

**The role of the newly discovered steroid receptor RNA
activator protein (SRAP) in the estrogen signaling pathway
and its implication in breast cancer**

By

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Abstract

In 1999, the discovery of the Steroid Receptor RNA Activator (SRA) was unprecedented in the field of steroid receptor co-regulator research. It was the first time that an RNA molecule was demonstrated to function similarly to its protein counterpart and modulate the activity of steroid receptors. This peculiar steroid receptor co-activator thus attracted the attention of numerous research groups. Over the years, studies were reported deciphering SRA mechanisms of action, its role in co-regulating nuclear receptors and its possible implication in human diseases.

While SRA was originally thought to exist solely as a non-coding RNA, our laboratory has identified longer SRA RNA isoforms with the theoretical capacity to encode for a protein. This discovery impelled us to investigate the existence of a Steroid Receptor RNA Activator Protein or SRAP. In this thesis, we first demonstrated the existence and function of endogenous evolutionary conserved SRA proteins. Based on these results we further explored SRAP expression in breast tumors. Interestingly, Western blot analysis of a small cohort of estrogen positive breast tumors suggested that SRAP expression correlates with a better overall survival in patients treated with tamoxifen. This observation prompted us to explore the biological role of SRAP. We found that MCF-7 cells stably expressing coding SRA isoforms had lower ligand dependent estrogen receptor alpha transcriptional activity. In order to dissect the function of the protein independently of its RNA counterpart, we separated the functions of the protein by introducing extensive silent mutations into the RNA sequence. Using this model, we established that SRAP, independent of its RNA counterpart, enhances estrogen receptor alpha activity in a ligand and response-element dependent manner. Furthermore, we

showed for the first time that SRAP physically interacts with multiple transcription factors and is recruited to specific promoter regions. Moreover, by artificially recruiting SRAP to the promoter of a luciferase reporter gene under the control of the strong transcriptional activator VP16, we observed a decrease in transcription. These latter results suggest that SRA could function as a repressor through direct association with promoters.

Overall, we believe that SRA is a very peculiar example of a bi-faceted system consisting of a functional RNA and its corresponding protein. Altogether our data suggest that SRAP, similarly to its RNA counterpart, is involved in many critical pathways that directly participate in gene expression regulation.

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List of Abbreviations

AF1	Activation function 1
AF2	Activation function 2
AIB1	Amplified in breast cancer 1
E2	Estradiol
EGFR	Epidermal growth factor receptor
ESR1	Estrogen receptor alpha
ESR2	Estrogen receptor beta
DCIS	Ductal carcinoma in situ
HDAC	Histone deacetylase
Hela	Henrietta Lacks
HER-2	Human epidermal growth factor receptor-2
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
LCIS	Lobular carcinoma in situ
MAPK	Mitogen activated protein kinase
MCF-7	Michigan cancer foundation-7
NCOR1	Nuclear co-repressor 1
SERM	Selective estrogen receptor modulator
SRA	Steroid receptor RNA activator
SRAP	Steroid receptor RNA activator protein
SMRT	Silencing mediator of retinoic and thyroid receptor

SRC1 Steroid receptor co-activator 1

SRC2 Steroid receptor co-activator 2

SRC3 Steroid receptor co-activator 3

Thesis Organization

My thesis narrates the story of the protein product of the Steroid receptor RNA activator (SRA) gene as it unfolded in Dr. Etienne Leygue's laboratory. To recount this story, I have divided this thesis into seven chapters as follows.

I have dedicated the first introductory chapter to a comprehensive literature review offering background information on breast cancer, the estrogen-signaling pathway and the SRA gene.

The subsequent four chapters (chapters 2 to 6) describe the story of SRAP story as it evolved in our laboratory. I have chosen to present these four chapters as *verbatim* reproductions of four peer-reviewed articles published as a direct result of my research conducted under the guidance of Dr. Etienne Leygue. Although published as separate entities, these articles are directly related to one another and are therefore reproduced in the chronological order of their publication date. It is important to note that each article is built on the results of the previous one. The article in chapter 2 recounts the establishment of SRAP existence. In chapter 3, we explore further SRAP expression in breast tumors and its possible association with outcome of patients under tamoxifen treatment. Based on these findings, we scrutinized further SRAP function on estrogen receptor alpha (ESR1) by separating the SRA RNA and protein actions in chapter 4. Finally, the article presented in chapter 5 explores emerging SRAP mechanisms of action where we identified potential target genes.

All four articles are self-contained and include their own Abstract, Introduction, Materials and Methods, Results and Discussion as well as Reference sections. A brief section underlining the continuity of SRAP's story has been inserted prior to each article.

The transition sections have been designed to amalgamate the four published articles and thereby emphasize the flow of information within this thesis.

Following SRAP's story, a concluding chapter 6 discusses the overall significance of my research and offers some future directions on the project. Chapter 7 is a list of references that encompasses the entire thesis.

Chapter 1: Introduction

1. Breast Cancer

Breast cancer is a disease that has affected women over the centuries and all over the world. It represents today the most common cancer in women, both in the developed and developing countries (www.breastcancer.org). Breast cancer has a considerable impact on individual lives and exerts significant financial burden on health care systems. A tremendous amount of research has therefore been undertaken in the hope to find better diagnostic methods and increase treatment options. It is important to note that our understanding of normal human breast physiology has been one of the bases responsible for the enhancements in diagnostic, preventative and therapeutic options in the fight against breast cancer.

1.1 Normal physiology of the mammary gland

1.1.1 Mammary gland development

There are several major phases in the development of the human mammary gland with specific structural changes occurring at each stage. The embryonic development is first initiated by the formation of a bilateral mammary ridge. Subsequently, two placodes formed at the site of each future nipple, penetrate the surrounding fat pad and form a rudimentary ductal tree. From birth until the onset of puberty, the breast will remain indistinguishable between males and females. At the onset of puberty, the increase in ovarian estrogen in females leads to the proliferation of the lactiferous ducts and an accumulation of adipose and connective tissues. At this time, the ducts further penetrate the fat pad and normal mammary gland structure is reached as depicted in figure 1.

Perhaps, the most remarkable mammary gland growth and differentiation occurs during pregnancy. The growth of the mammary gland during pregnancy is the result of the combined action of several hormones including estrogen and progesterone. The collective action of all these hormones results in a further development of the lobular alveoli and ducts. The alveoli develop into the active milk secreting structures. With the cessation of lactation, the alveoli developed during pregnancy undergo degeneration through apoptosis. The mammary gland alveoli and ducts then regress back to a resting state. Upon the onset of menopause, the decrease in estrogen levels results in the reduction of the glandular breast tissue (Howard and Gusterson 2000).

1.1.2 Mammary gland structure

The adult human female breast lies on the pectoralis major muscle between the 2nd and 6th rib. The mature mammary gland consists of 15-25 lobes that are separated from each other by dense connective and adipose tissues. The lobes are each composed of smaller structures called lobules. The lobules consist of clusters of alveoli that are the milk producing structures during lactation. Lobules and lobes are connected to each other by branching lactiferous ducts which themselves exit into the nipple (Keith L. Moore and Arthur F. Dalley, 2006). Figure 1 illustrates the basic anatomy of human mammary gland.

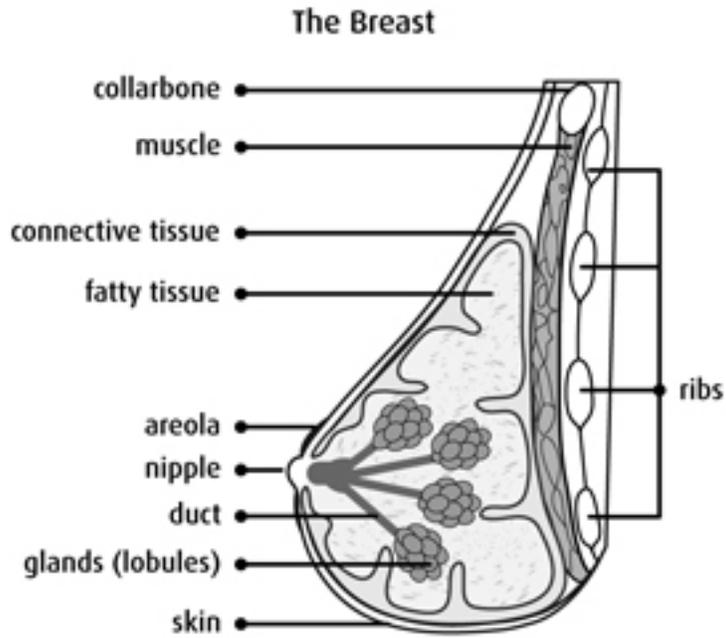


Figure 1: The basic anatomy of the human mammary gland. This schematic diagram from the www.breastcancer.org illustrates the basic anatomy of the human breast.

1.2 Breast cancer

1.2.1 Canadian breast cancer statistics

In 2010, an estimated 22700 Canadian women will be diagnosed with breast cancer and 5400 women will die from the disease (www.cancer.ca). Nonetheless, most recent Canadian cancer statistics also reports that the mortality due to this disease has steadily declined since the mid 1990's. This decrease is attributed to considerable advances in research that have allowed the development of new diagnostic as well as therapeutic strategies.

1.2.2 Risk factors

Breast cancer starts as a result of an unregulated proliferation of breast cells. While there is not a single exact cause, it is believed that a combination of genetic and environmental factors is responsible for the onset of breast cancer. Several risks factors with different degrees of severity have been established. For example, the inheritance within affected families of mutated genes is associated with a higher risk of developing familial breast cancer. The most common genetic anomalies connected with an increased risk occur in the breast cancer susceptibility gene 1 (BRCA 1) and breast cancer susceptibility gene 2 (BRCA2). Both of these genes encode for proteins involved in DNA repair and transcription regulation. It is however important to note that only 5 to 10% of the total number of breast cancers cases are thought to result from such inherited genetic mutations.

In addition to genetic susceptibility, several other risk factors have been defined. Age is one such factor as the odds to develop breast cancer increases greatly with age. Another factor is the number of full term pregnancy. Early full term pregnancy is considered to

confer protection against breast cancer. In this regard, nulliparous women are at a higher risk for developing breast cancer than early parous women. A third important risk factor is a personal history of breast cancer or proliferative breast diseases. These two conditions have both been associated with an increased risk of developing breast cancer in the future. In addition, a long reproductive window resulting from early menarche and/or late menopause is also associated with a higher risk for developing breast cancer. From a molecular biologist's perspective, this association is interesting as it indicates a link between the epidemiological aspects of breast cancer and the molecular mechanisms underlying breast cancer tumorigenesis and progression. Indeed it is believed that a long reproductive window results in an increased lifetime exposure to estrogen. This potent mitogen that increases the proliferation of breast epithelial cells is suspected to play an important role in the mechanisms underlying breast tumorigenesis and breast cancer progression.

1.2.3 Different types of breast carcinoma

There are two major categories of breast cancers: non-invasive and invasive. The non-invasive breast cancers can be further characterized into different sub-categories depending on their cellular origin. As outlined earlier, the breast tissue is composed of several cell types. While theoretically breast cancer can arise from any of these cells, the vast majority of breast cancers originate either from the epithelial cells located in either ductal or lobular structures of the breast. When cancerous cells arise and remain confined to the ducts, the tumor is referred to as ductal carcinoma *in situ*. When they arise and remain in breast lobules, the lesion is referred to as lobular carcinoma *in situ*. Paget's disease is another form of non-invasive breast cancer that affects the nipple area. While

most non-invasive breast cancers are not lethal *per se*, these can nonetheless progress into invasive breast cancers that have much higher degrees of complications.

Invasive breast cancers can also be subtyped into distinct categories depending on either the extent of invasion or the cytological characteristics of the cancer cells. Early invasive breast cancers refer to cancer cells that have grown through the walls of the milk ducts and glands into the normal fatty tissue of the breast. This form can progress into a locally advanced breast cancer where the cancer is growing into the skin or chest wall. Inflammatory breast cancer is an aggressive type of locally advanced breast cancer where cancer cells block the lymphatics and thus cause the breast to become red and inflamed. Metastatic breast cancer refers to cancer cells that have infiltrated the lymphatic and/or blood system and metastasized to distant part or parts of the body.

As stated earlier, invasive breast cancers can also be grouped into distinct categories depending on cytologic characteristics. The vast majority of invasive breast cancers originate from the epithelia of the breast glandular tissue and these cancers are therefore called adenocarcinomas. Invasive ductal carcinoma accounts for 75 % of all breast cancers while invasive lobular carcinoma is seen in 5 to 10% of cases. Invasive ductal carcinomas can be further subdivided into different types depending on cellular characteristics. Tubular carcinoma is characterized by tubule formation and is seen in less than 2% of cases. Medullary carcinoma presents extensive infiltration. Mucinous or colloid carcinoma is characterized by the accumulation of extracellular mucin. Although infrequent, invasive breast cancers arising from non-epithelial tissue type can occur. For example, cytosarcoma phylloides is a cancer that arises from the breast connective tissue.

1.3 Breast cancer diagnosis and treatments

1.3.1. Breast cancer diagnosis

Breast cancer diagnosis is an important aspect in the management of this disease. Most breast cancers are initially diagnosed as a result of self-examinations or physical examination by health care providers. In addition, in developed countries, the widespread availability of mammographic screening programs has greatly improved breast cancer diagnosis. Other imaging modalities such as ultrasound and magnetic resonance imaging although not used for screening purposes, are also beneficial in the evaluation of palpable lesions or in the assessment of regional lymph nodes.

Following physical and/ or mammographic evaluation of a suspected malignant mass, a fine needle aspiration or core needle biopsy is performed in order to obtain material for cytologic analysis. While both types of biopsies allow cytologic analysis, only core needle biopsy provides the pathologist with a core of tissue thus enabling the distinction between in situ and invasive carcinoma.

Following initial examination and biopsy analysis, two classification schemes are usually used to determine the extent or severity of breast cancer and prepare for treatment options. The tumor/ node/ metastasis (TNM) staging system takes into account the size of the tumor, whether lymph nodes are affected and whether cancer has metastasized. Based on these criteria, one of four cancer stages is assigned that are described in Table 1.

Stage	Tumor size	Node invasion	Metastasis
Stage 1	T1	N0	M0
Stage 2	T1 T2 T3	N1 N0 or N1 N0	M0
Stage 3	T-any T3 T4	N1 N0 or N1 N0	M0
Stage 4	T-any	N-any	M1

Table 1: The four stages of breast cancer using the TNM system. T1, T2, T3 and T4 represents tumors less than 2 cm, between 2 to 5 cm, more than 5 cm and tumors ulcerated or attached. N0 represents clear or negative nodes. N1 indicates cancerous or positive nodes. M0 indicates no sign of metastasis and M1 represents tumor metastasis.

A second system is used to determine a tumor grade based on the histological characteristics of cancer cells. This scheme uses four tumor grade categories. Grade one (G1) encompasses low-grade tumors that present well-differentiated cells. Grade two (G2) characterizes intermediate grade tumors that contain moderately differentiated cells. Grade 3 (G3) is assigned to poorly differentiated tumors while grade four (G4) describes undifferentiated tumors. G3 and G4 tumors are considered as high grade.

Biopsy samples from a suspected malignant lesion also allow pretreatment evaluation that includes the analysis for the presence of certain markers. Tumor markers are substances produced by the tumor cells or normal cells in the body as a response to the cancer. Most tumor markers are proteins that are present in the blood, urine, tumor or other tissue of the patients. These markers can be used for distinct purposes. These include screening patients, diagnosing the disease, determining the outlook of cancer

(prognostic markers), predicting the responsiveness to specific treatments (predictive markers), determining the effectiveness of treatments and diagnosing recurrent cancers.

With respect to breast cancer, today no tumor marker is yet clinically used to screen or diagnose early stage breast cancer. At the time of diagnosis, estrogen receptor is likely the single most valuable predictive marker for breast cancer. Indeed, breast cancer tumor tissues are routinely tested for estrogen (ER) and progesterone (PR) receptors as they indicate the likelihood of cancers to respond to hormonal therapies. In addition, tumor tissues are also tested for the presence of the human epidermal growth factor receptor 2 (HER-2) as this marker predicts the likelihood of cancers to respond to treatments targeting this receptor such as herceptin also known as trastuzumab. Several other markers are used to determine the effectiveness of treatments and possible cases of recurrence in advanced breast cancers patients. These include the cancer antigen 15.3 (CA 15.3) and carcinoembryonic antigen (CEA).

1.3.2. Molecular classification of breast cancers

As described earlier, molecular characteristics of breast tumor tissues provide insight into the effectiveness of certain treatments. Predictive markers such as ER, PR and HER-2 are routinely determined in order to identify the most effective choice of therapies. It has therefore been proposed that the identification of other such markers would increase the predictive value with respect to the current treatment options and therefore minimize unnecessary toxicity (de Ronde et al. 2010; Zepeda-Castilla et al. 2008). Retrospective studies examining protein profiles of tumors with known therapeutic outcome have allowed the identifications of new predictive markers. These types of studies have generated a possible new classification scheme for breast cancers. Based on molecular

signatures, Perou *et al* were the first ones to categorize breast cancers in four main molecular classes that have dissimilar therapeutic outcomes. These distinct categories consist of luminal A, luminal B, basal-like and HER-2 positive (Sorlie et al. 2001; Perou et al. 2000).

In a nutshell, luminal A subtype tumors express high amounts of estrogen receptors. In addition, these tumors express downstream estrogen receptor target genes including proteins expressed in luminal epithelial cells. Tumors within this category are usually low grade and sensitive to endocrine therapies. Patients with this type of tumor have a relatively good prognosis.

Luminal B subtype tumors also express estrogen receptors however at a lower rate. These tumors have usually a high grade and have a variable response to endocrine therapy. Patients with this type of tumor have a poorer prognosis than those with luminal A subtype tumors.

Basal like tumors are also known as triple negative since they are negative for the three markers: estrogen receptor, progesterone receptor or the human epidermal growth factor receptor 2 (HER2). Patients with this tumor type are therefore unlikely to respond to endocrine therapy or treatment targeting HER-2 signaling. While patients possessing these tumors are generally responsive to chemotherapy, they nonetheless have the shortest overall and disease free survival.

Tumors belonging to the HER-2 type have a high HER-2 expression often as a result of HER-2 gene amplifications. Patients presenting this tumor type are therefore candidates for treatments targeting this receptor.

Attempts to generate assays examining molecular signatures that can be used in clinical settings are being developed. The Food and Drug Administration in the United States has approved a 70-gene panel microarray called the MammaPrint that establishes whether a patient has a high or low risk for breast cancer reoccurrence. The MammaPrint is currently being validated in the MINDACT trial (Microarray in Node negative Disease may Avoid ChemoTherapy) (Kunz 2010). Another assay known as Oncotype Dx™ determines a 21-gene expression based recurrence score for ER positive, lymph node negative, tamoxifen treated breast cancer patients. This assay is currently being evaluated in the TAILORx trial (Trial Assigning Individualized Options for Treatment (Rx))(Kelly et al. 2010).

1.3.3 Breast cancer treatments

Improvement in breast cancer diagnosis methods and advances in our ability to characterize breast cancer at a molecular level have been paralleled with the development of more effective treatments options. Today, there are five standard treatments used that include surgery, radiation therapy, chemotherapy, endocrine therapy, and other targeted therapies. Depending on the cancer case, patients are advised of different combinations and/or sequences of treatments. Herein, a brief description of each of the five standard treatments is given.

1.3.3.A Surgery

Surgery and removal of the tumor was one of the first forms of therapy offered to breast cancer patients. The purpose of this treatment is to physically remove cancer cells from the patient and therefore limit the complications that would arise if tumor cells were left in the patient. In the past, mastectomies excising the entire breast tissue containing the

tumor were routinely performed. However, while this type of surgery is effective, it is nonetheless aggressive and does not accommodate cosmetic aspects important for the overall well being of the patient. Today, surgeries have therefore evolved and in the majority of cases breast conserving surgeries are performed and result in a more favorable cosmetic outcome. These include lumpectomy where only the tumor is removed, segmental or partial mastectomy where the tumor along with some breast tissue is removed.

The use of radiation and chemotherapy is an essential component behind the success of breast conservation therapy. The possible neoadjuvant use of these therapies shrinks breast tumors and consequently makes breast conservation surgeries feasible and optimal.

1.3.3.B Radiation therapy

Radiation therapy uses high-energy beams to cause DNA damage that ultimately results in cancer cells death. National Surgical Adjuvant Breast and Bowel Project (NSABP) study B-17 compared the benefits of lumpectomy alone to lumpectomy followed by breast irradiation in women with ductal carcinoma in situ. The study found that after eight years, patients treated with radiation following lumpectomy have significantly lower breast cancer recurrence (Fisher et al. 1999b).

Radiation therapy is a valuable approach that is employed prior to and or after surgery. Indeed as described earlier, the neoadjuvant use of radiation shrinks tumors and allows minimal surgical incisions. The use of radiation following surgery destroys cancers cells that could have been left behind.

1.3.3.C Chemotherapy

Today there is a multiplicity of chemotherapeutic drugs available to treat cancer. A combination therapy rather than with a single agent is thought to be more effective at reducing disease recurrence and death. The principle behind chemotherapeutic drugs is that they kill highly proliferating cells. These chemicals act in different ways but commonly interfere with the cells ability to divide or replicate DNA. For example, doxorubicin intercalates DNA thereby inhibits DNA synthesis and consequently replication. Paclitaxel is a mitotic inhibitor that interferes with normal microtubule breakdown and rearrangement. The chemotherapeutic drug 5 fluorouracil is a pyrimidine analogue that induces cell cycle arrest.

Chemotherapeutic drugs have significant advantage over surgery alone as they are able to permeate the entire body and thus are able to reach infiltrating and metastatic cancer cells.

1.3.3.D Endocrine therapy

As mentioned earlier, estrogen is a potent mitogen considered to promote breast cancer cell growth through the activation of the estrogen receptor. Endocrine therapies therefore aim at disrupting this signaling pathway and consequently restrain its mitogenic action on cancer cells. For this reason, only patients whose tumors express ESR1 are believed to benefit from these types of treatments. Today, two strategies are used to control estrogen action. These consist in either inhibiting estrogen action or reducing the overall amount of circulating estrogen in the body.

Compounds known as selective estrogen receptor modulators (SERMS) are used to compete against estrogen for the binding to their estrogen receptor and thereby inhibit its

action (Jordan 2007; Lewis-Wambi and Jordan 2005; Plouffe, Jr. 2000). Tamoxifen is one of such SERMS that has been widely used. The NSABP project B-24 reports the benefit of administering tamoxifen to women treated with lumpectomy followed by radiation therapy. This study demonstrates that the use of tamoxifen for five years has resulted in a significant fall in breast cancer recurrence and breast cancer related mortality (Fisher et al. 1999a). Tamoxifen has also been used to reduce the risk of breast cancer in undiagnosed women with a high risk of developing breast cancer.

In breast cells, tamoxifen not only competes with estrogen in binding to estrogen receptors but also alters the estrogen receptor structural conformation once bound thereby inhibits receptor activation (Pike et al. 2000; Brzozowski et al. 1997). Tamoxifen is therefore considered to act as an antagonist. Interestingly, tamoxifen can also act as a partial agonist mimicking estrogen in a cell specific context (Berry et al. 1990). The physiological consequence of this partially agonistic activity is desirable in some tissues. For example, tamoxifen has been shown to prevent bone loss. However undesirable consequences in other tissues have also been demonstrated as long-term tamoxifen use has been shown to increase the risk of endometrial cancer (Jordan et al. 2001).

Raloxifene is another SERM. As opposed to tamoxifen, raloxifene has been demonstrated to possess antagonistic activity in both the breast and uterine tissues. In addition, Raloxifene offers agonistic action on bone metabolism (Maximov et al. 2009).

Other ESR1 modulators such as fulvestrant (ICI 182,780) are considered pure inhibitors as they do not have any agonistic activity and act as antagonists in all tissue types (Howell 2000). Fulvestrant administered to postmenopausal patients with advanced

breast cancer that has progressed following anti-estrogen (tamoxifen) treatment is an effective treatment option (Robertson et al. 2003; Howell et al. 2002).

A second strategy to alter estrogen action in breast cancer is to decrease the amount of estrogen in the body. In post-menopausal women, estrogen is mainly generated by the aromatase enzyme present in adipose tissues. Aromatase inhibitors (AI) are therefore used to inhibit this enzyme and thus reduce the production of local estrogen. Several studies have validated the use of these compounds in different clinical settings. The ATAC trial (arimidex (anastrozole), tamoxifen, alone or in combination) compared the use of anastrozole (an aromatase inhibitor) alone or in combination with tamoxifen in women with localized breast cancer for five years (Duffy et al. 2010; Cella et al. 2006). The project reports that the use of anastrozole significantly prolongs disease free survival and significantly reduces distant metastases and contralateral breast cancers (Baum et al. 2003). Letrozole (another aromatase inhibitor) therapy after the initial five years of tamoxifen treatment significantly improved disease free survival (Goss et al. 2005). Therapy with Exemestane (another aromatase inhibitor) following an initial two to three year treatment with tamoxifen significantly improved disease free survival as compared to tamoxifen treatment for five years (Buzdar et al. 2008). Overall, these clinical data show that aromatase inhibitors are an important treatment strategy for post-menopausal women.

The pathways responsible for estrogen biosynthesis differ in pre and post menopausal women. In pre-menopausal women the primary source of estrogen is generated from the ovaries. Different endocrine therapy strategies are therefore used, as aromatase inhibitors cannot inhibit estrogen production from the ovaries. Drug mediated ovarian shutdown or

surgical removal of the ovaries are two possible strategies to reduce the level of estrogen in premenopausal women. Luteinizing hormone releasing hormone (LHRH) agonists such as goserelin, buserelin and triptorelin initially cause an increase in luteinizing hormone levels consequently leading to an increase in estrogen production. However, a continuous stimulation with these agonists leads to the down regulation of the receptors and thus ultimately a reduction in estrogen production. The clinical use of LHRH agonist is nonetheless limited, as these compounds do not prevent estrogen synthesis in tissues other than the ovaries and additional tactics such as tamoxifen and/or aromatase inhibitor have to be used concurrently (Tan and Wolff 2007).

