendered binding proteins from several different body functions outside of circadian rhythmicity. It is not surprising to find Per2 interaction with proteins outside of circadian rhythmicity because the circadian rhythm coordinates environment with internal physiology for the organized timing of biological functions. Significant binding partners for Per2 are clustered into functional groups to better visualize affected biological functions (Fig. 1A). Although 120 clones

were identified to be positive interactors for Per2, we chose to focus on molecules directly implicated in the onset and further development of breast cancer including the breast cancer associated protein, BRCA1.

Our results indicate that Per2 binds to the N- and C-terminal ends of BRCA1 (Fig.4.3.A.). Although Per2 can bind both regions, it is unknown whether Per2 binds both regions of BRCA1 simultaneously. Examples of similar binding models are found in BRCA1. In fact, the tumor suppressor p53 can mediate different functions by binding to distinct regions of BRCA1. The p53 protein binds to both the second BRCT domain (1670 to 1863) and residues 224 to 500 of BRCA1 resulting in different activated functions of BRCA1 [125]. The BRCT domain, alone, binds to p53 and activates the promoter of a potent cyclin dependent kinase inhibitor 1 (p21) necessary for the regulation of cell cycle progression of G_1 [126]. Interestingly, binding of p53 to BRCA1 residues 224 to 500 reduces BRCA1 binding affinity to DNA, suggesting distinct binding sites for Per2 on BRCA1 can carry out different functions [127]. In the event that Per2 binding to two distinct regions of BRCA1 results in different functions, one can speculate that the binding of Per2 to the RING finger domain prevents BARD to bind BRCA1 and result in the decrease of ubiquitin ligase activity for BRCA1 although additional experiments need to be done to prove (or disprove) this hypothesis. A main function for the C-terminal BRCT domain of BRCA1 is to recognize phosphorylated proteins in response to DNA damage and the binding of Per2 to this region may prevent BRCA1 to carry out this function, suggesting BRCA1 damage response can possibly be under Per2 [126]. An additional scenario arises if BRCA1 folding exposes both ends to bind to Per2. It is also possible that discrete bindings sites may uniquely alter BRCA1 function.

Our results show BRCA1 binds to Per2 in two distinct regions (residues 356 to 574 and 683 to 872) (Figure 4.3.B.). Residues 356 to 574 contain a nuclear export signal (NES) and residues 683 to 872 contain a nuclear localization signal. Binding to these regions might have a direct impact on Per2 shuttling into or out of the cell nucleus, however, BRCA1 is a nuclear protein and this would mean it would prevent Per2 from exiting the nucleus when bound to BRCA1 [128].

Heme has been shown to bind the PAS domain and to a novel heme regulatory motif (HRM) of Per2 exhibiting that it is possible to bind two locations of Per2, but not common [129, 130]. Further studies would be necessary to uncover the functional role of Per2 binding to BRCA1 under normal physiological conditions and to see how the function changes in cancerous tissues.

Although BRCA1 is typically associated with hereditary breast cancer because germline mutations of *brca1* increase the risk for breast tumors, BRCA1 does have a role in the transcriptional regulation of ERα and downstream signaling of ERα [3, 131]. Studies show the recruitment of BRCA1 by OCT-1 to *esr1* transcriptionally activates ERα in MCF-7 cells [3]. A survey of the OCT-1 response elements on ERα promoter A (this promoter is responsible for the upregulation of ERα in breast cancer tumors) yields only one site for OCT-1. Chromatin immunoprecipitation assays confirm OCT-1 DNA binding domain, POU is found at the ERα promoter site of interest [3] (Figure 4.4.A.).

The DNA binding domain, POU from OCT-1 binds to an octameric sequence ATGCATAT to regulate the expression of genes [132, 133]. POU is made up of two subdomains that contribute to the binding of DNA, the POU specific domain and the POU homeodomain [132]. The POU specific domain binds to the 5' end of the octamer