1.3.3.E Other targeted therapies

The identification of molecular players involved in tumorigenesis and breast cancer progression has led to the development of targeted therapies. Today, in addition to disrupting the estrogen signaling pathway, there are two other types of targeted therapies used in the clinic to treat breast cancer patients. Interestingly, both strategies aim at disrupting the human epidermal growth factor receptor 2 (HER-2) signaling pathway. HER-2 is a known proto-oncogene that encodes for a cell surface bound receptor tyrosine kinase. When activated, this receptor triggers down stream signaling that ultimately lead to cell survival and proliferation (Lurje and Lenz 2009). Interestingly, breast tumor analyses have shown that HER-2 gene is amplified in 20 to 25 % of breast cancers. It is therefore thought that this signaling pathway plays a significant role in breast tumorigenesis and progression. Consequently strategies disrupting these pathways could therefore be important avenues in treating breast cancer patients (Garnock-Jones et al. 2010). Herceptin (Trastuzumab) is a monoclonal antibody that binds to the HER2 growth

factor receptor and thereby blocks its function (Garnock-Jones et al. 2010). Lapatinib is a tyrosine kinase inhibitor that also blocks the effects of the epidermal growth factor receptor (EGFR) and the HER-2 signaling (Tevaarwerk and Kolesar 2009).

2. Estrogen-signaling pathway

2.1 Estrogen

As described earlier, estrogen is a potent mitogen that increases the proliferation of breast epithelial cells and plays an important role in promoting breast cancer. There are three naturally occurring estrogens: estradiol, estrone and estriol. Estradiol is the most prevalent estrogen in the adult female. The production of estrogen begins upon the onset of puberty as the gonadotropin releasing hormone is released from the hypothalamus and activates the secretion by the anterior pituitary of the follicular stimulating hormone as well as the lutenizing hormone. These two hormones target the ovaries where they participate in both oogenesis and steroidogenesis. The lutenizing hormone stimulates the follicular thecal cells to produce androgens and progesterone. The androgens diffuse to the granulosa cell layer where they are aromatized to estrogen. The aromatase responsible for this conversion is itself stimulated by the follicular stimulating hormone. A moderate constant level of circulating estrogen and progesterone in turn exerts a negative feedback on lutenizing and follicular stimulating hormones secretion while elevated estrogen levels exert a positive feedback on lutenizing hormone production (Wierman 2007).

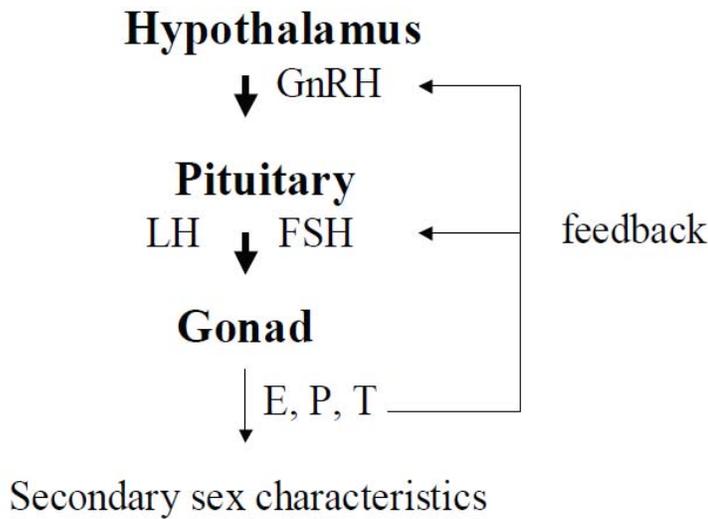


Figure 2: Schematic diagram illustrating the hypothalamic-pituitary-gonadal axis. GnRH: gonadotropin releasing hormone, FSH: follicular stimulating hormone, LH: lutenizing hormone, E: estrogen, P: progesterone T: Testosterone.

Estrogen exerts a number of physiological effects in different tissues. As described earlier, one of its main effects is the development of female secondary sexual characteristics including the mammary gland development. Estrogens have also been shown to play a role in bone, vascular system, central nervous system, gastro-intestinal tract, the skin, kidney and lung (Wierman 2007).

2.2 Estrogen receptors

It is known today that estrogen action is mediated mainly through two estrogen receptors, estrogen receptor alpha ($ER\alpha$ or ESR1) and estrogen receptor beta ($ER\beta$ or ESR2). These two receptors are encoded by distinct genes located on chromosome 6q25.1 (ESR1) and on chromosome 14q23.2 (ESR2). Both estrogen receptors bind estradiol but have separate physiological functions. This is illustrated by the distinct phenotypes reported in ESR1 knock out (ERKO) mice and ESR2 knock out (BERKO) mice models. For example, ERKO mice are infertile while BERKO are fertile but with fewer and smaller

litters. In addition, ERKO mice mammary gland pubertal development is non-existent. However, the BERKO mice have normal mammary gland development and are able to lactate (Korach et al. 2003; Krege et al. 1998).

The role of ESR2 in breast cancer remains controversial. One possibility for discrepancies in the results from different studies can be rooted in the existence of several ESR2 RNA variants with distinct C-terminal extremities. Specific ESR2 isoforms generate ESR2 proteins with different biological functions. Consequently distinct predictive and prognostic values are uncovered depending on the exact nature of the ESR2 protein analyzed (Fox et al. 2008). Generally however, ESR2 protein levels have been shown to decrease as breast cancer progresses (Fox et al. 2008). The majority of studies suggest that in tumors expressing ESR1, the presence of ESR2 (protein) correlates with a better response to adjuvant tamoxifen therapy indicating that ESR2 could be a good prognostic marker in these tumors. ESR2 has been shown to antagonize ESR1 dependent transcription and this antagonistic action could be involved with the mechanisms underlying a better response to hormonal therapies (Zhao et al. 2007a; Matthews and Gustafsson 2003). Nonetheless, in tumors not expressing ESR1, the presence of ESR2 (protein) correlates with poor clinical outcome. It is possible that the presence of other factors such as HER-2 independently of ESR2 expression could be responsible for poor prognosis in this subset of tumors (Maximov et al. 2009).

The role of ESR1 in breast cancer is indisputable. It is however interesting to note that ESR1 action differs between normal and cancer cells. Only approximately 10 % of normal breast epithelial cells express ESR1 (Ricketts et al. 1991; Palmieri et al. 2002). The normal epithelial ESR1 expressing cells do not proliferate but stimulate the

proliferation of surrounding cells (Ali and Coombes 2000). However, ESR1 expressing breast cancer cells are able to proliferate upon stimulation by estrogen.

Estrogen has long been shown to increase the expression of genes involved in cell proliferation. Since approximately 70 % of breast cancers express ESR1, estrogens exert a powerful mitogenic effect on breast cancer cells (Maximov et al. 2009).

Investigations were undertaken to decipher the mechanisms of action of these receptors. Today our increased knowledge on these receptors has evolved in the detailed understanding of their distinct domains with discrete functional implications.

2.2.1 Estrogen receptor alpha and beta structures

The structural organization of estrogen receptors consists of six functional domains that have various degrees of conservation between themselves and with other nuclear receptors (Figure 3). Understanding the functional aspects of each of these domains has promoted a better understanding of the estrogen receptors function and their mechanisms of action.

The A/B domain located at the N-terminal is poorly conserved among nuclear receptors. Furthermore, this region has only 17 % homology between the two ESRs. In ESR1 this region contains the autonomous ligand independent activation (AF1) domain that allows the receptor to activate transcription in a ligand independent manner. On the other hand, it appears that in ESR2, this region does not contain such an activity (Ellmann et al. 2009).

The C domain or DNA binding domain (DBD) of both ESR1 and ESR2 contains two zinc finger motifs that are involved in DNA binding as well as homo and hetero dimerization. There is a high degree of homology (90%) between ESR1 and ESR2

DBDs, highlighting the fact that both receptors can bind to similar DNA response element. The D domain is known as the hinge region and contains a nuclear localization signal (Ellmann et al. 2009).

The E region contains the ligand binding domain (LBD) and the ligand dependent activation domain (AF2). In addition, a portion of the receptor dimerization interface is also located within this domain. The AF2 domain consists of 12 helices that form a ligand binding cavity. Distinct structural conformations are observed depending on the nature of the ligand bound and are thought to dictate interaction with co-regulatory molecules. The interaction between estrogen receptors and co-regulatory proteins is often mediated via a leucine-X-X-leucine-leucine (LXXLL) motif present on these co-regulatory molecules.

F domain constitutes the carboxy- terminal end that has low homology between the two estrogen receptors (Ellmann et al. 2009)

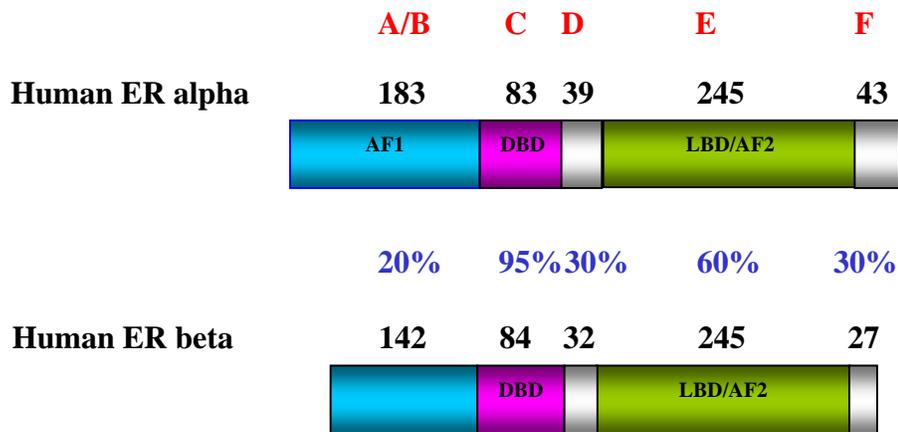


Figure 3: Schematic representation of human estrogen receptor alpha and beta. The six domains (A to F) are indicated. These domains include activation function 1 (AF1), DNA binding domain (DBD), ligand binding domain (LBD) and activation function 2 (AF2). The percentage homology between each domain of estrogen receptor alpha and estrogen receptor beta is indicated in blue (adapted from (Ellmann et al. 2009))

2.2.2 Genomic action

In the classical model of activation, estrogen binds to its receptor in the nucleus, which leads to a conformational change in the receptor structure and its release from heat shock protein (HSP) complexes. The receptors then dimerize and bind to specific DNA sequences (5'-GGTCAxxxTGACC-3') called estrogen responsive elements (ERE) located on the promoter or enhancer of classical target genes. ESRs subsequently recruit co-activators and members of the transcriptional machinery. This recruitment ultimately results in the transcription of specific target genes (Heldring et al. 2007).

ESRs also bind to response elements that differ slightly or extensively from the consensus ERE sequence described above. In fact the majority of ERE identified on ER target genes are non-perfect sites. It has been shown that the higher the differences from the consensus sequence the lower the estrogen receptor binding affinity. As such, ERE sequences influence estrogen receptor binding affinity, conformation, interaction with co-regulatory proteins and ultimately transcriptional activity (Klinge et al. 2004).

In addition to classical target genes that possess estrogen responsive elements in their promoter regions, ESR1 can also regulate genes that either contain distal ERE elements or do not possess any binding sites at all. As such, ESRs tether with transcription factors such as AP1 and SP1 and thereby indirectly associate with target gene promoters containing DNA binding elements specific to these transcription factors (Maximov et al. 2009).

In addition to their co-activating function, ESRs are also involved in the ligand dependent repression of genes. In fact, in MCF-7 cells more than 50% of estrogen regulated genes are repressed (Frasor et al. 2003). Emerging studies are now elucidating the mechanisms

behind such regulation. Active recruitment of repressive complexes containing co-repressors NCOR and SMRT is suspected to mediate such transcriptional repression (Higgins et al. 2008). In addition, positive regulation by E2 of molecules such as NRIP1 (nuclear receptor interacting protein1) that would subsequently repress the expression of other genes has also been proposed (Augereau et al. 2006a; Augereau et al. 2006b). Furthermore, novel mechanisms of actions are being uncovered. A recent study has shown that histone deacetylase 7 (HDAC7) and FoxA1 (forkhead box protein A1) are involved in estrogen regulated gene repression of the *Reprimo* gene. Estradiol treatment results in the release of RNA polymerase II from Reprimo's proximal promoter. HDAC7 and FoxA1 are both required for this action and these two molecules are recruited at the promoter through their interaction with ESR1 (Malik et al. 2010).

2.2.3 Non-genomic action

In addition to regulating gene expression in the nucleus, ESR1 located on the plasma membrane can initiate rapid intracellular signaling pathways that involve the activation of mitogen activated protein kinases MAPK, PI3K/AKT, release of calcium and secretion of prolactin. This non-genomic action has been termed membrane initiated steroid signaling (MISS) (Bjornstrom and Sjoberg 2005).

The nature of the estrogen receptors associated to the plasma membrane has been contentious. It had been proposed that a truncated 46 kDa as opposed to the wild type ESR1 was responsible for the non-genomic estrogen receptor action (Li et al. 2003). However, studies have shown that depending on cellular context, full length ESRs, or truncated 46 kDa ESR1 receptors can both be detected at the plasma membrane (Razandi et al. 1999).

Unlike some growth factors receptors, ESRs do not possess any transmembrane domains. Their localization near the plasma membrane should therefore be mediated via interaction with other proteins and ultimately through the formation of protein complexes. ESRs post-translation modifications could also play a role in plasma membrane localization (Song et al. 2006). For example, ESR palmitoylation facilitates ESR1 translocation to the membrane (Acconcia et al. 2005).

Protein complexes associated with ESR1 play an important role in conveying the non-genomic action of estrogen. These complexes include signaling molecules such as growth factor receptors, G proteins and kinases (Bjornstrom and Sjoberg 2005). In particular, estradiol bound ESR1 can activate two well known signaling cascades: the MAPK and PI3K/ AKT pathways. These signal transduction pathways may connect non-genomic action to genomic action by introducing post-translational modifications to transcriptional regulators and transcription factors (including ESR1 itself). Such changes would lead to a modification in the molecules functional activity and ultimately lead to changes in gene expression.

The non-genomic action of estrogen could also involve the membrane estrogen receptor GPR30 or GPER (G protein coupled estrogen receptor). GPR30 does not have much similarity to the estrogen receptors described earlier. In fact this receptor has seven transmembrane domains and is therefore an integral membrane protein. Nonetheless this receptor is able to bind estrogen and upon ligand binding, GPER can initiate a signaling cascade that activates the PI3K and MAPK pathways (Maggiolini and Picard 2010).

2.2.4 ESR1 post-translational modifications.

As described earlier, ligand binding, DNA binding and interaction with co-regulatory proteins ultimately determine the transcriptional activity of estrogen receptors. As outlined earlier, post-translational modifications play a crucial role in influencing the receptors function. Today modifications known to occur on estrogen receptor alpha include phosphorylation, acetylation, methylation, sumoylation, ubiquitination, O-linked N-acetylglucosamination, thiol oxidation, S-nitrosylation, and S-palmitoylation. These post translational modifications have been shown to affect the stability, cellular localization, dimerization as well as activity of both ESR1 and ESR2 (Lannigan 2003). Interestingly, ESR1 post-translational modifications have been linked to treatment outcome. For example, ESR1 phosphorylation status of breast tumors is currently under scrutiny. It has been proposed that phosphorylated ESR1 might generally indicate an intact estrogen receptor signaling pathway and could explain the better response to tamoxifen observed (Murphy et al. 2009). Discrete phosphorylated estrogen receptor alpha sites might nonetheless have distinct predictive value (Skiris et al. 2010).

2.3 Coregulators

Steroid receptors initially play a fundamental role in regulating the transcription of target genes by binding to their promoters. However, co-regulatory molecules recruited by these receptors ultimately orchestrate the critical enzymatic steps necessary for gene expression regulation. Estrogen receptor co-regulators are therefore essential partners implementing transcriptional regulation.

The eukaryotic DNA is packaged into a highly ordered structure termed chromatin. The nucleosome is the fundamental unit of the chromatin and consists of 147 bp of DNA

wrapped around an octamer composed of four core histone proteins (H3, H4, H2A and H2B) (Saha et al. 2006). This chromatin structure is dynamic, being modified during processes such as DNA replication, repair and transcription. It is thought that the highly ordered chromatin structure is repressive to transcription while an open chromatin structure is more permissive to the initiation of transcription (Kouzarides 2007). Through their enzymatic activities, nuclear receptor co-regulators are the effectors responsible for changes in chromatin structure regulating transcription control. In addition to the change in chromatin structure, transcription initiation also involves the recruitment of general transcription factors that can also be facilitated by co-regulators (O'Malley et al. 2008). It is important to note that transcription regulation is a highly dynamic and maybe cyclical process. Co-regulatory protein complexes bring a coordinated collection of functional activities in a timely fashion to ultimately achieve transcription activation or repression (Lonard and O'Malley 2007).

2.3.1 The functional diversity among co-regulators

The steroid receptor co-regulators form a vast functional family of genetically diverse molecules that comprises over 300 members. Several excellent reviews are available today discussing the various functional aspects of these molecules and exposing their huge repertoire of distinct mechanisms of action (McKenna et al. 1999; Robyr et al. 2000; Gao et al. 2002; Lonard et al. 2007; Lonard and O'Malley 2007; O'Malley et al. 2008; Thakur and Paramanik 2009). Interestingly, in order to present an organized account of these molecules, most reports classify members of this family into two broad functional categories, which respectively stimulate or inhibit receptor activity and thus are termed co-activators and co-repressors.

2.3.1.A Co-activators

Co-activators are defined as factors recruited by nuclear receptors that promote transcription activation. The large family of co-activators comprises members that are functionally diverse and usually act as multi-protein complexes that facilitate the expression of target genes. Members can be sub-grouped into several functional categories: chromatin remodelers, histone modifiers and general transcription factors adaptors.

Chromatin remodelers use the energy of ATP hydrolysis to perturb the structure of nucleosomes and thereby regulate the exposure of cis-DNA elements to transcription regulators. The current model for their mechanism of action suggests that chromatin remodeling complexes bind to nucleosomes and subsequently pump DNA loops thus ultimately disrupting the nucleosome structures. Chromatin remodelers participate in a variety of processes including DNA repair, replication, transcription regulation and elongation (Cairns 2009).

With respect to the estrogen-signaling pathway, several co-activators have been identified to function as chromatin remodelers. The human orthologues of the SWI/SNF family, namely BRG1 (Brahma related gene-1) and hBRM (human brahma) have been demonstrated to both interact with ESR1 (Ichinose et al. 1997). In addition BRG1 associated factor 57 (BAF 57) has been shown to also associate to ESR1 and to the p160 nuclear receptor coactivator. BAF 57 therefore acts as a bridging molecule that links chromatin remodeler and histone modifier complexes (Belandia et al. 2002). Indeed, chromatin remodelers often work in concert with histone modifiers to alter chromatin structure and thereby regulate transcription.

Histone modifications play a crucial role in the stability of chromatin structure. For this reason, histone modifying enzymes are important regulators of transcription. Today the covalent modifications associated with histones in the context of transcription regulation include phosphorylation, acetylation, methylation, ubiquitination, sumoylation, ADP-ribosylation, deimination and proline isomerization (Kouzarides 2007).

Among these modifications, the role of acetylation in nuclear receptor transcription activation has been extensively investigated. Traditionally histone acetylation was regarded as being favorable to transcription as it was thought to induce an open chromatin structure. On the other hand, histone deacetylation was perceived as intolerant to transcription as it leads to a compressed chromatin structure. This view is likely too simplistic as histone deacetylation has been associated with gene expression and acetylation with gene repression. It is therefore thought that the promoter context, site of acetylation/ deacetylation, neighboring post transcriptional modifications all participate in the final decision of transcriptional regulation.

Several NR co-activators have been shown to possess histone acetyl transferase (HAT) activity. These have been often grouped into three main families of proteins namely the P160, P300/ CBP and PCAF proteins. These proteins interact with each other and other transcriptional enhancers thereby ultimately mediating transcriptional activation.

The P160 family comprises three members: SRC1 (also known as NCOA1), SRC2 (also known as TIF2, GRIP1 and NCOA2) and SRC3 (also known as P/CIB, RAC3, AIB1, ACTR, TRAM1 and NCOA3). SRC1 (steroid receptor co-activator 1) was the first co-activator isolated using the PR AF2 ligand binding domain as bait. It was later determined that SRCs contain three leucine-X-X-leucine-leucine (LXXLL) motives that

form amphipathic alpha helices and mediate interaction with ligand bound nuclear receptors AF2 domain. Interaction between SRC molecules and steroid receptors AF1 domain has also been subsequently demonstrated. As such p160 family of proteins could promote a functional synergy between nuclear receptors AF-1 and AF-2, and recruit other co-regulators. The C-terminal domain of SRCs contains two activation domains. Activation domain 1 (AD1) can bind to the CBP/P300 acetyl transferases. The activation domain 2 (AD2) can interact with lysine methyl transferases: CARM1 (co-activator associated arginine methyltransferase 1) and PRMT1 (protein arginine N-methyl transferase 1). In addition to the domains described above, SRC1 and SRC3 also possess C-terminal intrinsic HAT region (Xu et al. 2009b). Through their multiple functional domains, members of the P160 family possess multiple means to regulate transcription.

Another important family of lysine deacetylases consists of the P300/CBP proteins that interact with nuclear receptors via P160 proteins. P300/CBP proteins are termed co-integrators as they act as scaffold proteins with multiple domains that allow the formation of protein complexes. P300 also associate with another acetyl transferase P/CAF. In addition, P300 and P/CAF proteins contain bromo domain shown to be involved in the recognition of acetylated lysines and are thus important in recognizing histones (Thakur and Paramanik 2009).

In addition to the chromatin remodelers and histone modifiers, there are several other transcription co-regulators that facilitate the formation of transcription initiation complex. One such example is the thyroid receptor associated protein (TRAP)/ Vitamin D receptor interacting protein (DRIP)/ activator recruited cofactor (ARC) protein complexes that contain similar subunits. These protein complexes are able to enhance the nuclear

receptor mediated transcription from naked (chromatin free) DNA in cell free *in vitro* assay. This observation thus suggests that the TRAP/ DRIP/ ARC co-activator complexes thus mediate their function independently of chromatin structure remodeling. Direct interaction between these complexes and nuclear receptors are mediated via LXXLL motifs contained by specific subunits (TRAP220, DRIP205, ARC205). The TRAP/DRIP/ARC complexes facilitate transcriptional activation by recruiting general transcription factors. For example, the TRAP/DRIP/ARC complexes recruit and phosphorylate the RNA polymerase II, thereby initiating transcription (Lonard and O'Malley 2006; Thakur and Paramanik 2009).

Several other co-regulatory molecules exert their effect on transcription activation through different means. The E3 protein ubiquitin ligases, E6-AP and the closely related RPF-1 are involved in protein ubiquitination and consequential degradation. The degradation process is crucial in maintaining the cyclical and dynamic nature of transcriptional initiation. Indeed the removal of factors ensures the active recruitment of transcriptional factors for subsequent activations (Lonard and O'Malley 2007).

2.3.1.B Co-repressors

As opposed to co-activators, co-repressors are defined as factors involved in the repression of gene expression. By definition, they counterbalance the action of co-activators through the inhibition of DNA bound nuclear receptor transcriptional activity in the absence of ligand. Furthermore, they also participate in the active repression of antagonist bound steroid receptors transcriptional activity. The mechanisms of action of co-repressors often mirror the enzymatic properties attributed to co-activators. Indeed, co-repressors act through chromatin remodeling, histone modification (deacetylation), or

prevention of the recruitment of basal transcription factors. In addition to these mechanisms, co-repressors also repress transcription by competing with co-activators, interfering in steroid receptor dimerization, altering receptor stability, sequestering receptors to the cytoplasm and preventing receptors from binding target gene promoters (Dobrzycka et al. 2003).

The nuclear co-repressor 1 (NCOR1) and the silencing mediator for retinoic acid or thyroid receptor (SMRT) (also known as NCOR2) are the products of two distinct genes but share a high degree of homology and assemble into similar complexes. NCOR and SMRT were first isolated as factors binding to the thyroid receptor and the retinoic acid receptor when these receptor are associated to the DNA. In the absence of ligand, the recruitment of these co-factors is responsible for the transcriptional repression of thyroid receptor and retinoic acid receptor target genes. Subsequent studies have shown that upon tamoxifen treatment, these NCOR1 and SMRT can also repress transcription of ESR1 target genes. Tamoxifen bound ESR1 can recruit these factors. The interaction between NCOR/SMRT and nuclear receptors is mediated via L/IXXI/VI motif that resembles the LXXLL motif of co-activators.

Research into their mechanisms of action has suggested that NCOR1 and SMRT do not harbor intrinsic repressive enzymatic properties. Rather, these two co-repressors can associate with other co-repressive molecules and recruit transcriptional repressive complexes. For example, SMRT interacts with SIN3A that acts as a bridging molecule and recruits histone deacetylase 1 (HDAC-1). NCOR can also associate with the NURD chromatin remodeler complex that contains HDAC1 and HDAC2. The conscription of

HDAC activity results in deacetylated and therefore closed chromatin structure that is unfavorable to transcription.

Unlike NCOR1 and SMRT, other co-repressor such as ligand dependent co-repressor (LCOR) and nuclear receptor interacting protein 140 (RIP140) associate to NR through perfect LXXLL motives. LCOR and RIP 140 have been shown to repress the activity of ligand bound ESR1. Studies into their mechanism of action suggest that these repressors recruit HDACs as well as other repressive molecules such as C-terminal binding protein (CTBP) and polycomb proteins to mediate gene repression (Augereau et al. 2006a).