sequence, ATGC and the POU homeodomain makes contacts with the 3' end of the octamer sequence ATAT [133]. The POU specific domain and homeodomain are made up of 4 α-helices and 3 α-helices, respectively, and it is the third α-helix of each subdomain that makes contact within the major groove of DNA [132, 133]. Helix two and three of the POU specific domain contain highly conserved glutamines at the beginning of each helix [132, 133]. Glutamine-27 is the highly conserved glutamine in helix 2 of the POU specific domain (Figure 4.5.A.). It is this glutamine that we decided to mutate to alanine because glutamine-27 stabilizes the hydrogen binding of three other highly conserved residues, glutamine-44, glutamic acid-51 both on helix 3 and arginine-20 on helix 1 resulting in a net of hydrogen binding for POU to DNA. In result, the mutation of glutamine-27 to alanine disrupts the network of hydrogen bonding between highly conserved residues with DNA and abrogates POU's binding to the ERα promoter (Figure 4.5.C.) [132, 133]. It is for the same reason that we mutated arginine-20 of the POU specific domain to alanine; the mutation causes a disruption to the network of hydrogen binding of POU to the ERα promoter (Figure 4.5.C). Valine-47 was the only residue mutated to alanine on the POU homeodomain (Figure 4.5.C.). This mutation was interesting because valine and alanine share certain properties: they are both hydrophobic and small amino acids, and the biggest difference between the two is that valine contains one more methyl group than alanine. Valine-47 is located on helix 3 (the helix that makes contact with the DNA major groove) of the POU homeodomain and it is suggested that the disruption of Van der Waal contacts between valine-47's side chain with the DNA results in an incorrect fit of the third helix in the DNA major groove [133]. Interestingly, valine-47 has been suggested to be the key residue for POU's sequence specific affinity

to DNA [134]. The binding affinity of POU to DNA can be changed through the single mutation of valine-47 depending on the residue that takes the place of valine-47 [134]. Stephchenko *et. al* found that the mutation of valine-47 to alanine reduces the affinity of POU to DNA even though the change does not create any added steric hindrance [134]. In summary, these single mutations abrogate POU binding to the ERα promoter. The loss of OCT-1-POU binding to DNA can affect other genes regulated by OCT-1 and affect other cellular responses such as the development of organs and tissues, control of cell cycle progression and response to DNA damage [135-137]. One of the potential downstream physiological effects of diminished POU binding is the inability to respond to genotoxic stress [135]. OCT-1 affinity to DNA is enhanced in the event of genotoxic stress, independently of p53 activation [135]. If the POU domain is mutated on just one conserved residue, OCT-1 will be unable to respond to genotoxic stress leaving p53 and other DNA damage proteins with the sole responsibility of activating DNA damage response pathways.

The *gadd45* promoter is regulated by BRCA1 via OCT-1 [123]. In the response to genotoxic stress, Gadd45 activates cellular responses to DNA damage [123]. Although BRCA1 and OCT-1 work together for the transcriptional regulation of genes, it was unknown whether POU directly bound BRCA1. We found that POU directly binds on the N- and C- terminal ends of BRCA1. Curiously, these are the same binding sites Per2 binds to BRCA1.

Shared binding sites between OCT-POU for Per2 and BRCA1 raises the question whether POU can activate and repress the same gene, in this case *esr1*, in the presence of different transcription co-regulators. The notion that POU can transcriptionally activate

and repress the same gene is not unlikely [138]. For example, overexpression of silencing mediator for retinoid and thyroid hormone receptor (SMRT) competes with POU bound OCT-1 associated coactivator (OCA-B) [138]. Furthermore SMRT and OCA-B can form a complex suggesting a regulated balance between the two proteins to determine OCT-1 activity [138]. When OCA-B is bound to OCT-1 the transcription of the OCT-1 response element is activated; however, when SMRT is overexpressed the activation is repressed [138]. Both transcription factors, SMRT and OCA-B were found to bind POU, the DNA binding domain of OCT-1 [138]. Another POU containing transcription factor, Pit-1, has the same bifunctional transcriptional activity as OCT-1. Pit-1 bound to CREB-binding protein (CBP) transcriptionally activates the response element, however SMRT competes with CBP and represses transcriptional activation of Pit-1 [139].

In the same way that POU mediates BRCA1 transcriptional regulation, we speculate the possibility of POU also mediating Per2 transcriptional regulation of ERα. We found that POU is able to bind to Per2 residues 683 to 872. POU also binds a proline rich area that is predicted to give Per2 its flexibility. Furthermore it has been suggested that this flexibility can allow a docking station for Per2 to bind more than one protein at a time [76]. If this were true, the binding of POU to this region could lessen Per2 flexibility and prevent other proteins to bind once POU was bound.

Our EMSA results show that bound OCT-1-POU to the *esr1* is competed off by Per2 (683 to 872) (Figure 4.7.C, D). This would suggest that the removal of POU from the promoter by Per2 modulates ERα transcription. Further studies were done to determine whether Per2, via POU, did indeed modulate the ERα activity (Figure 4.9.B.).