In addition to mechanisms involving chromatin remodeling and histone modification, co-repressors can achieve transcriptional repression by other means. Repressor of estrogen receptor activity (REA) and small heterodimer partner (SHP) compete with co-activator TIF-2 for ESR1 binding. In addition SHP prevents ESR1 dimerization and consequently transcription activation. A short form of metastasis associated protein 1 (MTAs) has been shown to bind to ESR1 and sequester it into the cytoplasm and thereby preventing the receptor to mediate its genomic action. In similar fashion, carreticulin interacts with ESR1 DBD and thus prevents the receptor from binding to the DNA and thereby initiating transcription (Lonard and O'Malley 2007; Lonard and O'Malley 2008).

2.3.2 Co-regulators as master regulators

Increased understanding of co-regulator functions suggests that classifying co-regulator as either co-activator or co-repressor might lead to an inaccurate perception of their versatile functions. The co-activator/co-repressor classification is based on the initial studies defining the action of a given co-regulator on particular nuclear receptors in a specific cell system. Many a time, subsequent studies have demonstrated that a co-

regulator can have different or even opposite actions depending on the cell line used or target gene considered. For example, the co-activator independent of AF2 function (CIA) possesses both co-activating and co-repressing functions (Sauve et al. 2001).

The nuclear co-repressor (NCOR) protein is classically associated with transcription repression. However peptides resulting from the translation of specific splice variants have been shown to activate thyroid receptor alpha in a promoter context dependent manner (Meng et al. 2006).

The boundary between co-activator and co-repressor is increasingly becoming fluid, as these co-regulators are appreciated as nuanced regulators with versatile functions rather than rigid predictable effectors (O'Malley and McKenna 2008).

Potential additional functions fulfilled by these regulatory molecules further complicate the perception of a positive or negative role in gene regulation. For example, in addition to its classical nuclear receptor co-activating function, SRC-3 binds to and represses cytokine mRNA translation (Yu et al. 2007). DEAD box (a motif named after its amino acid sequence Asp-Glut-Ala-Asp) RNA binding helicases protein p68 co-activates ESR1 transcriptional activity but is also implicated in RNA metabolism including RNA splicing, export and translation (Fuller-Pace 2006). Co-regulators therefore often have much broader roles than just regulating transcription. From this perspective these molecules should be appreciated as “master regulators” implicated in the various processes of gene expression (O'Malley et al. 2008).

2.3.3 Post-translational modifications

When considering co-regulators as master regulators, a question naturally arises: how do these molecules coordinate their diverse functions? One avenue that gives co-regulators their functional versatility is through regulation of their post-translational modifications. Post-translational modifications modulate co-regulators protein-protein interaction, their subcellular localization and degradation, altogether affecting their activities and functions. These observations lead to the notion of “co-regulator codes” similar to the histone code (O'Malley et al. 2008). It is thought that cells respond to cellular changes through the use of signaling cascades that introduce post translational modifications to co-regulators thereby fine tuning their activity in order to mediate coordinated changes in gene expression. Post-translational modifications might therefore not only be accountable for the switch in co-activating/co-repressive (and vice versa) functions but also responsible for the change in function, from transcriptional control to RNA splicing for example (O'Malley et al. 2008).

2.3.4 Co-regulators and diseases

Nuclear receptor co-regulators have versatile functions and it is therefore not surprising that they have been implicated in a wide array of human diseases. Based on literature reviews, a report indicates that 102 unique co-regulators are involved in at least one human pathology (Lonard et al. 2007). These include co-regulators that are mutated, over or under expressed (Lonard et al. 2007). Considering the functional intertwining between steroid receptors and co-regulators, it is expected that co-regulators would be heavily implicated in endocrine related cancers such as breast, prostate, uterine and ovarian cancers. In most cases, co-regulator misexpression might not always play a causal role in the genesis of endocrine related cancers. The change in expression could rather be a

reflection of the global change in gene expression occurring in cancer cells as a result of their adaptation (Lonard et al. 2007). Nonetheless, a change in co-regulators expression or activity could significantly influence the mechanisms underlying cancer progression. It might also affect the effectiveness of therapeutic strategies (Lonard et al. 2007; Thakur and Paramanik 2009). Increasing our understanding of co-regulator functions might therefore provide new targets for fighting cancer and in particular breast cancer. It is widely believed that improving our knowledge of co-regulator biology could lead to the development of novel treatment strategies. With this purpose in mind, countless studies are currently being devoted towards elucidating the mechanisms of action of known co-regulators. Furthermore, an intense search for new co-regulators has also been undertaken.

3 The Steroid Receptor RNA Activator

3.1 The Steroid Receptor RNA Activator: an atypical co-activator

In an attempt to identify co-regulators physically interacting with the progesterone receptor AF1 domain, Lanz *et al* used the yeast two hybrid system (Lanz et al. 1999). They isolated a new positive clone from a human B-lymphocyte library that they named steroid receptor RNA activator or SRA. The authors subsequently screened 3 different human cDNA libraries from skeletal muscle, heart and Hela cell line in order to obtain a full length SRA cDNA. Three SRA isoforms were identified that have sequences identical in their central region but with distinct 5' and 3' extremities. Subsequent analyses reveals interesting peculiarities about SRA, pointing towards the fact that SRA

was acting as an RNA molecule rather than a protein. Such discovery was unprecedented as all other known co-regulators are proteins.

In their initial study, the authors highlighted observations indicating that SRA was functioning as a RNA. First, analysis of the SRA sequence isolated from the GAL/SRA fusion clone used for the yeast two hybrid screen contained an in frame stop codon prematurely terminating the translation of gal/SRA fusion product at the 5'end. This suggested that a gal-SRA fusion protein could not have been generated. A peptidic product was therefore not likely responsible for the obtention of a positive clone in the yeast two hybrid screening of molecules interacting with PR AF1 domain. Although the yeast two hybrid screening system is originally based upon identifying protein–protein interaction, the authors had to disregard the possibility of an interaction between a translation product of *SRA* and PR.

Subsequently, attempts to generate SRA protein products *in vitro* were only successful if the SRA sequence was fused at the N-terminal with GST or GAL4. This reflects the need of an initiating methionine codon to initiate translation of the cloned RNAs. Such an initiation codon was obviously absent in the three identified SRA sequences. Finally, mouse monoclonal antibodies generated against a peptide encoded by the C-terminal extremity of SRA open reading frame failed to detect any endogenous protein in COS cell extracts and other lysates. This observation suggested the absence of an endogenous SRA protein.

The authors thus concluded that the SRA cDNA did not encode any viable translation product and the positive selection of the SRA clone was the result of transcriptional activation due to interaction between PR and the SRA transcript. The authors themselves

thus reported their initial finding as “fortuitous” as the yeast two-hybrid system is based on identifying protein-protein interaction. They speculated that the interaction between SRA RNA transcript and the PR AF-1 bait might have been supported by intermediate yeast proteins with transcription mediator like function (Lanz et al. 1999).

Because SRA was the first co-activating RNA, the authors presented several additional strong evidence demonstrating that SRA co-activates steroid receptors not as a protein but as an RNA transcript. They first showed that SRA was able to co-activate PR in an open reading frames independent manner. They demonstrated that the three potential SRA open reading frame fused to the translation initiation region of the herpes simplex virus -thymidine kinase sequence were equally able to co-activate the PR transcriptional activity in a reporter vector assay.

The authors also reported that the introduction of point mutations changing any putative open reading frame and adding translation stop codons did not affect SRA ability to co-activate PR mediated transactivation. They further demonstrated that only SRA and not known peptidic co-activators such as SRC-1 or CBP, was capable of potentiating GR-mediated transcription in the presence of cyclohexamide, a *de novo* protein inhibitor.

All these data strongly point that the identified SRA is acting as a RNA transcript rather than a protein product (Lanz et al. 1999).

3.2 The function of SRA

To determine the functional role of SRA, Lanz *et al* assayed the effect of SRA on nuclear receptors mediated transactivation. Using a chloramphenicol acetyl transferase reporter assay, the authors showed that SRA was selectively enhancing the activity of steroid receptors (AR, PR, ER, GR) while the activity of other nuclear receptors remained

unaffected in their experimental conditions (Lanz et al. 1999). The authors thus defined SRA as a new **specific** co-activator for steroid receptors. Subsequent studies performed by other groups have however demonstrated that SRA co-activates the ligand bound action of thyroid receptor alpha and beta (Hatchell et al. 2006; Xu and Koenig 2005), peroxisome proliferator receptor gamma (PPAR gamma) (Hatchell et al. 2006), retinoic acid receptor (RAR)(Zhao et al. 2004; Zhao et al. 2007b), vitamin D receptor (VDR) (Hatchell et al. 2006), myogenic differentiation 1 (MYOD) transcription factor (Caretti et al. 2006) as well as the steroidogenic factor 1 (SF1). These results thus suggest that SRA has a role much broader than originally expected (Leygue 2007).

In their initial study, Lanz *et al* stated that steroid receptors AF1 domain is necessary for SRA coactivation as removal of PR AF1 resulted in SRA inability to co-activate PR. While AR and PR AF1 domain directly or indirectly participates in SRA/ nuclear receptors interaction, SRA has been also shown to interact directly with the DNA binding domain of the Thyroid receptor (Lanz et al. 1999; Xu and Koenig 2004). These results thus suggest that SRA is capable of interacting with different nuclear receptor domains. In addition, SRA was shown to enhance ESR1 and ESR2 AF2 activities (Coleman et al. 2004; Deblois and Giguere 2003). Altogether, these studies have demonstrated that SRA co-activating function, one likely mediated via a variety of mechanisms and the presence of the steroid receptor AF1 domain seems to be essential for only some nuclear receptors.

3.3 Importance of the secondary structures of SRA

Lanz *et al* determined through successive deletions at the 5' and 3' end that a 672 bases SRA core region (starting at exon 2 and ending at exon 5) common to all transcripts

identified is necessary and sufficient for its function. Removal of small sections within this core is sufficient to diminish SRA co-activating property. However the removed sequences independently don't harbor co-activating properties. These data thus suggested the presence of various domains within the RNA sequence that in concert are able to modulate transcriptional co-activation (Lanz et al. 2002; Lanz et al. 2002).

Through the use of low-resolution structure modeling software MFOLD (Zuker 2003), Lanz *et al* predicted the presence of twelve substructures in SRA RNA putative secondary structure (Zuker 2003; Lanz et al. 2002) (Figure 4). The introduction of silent mutations in SRA sequence determined that six substructures are critical for SRA to co-activate the progesterone receptor. Indeed, the independent modification in each of these six substructures resulted in a reduction in SRA co-activation ability. Interestingly the simultaneous alteration of substructures 1 and 7 nullifies the stimulating effect of SRA on PR activity (Lanz et al. 2002).

Zhao *et al* have shown that SRA is pseudouridylated by two distinct enzymes Pus1p and Pus3p (Zhao et al. 2007b; Zhao et al. 2004). Pseudouridylation is a post-transcriptional modification that isomerises uridine to pseudouridine. This post-transcriptional modification has been shown to occur in non-coding RNAs such as tRNA, rRNA, snRNA where the RNA structure is intimately linked to its function. Pseudouridylation events are indeed expected to play a role in altering the structure and rigidity of RNA molecules, thereby playing a role in RNA/RNA as well as RNA/protein interactions. Interestingly, Pus1p and Pus3p pseudouridylate SRA at common as well as distinct uridine sites. Thus far only one *in vivo* pseudouridylation target site common to both Pus1p and Pus3p has been identified (Figure 4). Interestingly the mutation from U to A at

this specific site results in a hyperpseudouridylated state that renders SRA into a co-repressor for AR and ER as measured by luciferase reporter assays (Zhao et al. 2007b). Overall, SRA is today the only known co-activator to function as an RNA molecule. Similarly to other functional RNAs such as tRNA, rRNA, SRA relies on its secondary structure to function.

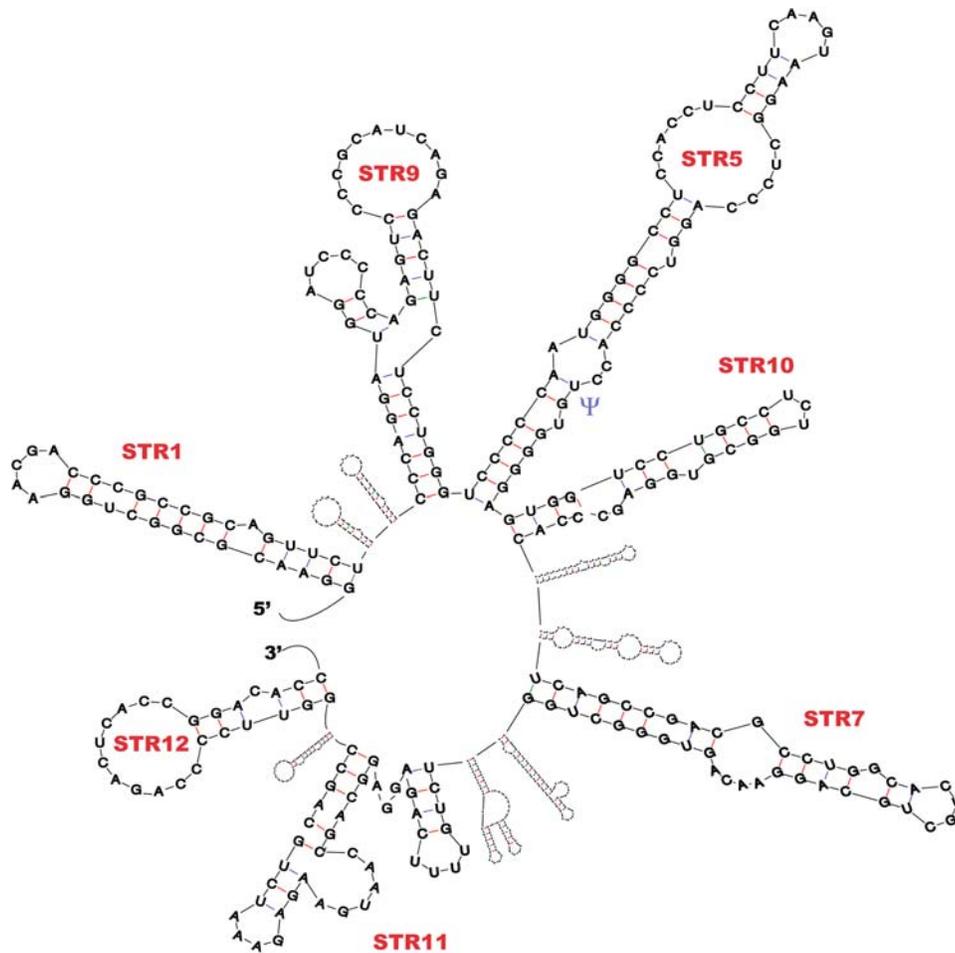


Figure 4. Schematic diagram illustrating SRA secondary structures. Secondary structures were predicted by the MFOLD software (Leygue 2007). The structures important for SRA transactivation function as determined by Lanz et al are indicated as structures 1 to 12 (STR1 to 12)(Lanz et al. 2002). The common uridine site demonstrated to be pseudouridylated by Pus 1 and Pus 3 is indicated by the ψ symbol.

3.4 SRA interacting proteins

Upon its discovery, SRA was defined as a steroid receptor activator involved in enhancing the transcriptional activity of steroid receptors. As described earlier, subsequent studies have demonstrated that SRA has a much broader impact as it also modulates the transcriptional activity of other nuclear receptors as well as the transcription factor MYOD. Several studies have emerged identifying proteins interacting with SRA. These proteins include a number of known co-regulators indicating that SRA participates in transcriptional regulation as a component of ribonucleo-protein complexes. The identification of specific co-regulators interacting with SRA provides insight into its mechanisms of action. Interestingly SRA interacts with both known co-activator and co-repressors. Interestingly, depending on the nuclear receptor or transcription factor, SRA seems to associate with distinct co-regulatory molecules and thus likely possesses a repertoire of distinct modes of action.

With respect to nuclear receptor activity, SRA's mechanism of action seems to be mediated by its interaction with histone modifying P160 family member SRC1. SRA has been suggested to perhaps serve as a "gasket" facilitating the interaction between co-regulatory molecules (Leygue 2007). In agreement with this hypothesis, it is important to note that SRA in fact interacts with several co-regulatory molecules. For instance, the DEAD box containing RNA helicases P68 and P72 abilities to co-activate ESR1 are dependent upon their interaction with SRA (Carette et al. 2006). Similarly, the presence of SRA is also essential for DAX1 (dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on X chromosome gene 1) to co-activate SF1 (the steroidogenic factor 1). Indeed, even in the presence of DAX-1, SRA knock down results in the

reduced expression of two SF-1 target genes: StAR (steroidogenic acute regulatory protein) and melanocortin 2 receptor (Xu et al. 2009a). These observations possibly underline SRA as an essential component of a bridge acting as an adaptator molecule facilitating the formation of multiprotein co-regulatory complexes (Caretti et al. 2006; Caretti et al. 2007; Watanabe et al. 2001).

In addition to its association with transcriptional co-activators, SRA also interacts with co-repressors and therefore perhaps partakes in transcriptional repression. SRA interacts directly with SMRT/HDAC1 associated repressor protein (SHARP) that is a potent repressor of retinoic acid receptors (RAR) transcriptional activity in the absence of ligand. One of the mechanisms through which SHARP represses transcription is by recruiting co-repressors such as SMRT and HDACs (Shi et al. 2001). In addition to and independently from this system, SHARP has been shown to repress ER and GR transcriptional activities by sequestering SRA and its associated co-activating factors away from these steroid receptors (Shi et al. 2001).

SRA is also able to directly interact with another co-repressor known as the SRA stem-loop interacting RNA binding protein (SLIRP). Interestingly, similarly to SRA/SHARP interaction, SRA/SLIRP interaction is also mediated via its substructure 7. However these two co-repressors do not act competitively but rather function in an additive manner to repress ESR1 activity. Interestingly, SLIRP potentiates the antagonistic action of tamoxifen as well as ICI 182780. In addition to ESR1, SLIRP attenuates SRA-mediated transactivation of a wide range of nuclear receptors including GR, AR, TR, VDR and PPARgamma. SLIRP likely represses transcription by competing out the interaction between SRA and SRC1. In addition, siRNA mediated SLIRP knock down

reduces nuclear corepressor NCOR recruitment to TFF1/pS2 promoter in the absence of E2. These results suggest that part of SLIRP participates in recruiting NCOR to the target promoter. Interestingly, SLIRP is a predominantly mitochondrial protein indicating a possibility for its role in this organelle (Colley and Leedman 2009; Colley et al. 2008; Hatchell et al. 2006).

It is interesting to note that the role of SRA in repression is intimately linked with its ability to facilitate co-activation. Indeed essentially, co-repressors sequester SRA and perhaps its associated factors away from nuclear receptors and/or other co-activators thereby achieving repression.

3.5 SRA and the estrogen signaling pathway

SRA activates the transcriptional activity of ESR1 and ESR2 in the presence of estradiol (Coleman et al. 2004; Deblois and Giguere 2003). ESR1 and ESR2 AF2 domain are sufficient for the induction of the ligand dependent co-activation by SRA. This effect appears to be independent of the AF1 domain as ESR1 and ESR2 AF1 deleted mutants remain co-activated by SRA. However, these results are in direct contrast with the initial results by Lanz *et al.* demonstrating SRA co-activation of the PR AF1 domain. Furthermore, treatment with a MAPK inhibitor (PD98059) completely abolishes the ligand dependent effect of SRA on full length ESR1 suggesting an important role of MAPK in SRA mediated ESR1 transactivation (Deblois and Giguere 2003). As illustrated earlier, estradiol has been shown to activate the MAPK that in turn activates ESR1.

In addition to this AF2 activation, the transcriptional activation of ESR1 but not ESR2 could be enhanced by SRA through the AF-1 domain. The roles of estradiol treatment as

well as ESR1 phosphorylation in this AF1 coactivation are contentious. Deblois *et al* have shown that SRA co-activates an ESR1 mutant construct containing the AF1 and DBD (omitting the AF2 domain) only in the presence of estradiol. Similar results were obtained when using an ESR1 construct containing a mutation (L539A) that abolishes ESR1 AF2 function. In addition, the authors also showed that ESR1 S118 is necessary for SRA co-activating function. S118 has been demonstrated to be phosphorylated by the mitogen activated protein kinase (MAPK) and was shown to play an influential role in the ligand independent ESR1 activity. The authors thus propose that E2 treatment activates the MAPK pathway that in turn phosphorylates ESR1 S118 that is necessary for SRA to fully co-activate ESR1 AF1. In support of this hypothesis, the authors demonstrate that the H-Ras mediated MAPK activation leads to ESR1 activation that is further enhanced by SRA expression (Deblois and Giguere 2003).

In contrast with these results, Coleman *et al* have shown that SRA co-activates ESR1 in the presence or absence of estradiol treatment. The authors also demonstrate that SRA also increases the agonistic activity of tamoxifen. Furthermore, using a chimeric molecule consisting of the ESR1 AF1 domain fused to the GAL-4 DBD, the authors show that mutating serine 118 to alanine does not affect SRA ability to co-activate the chimeric GAL DBD/ESR1 AF1 molecule. Similar results are also reported when mutating serines 104 and 106 to alanines. Only simultaneous mutations in all three serines (104, 106 and 118) reduce SRA's ability to co-activate ESR1 AF1 by half. These results thus suggest that ESR1 phosphorylation plays a role but is not crucial in SRA mediated AF1 co-activation (Coleman et al. 2004).

The apparent discrepancy between results obtained by the two groups could be due to the differences in constructs used. Deblois *et al* used AF1/ DBD ESR1 construct while Coleman *et al* used ESR1 AF1 fused to GAL DBD to explore SRA function on ESR1 AF1 domain. The main difference in the two constructs used, is the presence in Deblois *et al.* study (or lack in Coleman *et al.* study) of the ESR1 DBD (Leygue 2007). The inconsistency in the results might also emerge from the influential role played by the ERE sequence in ESR1 structural conformation. The structural conformation of the AF1 domain might be different when bound to the DNA via the gal-4 DNA binding domain as opposed to the ESR1 DNA binding domain. The change in structure might consequently influence interactions with co-regulator complexes thus ultimately altering ESR1 activation.

As described earlier, the ERE sequence found in estrogen receptor target genes greatly differs from one another. The ERE sequence has been proposed to act as an allosteric factor influencing estrogen receptor's DNA binding affinity, conformation, protein-protein interaction and consequently activity. Klinge *et al* used various ERE sequences with different association kinetics to demonstrate that ERE sequences dictate co-regulators ability to modulate estrogen receptor function. They specifically demonstrated that in the absence of ligand SRA activates ESR1 and ESR2 activities solely when the receptors are bound to particular response elements (see table 2). In addition, SRA modulates ESR1 and ESR2 ligand dependent activities also in an ERE context specific manner (see table 3). When coexpressed with other co-regulators (SRC1, GRIP, ACTR) SRA is able to further increase the selective co-activation of ESR1 or ESR2 is also dependent on the nature of the response element (see table 2).

In summary, SRA co-activates both estrogen receptors. SRA likely regulates estrogen receptor transcriptional activity differently than the way it modulates AR and GR activities. In fact, at least two separate mechanisms affecting ESR1 AF1 and AF2 domains are probably in play. These mechanisms are cell line, promoter (ERE) specific and dependent on the concurrent presence of other co-regulators.

		C38	C13	PS2	PR	FOS
No ligand	ESR1	↑	↑	↑	↑	↑
	ESR2	↑	↑	0	↑	↑
Estradiol	ESR1	0	↑	0	↓	0
	ESR2	0	0	↑	0	↑

Table 2: Summary of SRA's co-regulatory action on ESR1 and ESR2 transcriptional activities on C38, C13, PS2, PR and FOS EREs. Green arrow pointing upward symbolizes the ability of SRA to co-activate. Red arrow pointing downward symbolizes the ability of SRA to co-repress. 0 signifies no change in transcription is detected upon SRA expression. Source: (Klinge et al. 2004)

		C38	C13	PS2	PR	FOS
SRC1	ESR1	0	0	0	0	0
	ESR2	0	↑	0	0	0
GRIP	ESR1	0	↑	0	0	↑
	ESR2	↑	↑	0	0	↑

Table 3: Effect of SRA action on the SRC1 or GRIP mediated co-activation of ESR1 or ESR2 on the C-38 C13, PS2, PR FOS EREs. Green arrow pointing upward symbolizes the ability of SRA to co-activate. Red arrow pointing downward symbolizes the ability of SRA to co-repress. 0 signifies no change in transcription is detected upon SRA expression.

3.6 SRA physiological roles

Originally Lanz *et al* had identified three SRA isoforms with distinct 5' and 3' extremities. In addition, by Northern blot analysis using poly (A) RNA from different human tissues, these authors determined the existence of SRA transcripts of several different sizes. The authors reported the presence of predominant 0.7- 0.85 Kb and less abundant 1.3-1.5 kB transcripts (Lanz *et al.* 1999; Leygue 2007; Lanz *et al.* 2003). Furthermore the authors also demonstrated that SRA transcripts are differentially expressed in human tissues. SRA is highly expressed in liver, skeletal muscle, adrenal gland and the pituitary gland. Intermediate expression is seen in the placenta, lung, kidney, and pancreas. Low levels of SRA are detected in the prostate, breast, brain, uterus and ovary (Lanz *et al.* 2003; Lanz *et al.* 1999). A predominant 0.8 Kb SRA transcript is observed in all cancer cell lines. Interestingly, in addition to the 0.8 Kb transcript, a 0.75 Kb transcript is specifically expressed at higher level in breast cancer cells (MCF-7 and T47D cells) (Lanz *et al.* 1999).