However, when chromatin immunoprecipitation assays were performed to determine POU recruitment of BRCA1 and PER2 to the ERα promoter, results indicate that POU is able to recruit both molecules. It is possible that Per2's inhibition of POU in the EMSA could be an effect of using a fragment of Per2 (683 to 872) instead of full-length Per2, furthermore the in-vitro study is done with annealed oligonucleotides that only span approximately 23 nucleotides of the ERα promoter, resulting in a limited scope of the regulatory effect Per2 has on POU's binding to the ERα promoter. In addition, the ChIP assay shows that POU is able to recruit Per2 to the ERα promoter, however it does answer the question whether another protein or a complex of proteins are involved in stabilizing this association on the ERα promoter.

In agreement with previous studies, the recruitment of BRCA1 to *esr1* by OCT-1-POU (ChIP assay) suggests that BRCA1 associates with OCT-1-POU on the ERα promoter [3]. This result is a validation of the ChIP assay and corroborates the recruitment of Per2 by POU to *esr1*. In an attempt to determine whether BRCA1 changes the binding of POU to the ERα promoter, EMSAs were performed with the N- and C-terminal ends of BRCA1. However, our results show the binding of POU to the ERα promoter wasn't altered by addition of either terminal end of BRCA1. This suggests that full length BRCA1 is necessary for the binding of POU at the *esr1*.

Studies show BRCA1 activates ERα transcription when recruited by OCT-1 to *esr1* [3], and this had us asking whether Per2 counteracts BRCA1 transcriptional activation by repressing the transcription of ERα *via* POU. Initial promoter activity assays in CHO cells provided insight to the effect Per2 has on the transcription of ERα. Our results show repression of the ERα promoter with increasing concentrations of

transfected *per2*, in CHO cells. We picked CHO cell line for our studies because it provides a simpler cell system compared to breast cancer cell lines, while still expressing ERα, Per2, and BRCA1 and is estradiol responsive [140, 141]

Although CHO cells have been used in studies to determine a putative role for Per2 in the regulation of the ERα (our results), it was necessary to define the regulatory role in a cell line that generated ERα from promoter A [33]. The expression levels of ERα mRNA decrease when Per2 is transfected in MCF-7 cells (Figure 4.9.C.), however there was no change in the expression levels of ERα mRNA when cells were transfected with POU alone and when cells were co-transfected with POU and PER2 (Figure 4.9.C.). A known repressor of the *esr1* is the gene product, ERα, with the aid of a recruited repressor protein, Sin3A [142]. ERα is activated by estradiol and binds to the ERα promoter recruits Sin3A and resulted an approximately 40% decrease in ERα mRNA expression levels [142]. Similar to Per2 repression of the ERα transcription, the mRNA expression level of ERα in MCF-7 cells was decreased approximately 40% when the cells were stimulated with E_2 to activate ERα. Our work is supported by Rosetti *et al*. where they confirmed that Per2 and ERα expression counter oscillate with one another and the mRNA expression levels of each one lose rhythmicity in breast cancer cell lines. Our work supports that Per2 drives the counter oscillation with ERα.

In summary, our work demonstrates the molecular binding of BRCA1 to POU and identifies the residues involved in the binding of BRCA1 to Per2. In addition, POU mediates not only BRCA1 transcriptional activation of ERα, but also at the same location of the ERα promoter, transcriptional repression of Per2.

Chapter 6

Conclusions and future directions

In conclusion we found that a key circadian clock protein Per2 binds to BRCA1. Necessary for this interaction, BRCA1 binds to a region of amino acids 356-574 on Per2. This region on Per2 overlaps with the NLS region. Interestingly, the region through which BRCA1 binds to Per2 is also on the NLS, suggesting that BRCA1 and Per2 may have the ability to sequester one another. Given this interaction between BRCA1 and Per2 it would be interesting to determine whether or not BRCA1 and Per2 prevents movement out of the nuclear membrane. Furthermore, identification of key molecules or proteins that regulate this interaction may elucidate more or less circadian regulation of the cell cycle and external factors that may initiate such control.

For the purpose of my work it will be interesting to see whether BRCA1 and Per2 balance each other to regulate transcription of ERα. In addition, it would also be interesting to determine the factors that could disrupt BRCA1 binding to Per2. These disruptions potentially could be the germline mutations of *brca1* that have been previously seen to increase the risk of tumorigenesis in breast tissue.

Per2 has been shown to bind POU through an unstructured region of Per2. The binding of both of these may in fact allow for the interaction between both Per2 and BRCA1 through their NLS regions, thus forming a complex between all three proteins.

Moreover, BRCA1 transcriptionally activates the expression of ERα. Determination of whether or not all three proteins, POU, Per2, and BRCA1 interact with one another may reveal mechanistic insight for how the interplay between all three molecules affects ERα transcriptionally.

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