Together the wide expression of SRA transcripts and its established role in modulating transcription suggest that this RNA has likely several important physiological roles. In order to identify possible pathways involving SRA, a recent report analyzed the global change in gene expression resulting from siRNA mediated SRA knock down in cancer cell lines (Foulds *et al.* 2010). Interestingly, upon SRA knock down, the vast majority of genes are down regulated suggesting that SRA widely participates in transcriptional co-activation. Gene ontology analysis of the change in gene expression suggested that SRA participates in several different biological pathways including glucose uptake, fatty acid

synthesis, cell mobility, heart contraction, thyroid hormone metabolism, invasion and metastasis (Foulds et al. 2010).

3.6.1 SRA's role in human dilated cardiomyopathy

Two studies have reported SRA knock out animal models. Interestingly, the SRA knock out mouse model does not present any phenotype perhaps suggesting SRA's functional redundancy in this animal (Lanz et al. 2003). It is also possible that while the lack of SRA expression weakens physiological systems it might be involved in, noticeable phenotypic changes would only occur if these systems were further compromised.

Unlike the SRA knockout mouse model with no noticeable phenotypic change, a recent study has reported that the lack of SRA expression results in myocardial contractile dysfunction in a zebra fish model (Friedrichs et al. 2009). The biological role of SRA responsible for this phenotype is poorly understood and needs further investigation (Friedrichs et al. 2009).

Human dilated cardiomyopathy is a condition where the myocardium becomes enlarged and the systolic functions of the ventricles are impaired. Consequently the heart does not pump blood efficiently. Eventually, dilated cardiomyopathy could lead to heart failure. While in the majority of cases no cause for this disorder is apparent, in a subset of patients genetic factors have been identified to play a role in the pathogenesis of this disease. SRA therefore seems to be one of such factors. Further studies are needed to decipher SRA's important role in the normal physiological function of the heart.

3.6.2 SRA role in cancer

As previously outlined, co-regulators are suspected to participate in the development of endocrine related cancers. Using RT PCR targeting the core SRA sequence, SRA expression was increased in breast, uterus and ovarian tumors (Leygue et al. 1999; Murphy et al. 2000; Hussein-Fikret and Fuller 2005). Interestingly within these different cancers selective subgroups have different levels of SRA expression. For example, serous ovarian tumors express higher SRA levels than other ovarian tumor types (Hussein-Fikret and Fuller 2005). SRA levels also correlate with ER and PR levels in specific subgroups of breast tumors. Higher SRA levels are indeed observed in ER + PR- tumors compared to ER+ PR+ tumors. On the other hand in ER- tumors SRA levels positively correlate with PR levels in that ER- PR+ tumors express higher SRA levels than ER- PR- tumors. Interestingly ER-PR+ tumors are more likely to respond to endocrine therapy than ER-PR- tumors. It has therefore been proposed that SRA might participate in the mechanisms dictating the responsiveness to hormonal therapy (Leygue et al. 1999). Tamoxifen sensitive and resistant breast tumors express similar levels of SRA suggesting that SRA might not directly participate in the mechanisms underlying resistance to hormonal therapies (Murphy et al. 2002). A unique SRA isoform namely SRA $\Delta 3$ (where the exon 3 is deleted) is also detected in breast tumor tissues. The expression of this particular SRA isoform positively correlates with a higher tumor grade (Murphy et al. 2000; Leygue et al. 1999). SRA $\Delta 3$ is missing a significant portion of the SRA core sequence necessary for its function. This transcript is therefore unlikely to function as an RNA molecule and has been proposed to interfere with SRA RNA activity and consequently

disrupting estrogen receptor signaling thereby conferring a more aggressive behavior to tumors highly expressing this form of SRA (Leygue et al. 1999).

In order to assess a potential action of SRA in mouse mammary tumorigenesis, Lanz et al generated a MMTV driven SRA transgenic mouse model. These transgenic mice revealed that SRA expression is not sufficient in itself for mammary gland tumorigenesis (Lanz et al. 2003). However, SRA overexpression coincides with several pathological features in tissues sensitive to estrogen, progesterone and testosterone. SRA overexpression in female SRA transgenic mice results in ductal ectasia, ductal epithelial hyperplasia as well as the appearance of preneoplastic lesions. In addition, SRA overexpression also enhances the generation of brown adipose tissue (Lanz et al. 2003).

Interestingly PR levels are also significantly higher in the mammary gland of virgin SRA transgenic mice. SRA co-activation of ESR1 signaling might be responsible for the increased PR expression. Higher PR could potentially account for the proliferative phenotypes observed in SRA transgenic mice since similar phenotypes have been observed in PR A transgenic mice. SRA transgenic mice also exhibit a high level of apoptosis that could counteract epithelial cell hyperplasia and thus prevent the formation of palpable tumors (Lanz *et al.* 2003). Interestingly, SRA/RAS bitransgenic mice show a significantly lower rate of tumor formation suggesting an antitumorigenic potential for SRA. SRA therefore seems to participate both in proliferative and anti-proliferative mechanisms (Lanz *et al.* 2003).

3.7 Coding and noncoding SRA isoforms and the emergence of a new face.

Today, several SRA sequences can be found in the NCBI database. Analysis of these sequences reveals the existence of 14 distinct SRA transcripts (figure 5). Differences

between SRA sequences include distinct 5' and 3' extremities, presence of base pair substitutions and a full codon insertion. An SRA isoform with the exon 3 deletion has also been reported in breast tumor tissues (Leygue *et al.* 1999) (see figure 5). One additional striking difference between these transcripts is the presence or absence of the full or partial intron 1.

Our laboratory was the first to identify SRA transcripts with an extended exon-1 containing two methionine start codons in frame with a 236/237 amino acids open reading frame (Emberley *et al.* 2003). This observation opened up an exciting possibility indicating that certain SRA transcripts might have a protein coding capacity. These longer isoforms are indeed able to encode for a protein both *in vitro* and *in vivo* (Emberley *et al.* 2003). We therefore suspected that the *SRA* gene had a bifaceted aspect involving a functional RNA as well as a protein. However, SRA was at this time widely considered as a **non-coding** RNA and we had first and foremost the responsibility of establishing the existence of an endogenous SRA protein.

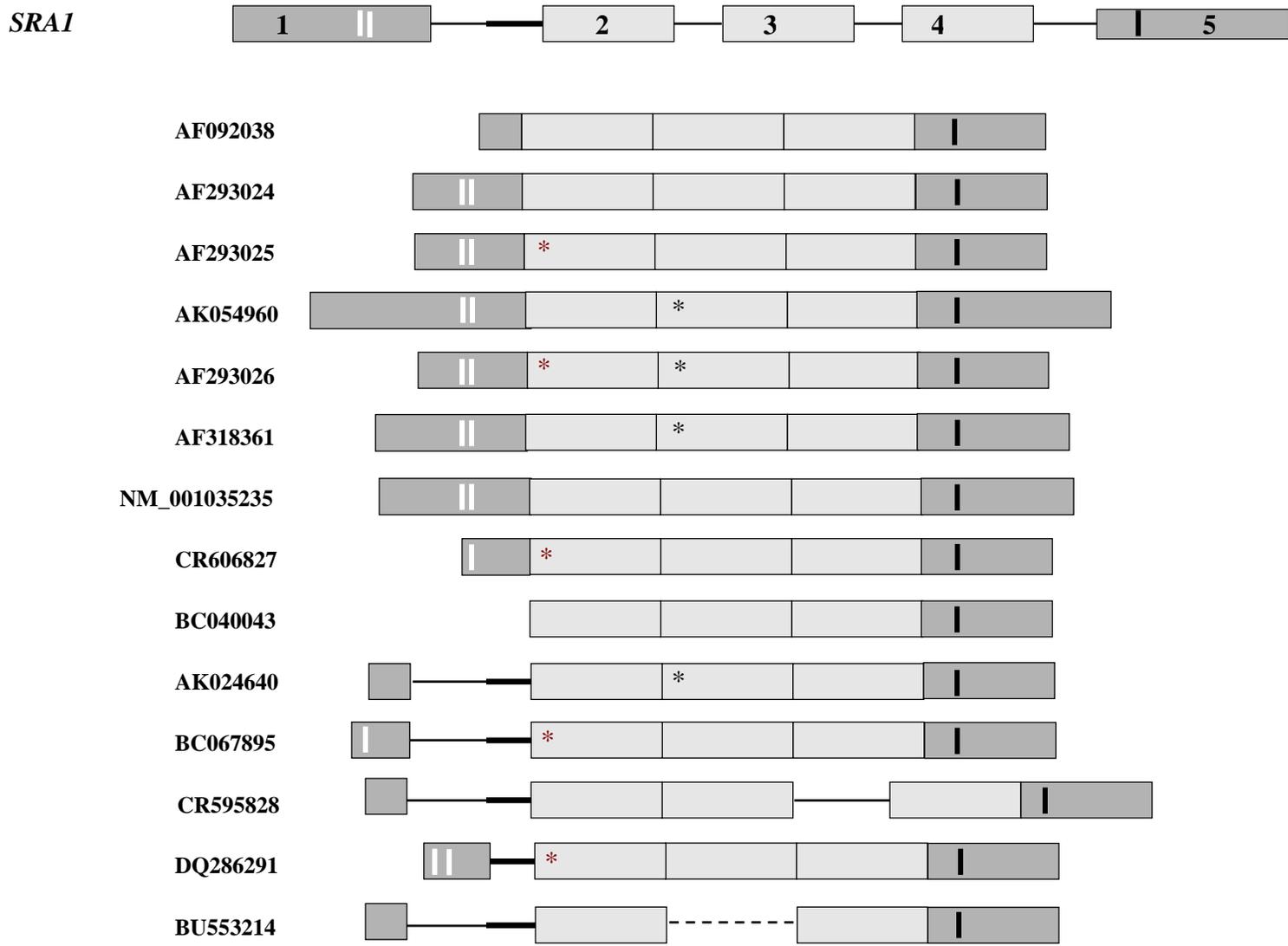


Figure 5: Schematic diagram of the *SRAI* gene and its various different transcripts. Exons and intron are indicated with gray boxes and line respectively. Stars indicate a mutation with respect to the AF293024 sequence. The pink star indicates a mutation in exon 2 from U to C and the green star indicates a base pair mutation followed by a codon insertion G to CGAC. The black bar indicates the stop codon terminating the potential SRAP open reading frame. The white bars indicate potential start codons for the SRAP ORF. The dotted line indicates the absence of exon 3 (Leygue 2007).

4 Thesis Rationale and Objectives

The steroid receptor RNA activator was originally identified as a non-coding RNA able to co-activate the activity of steroid receptors. Today, SRA still remains the only known co-activator to act as an RNA molecule. The identification of longer SRA transcripts with protein coding capacity opened up a new and exciting possibility for the existence of a SRA protein (SRAP). At a time when SRA was widely considered as a **non-coding** RNA, we hypothesized the existence of an endogenous SRA protein.

The work presented in this thesis started with the intent to prove this hypothesis. Subsequently, similarly to unpeeling the layers of an onion, we proceeded to also uncover a possible role of SRAP in breast cancer and its possible function in the estrogen signaling pathway.

4.1 Thesis Hypothesis

The Steroid receptor RNA activator (*SRA1*) gene is able to also encode for a SRA protein with a role in the estrogen signaling pathway and breast cancer.

4.2 Thesis Aims

1. Establish the existence of the steroid receptor RNA activator protein (SRAP).

SRA was identified as an RNA molecule able to co-activate the activity of steroid receptors. Subsequent study identified the existence of SRA transcripts with an extended 5' end with the ability to encode for a SRA protein. Dr. Leygue's laboratory had generated an antibody targeted against the putative human SRAP and had detected a putative endogenous SRA protein by Western blot analysis in breast cancer cell lysates. It was therefore necessary first and foremost to confirm

that the band detected by our antibody was indeed SRAP and thereby unequivocally confirm the existence of an endogenous SRAP.

2. **Investigate SRAP expression in breast tumor tissues.** We had established SRAP existence. Interestingly, SRA RNA expression had been suggested to participate in the mechanisms underlying breast cancer tumorigenesis and progression (Murphy et al. 2000; Leygue et al. 1999). We had detected SRAP expression in all cancer cell lines analyzed including mammary cancer cells. Our second objective was therefore to determine whether SRAP was also expressed in breast tumors and whether there was any correlation between its expression and clinical outcome.
3. **Characterize the role of SRAP in ESR1 transcriptional activity in a ERE context dependent manner.** MCF-7 cells stably expressing SRAP have a lower sensitivity to estradiol. The SRA RNA responsible for SRAP overexpression contains the core SRA sequence necessary for the RNA to be active. Our third objective was to dissect SRA RNA and protein function and to determine the effect of SRAP expression independently of its RNA expression on ESR1 activity. We used an ERE-driven luciferase reporter assay to monitor ESR1 activity. Since EREs have been shown to influence ESR1 function we used three distinct EREs to determine the effect of SRA RNA and protein on ESR1 activity.
4. **Explore SRAP emerging mechanism of action.** We demonstrated that SRAP independently of its RNA is involved in modulating ESR1 transcriptional activity. We therefore wanted to further explore SRAP function. We questioned whether SRAP was able to associate with DNA and subsequently identified possible

SRAP target gene promoter regions. SRAP function is likely not limited to ESR1, therefore we interrogated SRAP's ability to interact with other transcription factors. In order to determine SRAP effect on transcription, we artificially recruited SRAP in close proximity to the VP16 transcription activator on a promoter and monitored its effect using a luciferase reporter assay.

Chapter 2: The steroid receptor RNA activator is the first functional RNA encoding a protein

In 1999, SRA's discovery was unprecedented in the field of steroid receptor co-regulators research. It was the first time that an RNA molecule was demonstrated to function similarly to its protein counterparts and modulate the activity of steroid receptors. This finding was therefore very exciting and intriguing. SRA subsequently captured the attention of scientists and numerous research groups undertook the task to dissect SRA mechanisms of action and determine its action on specific receptors.

SRA was widely considered as a **non-coding** functional RNA until Dr. Leygue's laboratory identified longer SRA transcripts with a 236/237 amino acids open reading frame. In fact in 2000, Dr Leygue had depicted the *in vitro* coding capacity of these sequences. Although the existence of a putative SRA protein did not challenge the highly investigated and established functional aspect of SRA RNA, it certainly added some complexity with regards to the global function of the *SRA* gene. In order to expose the suspected complexity of *SRA*, we first had the task to ascertain SRA protein's endogenous existence. The preliminary steps taken in this direction proved to be successful. Indeed we were able to *in vitro* and *in vivo* translate these longer isoforms and detect a SRA protein. These early results encouraged us to generate an antibody targeted against the N-terminal domain of the putative human SRA protein (SRAP) sequence. Upon Western blot analysis of protein lysates from various cell lines, we detected a band at around 32 kDa using this newly made antibody. Thus, these data highly indicated the

existence of an endogenous SRA protein (SRAP). These exciting results were at the starting point of the work presented here.

Although our preliminary results put forward the existence of endogenous SRAP, we needed to unequivocally demonstrate that the peptide recognized by our antibody was indeed SRAP. The experimental approaches and results to confirm SRAP's existence are detailed in the article entitled **The steroid receptor RNA activator is the first functional RNA encoding a protein**. This article was published on May 21st 2004 in FEBS letters volume 1-3 pages 43 to 47. I have chosen to insert this paper *verbatim* in this chapter. Copyright permission to reproduce this article in this thesis has been obtained.

This article explains how we designed an RNA interference assay targeted against the putative human SRAP sequence. We used this technique to successfully suppress the expression of the protein specifically recognized by our antibody targeting the N-terminal domain of the human SRAP sequence. We thereby unequivocally confirmed that the protein recognized by our antibody is encoded by SRA RNA and consequently confirmed the existence of an endogenous SRAP.

After establishing the existence of SRAP, we subsequently wanted to determine whether SRAP was phylogenetically conserved. We believed that if SRAP sequences were evolutionary conserved, this finding would not only reinforce its existence in the scientific community but perhaps also suggest its important cellular role. Using database searches and Western blot analysis, we showed that SRAP is highly conserved among chordate. Overall the results presented in our article indicate that SRA is the first example of a new class of functional RNAs also able to encode for a protein.

Today, with the development of high throughput sequencing, more expressed sequence tags (ESTs) from many more species are available. SRAP sequence is conserved in additional species such as *Bombyx Mori* (a butterfly), *Brachionus plicatilis* (plankton), *Lottia gigantea* (a snail), *Daphnia pulex* (water flea), *Nematostella vectensia* (sea anemone) as well as perhaps the simplest animal *Trichoplax adhaerens*.

The steroid receptor RNA activator is the first functional RNA encoding a protein

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Abstract The steroid receptor RNA activator (SRA) has previously been characterized as belonging to the growing family of functional non-coding RNAs. However, we recently reported the Western blot detection of a putative endogenous SRA protein (SRAP) in breast cancer cells. Herein, we successfully suppressed the expression of this protein through specific RNA interference assay, unequivocally confirming its existence. Moreover, using database searches and Western blot analysis, we also showed that SRAP is highly conserved among chordata. Overall, our results suggest that SRA is the first example of a new class of functional RNAs also able to encode a protein.

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Keywords: Steroid receptor RNA activator; Non-coding RNA; RNA interference; Steroid receptor RNA activator homologue; Steroid receptor RNA activator protein

1. Introduction

Even though RNAs have long been thought to be either messenger RNAs (mRNAs), transfer RNAs or ribosomal RNAs, it has become apparent in the last 20 years that many RNAs do not belong to any of these three subgroups [1]. The family of non-coding RNAs (ncRNAs), which groups all RNAs unable to encode a protein, is increasing exponentially [2]. Data for the new members of this growing family are actively gathered and corresponding information sorted on several websites such as <http://biobases.ibch.poznan.pl/ncRNA/>, <http://rfam.wustl.edu/index.html> or <http://indiana.edu/~tmrna/>. These RNAs which have no protein coding capacity have been shown to regulate several cellular processes as diverse as the subcellular distribution of RNAs and pro-

teins, the modulation of protein function, or the transcriptional and translational regulation of gene expression [1,2]. The steroid receptor RNA activator (SRA) has recently been characterized as one such ncRNA that modulates steroid receptor transcriptional activity [3].

The originally described SRA sequences differed in their 5' and 3' ends, but were conserved in their central core region [3]. The core region was shown to be necessary and sufficient to increase the ligand-dependent transcriptional activation of target genes by steroid receptors. None of these original SRA sequences were successfully translated in vitro or in vivo [3], and SRA is still currently classified as belonging to the expanding family of functional ncRNAs [4]. Since 1999, data have accumulated regarding the possible mechanisms of action of SRA RNA. SRA RNA interacts with other proteins such as the co-repressor Sharp and the AF-1 specific activator p72/p68 protein to modulate steroid receptor activity [5,6]. Moreover, SRA RNA potentiates the estrogen-induced activation of both estrogen receptors α and β [7]. By introducing mutations in the SRA RNA sequence, Lanz et al. recently identified motifs participating in the RNA secondary structure that are involved in its ability to co-activate progesterone receptor [8,9].

We recently identified three new SRA RNA isoforms which corresponded to SRA except for an additional 37 nucleotides in the 5' region [10]. This 5' region contains two putative ATG codons, close together in the same open reading frame, that could encode putative 236/224 amino-acid SRA proteins (SRAPs). These isoforms, which contain the functional core region, encoded a stable protein both in vitro and in vivo. Using reverse polymerase chain reaction of RNA extracts, we were able to confirm the presence of these endogenous coding isoforms in breast cancer cell lines [10]. Furthermore, using an antibody raised against a peptide corresponding to amino acids 20–34 of the putative human SRAP, we were able to specifically detect a doublet at 30 kDa by Western blot analysis of total protein lysate from these same cell lines [10].

To date, all other reports describe and discuss human SRA as a ncRNA molecule. To our knowledge, no functional RNA has been described to have a protein coding capacity. It, therefore, became important to confirm unequivocally the existence of such an SRAP. In the present study, we demonstrate that the human SRA gene not only encodes for a protein but that the sequence of this protein is conserved among vertebrates.

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Abbreviations: SRA, steroid receptor RNA activator; RNAi, RNA interference; ncRNA, non-coding RNA; SRAP, steroid receptor RNA activator protein

2. Materials and methods

2.1. RNA interference (RNAi) vector construction and SRAP knockdown assay

pSuper.retro-SRA construct was generated by hybridization of oligonucleotides SRARNAif (5'-cccccaagttccctcagagcttcaagagagactc actgggaaactgtttt-3') and SRARNAir (5'-aaacaagttcccaagctcagctctctt gaagactcagctgggaaactgggg-3'), which were then cloned between the BglII and HindIII sites of the pSuper.retro vector (Oligoengine, Seattle, WA). The SRARNAi oligonucleotide sequence was selected following analysis with the Oligoengine RNAi design tool (<http://www.oligoengine.com/>). HeLa cells were transiently transfected with either the pSuper.retro-SRA construct or pSuper.retro vector (empty vector) using the transfection agent Effectene (Qiagen, Mississauga, ON) according to the manufacturer's protocol. At indicated times, cells were washed in 1× PBS and lysed with 2× SDS buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 0.1% bromophenol blue and protease inhibitor cocktail (Roche, Laval, QB). Protein sample concentration was determined by the Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Protein samples were analyzed by Western blot.

2.2. Western blot analysis

Proteins were extracted from skeletal tissue [11] or cell lines [10] as described previously. We chose total skeletal muscle as Lanz et al. [3] had shown that SRA RNA is highly expressed in human skeletal muscle. Protein concentration for each sample was determined using Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) and equal amounts of total protein were analyzed by SDS-PAGE and immunoblotting. Supernatant samples containing equal amount of total protein were mixed 1:1 with sample buffer [1.25 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.02% (w/v) bromophenol blue and 20 mM DTT], boiled for 5 min, electrophoresed using a 5% stacking gel and a 10% resolving polyacrylamide gel, and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (pH 7.6) and incubated with rabbit polyclonal antibodies raised against amino acids 20–34 of human SRA sequence [10] at a dilution of 1:1000 in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween. Secondary horseradish peroxidase-linked goat antirabbit antibodies (1 µg/µl, Bio-Rad) were then used and signals were analyzed by SuperSignal West Pico Chemiluminescent Substrate (Pierce). Chemiluminescence signal was captured by video image analysis using the Quantity One system (Bio-Rad). To determine equal loading, the SDS-PAGE gels were either stained in Coomassie blue solution (50% methanol, 10% acetic acid and 0.1% w/v Coomassie powder) following transfer or blots were stripped and reprobed with anti-β-actin antibody (Sigma, Oakville, ON). In order to detect the specificity of the detection in the protein extracts from different species, we incubated duplicate blots with anti-SRA antibody premixed with the peptide used to raise it (1/10 v/v).

2.3. Sequence analysis and database searches

Search of the NCBI protein database (www.ncbi.nlm.nih.gov) led to the identification of the SRA sequences for three species: *Homo sapiens* (GenBank Accession Nos. AF293024, AF293025 and AF293026), *Mus musculus* (GenBank Accession No. NP_079567) and *Rattus norvegicus* (GenBank Accession Nos. NP_000035 and AAG02116). Upon examining the mouse and rat expressed sequence tag (EST) database, a longer 5' *M. musculus* sequence (GenBank Accession No. CB274276) and a *R. norvegicus* sequence (GenBank Accession No. CB771552) were identified. These sequences were used to deduce the theoretical sequences for the rat and mouse SRAPs. Search of the Unigene database and ProtESTs (<http://www.ncbi.nlm.nih.gov/UniGene/prot-test.cgi?SORT=4&ORG=Hs&XID=114234>) led to the identification of SRA EST sequences for the following species: *Xenopus laevis* (GenBank Accession Nos. BG364002.1, BG551872.1 and AW642449.1), *Silurana tropicalis* (GenBank Accession No. AL969036.1), *Sus scrofa* (GenBank Accession Nos. CF366666 and CF368085), and *Oryzias latipes* (GenBank Accession No. AU170197.1). We blasted the human SRA3 amino-acid sequence against the translated EST nucleotide sequences using tblastn, limiting the search to vertebrates and excluding mouse and human sequences. This search identified ESTs for the following additional species: *Macaaca mulata* (GenBank Accession No. CD766957), *Equus caballus* (GenBank Accession Nos. B1961443 and B1961063), *Bos taurus* (GenBank Accession Nos. CB422540, AW654516, CB450664 and

CB457765P), *Danio rerio* (GenBank Accession Nos. BQ258955, CB352395), *Occhornhyncus Mykiss* (BX860673) and *Gallus gallus* (GenBank Accession Nos. CR338992, CR338823, CR338795). Species-specific sequences were assembled using an EST assembler tool (<http://bio.ifom-firc.it/ASSEMBLY/assemble.html>) giving contigs that were translated using the DNA to amino-acid translational tool (http://ca.expasy.org/cgi-bin/dna_aa.html). All the putative SRAP sequences were aligned using the Multalin alignment tool (<http://prodes.toulouse.inra.fr/multalin/>) and two conserved regions were identified. These two conserved sequences were used to identify the SRA analogue in Fugu fish by doing a Blast search against the Fugu fish genome. Blast search using the human SRA1 sequence against the ascidian *Ciona intestinalis* genome in the TIGR database (<http://tigrblast.tigr.org/tgi/>) resulted in the identification of an SRA analogue (GenBank Accession No. BW276199) in *C. intestinalis*. Blast searched at the NCBI site http://www-genome.wi.mit.edu/cgi-bin/annotation/ciona/blast_page.cgi using the *C. intestinalis* SRA sequence resulted in a *Ciona savignyi* SRA analogue. Nuclear localization signals were search on each individual sequence using Psort II (<http://psort.nibb.ac.jp/form2.html>).

2.4. PCR cloning and in vitro translation of rat SRA cDNA

PCR primers (5'-agtgagctaccaccggaa-3' and 5'-tatagaagctgtg-agggt-3') designed by analyzing the rat theoretical SRA sequence were used to amplify cDNA from rat skeletal muscle. The resulting product was sequenced (GenBank Accession No. AY542868) and cloned in pcDNA3.1 expression vector. RNA isolation from rat skeletal tissue and reverse transcription-polymerase chain reaction were conducted as described previously [9]. pcDNA3.1 (Invitrogen, Carlsbad, CA) expression plasmid containing either human SRA cDNA or rat SRA cDNA was used for in vitro translation/transcription reaction. [³⁵S]methionine labelled SRAPs were generated using wheat germ lysate coupled transcription/translation reactions by the TnT System (Promega, Madison, WI) according to the manufacturer's instructions. The V5-tagged human SRA cDNA construct, previously [10] cloned in pcDNA3.1/V5-His[®] (Invitrogen, Carlsbad, CA), was used as a positive control. Lysates were then subjected to SDS-PAGE separation, after which gels were dried and [³⁵S]methionine labelled protein bands visualized by exposing overnight to a Molecular Imager[™]-FX Imaging screen (Bio-Rad) and subsequently scanned using a Molecular Imager[™]-FX (Bio-Rad).

3. Results and discussion

We were previously able to detect a putative endogenous SRAP in breast cancer cell lines with an antibody targeted against amino acids 20–34 of the hypothetical human SRAP sequence [10]. As we are still today the only ones to have reported the existence of such a putative protein, it became essential to definitively confirm the identity of the protein recognized by our antibody. In order to do so, we used the recently developed RNAi technology in an attempt to knockdown its expression. Indeed, we reasoned that the specific degradation of SRA RNA should result in a decrease in the expression of the protein recognized by our antibody. Upon 24 and 48 h, transfection of HeLa cells with a RNAi specifically targeted against SRA RNA resulted in a significant decrease in the doublet detected by Western blot and believed to correspond to the SRAP (Fig. 1A). In contrast and as expected, RNAi treatments had no significant effect on β-actin levels (Fig. 1B) as well as a non-specific protein detected by our anti-SRA antibody (Fig. 1A, 45 kDa). This result links the expression of the SRA gene to the detection of the suspected SRAP by Western blot. This unequivocally confirms for the first time the existence of an endogenous SRAP in human cells. It should be stressed that no noticeable phenotypic changes were observed in cells treated with SRA-specific RNAi over the 48-h post-transfection. This absence of apparent effect

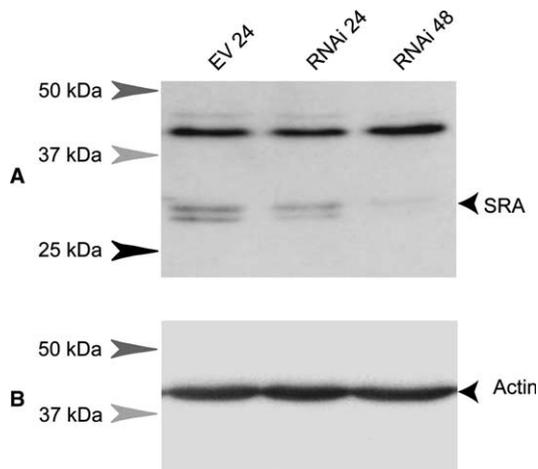


Fig. 1. Decrease of SRAP expression by RNAi specifically targeted against SRA RNA. HeLa cells were transiently transfected with either SRA RNAi (RNAi) or control (EV) vectors, and SRA or actin protein expressions assessed by Western blot 24 and 48 h after transfection as described in Section 2. Positions of the molecular size markers are indicated on the left.

likely results from the short length of time and the transient nature of these experiments have been conducted. Further experiments are needed to investigate longer term effects of the knockdown of SRA gene on phenotypic changes such as growth rate.

We have then investigated the possible existence of this SRAP in other species. As of today, Blink, the NCBI software which groups putative species homologues (<http://www.ncbi.nlm.nih.gov/sutils/blink.cgi?pid=9930614>), only gathers human, rat and mouse SRA sequences. Rat SRA sequences (GenBank Accession Nos. NP_000035 and AAG02116) correspond to the recently published 146 amino-acid rat SRAP sequence [12]. This putative rat SRAP sequence was successfully translated in vitro and also expressed in vivo when fused with the C-terminal extremity of green fluorescence protein. SRAP is much smaller (16 kDa) than the putative human SRAP we identified and its endogenous existence has not yet been demonstrated. Close analysis of the SRAP mRNA sequence revealed that it is analogous to the human SRA sequences starting at exon 2. We thus suspected the SRAP mRNA sequence described so far to be incomplete. Indeed, analysis of the rat chromosome 18 sequence revealed the presence of a putative additional exon 1 present in a single rat EST sequence (GenBank Accession No. CB771552). To confirm the existence of this longer SRA sequence, we PCR amplified rat muscle cDNA with specific primers as described in Section 2. We cloned a new rat cDNA sequence (GenBank Accession No. AY542868), which unlike the shorter SRAP sequence contains two possible methionines possibly initiating the translation of a 222/230 amino-acid proteins. As shown in Fig. 2, this sequence is translatable in vitro, generating a visible doublet at 31/32 kDa. As expected and as a result of an additional V5-tag (4.8 kDa), in vitro translated SRA-V5 protein (35 kDa) has a higher apparent molecular size than the non-tagged human SRA doublet (30/31 kDa) and rat SRA doublet (31/32 kDa). As previously described [10], the observed molecular masses (35, 30/31 and 31/32 kDa) are slightly higher than those predicted (30.5, 25.7 and 25.3 kDa for the V5-tagged human, non-tagged human SRAP and rat SRAP,

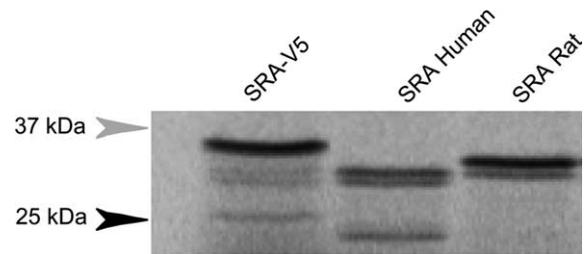


Fig. 2. In vitro translation of human and rat SRA cDNAs. In vitro transcription/translation reactions were performed using V5-tagged human SRA cDNA as control (SRA-V5), human SRA cDNA (SRA Human) and rat SRA cDNA (SRA Rat) as described in Section 2. Positions of the molecular size markers are indicated on the left.

respectively). We hypothesize that the doublet seen for human and rat SRAPs results from the alternative use of one initiating methionine instead of another (1 versus 12). Weaker lower molecular size bands (around 25 kDa) are likely resulting from translation at internal downstream methionines present in both the human and rat SRA sequences (see Fig. 3).

Through database analysis, we identified a *M. musculus* sequence (GenBank Accession No. CB274276), slightly longer than the one present in the protein database. When translated, this sequence contains an additional stretch of 12 N-terminal amino acids, 10 out of which are identical to their corresponding human counterparts (Fig. 3).

In an attempt to identify SRA analogues in other species, we searched several databases as described in Section 2. Obtained cDNA sequences were translated and aligned (Fig. 3). The alignment of putative SRA sequences from different species shows proteins of similar lengths, highly conserved in discrete domains and in two main regions (amino acids 15–39 and 180–208 of the human sequence). We have previously demonstrated that the stably transfected SRAP localizes to the nucleus in MCF-7 cells [10]. Analysis of the SRA sequences (Psort II), for nuclear localization signal domains, revealed a conserved pat-7 (P-x-[RK]-[RK]-[RK]) nuclear localization motif in *Gallus* (chicken), *Oryzias* and *Oocorhyncus* (two fish) and all the mammalian putative SRAP sequences (amino acids 155–160). Interestingly, the two *Ciona* species which do not contain pat-7 motif at this position, contain however another nuclear localization pat-4 motif (P-[RK]-[RK]-[RK]) at amino acids 39–42 with respect to the human SRA sequence.

Overall, putative SRAPs were found in all vertebrates in which SRA-related EST sequences could be detected. In addition, putative SRAP analogues were identified in two *Ciona* species, *Ciona savignyi* and *C. intestinalis*. *Ciona* belong to the urochordata subphylum, which together with Cephalochordata and Craniata (contains Vertebrata) subphyla, defines the *Chordata* phylum. We were unable to find any sequences closely related to SRA in any other phyla such as Arthropoda (*Drosophila melanogaster*), Nematoda (*Caenorhabditis elegans*) or Protobacteria (*E. coli*). Conservation of the SRAP sequence from an invertebrate Chordata (*Ciona*) to a higher vertebrate (human) suggests an important role possibly played by this molecule. Furthermore, the conservation of a nuclear localization signal in most of the SRAPs suggests a nuclear localization for this putative conserved function. In addition, the apparent absence of SRA homologues in all non-chordata phyla suggests that this protein might have been involved in the emergence of early Chordata.

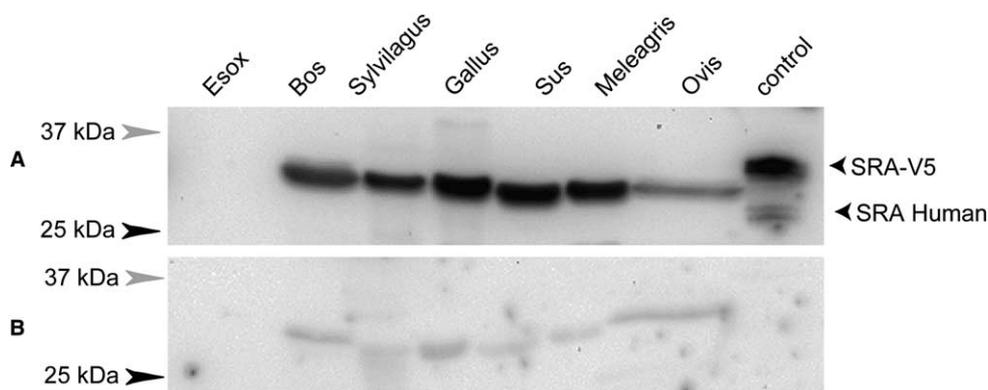


Fig. 4. SRAP expression in skeletal muscle of various species. As described in Section 2, Western blots of total protein extracts from the muscle of different species were performed in the absence (A) or presence (B) of neutralizing peptide. Positive control consists of protein extracts from MCF-7 cell line stably expressing V5-tagged human SRA. Positions of the molecular size markers are indicated on the left.

loaded as confirmed by Coomassie blue staining of the gels after transfer (data not shown). Protein extracts from MCF-7 cells stably expressing V5-tagged SRA were used as positive control (right lane). An upper band corresponding to the transfected V5-SRA is seen at 35 kDa. In addition, a doublet, corresponding to the endogenously expressed SRA is seen at 30/31 kDa. No signals were observed when the antibody was pre-neutralized by the corresponding peptide (Fig. 4B). Interestingly, only one band is detected in muscle extracts, suggesting that only one AUG codon is used to initiate SRAP translation in these tissues. This may result either from the existence of only one such codon in the *Sus*, *Bos*, *Gallus* putative mRNA sequences (see Fig. 3), or from the preferential tissue-related use of one codon over the other.

No SRAP was detected in the *Esox* (pickerel) protein extract. A careful examination of the four hypothetical fish sequences we gathered (*Fugu*, *Oryzias*, *Occhonrhyncus* and *Danio*) shows that they are similar to the human sequence in the region recognized by the antibody used (20–34, referenced to human SRA) except for the presence of a glutamine instead of a lysine at amino acid 23 (Fig. 3). The *Esox* SRA sequence, which remains unknown to date, should, if similar to the other fish sequences in this particular region, also contain this particular amino-acid substitution. Interestingly, our antibody was able to recognize SRAPs from the *Bos* (cow) and *Gallus* (chicken) tissue extracts despite the presence of a threonine (*Bos*) and an alanine (*Gallus*) instead of a lysine at this same residue 23. Lysine, alanine and threonine as opposed to glutamine are all hydrophobic amino acids. We therefore suspect that a switch in the hydrophobicity of the amino acid at residue 23 may be responsible for the impaired recognition of *Esox* SRAP by our antibody.

In this study, we have demonstrated that the human SRA gene encodes for a protein conserved among vertebrates. The high conservation of SRAP sequence underlines the possible important role played by this protein. Previous studies have shown that SRA RNA is fully functional independently of its protein coding capacity. SRA therefore appears to be the first example of a new class of functional RNAs also able to encode a protein.

The existence of an SRAP raises several important questions waiting to be addressed. What is the role of SRAP? What are the implications of SRAP on SRA RNA function? How are the expressions of SRA RNA and SRAP regulated? What other molecules function at dual protein/RNA levels? Since all of the functional studies on human SRA described to date ignore the existence of a protein, addressing the above questions is critical to fully understand SRA function. More importantly, development in our understanding of SRA RNA and protein function is in turn critical for a change in the current perspective of functional “non-coding” RNA molecules.

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Chapter 3: The steroid receptor RNA activator protein is expressed in breast tumor tissues

SRA was widely accepted as a non-coding RNA co-activating steroid receptor activity. As described in the previous article, we have nonetheless exposed a more complex facet of the SRA gene by indisputably confirming the existence of a SRA protein (SRAP). In addition, we demonstrated that the SRAP protein sequence has two highly evolutionary conserved domains perhaps suggesting its important cellular role.

Concurrently reports had indicated that SRA RNA modulates the estrogen signaling pathways. Furthermore SRA RNA expression in breast tumors was also investigated. SRA RNA expression might have implications in breast cancer tumorigenesis as well as tumor progression. We thus suspected that perhaps similarly to its RNA counterpart, SRAP could also be involved in the mechanisms underlying breast cancer. We therefore started to direct our research in investigating SRAP expression in breast tumors.

The experimental approaches and results to examine SRAP expression in breast cancer are detailed in the article entitled **The steroid receptor RNA activator protein is expressed in breast tumor tissues**. This article was published on February 15th 2006 in International Journal of Cancer volume 118 issue 4 pages 1054 to 1059. I have chosen to insert this paper verbatim here. Copyright permission to reproduce this article in this thesis has been obtained.

In this article, we investigated SRAP expression by Western blot analysis of protein extracts from 74 primary breast tumors corresponding to patients subsequently treated with tamoxifen. We were able to specifically detect two bands at 25 kDa and 32 kDa. Interestingly, we found that patients whose primary tumors were positive for the 32 kDa

SRAP expression had a significantly lower likelihood of dying from recurrent disease than SRAP-negative patients.

The observed correlation between SRAP expression and overall better survival in ER-positive patients prompts us to examine further the impact of SRAP on the ER signaling pathway. We generated two MCF-7 breast cancer cell lines stably overexpressing coding SRA and consequently SRAP. Transient transfection experiments, performed using a luciferase reporter gene under the control of an estrogen-responsive element, revealed decreased sensitivity to estradiol but no additional sensitivity to tamoxifen in SRAP-overexpressing cells. Overall, our data suggest that the presence of both coding SRA RNA and its corresponding SRAP modifies the activity of estrogen receptor alpha in breast cancer cells and that SRAP could be a new clinical marker for breast cancer.

SHORT REPORT

The steroid receptor RNA activator protein is expressed in breast tumor tissues

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The steroid receptor RNA activator (SRA) was originally described as the first functional non-coding RNA able to specifically coactivate the activity of steroid receptors. We previously demonstrated the existence in breast cancer cell lines of new SRA isoforms that, as opposed to the first cloned SRA RNA, encode for a 236-amino acid protein, SRAP. To investigate the possible implications of the coding SRA RNA and SRAP expression on breast cancer progression, we examined by Western blot analysis 74 primary breast tumors of patients subsequently treated with tamoxifen. Patients whose primary tumors were positive for SRAP expression ($n = 24$) had a significantly (Kaplan-Meier survival curve $p = 0.047$) lower likelihood of dying from recurrent disease than SRAP-negative patients ($n = 50$). We generated 2 cell lines, SRAP-V5-High.A and SRAP-V5-High.B, by stably overexpressing SRAP in the estrogen receptor-positive MCF-7 breast cancer cell line. Transient transfection experiments, performed using a luciferase reporter gene under the control of an estrogen-responsive element, revealed decreased sensitivity to estradiol but no additional sensitivity to tamoxifen in SRAP-overexpressing cells. Overall, our data suggest that the presence of both coding SRA RNA and its corresponding SRAP modifies the activity of estrogen receptor in breast cancer cells and that SRAP could be a new clinical marker for breast cancer. Further studies are needed to define the respective mechanisms of action and the roles of SRA RNA and protein in breast tumorigenesis and tumor progression.

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Key words: steroid receptor coactivator; human breast tumor; steroid receptor coactivator protein

Through its action on breast epithelial cells, estrogen not only controls the growth and the development of normal mammary gland but also promotes breast tumorigenesis and breast cancer progression.¹ The biological action of estrogen is mainly mediated through two ERs, α and β , which act as ligand-dependent transcription factors.^{2,3} While estrogen initially plays a pivotal role in the activation of ERs, the transcriptional activation of target genes is ultimately determined by interactions between receptors and regulatory molecules known as coactivators and corepressors, which respectively stimulate or inhibit ER activity.⁴ SRA differs from all previously characterized coactivators as it was originally identified as a functional non-coding RNA molecule.⁵ SRA mechanisms of action have since become the focus of extensive investigation. SRA was shown to contain a core RNA sequence necessary and sufficient to mediate steroid receptor activity⁶ through interactions with several proteins including the coactivator/corepressor SHARP,⁷ SRC1,⁵ and the AF-1-specific activator p72/p68 protein.⁸ Post-transcriptional modifications of SRA have also been shown to participate in the ability of this RNA to modulate receptor activity.⁹ We established that SRA RNA was differentially expressed in normal and in breast tumor tissue and suggested that SRA RNA could be involved in mechanisms underlying breast tumorigenesis and breast tumor progression.¹⁰ The observation by Lanz *et al.*¹¹ of multiple proliferation anomalies in the overexpressing noncoding SRA RNA mammary glands of transgenic mice corroborates this hypothesis.

While all these studies refer to SRA as a noncoding RNA, we have previously demonstrated the existence of coding SRA RNA isoforms and corresponding endogenous SRA proteins,¹² highly conserved in vertebrates and expressed in breast cancer cell lines.^{13,14} To date, no data are available on the possible role of the coding SRA RNA or SRAP in breast cancer cells or on their

expression in human breast tumor tissues. Here, we investigated SRAP expression in a cohort of ER-positive primary breast tumors from patients subsequently treated with tamoxifen and examined the effect of SRAP overexpression on ER α activity in MCF-7 mammary tumor cells.

Material and methods

Human breast tissues and cell lines

Seventy-four primary breast tumors were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Tumors corresponded to cases associated with node-negative status that were treated by surgery with or without radiation therapy and then tamoxifen endocrine therapy. All tumors were ER-positive (ER levels ranging 4–247 fmol/mg protein, median 45.5) and spanned a wide range of PR levels (2.4–444 fmol/mg protein, median 31). Nottingham grade was known for 66 tumors, which were assigned to low ($n = 23$, scores 3–5), moderate ($n = 35$, scores 6–7) or high ($n = 8$, scores 8–9) categories. MCF-7 cells were stably transfected with the pCDNA.3.1-V5-His expression vector alone (control cell line) or containing a coding SRA cDNA to generate the SRAP-V5-High.A, SRAP-V5-High.B and SRAP-V5 low cell lines, as previously reported.¹⁴

Western blot analysis

Total proteins were extracted from cells¹⁴ or breast tumor tissues¹⁵ and analyzed by Western blot as previously described.^{13–15} Four primary antibodies, a rabbit polyclonal anti-SRAP antibody,¹⁴ a mouse anti-ER MAb (NCL-ER 6F11/2; Novocastra, Newcastle, UK), a mouse anti-PR MAb (NCL-PGR 312, Novocastra) and a mouse anti- β -actin MAb (A5441; Sigma, Oakville, ON), were used at dilutions of 1:1,000, 1:1,000, 1:1,000 and 1:5,000, respectively. Preincubation of the primary anti-SRAP with its corresponding peptide was performed as described previously.¹⁴

To examine PR expression, MCF-7 cont, SRAP-V5-High.A, SRAP-V5-High.B and SRAP-V5-Low were grown for 6 days in serum-free phenol red-free DMEM supplemented with apotransferrin and BSA. Cells were then treated with either ethanol (vehicle) or estradiol (10^{-8} M) for 4, 24 and 48 hr. Cells were then lysed, and identical amounts of total protein extracts were analyzed as described above. To ensure equal loading, gels were stained with Coomassie blue.

Abbreviations: ER, estrogen receptor; ERE, estrogen-responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAb, monoclonal antibody; PR, progesterone receptor; SHARP, SMRT/HDAC-associated repressor protein; SRA, steroid receptor RNA activator; SRAP, protein encoded by the steroid receptor RNA activator; SRC1, steroid receptor coactivator-1; TFF1, trefoil factor 1.

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RT-PCR analysis

MCF-7 cont, SRAP-V5-High.A, SRAP-V5-High.B and SRAP-V5-Low cells were grown for 6 days in serum-free phenol red-free DMEM supplemented with apotransferrin and BSA and subsequently treated with either ethanol (vehicle) or estradiol (10^{-8} M) for 15 and 60 min. Total RNA was isolated from these cells using the Eppendorf (Hamburg, Germany) RNA isolation kit. cDNA was synthesized using MMLV reverse transcriptase (Invitrogen, Burlington, ON) as described in the manufacturer's instructions. cDNAs were then amplified with platinum Taq polymerase (Invitrogen) as previously described.¹⁶ Primers to TFF1 were upper 5'-CTGGGGCACCTTGCCATTTTCC-3' and lower 5'-CGGGGGGCCACTGTACACGTC-3',¹⁷ and those to GAPDH were upper 5'-ACCCACTCTCCACCTGG-3' and lower 5'-CTCTGTGCTCTTGCTGGG-3'.

PCR products were separated electrophoretically on 2% agarose gels and subsequently stained with ethidium bromide. Gels were visualized under UV light on a GelDoc2000/ChemIDoc System (Bio-Rad, Richmond, CA).

Cell transfection experiments

Transfection experiments were performed as previously described¹⁸ with small modifications. Briefly, cells grown in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS for 48 hr were transfected for 4 hr with 1 μ g of ERE-luciferase plasmid (containing a vitellogenin ERE, GGTCACCTGTGACC site upstream of the firefly luciferase cDNA) and 0.1 μ g of renilla luciferase reporter vector (Promega, Madison, WI) using the lipofectamine reagent (Invitrogen). Cells were then treated with either ethanol (vehicle), estradiol (10^{-6} – 10^{-10} M) and/or 4-hydroxytamoxifen (Sigma, 10^{-6} – 10^{-9} M) for 24 hr. Cells were lysed in 200 μ l of cell culture lysis buffer (Promega), and lysates were analyzed for luciferase and renilla luciferase activities according to the manufacturer's protocol (Promega).

Statistical analysis

Transfection results were normalized by dividing ERE luciferase activities by their corresponding renilla luciferase activities. For each treatment, the relative luciferase activity was used to calculate fold induction (ratio of value for a treatment and the corresponding value for ethanol treatment). Results are representative of at least 3 independent experiments. Significant differences were assessed using Student's *t*-test. Error bars represent SEM.

Tumor cases were classified as SRAP-positive or SRAP-negative following independent assessment of the corresponding Western blot signal by 2 investigators. Statistical differences in ER/PR or Nottingham grade between the 2 groups were tested using the Mann-Whitney test (2-tailed) or the χ^2 test, respectively. Relapse-free survival was defined as the time from initial surgery to the date of clinically documented local or distant disease recurrence or death attributed to breast cancer. Overall survival was defined as the time from initial surgery to the date of death attributed to breast cancer. Deaths caused by other known or unknown causes were censored. The association between SRAP expression and relapse or survival was assessed by the Kaplan-Meier method.

Results

Differential expression of SRAP in breast tumor tissues

To investigate the possible relationship between SRAP expression and known prognostic markers, we performed Western blot analysis on a series of proteins extracted from 74 different ER-positive breast tumors. A strong background signal (50 kDa), still present with the neutralizing peptide, was observed in all tumors (Fig. 1a,b).

SRAP signal, which disappeared when the antibody was preincubated with the corresponding peptide, was observed in some (24 tumors, e.g., lanes 1, 2, 8), but not all (50 tumors, e.g., lanes 3, 4, 5, 7), tumors (Fig. 1a,b). As expected, the size of the bands varied around 30 kDa, likely as a result of the genetic background

(homozygous/heterozygous for the different alleles) of the patients.¹⁴ A band of approximately 25 kDa, not previously seen in breast cancer cell lines,¹⁴ was also specifically recognized in 34 cases (e.g., lanes 1, 2, 4–8 of Fig. 1a).

Neither ER nor PR levels were significantly different (Mann-Whitney rank sum test $p > 0.05$) between SRAP-positive ($n = 24$, median ER 74.5, PR 30.5 fmol/mg protein) and SRAP-negative ($n = 50$, median ER 39, PR 32.5 fmol/mg protein) subgroups. Similarly, no significant relationship (χ^2 test $p > 0.05$) was found between SRAP expression and histologic tumor grade distribution (low grade $n = 7$, $n = 16$; moderate grade $n = 9$, $n = 26$; high grade $n = 5$, $n = 3$ for SRAP-positive and -negative subgroups, respectively).

When SRAP expression was considered in relation to recurrence, no significant difference was seen ($n = 9$ events for SRAP negative patients and 3 events for SRAP-positive patients, Fig. 1c). In contrast, when considered in relation to outcome (Fig. 1d), a significant (Kaplan-Meier $p = 0.044$) association was found with undetectable level of SRAP expression and poor survival ($n = 7$ events for SRAP-negative and 0 events for SRAP-positive).

No correlation was found between expression of the 25 kDa band and any tumor or patient characteristics (data not shown).

Clones stably expressing SRAP-V5 recombinant protein

We stably transfected MCF-7 mammary cancer cells, known to express high levels of endogenous ER, with a construction consisting of coding SRA RNA able to encode a fusion protein, SRAP-V5-tag.¹⁴ Several clones were selected and protein extracts analyzed by Western blot using an anti-SRA antibody previously shown to recognize both endogenous SRAP (approx. 30 kDa) and V5-tagged SRAP (approx. 35 kDa).¹⁴ As shown on Figure 2a, clones expressing detectable levels (SRAP-V5-High.A and SRAP-V5-High.B) or not detectable levels (SRAP-V5-Low) of the recombinant SRAP-V5 protein were obtained. All cell lines, including control MCF-7 stably transfected with vector alone, expressed identical levels of endogenous SRAP (Fig. 2a), ER (Fig. 2b) and PR isoforms A (112 kDa) and B (83 kDa) (Fig. 2c), as assessed by Western blot.

Decreased ligand-dependent transcriptional activity of ER in SRAP-V5-overexpressing cells

To establish whether the ER signaling pathway was altered in cells overexpressing SRAP, an ERE-luciferase reporter vector was transiently transfected in all 4 cell lines described above. Cells were then treated with ethanol (vehicle), estradiol (10^{-8} M) or 4-hydroxytamoxifen (10^{-6} M) for 24 hr and luciferase activity was measured, as described in Material and methods. Luciferase activities of the 4 cell lines were identical when cells were treated with ethanol or 4-hydroxytamoxifen (data not shown and Fig. 3a). However, upon estradiol (10^{-8} M) treatment, cells overexpressing SRAP-V5 (SRAP-V5-High.A and SRAP-V5-High.B) showed significantly lower induction (approx. 19-fold, Student's *t*-test $p < 0.03$) of ER α transcriptional activity compared to control cells (approx. 39-fold, Fig. 3a). In contrast, even though the reporter gene induction was slightly lower (approx. 32-fold) in SRAP-V5-Low cells compared to control cells (approx. 39-fold), this difference did not reach statistical significance ($p > 0.05$).

To establish whether the decreased activation of ER in SRAP-V5-overexpressing cells was dependent on the dose of estradiol used, we transiently transfected all 4 cell lines with an ERE reporter gene and treated them with increasing amounts of estradiol (10^{-6} – 10^{-10} M). Lower activation of the reporter gene in SRAP-V5-overexpressing cells compared to control cells (Fig. 3b) was observed for all concentrations of estradiol used. Differences between the SRAP-overexpressing and control cell lines were statistically significant ($p < 0.02$) at 10^{-8} , 10^{-9} and 10^{-10} M. We suspect treatment with higher concentrations (10^{-6} and 10^{-7} M) had toxic effects on cells that could have led to lower reproducibility between experiments.

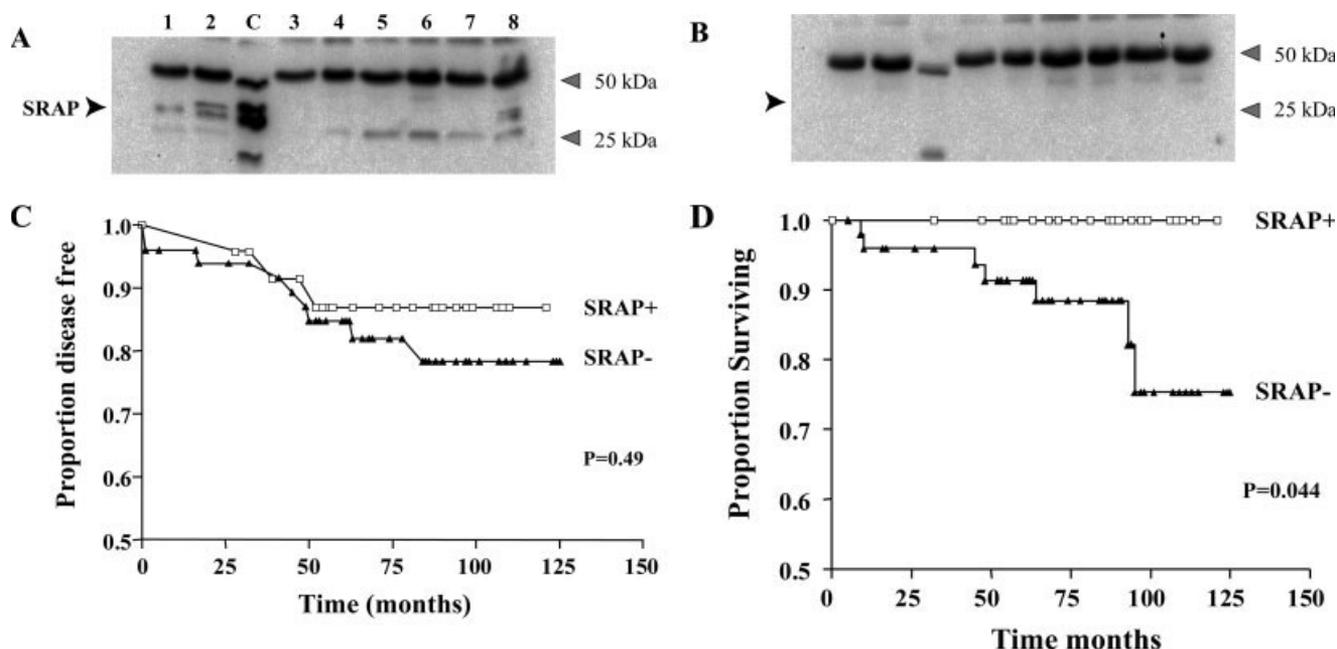


FIGURE 1 – SRAP expression correlates with overall better survival in ER-positive breast cancer patients. Proteins were extracted from a cohort of 74 ER-positive tumors and analyzed by Western blot for SRAP expression as described in Material and methods. (a) Representative panel showing Western blot for tumors 1–8. C, SRAP-V5-High.A cells. (b) Western blot performed in parallel and incubated with an anti-SRA antibody premixed with the neutralizing peptide. (c,d) Kaplan-Meier graphs for time to progression and overall survival, respectively, with regard to SRAP expression.

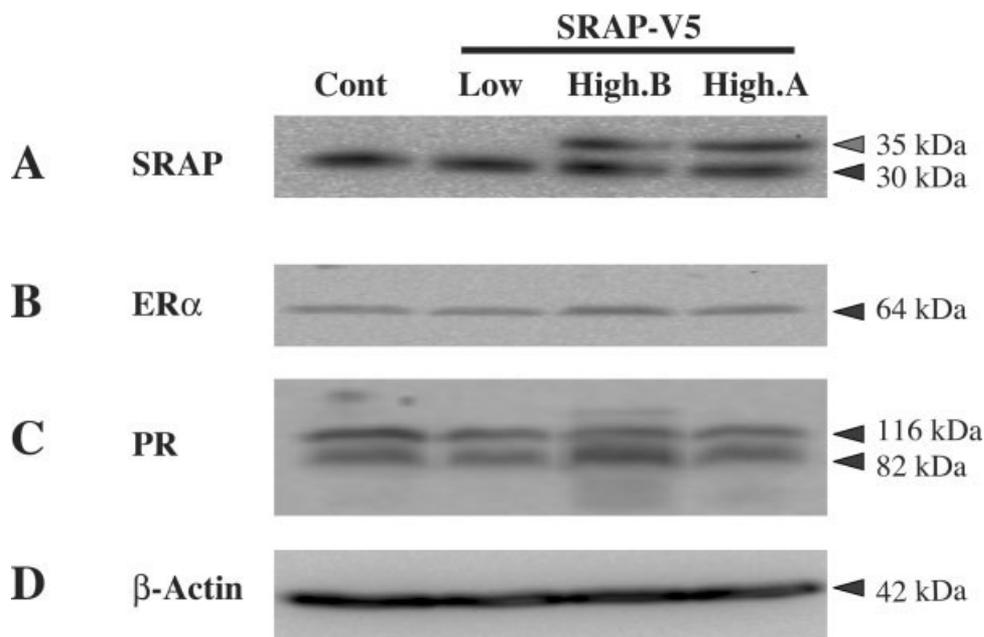


FIGURE 2 – Western blot analysis of MCF-7 cells stably transfected with SRAP-V5 cDNA. MCF-7 breast cancer cells were stably transfected with SRAP-V5 cDNA (SRAP-V5) or empty vector, and total protein extracts were analyzed by Western blot as described in Material and methods. Antibodies consisted of anti-SRAP (a), anti-ER α (b), anti-PR (c) and anti-actin (d). Two high (High.A and -B) and one not detectable (Low.A) SRAP-V5 expressors were selected for further analysis.

Similar tamoxifen sensitivity in SRAP-V5-overexpressing and control cell lines

To determine whether SRAP overexpression potentiates the inhibitory effect of 4-hydroxytamoxifen, SRAP-V5-High.A, SRAP-V5-High.B, SRAP-V5-Low and control cells transiently transfected with an ERE-luciferase reporter vector were treated with 10^{-8} M estradiol supplemented with increasing amounts of 4-hydroxytamoxifen (10^{-9} – 10^{-6} M). Reporter gene activities were lower in SRAP-overexpressing cells compared to activities observed for the corresponding treatment in control cells (Fig. 3c).

However, in all cell lines, the first efficient concentration of 4-hydroxytamoxifen able to significantly ($p < 0.05$) decrease estradiol-dependent induction was 10^{-7} M (Fig. 3c).

Higher PR expression upon estradiol stimulation in SRAP-V5-overexpressing cells

It was important to determine whether the difference in the response to estradiol observed between high and low SRAP-expressing MCF-7 cells seen with the luciferase reporter assay could also be observed during the induction of known ER target

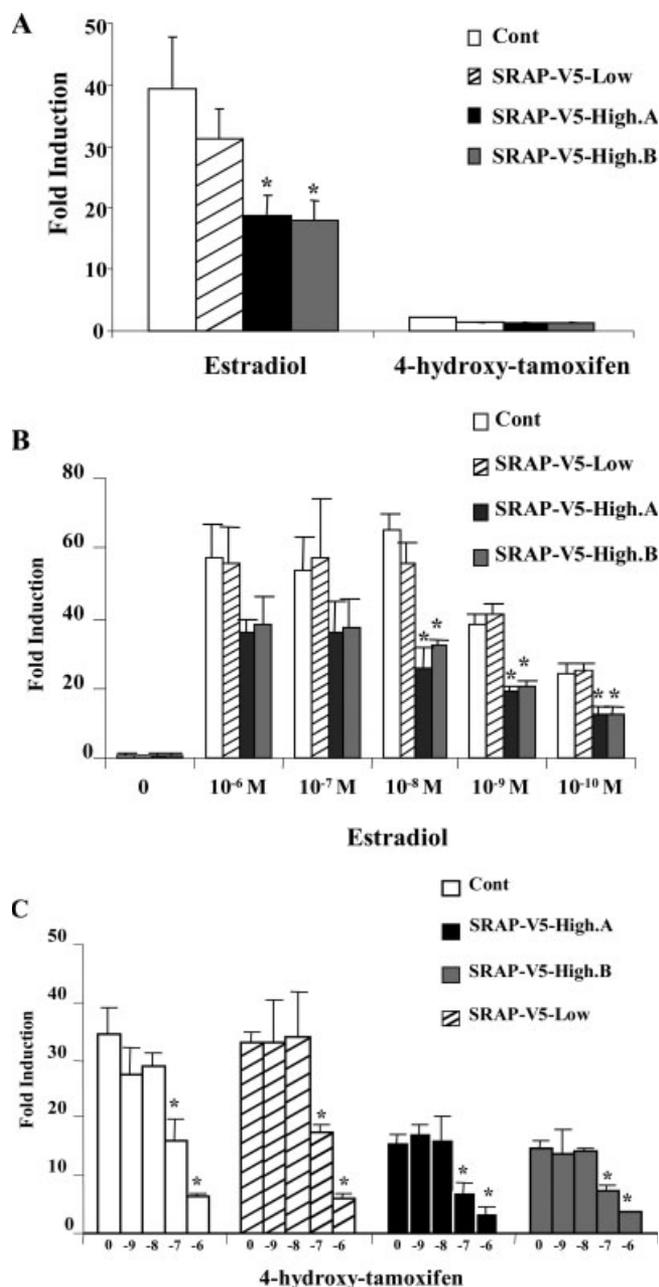


FIGURE 3 – Decreased activation of an ERE-luciferase reporter gene in SRAP-V5-overexpressing cells. MCF-7 cells expressing detectable (SRAP-V5-High.A and -B) or not detectable (SRAP-V5-Low and Cont) levels of SRAP-V5 recombinant protein were transiently transfected with an ERE-luciferase reporter gene and subsequently treated with (a) ethanol, 10⁻⁸ M estradiol or 10⁻⁶ M 4-hydroxytamoxifen; (b) 10⁻⁶–10⁻¹⁰ M estradiol; or (c) 10⁻⁶ M estradiol 10⁻⁹, 10⁻⁸, 10⁻⁷ or 10⁻⁶ M 4-hydroxytamoxifen for 24 hr. Results correspond to the average fold induction (ratio between luciferase values during ligand treatment and corresponding ethanol treatment) of at least 3 independent experiments. Bars \pm SEM. Cont, MCF-7 cells stably transfected with vector alone. *(a,b) Statistically significant difference ($p < 0.05$, Student's t-test) between the fold induction obtained for SRAP-V5-overexpressing clones and the corresponding fold induction for control cells. (c) Fold inductions for specific tamoxifen concentrations that were statistically lower ($p < 0.05$, Student's t-test) than the corresponding fold induction at 10⁻⁸ M estradiol for each cell line.

genes. To address this question, SRAP-V5-High.A, SRAP-V5-High.B, SRAP-V5-Low and control cells were grown in serum-free media and subsequently treated with 10⁻⁸ M estradiol for 4, 24 and 48 hr. Proteins were extracted and identical amounts of total protein lysates were analyzed by Western blot for PR expression, as described in Material and methods. As shown in Figure 4a, PR protein was extremely low or even undetectable in all 4 cell lines when grown in serum-free medium for 6 days (Fig. 0) and when treated with vehicle alone. Interestingly, upon 24 and 48 hr of estradiol stimulation, SRAP-V5-High.A and -High.B expressed noticeably higher PR levels than the control cell line¹⁹. To ensure equal loading, SDS-PAGE gels were stained with Coomassie blue, and the intensity of the staining was shown to be identical in all cell lines (data not shown). As seen in Figure 4, the SRAP-V5-Low cell line also had a higher PR induction at 24 and 48 hr than the control cell line. However, PR expression was considerably lower in the SRAP-V5-Low cell line compared to the SRAP-V5-High.A and -High.B cell lines at 48 hr.

Similar estradiol-dependent TFF1 mRNA induction in SRAP-V5-overexpressing and control cells

TFF1 is another well-characterized ER target gene, expression of which was increased by estradiol as early as 1 hr after treatment of MCF-7 cells.²⁰ To determine whether the estradiol-dependent induction of TFF1 is differentially regulated in SRAP-overexpressing cells, SRAP-V5-High.A, SRAP-V5-High.B, SRAP-V5-Low and control cells were grown in serum-free media and subsequently treated with 10⁻⁸ M estradiol for 15 and 60 min. Total RNA was extracted, reverse-transcribed and analyzed by RT-PCR using primers recognizing TFF1 cDNA as described in Material and methods. As shown in Figure 5, a similar increase in TFF1 mRNA levels was observed in all 4 cell lines upon 60 min of 10⁻⁸ M estradiol treatment.

Discussion

To date, all functional studies on SRA have focused only on its RNA aspect and were performed in transient expression systems. Here, we establish the existence of the corresponding SRAP in breast tumor tissues and examine the possible implication of SRAP expression on the ER signaling pathway.

SRAP was detected by Western blot analysis in 24 of 74 (32%) cases, migrating at around 30 kDa and appearing as either a single band or a doublet. We suspect that the diverse band pattern observed in breast tumor tissues results from the different genetic background of the patients. Indeed, we have previously demonstrated the existence of 3 SRAP isoforms, with SRA isoform 3 migrating slightly slower than the other two¹⁴. The differences in SRAP migration in the tumor samples could therefore be due to homozygosity/heterozygosity for the different SRA isoforms and/or the differential use of either the first or second methionine as described previously¹³.

In addition to the expected 30 kDa band, we observed in 34 of 74 cases (45%) a 25 kDa band specifically recognized by our antibody. Although the theoretical size of SRAP is 25 kDa, we had previously never detected a 25 kDa SRAP in any of the breast cancer cell lines grown in vitro and analyzed by Western blot.⁴ This new form of SRAP seen in breast tumor tissues cannot be attributed to an alternative translation starting at the second methionine as a 12 amino acid difference would not account for a shift in migration by 5 kDa. Similarly, the 25 kDa form cannot correspond to an alternative translation starting at the third methionine (at amino acid position 75) since this form of SRAP would not be detected by our antibody targeted against amino acids 20–34. We suspect this 25 kDa band has distinct posttranslational modifications from the ones observed on the 30 kDa SRAP. Whether both are expressed by the same cells remains to be determined. Antibodies directed against different regions of SRAP will be generated to address these issues.

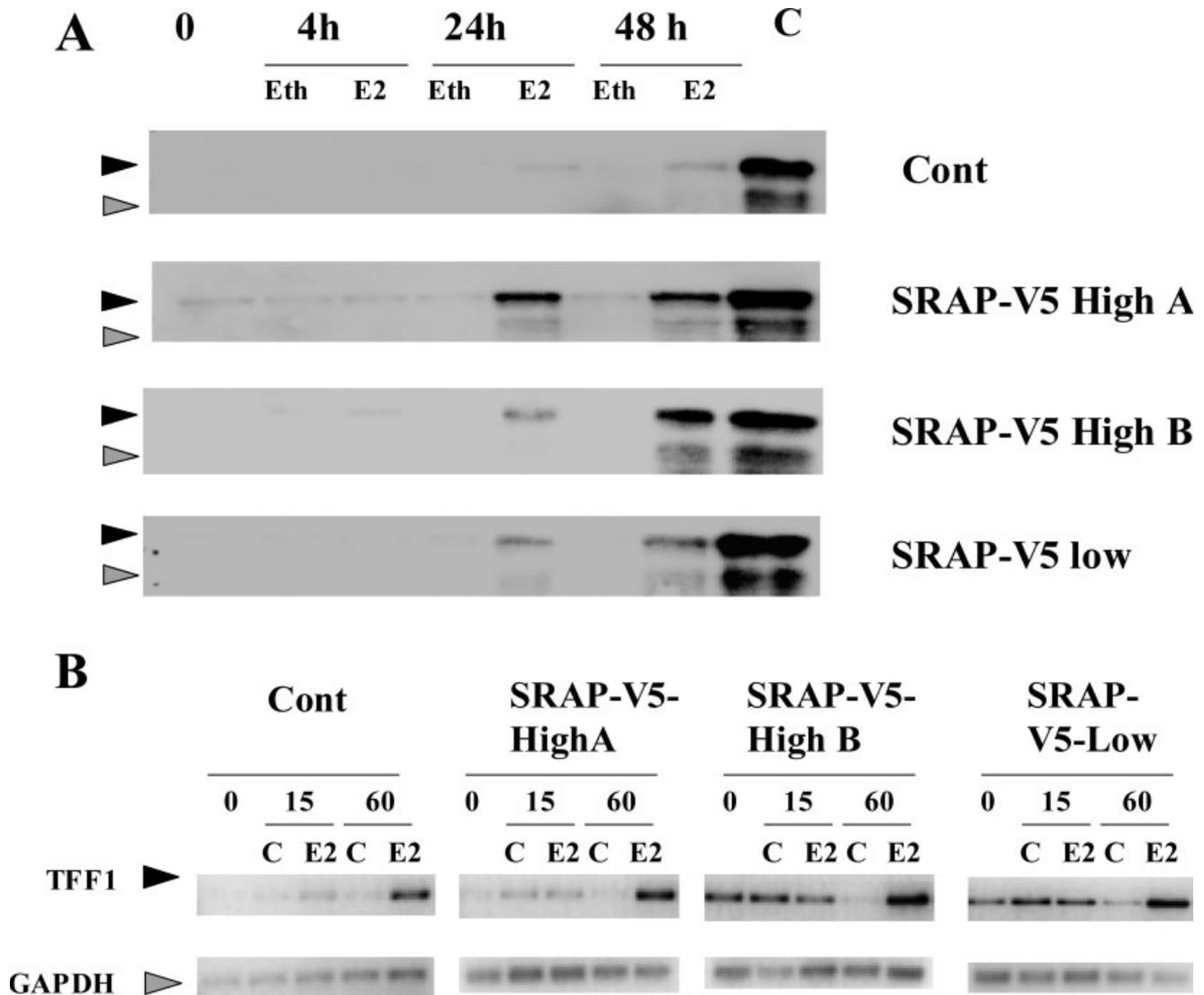


FIGURE 4 – SRAP-V5-overexpressing cells have higher PR, but not TFF1, expression upon estradiol stimulation. MCF-7 cells expressing detectable (SRAP-V5-High.A and -.B) or not detectable (SRAP-V5-Low and Cont) levels of SRAP-V5 recombinant protein were grown in serum-free medium for 6 days and subsequently treated with ethanol or 10^{-8} M estradiol for (a) 4, 24 and 48 hr or (b) 15 or 60 min as described in Material and methods. (a) Cells were lysed, and identical amounts of total protein extract were analyzed by Western blot for PR expression. An identical amount (100 μ g) of a lysate positive for PR expression was used as positive control (C). Black arrows indicate PR isoform A (112 kDa); gray arrows indicate PR isoform B (82 kDa). (b) Cells were lysed, and extracted RNAs were reversed-transcribed and then analyzed for TFF1 and GAPDH expression by PCR as indicated in Material and methods.

No significant correlations were found between the detection of any SRAP (30 kDa or 25 kDa) and levels of ER or PR or tumor grade. Similarly, no correlation was observed between the reoccurrence of disease and SRAP expression. We have, however, found that expression of the 30 kDa SRAP correlated with overall better survival in ER-positive patients subsequently treated with tamoxifen. This suggests that SRAP could be a new independent prognostic marker that might predict disease outcome. In other words, detection of SRAP in the primary tumor could be a marker of a “less aggressive” form of cancer. Further analyses, performed on larger cohorts of patients associated with different tumor subgroups, are needed to corroborate this hypothesis.

The observed correlation between SRAP expression and overall better survival in ER-positive patients prompts us to examine further the impact of the coding SRA RNA and consequently SRAP on the ER signaling pathway. Here, we show that overexpression of SRAP in breast cancer MCF-7 cells results in decreased respon-

siveness to estrogen (for all concentrations used), as assessed by activation of a transiently expressed ERE-luciferase reporter gene. This decrease cannot be attributed to a change in ER- α expression since similar levels of this receptor are detected in control and SRAP-overexpressing cells.

SRA RNA overexpression had previously been shown to potentiate ER and PR transcriptional activities.^{5,18} We therefore did not expect to observe decreased ER activity in cells stably overexpressing SRA RNA. To our knowledge, all the SRA sequences used by others when investigating SRA RNA function lacked the first 2 starting methionines and were consequently unable to encode SRAP.^{5-7,11} The SRA sequence used here contains 32 additional 5' end base pairs with 2 putative starting methionines and therefore has the capacity to initiate the translation of either a 236- or a 224-amino acid SRAP.¹⁴ This coding RNA is expected to function as an ER-activating RNA since it contains an intact SRA core sequence previously shown to be necessary and suffi-

cient for SRA RNA to function as a non-coding RNA.⁵ Our luciferase reporter assays therefore indicate that the concurrent coding SRA RNA and protein expressions result in a significant reduction of ER activity. We observed such opposite action of the coding SRA RNA from the previously reported steroid receptor coactivating function of the noncoding SRA RNA. We therefore suspect that SRAP expression is responsible for this apparent lower ER activity. Further studies are, however, needed to demonstrate this hypothesis. In addition, our results suggest that the concurrent expression of coding SRA RNA/SRAP selectively modifies the activity of estradiol-stimulated ER but does not affect the ER sensitivity to tamoxifen. This fits the observation that patients whose primary tumors expressed SRAP did not have a lower incidence in reoccurrence of the disease. Indeed, in light of our reporter gene assay, tumor cells expressing high levels of SRAP are not suspected to respond better to tamoxifen.

Similar PR levels were observed in control and SRAP-overexpressing cells when grown in complete medium. PR is an ER target gene, and an apparent decrease in ER activity, through overexpression of SRAP, was expected to lead to lower PR levels in these cells. Surprisingly however, when cells were grown in serum-free medium and subsequently treated with estradiol, PR expression was induced faster in the SRAP-V5-overexpressing cells. Although noncoding SRA RNA has previously been shown to increase PR expression and activity,⁵ it is premature to attribute the increased PR levels to expression of either the RNA or protein. Indeed, our MCF-7 cell model reflects a more complex system with concurrent actions of both SRA RNA and protein. This model, although more comprehensive, does not allow separation and analyses of the SRA RNA and protein functions. In addition, it is now increasingly apparent that coregulating molecules alter-

ing ER activity do not have a global effect but rather distinct outcomes on individual target genes.²¹ In support of this concept, we observed that the estradiol-mediated regulation of the 2 ER target genes studied (*PR* and *TFF1*) was dissimilar in the SRAP-V5-overexpressing and control cells. Indeed, while the estradiol-mediated induction of PR was evidently distinct between the SRAP-overexpressing and control cell lines, no difference in TFF1 induction was observed between the cells. Furthermore, although noncoding SRA RNA has been shown to act as an ER activator, a recent study has shown that it is only able to activate distinct ER target gene promoters.²¹ Additional studies are needed and will be performed to dissect separately the exact mechanisms of action of the SRAP and SRA RNA and subsequently analyze their respective actions on individual ER target genes. Nonetheless, our reporter assays and analyses of PR expression have demonstrated that expression of the coding SRA RNA leads to alteration in the ER signaling pathway distinct from the previously reported effect of the noncoding SRA RNA.

To date, all functional studies on SRA have focused only on its RNA aspect. Here, we establish the existence of the corresponding SRAP in breast tumor tissues and examined the possible implication of a coding SRA RNA and consequently SRAP expression on the ER signaling pathway and breast cancer progression. The discovery that SRAP might itself also be implicated in the ER signaling pathway and that its expression correlates with disease outcome emphasizes the need to actively probe the exact mechanisms of action of this increasingly complex but promising bifaceted molecule. Indeed, additional studies, examining the separate and concurrent functions and regulations of SRA RNA and SRAP, are essential to establish the clinical potential of these bifaceted molecules in the treatment of breast cancer.

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Chapter 4: The protein encoded by the functional Steroid receptor RNA activator is a new modulator of ER alpha transcriptional activity.

SRA RNA has been shown to co-activate the ligand dependent and independent action of estrogen receptor alpha activity. As described by the previously presented article, the expression of coding SRA transcripts (and consequently SRAP) led to decreased sensitivity to estradiol in MCF-7 cells. These results thus suggest that similarly to SRA RNA, SRA proteins might also participate in modulating estrogen receptor alpha activity. However while SRA RNA action on ER activity was well accepted, the role of SRAP in this pathway needed validation. The possible concurrent action of two genetically linked molecules (RNA and protein) on the same signaling pathway presented a challenge in establishing SRAP's action autonomously of its RNA counterpart.

We addressed this issue in the article entitled **The protein encoded by the functional Steroid receptor RNA activator is a new modulator of ER alpha transcriptional activity**. This article described the experimental approaches and results conducted to investigate SRAP function independently of its RNA. This article was published on March 6th 2010 in FEBS letters. I have chosen to insert this paper *verbatim* in this thesis in this chapter. Copyright permission to reproduce this article in this thesis has been obtained.

In this article, we generate two separate models to study SRAP independently of SRA RNA action. As outlined earlier, Lanz et al. showed that two series of silent mutations (SDM1 and SDM7) drastically reduced SRA-RNA ability to co-activate PR. We exploited this property and designed distinct SRA constructs allowing the discrimination

between SRA-RNA and protein functions. The constructs containing these silent mutations were thus predicted to express RNA transcripts unable to function as co-activators but encoding for SRAP. The second model followed the same strategy but to its extreme. We indeed generated a new extensively mutated artificial SRA sequence where silent mutations were introduced in every possible wobble position and thus solely expressing SRAP. Using these two models, we determined that SRAP independently of its RNA counterpart was able to coactivate ligand dependent estrogen receptor alpha activity in an ERE dependent context.



The protein encoded by the functional steroid receptor RNA activator is a new modulator of ER alpha transcriptional activity

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ABSTRACT

The steroid receptor RNA activator gene (SRA1) encodes for a functional RNA (SRA) as well as a protein (SRAP). While several groups reported on SRA-RNA mechanism of action, SRAP exact function remains to be elucidated, mainly due to a lack of studies investigating the function of the protein independently of its RNA counterpart. Using two independent models to examine its specific functions, SRAP was found to enhance estrogen receptor alpha activity in a ligand and response-element dependent manner. Our data therefore suggest that both transcript and protein products of the SRA1 gene co-modulate the transcriptional activity of steroid receptors.

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1. Introduction

The steroid receptor RNA activator (SRA) was characterized as a non-coding transcript co-activating steroid receptors [1], nuclear receptors [2–4] and the myogenic differentiation factor MyoD [5]. In vivo experiments showed that a decrease in SRA expression lead to a phenotype of myocardial contractile dysfunction in a zebrafish model [6], whereas an increase in mouse mammary gland stimulated proliferation and apoptosis [7]. Altogether, SRA is suspected to participate in normal and pathological events such as tissue differentiation and tumorigenesis.

SRA acts embedded in ribonucleo-protein complexes recruited at the promoter of target genes [1,8–10]. The ribonucleotide regions involved in SRA/protein interactions have been mapped to multiple predicted loops within the SRA core sequence [8,11]. SRA core (exon-2 to exon-5) defines the sequence necessary and sufficient for its co-activating function [1]. Lanz et al. showed that silent mutations, which alter the predicted folding of specific loops

Abbreviations: SRA, steroid receptor RNA activator; SRAP, steroid receptor RNA activator protein; ER α , estrogen receptor alpha; STR, substructure

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(STRs), decrease SRA ability to co-activate the progesterone receptor (PR) activity [11]. In particular, mutations (SDM1 and SDM7) in the predicted substructures STR1 and STR7 almost suppressed SRA activity (Fig. 1A).

Several alternatively spliced SRA transcripts have now been characterized, differing from the originally identified SRA-RNA by an extended 5' extremity [9,12,13]. In particular, we have identified coding SRA transcripts, where exon-1 harbors a methionine codon initiating an extended reading frame terminated in exon-5 [14]. The corresponding 236 amino-acids long protein (SRAP) has been detected in multiple tissues and cell lines [15]. SRAP peptides are differentially expressed in breast tumor tissue [16,17] and their expression is associated with a poor prognostic in specific patients subsets. This raises the possibility that SRAP might also be involved in breast cancer progression.

Little is known about SRAP putative functions. It has nonetheless been proposed that SRAP physically interacts with the androgen receptor and increases its activity in prostate cells [18,19]. More recently, Borth et al. reported that CT441, a Chlamydia protease, interacted with SRAP and had the ability to retain this protein in the cytoplasm [20]. They proposed that a decrease in estrogen activity might result from this sequestration of SRAP outside the nucleus. Inversely, a decrease in estrogen receptor activity was observed in breast cancer cells stably transfected with coding

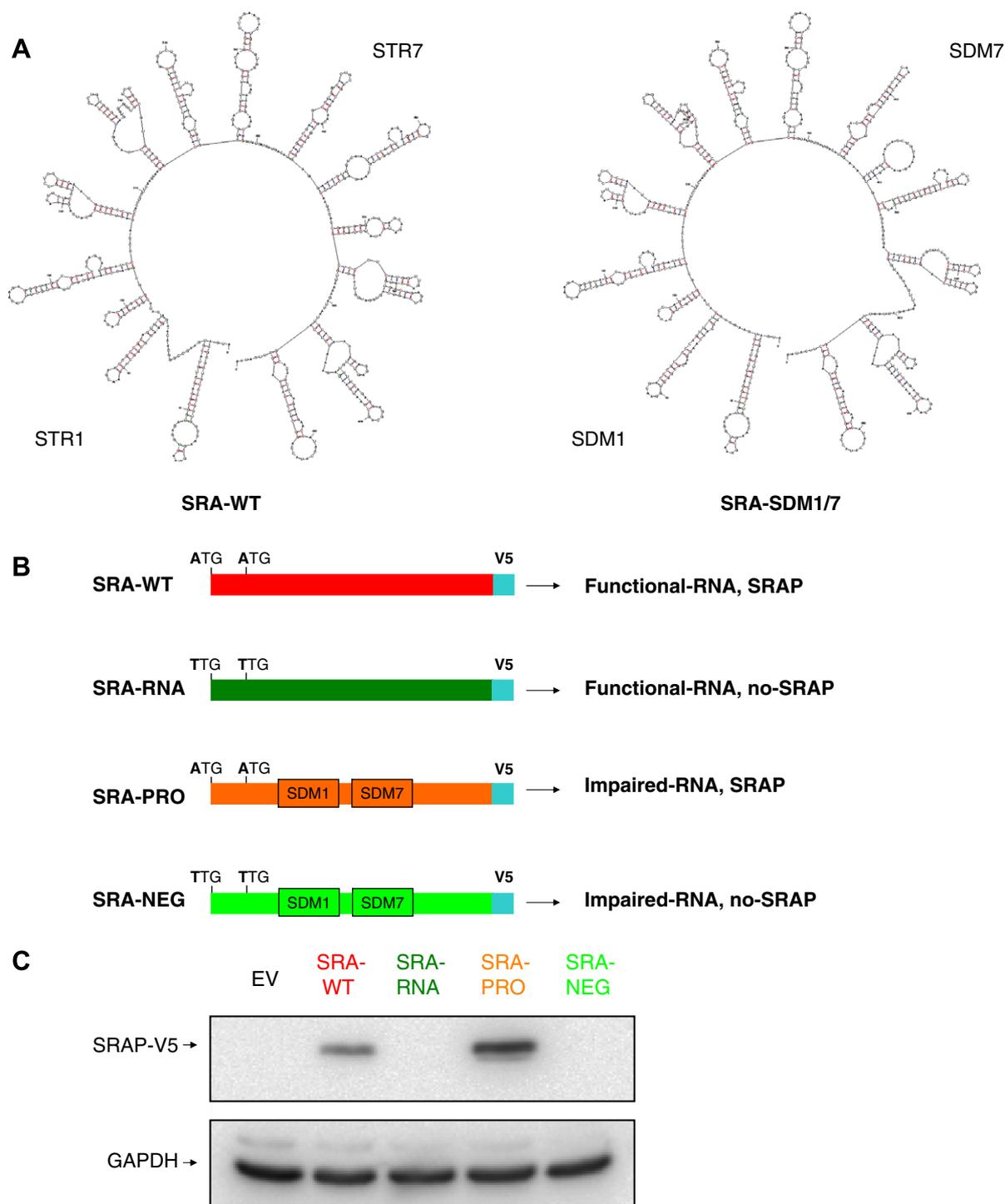


Fig. 1. Modelization of SRA secondary structures of the wild-type (WT) and STR 1 and 7 mutated (SRA SDM1/7) SRA sequences. (A) SRA substructures were visualized using the mfold software. (B) Schematic diagram representing the different SRA constructs designed to discriminate between SRA and SRAP functions. (C) Western blot analysis of HeLa cells transiently transfected with the constructs described in (B).

SRA transcripts [16]. Both SRA1 gene products, RNA as well as protein, might therefore regulate the activity of steroid receptors. It should however be stressed that many coding-SRA sequences used in previous studies contained an intact core sequence. The possibility that SRA-RNA itself could participate in the effect potentially attributed to SRAP cannot therefore be discarded.

To establish whether SRAP has the potential, independently of its RNA, to modulate the activity of steroid receptors, we herein have used two different approaches.

2. Materials and methods

2.1. Plasmids used

The pS2-ERE-luciferase and PR-ERE-luciferase vectors were kindly provided by Dr. Klinge [21]. The vitellogenin-ERE-luciferase reporter construct and the PCDNA-V5-his-SRA1 (SRA-WT) construct were previously described [16]. Sequences of EREs can be found in [Supplementary data](#). The site directed mutants, namely

SRA-RNA, SRA-PRO and SRA-NEG were generated by using synthetic oligonucleotides and the Quick change site directed mutagenesis kit (Stratagene, La Jolla, CA) using the SRA-WT vector as template following manufacturer's protocol. The oligonucleotide sequences are outlined in the [Supplementary data section](#). SRA-NEW vector sequence is outlined [Fig. 3A](#). This construct was commercially generated by GeneCopoeia Inc (Rockville, MD).

2.2. Cell culture and luciferase assays

Human cervical cancer (HeLa) cells were grown in DMEM supplemented with 5% FBS. Transfection experiments were performed as previously described [18]. Detailed protocol and statistical analysis are described in the [Supplementary data section](#).

2.3. Western blot analysis

Total cell lysates were analyzed by Western blot analysis. Transfected SRAP-V5 was detected using a mouse anti-V5 antibody in conjunction with a goat anti-mouse HRP (Sigma, St Louis, MO) antibody at dilutions of 1/5000 and 1/3000 respectively.

2.4. Low resolution structure modeling and sequence analysis

Michael Zuker's Mfold program (<http://mfold.bioinfo.rpi.edu>) was used to generate low resolution structure models. Computed SRA sequences corresponded to the exact coding sequence used, starting at the first ATG (exon-1) and ended at the TAA stop codon. The maximum distance allowed between paired bases was assigned to 54 bases.

3. Results and discussion

As outlined earlier, Lanz et al. showed that two series of silent mutations (SDM1 and SDM7) drastically reduced SRA-RNA ability to co-activate PR [11]. We exploited this property and designed four distinct SRA constructs allowing the discrimination between SRA-RNA and protein functions ([Fig. 1B](#)). SRA Wild-type construct (SRA-WT) possesses an unmodified SRA sequence, from the first initiating ATG codon in exon-1 (61 nucleotides upstream of exon-2) to the stop codon in exon-5. SRA-WT is therefore able to encode for a functional RNA (intact core) as well as for the SRAP protein. SRA-protein construct (SRA-PROT) contains a SRA sequence modified with the previously described series of silent mutations SDM1 and SDM7. This construct, whose functional RNA should be impaired, however encodes for SRAP. SRA-RNA encodes for a wild-type SRA sequence where the first two ATG are mutated to TTG in order to lose the ability to encode for SRAP. This construct encodes for a functional RNA but no SRAP protein. The fourth construct, SRA-NEG contains both mutated initiating codons as well as SDM1 and SDM7 mutations. This construct should encode for a functionally altered RNA unable to produce SRAP. All four SRA constructs contained a C-terminal V5 epitope in frame of the SRAP coding sequence in order to monitor for protein coding capacity.

Upon transient transfection, we detected the expected SRAP-V5 protein (36 Kda) solely in cells transfected with the two SRAP coding constructs, namely SRA-WT and SRA-PRO ([Fig. 1C](#)). No other proteins were detected that might have been initiated at any of the downstream five ATG codons present in SRA core sequence.

The effect of each of these constructs on ligand dependent estrogen receptor alpha transcriptional activity has then been assessed by measuring luciferase activity driven by three different estrogen receptor responsive elements (EREs). Elements chosen, found in human PR, human pS2, and *Xenopus laevis* vitellogenin

A2 genes are known to be recognized by E2-bound estrogen receptor with increasing affinity; $K_d = 3.3 \pm 0.3$ nM, $K_d = 1.6 \pm 0.02$ nM, and $K_d = 0.11 \pm 0.02$, for PR-ERE, pS2-ERE and Vit-ERE, respectively [21].

In the absence of ligand, none of the SRA constructs had any effect on the luciferase activity driven by PR-ERE or pS2 ERE ([Fig. 2A and B](#)). In contrast, when investigating vitellogenin-ERE driven reporter activity, cells co-transfected with SRA-WT, but not with other constructs, had a significantly ($P = 0.02$) higher basal ESR1 activity than cells transfected with empty vector ([Fig. 2C](#)). This suggests that the co-expression of SRAP and its functional RNA can lead, on specific EREs, to a co-activation of ESR1 in the absence of ligand.

Upon estradiol treatment, cells co-transfected with SRAP encoding SRA-WT or SRA-PROT had, for each ERE, a significantly higher ESR1 transcriptional activity (Student's *T*-test, two-sided, $P < 0.05$) than cells co-transfected with empty vector ([Fig. 2A–C](#)). For EREs with lower affinity, namely PR-ERE ([Fig. 2A](#)) and pS2-ERE ([Fig. 2B](#)), this effect was not seen upon co-transfection with non-coding SRA-RNA or SRA-NEG. The selective removal of the first

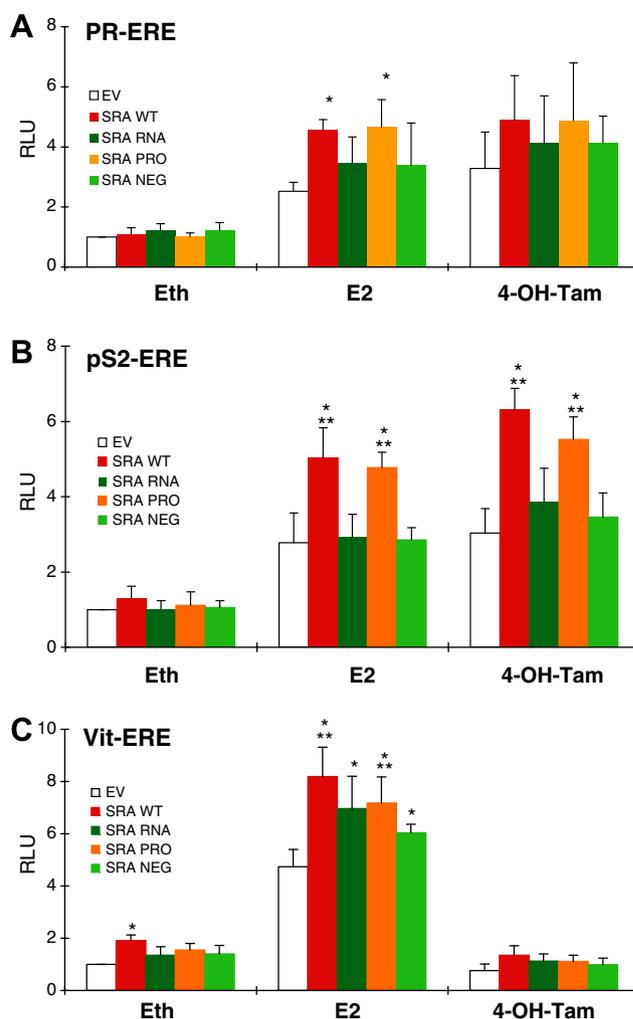


Fig. 2. SRAP differentially upregulates unbound and agonist/antagonist bound estrogen receptor alpha activity in an ERE dependent manner. (A) HeLa cells were co-transfected with the PR-ERE-luciferase reporter vector together with the SRA constructs illustrated in [Fig. 1B](#) as described in the materials and method section. Bars and stars represent standard deviations and * and ** represent significant (Student's *t*-test, $P < 0.05$) difference with EV control or SRA-NEG, respectively. (B) Same experimental procedure as in (A) but using the pS2-ERE-luciferase vector. (C) Same experimental procedure as in (A) but using the vitellogenin-ERE-luciferase vector.

two ATG is therefore sufficient to abolish the co-activation properties of the products of SRA-WT and SRA-PROT. These data fit with results obtained by Klinge et al. who found that SRA-RNA was unable to co-activate ESR1 pS2- nor PR-ERE driven activity [21]. These results also establish that SRAP as opposed to SRA-RNA potentiates Estrogen bound estrogen receptor transcriptional activity assessed

through the expression of these lower affinity ERE-luciferase reporter vector.

Non-coding SRA-RNA and SRA-NEG construct however significantly enhanced estrogen liganded ESR1 activity measured on the higher affinity vitellogenin-ERE (Fig. 2C). The co-activation seen in the absence of encoded protein confirm that SRA, as an

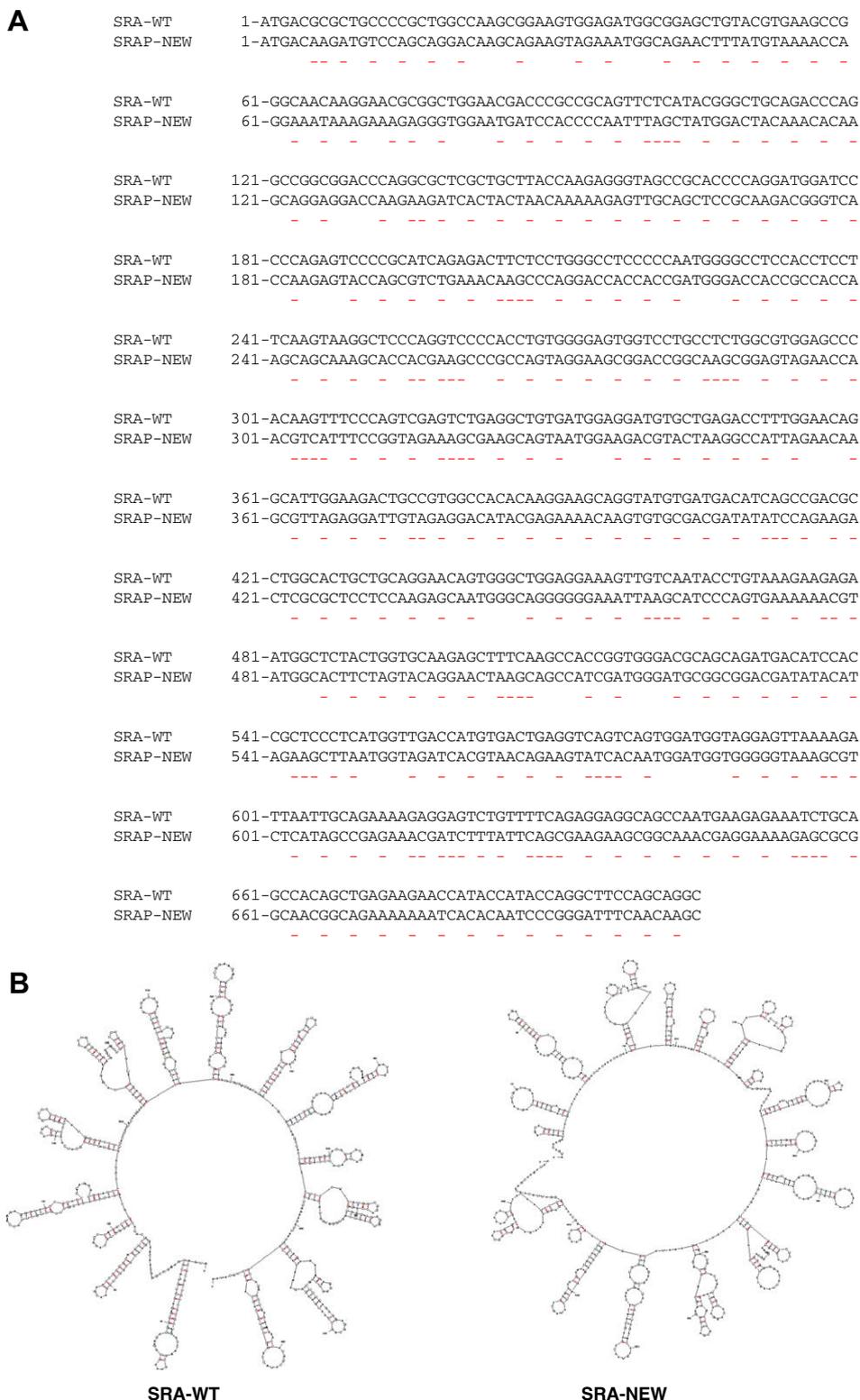


Fig. 3. A SRA-NEW sequence encoding solely for SRAP. (A) Alignment of the wild-type (WT) and the new artificial (SRA-NEW) sequence containing extensive silent mutations. (B) Modelization of the impact of these mutations on SRA-RNA structure.

RNA, has the ability to co-activate ESR1 activity [1,21,22]. Surprisingly, SRA-NEG construct, originally designed to encode for a non-functional RNA, was still able to enhance estrogen bound ESR1 activity. Alterations in the STR-1 and STR-7 are therefore not sufficient to completely abolish SRA-RNA effects in this high affinity vitellogenin-ERE context. As previously shown for several co-activators, including SRA, it appears that co-activation abilities are potentially linked to the binding affinity of the receptor considered [21]. Further studies are needed to clarify this issue. SRA-RNA effect does not allow to unequivocally establish whether SRAP has the potential to co-activate vitellogenin-ERE driven estrogen receptor activity. It should however be noted that the previously described effect of SRA-WT only on ligand independent estrogen receptor alpha activity (Fig. 2C, ethanol) suggests that a complementation might happen between RNA and protein activity.

We found that 4-OH-Tam had an agonistic effect and increases ESR1 activity on both PR- and pS2-ERE reporter (Fig. 2A and B). This agonistic effect was not significantly modified by either SRA-RNA or SRAP when assessing PR-ERE luciferase activity

(Fig. 2A). In contrast, we found that constructs encoding for SRAP (SRA-WT and SRA-PRO) but not SRA-RNA (SRA-RNA and SRA-NEG) significantly enhanced tamoxifen bound ESR1 activity as compared to the empty vector and compared to SRA-NEG (Fig. 2B). This suggests that SRAP has the ability to potentiate some of the agonistic effects of tamoxifen. Interestingly, we have recently shown that high expression of different endogenous SRAP peptides in estrogen receptor positive breast tumors was a poor prognostic marker in breast cancer patients treated with tamoxifen [17]. It is therefore possible that SRAP peptides, through increasing the agonistic tamoxifen action on selective ER target genes, might contribute to a “more aggressive tumor phenotype”. Further studies are needed to establish the validity of this hypothesis.

To determine the exact contribution of SRAP on the co-activation of ESR1 effect on the vitellogenin-ERE reporter, we designed a new extensively mutated artificial SRA sequence (SRA-NEW) where silent mutations were introduced in every possible wobble position (Fig. 3A). This led to a drastic change (Fig. 3B) in all predicted SRA secondary structures. SRA-NEW construct is therefore

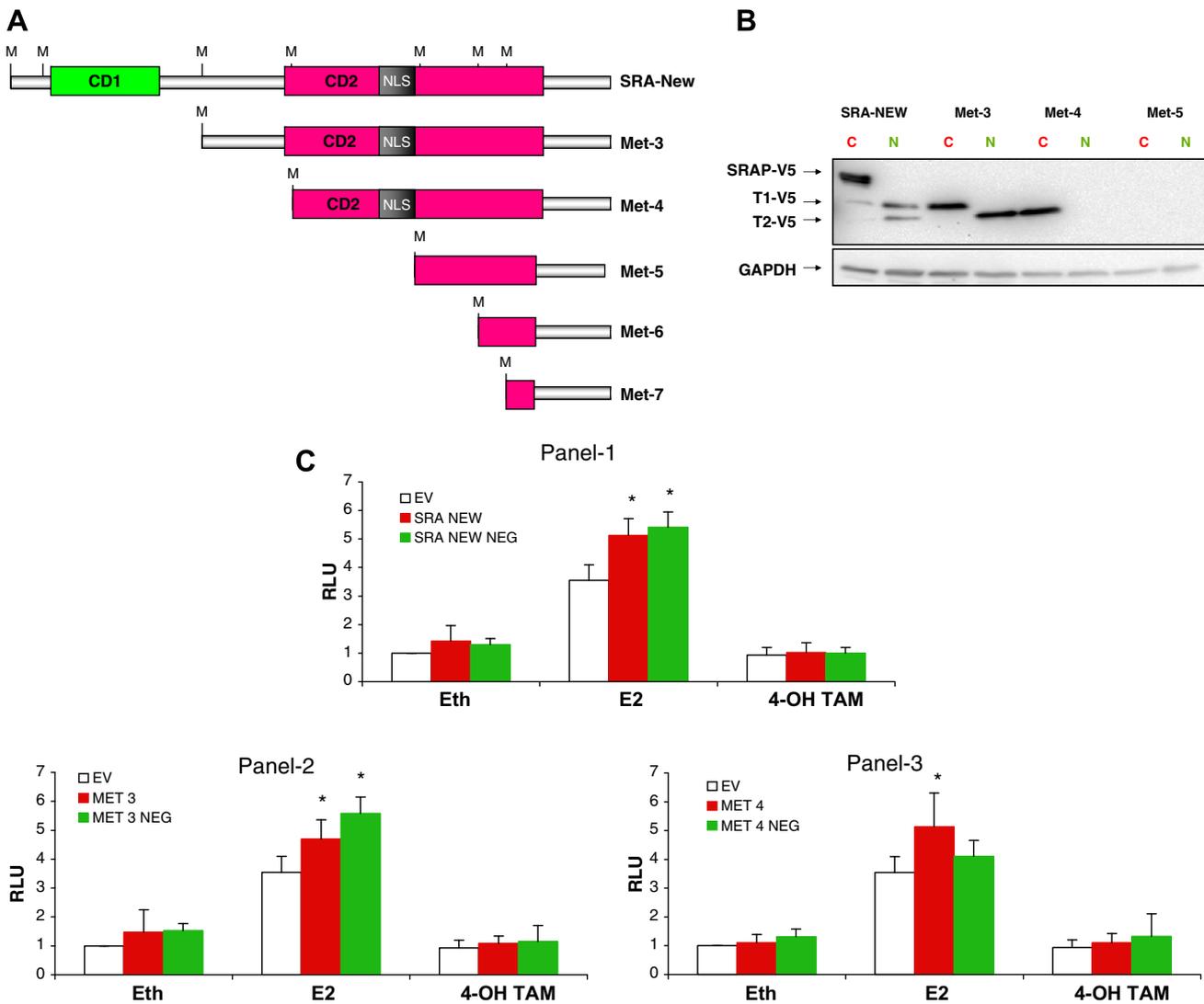


Fig. 4. SRAP potentiates ESR1 transcriptional activity. (A) Schematic diagram of the new full length and truncated SRAP constructs. The two conserved domains are indicated by CD1 and CD2. NLS: nuclear localization signal. (B) Western blot analysis of HeLa cells transfected with the different SRAP-NEW constructs. C and N indicate constructs with intact ATG and ATG mutated to TTG respectively. T1 and T2 refer to the 26 and 23 kDa truncated SRAP-V5. (C) Full -length SRAP as well as the two truncated SRAP starting at methionine 3 and 4 enhance ESR1 ligand dependant activity on the vitellogenin reporter vector. Panel1, panel 2 and panel 3 illustrate results for the SRA-NEW and SRA-NEW-NEG, Met-3 and Met-3 Neg and Met-4 and Met-4 Neg constructs respectively. Bars represent standard deviations. * represents significant (Student's *t*-test, *P* < 0.05) difference with EV control.

predicted to produce a RNA very unlikely to be functional but still encoding for SRAP. As control, a similar construct (SRA-NEW-NEG) where the two initiating ATG codons have been mutated was obtained.

Transient transfection led to a 36 kDa SRAP-V5 tagged protein (Fig. 4B lane 1). Two additional fainter bands, migrating at 26 and 23 kDa, were also detected. Similar bands were detected when cells were transfected with SRA-NEW-NEG (Fig. 4B lane 2). As depicted Fig. 4A, SRAP sequence contains seven in frame ATG codons. We suspect that in the absence of the first two ATGs, downstream ATG codons can act as alternative start codons in this extensively mutated SRA sequence. It should be noted that in the original wild-type SRA sequence, this phenomenon does not occur (Fig. 1C). A series of 5' deleted mutants starting at each in frame methionine codon (MET-3 to MET-7) was generated (Fig. 4A). Corresponding negative controls were obtained by mutating the initiating ATG to TTG. As shown Fig. 4B, mutating the third methionine resulted in the loss of the 26 kDa protein but did not affect the presence of the 23 kDa SRA protein. Only mutation of the fourth ATG to TTG however led to the loss of this peptide.

Altogether, we believe that detected truncated proteins resulted from the use of alternative starts at methionines 3 and 4. No detectable protein starting from methionine 5, 6 or 7 was detected (Fig. 4B and data not shown).

The effect of each of these constructs on ESR1 transcriptional activity on vitellogenin-ERE was assessed as described earlier. Both SRA-NEW and SRA-NEW-NEG significantly enhanced ESR1 transcriptional activity as compared to the empty vector ($P = 0.0006$ and $P = 0.0001$, respectively) (Fig. 4B panel 1). This suggests that the full-length SRAP as well as the two shorter SRA peptides are able to co-activate estradiol dependent ESR1 activity. SRA-MET-3, SRA-MET-3-NEG and SRA-MET-4 indeed significantly enhanced estradiol dependent ESR1 transcriptional activity ($P = 0.008$, $P = 0.00008$ and $P = 0.01$, respectively). Removal of the fourth methionine (SRA-MET-4-NEG), which led to a loss of any detectable protein expression, also resulted in the suppression of ESR1 co-activation. SRA-MET-5, SRA-MET-6, and SRA-MET-7 did not express any detectable protein nor co-activate estradiol bound ESR1 transcriptional activity (data not shown). Overall, our results demonstrate that SRAP can potentiate ESR1 activity on the vitellogenin-ERE independently of SRA. Furthermore, the C-terminal SRAP sequence starting from the fourth methionine is sufficient for this effect. In support of this hypothesis, Kawashima et al. have shown that a short rat SRAP (starting at methionine 3) was able to co-activate androgen receptor's transcriptional activity [18]. As shown Fig. 4A, the region sufficient in potentiating ESR1 activity contains the second conserved SRAP domain [9,15]. Interestingly, this domain contains a nuclear localization (NLS) motif as well as a LXXAL motif, shown on another nuclear receptor co-activator NcoA62 to be responsible for its direct interaction with nuclear receptors [23]. Further functional studies are needed to examine the exact contribution of the NLS and the LXXAL motifs in SRAP's ability to potentiate E2 dependent ESR1 activity. It should be noted that recent work by Borth et al. suggests that SRAP nuclear localization could be impaired by binding of SRAP to the Chlamydia protease CT441 [20]. These authors proposed that it might provide a new way to address SRAP function independently of its RNA.

Overexpression of both SRA-RNA and protein in MCF-7 cells resulted in a decreased ESR1 transcriptional activity as measured by the same vitellogenin-ERE luciferase vector as the one used in this study [16]. We had attributed the decrease in ESR1 activity to the concomitant expression of both SRA and SRAP. The disparity in SRAP function (repressor versus activator) observed between the previous and current studies could be due to the difference in cell lines used (MCF7 versus HeLa) and/or the transfection techniques used (stable versus transient SRA transfection). Moreover, increas-

ing evidence suggests that target gene promoter, tissue and cell lines contexts can lead to functional inversion among co-regulators [24]. As such, co-activators can become co-repressors and vice versa.

In this study, we are for the first time demonstrating that SRAP increases ESR1 transcriptional activity independently of its RNA counterpart. SRAP and SRA functions therefore appear intertwined and both involved in the regulation of the same signaling pathway. SRA1 gene belongs to a recently discovered class of genes encoding both functional RNA and protein [25–27]. We will only understand the full implication of such systems when both of their faces are fully explored.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2010.02.024](https://doi.org/10.1016/j.febslet.2010.02.024).

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Chapter 5: The steroid receptor RNA activator protein is recruited to promoter regions and acts as a transcriptional repressor.

Products of the steroid receptor RNA activator (SRA1) gene have the unusual property to function both at the RNA and the protein levels. SRA-RNA has long been known to increase the activity of multiple nuclear receptors (Leygue 2007). As discussed in the previously presented article, it has more recently been established that the steroid receptor RNA activator protein (SRAP) also modulates steroid receptors activity. There is consequently a need to explore SRAP possible mechanisms of action. With this goal in mind we directed our research to explore the emerging mechanisms of SRAP action.

The experimental approaches and results to survey SRAP mechanisms of action are detailed in the article entitled **The steroid receptor RNA activator protein is recruited to promoter regions and acts as a transcriptional repressor**. This article was published on June 3 2010 in FEBS letters volume 584 issue 11 pages 2218 to 2224. I have chosen to insert this paper verbatim in this chapter. Copyright permission to reproduce this article in this thesis has been obtained.

In this article, we show for the first time that SRAP physically interacts with multiple transcription factors with distinct binding affinities. We have also shown for the first time that SRAP associates to the chromatin and we have identified specific promoter regions bound to SRAP. In order to determine the effect of SRAP on transcription we artificially recruited SRAP to the promoter of a luciferase reporter gene under the control of the strong transcriptional activator VP16. SRAP's recruitment in this model led to a decrease in transcription. Altogether our results therefore suggest that SRAP could be a new

transcriptional regulator, able to function as a repressor through direct association with promoters.



The steroid receptor RNA activator protein is recruited to promoter regions and acts as a transcriptional repressor

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ABSTRACT

Products of the steroid receptor RNA activator (SRA1) gene have the unusual property to function both at the RNA and the protein levels. SRA-RNA has long been known to increase the activity of multiple nuclear receptors. It has more recently been proposed than steroid receptor RNA activator protein (SRAP) also modulates steroid receptors activity. Herein, we show for the first time that SRAP physically interacts with multiple transcription factors and is recruited to specific promoter regions. Artificially recruiting SRAP to the promoter of a luciferase reporter gene under the control of the strong transcriptional activator VP16 leads to a decrease in transcription. Altogether we propose that SRAP could be a new transcriptional regulator, able to function as a repressor through direct association with promoters.

Structured summary:

MINT-7761068: SRAP (uniprotkb:Q9HD15) physically interacts (MI:0915) with HDAC2 (uniprotkb:Q92769) by anti bait coimmunoprecipitation (MI:0006)

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1. Introduction

The steroid receptor RNA activator (SRA) has originally been characterized as a non-coding transcript specifically co-activating steroid receptors [23,25]. This transcript acts embedded in ribonucleo-protein complexes containing steroid receptors as well as other proteins, such as the RNA helicase p68 or the SRA stem-loop interacting RNA binding protein (SLIRP), physically interacting with SRA and acting either as positive or negative functional regulators [2,9,25]. Secondary sub-structures (STRs) located within SRA core sequence (exons 2–5) are critical for these physical interactions to occur and therefore directly contribute to the co-activator role of this messenger [24]. It has now been demonstrated that SRA-RNA regulates the activity of additional transcription factors, including other nuclear receptors (such as the vitamin D (VDR)

Abbreviations: SRA, steroid receptor RNA activator; SRAP, steroid receptor RNA activator protein; HDAC, histone de-acetylase; TSA, trichostatin A

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and the retinoic acid (RAR) receptors) and the myogenic differentiation factor MyoD [25]. This functional transcript therefore appears to have a much broader role than originally anticipated, participating in multiple normal and pathological events including tissue differentiation and tumorigenesis [2,9,25].

Coding SRA-RNAs, differing from the originally described non-coding RNA by an extended exon-1, have now been characterized [5,11,25]. This additional sequence contains two methionine codons, respectively, initiating a 236 and a 224 amino-acids long open reading frame terminated in exon-5. The corresponding endogenous proteins steroid receptor RNA activator proteins (SRAPs), that has been detected in multiple tissues and cell lines [6,22,34], contain two phylogenetically conserved domains (amino-acids 15–52 and amino-acids 135–204 [25]). This strong conservation suggests that both domains contribute to SRAP potential functions.

Most studies only focused on SRA-RNA and little is known about SRAP putative functions. It has nonetheless been proposed that SRAP, as its RNA counterpart, might also modulate the activity of steroid receptors [1,7,20,22]. SRAP was indeed found to physically interact with the androgen receptor and to enhance its ligand-induced transcriptional activity in prostate cells [20,22]. Transient

transfection experiments and reporter assays have further shown that SRAP could also enhance the transcriptional activities of estrogen (ESR1), glucocorticoid (GR) and peroxisome proliferator-activated receptor gamma (PPAR γ) receptors [7,20,22]. Altogether, SRAP is currently suspected to act as positive regulator of several nuclear receptors.

The potential involvement of SRAP in mechanisms underlying transcriptional regulation is further substantiated by the identity of proteins characterized as co-immunoprecipitating with SRAP in HeLa cells [19,25,28]. Known transcriptional regulators, such as SMARCC2 (member of the SWI/SNF chromatin remodeling complex, [35]) or the RNA helicase p68 [16], have indeed been identified as forming complexes with SRAP [19,28]. Most interestingly, the identification of the myocyte enhancer factor 2A (MEF2A) as one of SRAP protein partners further suggested that, beside androgen receptor, SRAP might also physically interact with other transcription factors. This would imply that SRAP, as its RNA, might modulate the transcriptional activity of a much wider range of transcription factors than initially predicted. Herein, we first used protein array to investigate SRAP potential interactions with multiple transcription factors and Chromatin immunoprecipitation (ChIP) to identify promoter regions potentially recruiting SRAP. We further assessed the potential effect of recruiting SRAP to the promoter region of an actively transcribed reporter gene.

2. Materials and methods

2.1. Plasmids

CMV-Renilla-luciferase, LexA-VP16, and L8G5-luciferase plasmids were previously described [3,6,17]. SDM1 and SDM7 mutations, impairing SRA-RNA action [24], were introduced in SRAP coding sequence using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) to generate SRAP-SDM as described [7]. GAL4-SRAP-SDM, GAL4-SRAP-N and GAL4-SRAP-C were obtained by, respectively, cloning full-length SRAP-SDM, aa 1–100, and aa 101–236 in frame with an existing GAL4 coding plasmid as described for the Receptor-Interacting Protein 140 [3].

2.2. Reporter assays

LexA-VP16 Luciferase reporter assays were performed using HeLa cells as previously described [3,17]. Briefly, HeLa cells were co-transfected with 0.1 μ g of L8G5-luciferase, 0.05 μ g LexA-VP16, 0.025 μ g Renilla luciferase and 0.1 μ g of GAL4, GAL4-SRAP-SDM, GAL4-SRAP-SDM-N, or GAL4-SRAP-SDM-C. Trichostatin A (TSA, Sigma, St. Louis, MO) was added to the indicated concentrations 16 h before lysis. Cells were harvested 24 h after transfection and Renilla luciferase and luciferase activities assessed and analyzed as previously described [3,17]. Following normalization to Renilla luciferase (accounting for transfection efficiencies), relative luciferase activity (RLU) of cells transfected with GAL4 alone with LexA-VP16 was arbitrarily assigned as 1. Luciferase activities within cells transfected with other vectors were expressed relatively. Results represent the average of at least three independent experiments performed in triplicate. Standard deviations were calculated and differences between results obtained with the various constructs and control (GAL4) were tested using the Student's *t*-test (two-tailed distribution, two-sample equal variance).

2.3. Immunoprecipitation and histone de-acetylase (HDAC) activity assay

Human breast cancer cell lines expressing SRAP-V5-tagged (MCF-7-SRAP-V5-High.A) and control MCF-7-SE cells were cultured as previously described [6]. Nuclear proteins were isolated

(Panomics, Redwood City, CA) and incubated with agarose-beads-anti-V5 antibodies (Sigma, St. Louis, MO) according to the manufacturer's instructions to immunoprecipitate SRAP-V5 containing complexes. As an additional control, MCF-7-SRAP-V5-High.A extract was immunoprecipitated in the presence of V5 peptide (350 excess binding capacity of beads). HDAC activities were assayed using the fluorometric HDAC assay kit (Abcam, Cambridge, MA) as indicated by the manufacturer. For each experiment ($n = 4$) and each cell type, the immunoprecipitated HDAC activity was expressed as a percentage of the value before immunoprecipitation and normalized to the average residual background signal obtained with MCF-7-SE cells. Results correspond to the average immunoprecipitated HDAC activity expressed as percentage of total activity. Significant differences ($P < 0.05$) between samples were assessed using the Student's *t*-test (two-tailed distribution, paired).

Nuclear extracts from MCF-7 breast cancer cells were prepared and endogenous SRAP immunoprecipitated using a rabbit polyclonal anti-SRAP antibody (#A300-743A, Bethyl Laboratories Inc., Montgomery, TX) as described [11,33]. Irrelevant isotype matched antibodies (rabbit anti-goat IgGs, Jackson ImmunoResearch Lab-Inc., West Grove, PA) were used as non-specific control. Presence of HDAC-2 in co-immunoprecipitated lysate was assessed by Western blot (Cell Signaling Technology Inc., Boston, MA) as previously reported [11,33].

2.4. Transcription factor protein array

Transcription factors protein-array analysis (TF-array, Panomics, Redwood City, CA) was performed as per manufacturers' instructions. Briefly, TF-array-I was incubated with 5 μ g of recombinant SRAP protein (ProMab Biotechnologies Inc., Albany, CA) or without (control blot) for 2 h at room temperature. Immunodetection was performed using a primary anti-SRAP antibody (targeting aa 20–34) and chemiluminescence signals were captured and analyzed as described [6]. For both control and samples blots, intensity of the area (in counts/mm²) encompassing the two spots corresponding to each transcription factor was first corrected by subtracting the signal of an immediately negative adjacent area (local background). For each transcription factor, the relative interaction (RI) was then determined by subtracting signal-control from signal-sample. Interactions were arbitrarily classified as strong (+++, RI > 15 000 counts/mm²), positive (++, 1000 < RI < 10 000 counts/mm²), intermediate (+, 400 < RI < 1000 counts/mm²) and weak/negative (RI < 400 counts/mm²).

2.5. Isolation of DNA-associated proteins by formaldehyde cross-linking

DNA-associated proteins from MCF-7-SRAP-V5-High.A cells were isolated as previously described [31]. As a control for non-specific precipitation by hydroxy-apatite, not cross-linked cells extracts were used. Proteins precipitated with hydroxy-apatite were detected by Western blot using Anti-SRAP antibodies, anti-SP3 and anti-GAPDH antibody as described [6].

2.6. Chromatin immunoprecipitation assays and analysis

Chromatin immunoprecipitation assays were conducted as described [33]. Briefly, MCF-7 cells stably expressing SRAP-V5 cells were cultured in complete DMEM supplemented with 5% FBS. Cells were treated with formaldehyde in order to cross-link protein to DNA. Cells were then lysed and nuclear extracts were sonicated. The supernatants were then incubated overnight with a mouse anti-V5 antibody (Invitrogen) or the V5 antibody pre-incubated with the V5 peptide (negative control) (In a 100-fold excess). The immuno-complexes were collected by addition of protein

G-Sepharose (Amersham). The proteins were digested with proteinase K. DNA fragments were purified with a QIAquick Spin Kit (Qiagen, CA) and amplified by using the WGA amplification kit (Sigma, St. Louis, MO) according to the manufacturers protocol. The precipitated samples were sent to Nimblegen for Chromatin immunoprecipitation on CHIP (ChIP on CHIP) analysis using the human ChIP 385K Refseq promoter array (Nimblegen). Promoter regions that were enriched by at least 4-folds (peak score >2) in the SRAP-V5 precipitated sample compared to the negative control were further analyzed. Using the Genomatix RegionMiner software (Ann Arbor, MI), we determined the frequency of transcription factor binding sites and their over-representation against an average corresponding to the population of all annotated promoter regions. Obtained Z-score corresponds to the distance from the promoter population mean in units of the population standard deviation.

3. Results and discussion

3.1. SRAP interacts with multiple transcription factors

The fact that SRAP was found to directly interact with the androgen receptor [20] and form complexes with another known transcription factor MEF2A [19,28], led us to investigate whether it could also interact with other transcription factors. Using protein array (Fig. 1), we found that 29 out of 48 different transcription factors tested directly interacted with recombinant SRAP. The strength of these interactions varies upon the transcription factor considered. As such, SRAP appears to more strongly interact with ESR2 than with ESR1 (Table 1). This raises the interesting concept

that in specific contexts, the relative amount of estrogen receptor beta might interfere with the formation of complexes between SRAP and estrogen receptor alpha. This possibility might become of particular interest when considering the differential expression and roles played by these two receptors in breast tumorigenesis and tumor progression [8,30]. The observation that SRAP directly interacts with estrogen and glucocorticoid receptors fits with previous data from Kawashima et al. showing a direct interaction between SRAP and androgen receptor. This suggests that SRAP has the ability to modulate the action of steroid receptors (ESR1, AR and GR) likely through direct interactions.

Beside steroid receptors, several other transcription factors such as FOS [27], GATA1 [32] and ETS1 [10], known to participate in critical normal developmental steps or to events underlying tumorigenesis, directly interact with SRAP (Table 1). It is of interest that an interaction has been observed between SRAP and HAND1, essential to heart development [13]. Indeed, a recent study has

Table 1

Transcription factors analyzed for their direct physical interaction with SRAP.

Transcription factor description		Relative interaction ^a
AES	Amino-terminal enhancer of split	61 –
AP2A	Transcription factor AP-2 alpha	2346 ++
ASH2L	Ash2 (absent, small, or homeotic)-like	316 –
ATF1	Activating transcription factor 1	810 +
ATF2	Activating transcription factor 2	996 +
ATF3	Activating transcription factor 3	595 +
ATF4	Activating transcription factor 4	1158 ++
BLZF1	Basic leucine zipper nuclear factor 1	965 +
BTG2	B-cell translocation gene 2	465 +
C/EBPα	CCAAT/enhancer binding protein alpha	739 +
CART1	Cartilage paired-class homeoprotein	840 +
CBFB	CBFB: core-binding factor, beta subunit	0 –
CDX2	CDX2: caudal type homeo box transcription factor 2	0 –
CERM	cAMP responsive element modulator	146 –
CREB1	cAMP responsive element binding protein 1	1051 ++
CREBL2	cAMP responsive element binding protein-like	0 –
CRSP9	Cofactor-required for Sp1 transcriptional activation, subunit 9	0 –
DDIT3	DNA-damage-inducible transcript 3	2594 ++
DLX4	Distal-less homeobox 4	172 –
DMTF1	Cyclin D binding myb-like transcription factor	2663 ++
DR1	Down-regulator of transcription 1, TBP-binding	326 –
E2F3	E2F transcription factor 3	3286 ++
E2F4	E2F transcription factor 4	0 –
E2F5	E2F transcription factor 5	0 –
E2F6	E2F transcription factor 6	0 –
EGR1	Early growth response 1	3373 ++
EGR2	Early growth response 2	913 +
EGR4	Early growth response 4	398 –
ELK	ELK1, member of ETS oncogene family	0 –
ESR1	Estrogen-related receptor gamma	2805 ++
ESR2	Estrogen receptor alpha	25 289 +++
ERRg	Estrogen receptor beta	1954 ++
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1	1607 ++
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2	435 +
F2RL1	Coagulation factor II (thrombin) receptor-like 1	0 –
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	1126 ++
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	1468 ++
FOSL1	FOS-like antigen 1	0 –
FOSL2	FOS-like antigen 2	2097 ++
GATA1	GATA binding protein 1	2215 ++
GCNF	Nuclear receptor subfamily 6, group A, member 1	125 –
GMEB1	Glucocorticoid modulatory element binding protein 1	0 –
GR	Glucocorticoid receptor	735 +
GTF2B	General transcription factor IIB	5478 ++
GTF2H2	General transcription factor IIIH, polypeptide 2	578 +
GTF2I	General transcription factor II, I	0 –
GTF3C5	General transcription factor IIIC, polypeptide 5	1195 ++
HAND1	Heart and neural crest derivatives expressed 1	16 784 +++

^a High (+++), positive (++) , intermediate (+) and weak/negative (–) interaction.

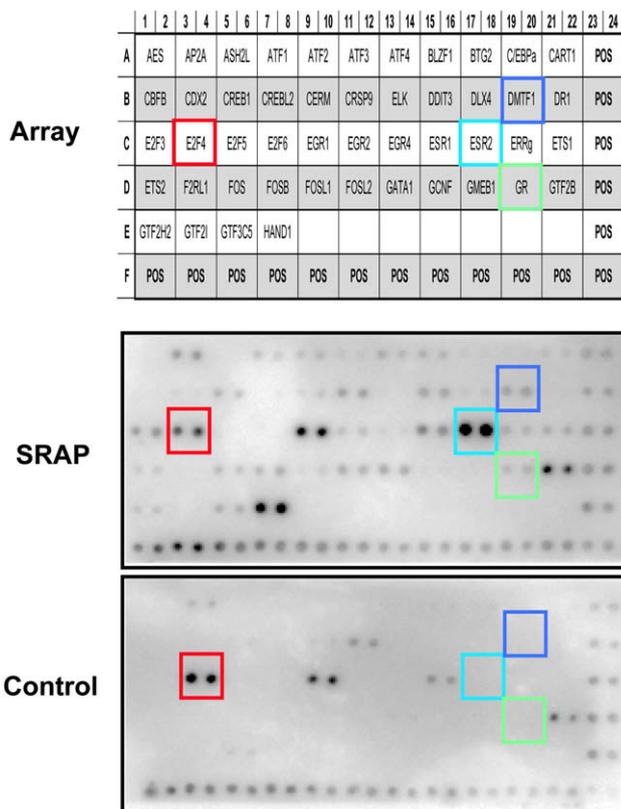


Fig. 1. SRAP directly interacts with transcription factors in vitro. A protein array containing 48 transcription factors spotted in duplicate was incubated with human recombinant SRAP (SRAP) or no protein (control) before immunodetection with anti-SRAP antibodies as outlined in Section 2. Following signal analysis, interactions with SRAP were qualified as high (+++, light blue box), positive (++, dark blue box), intermediate (+, green box) or weak/negative (–, red box). Relative interactions are detailed in Table 1.

shown that knocking down SRA gene led to myocardial contractile dysfunction in zebrafish, linking for the first time SRA and heart diseases [15]. The wide range of transcription factors identified as interacting with SRAP suggests that many normal and pathogenic events might involve this newly described protein.

In normal conditions, most transcription factors and nuclear receptors are localized and exert their functions in the nucleus. In breast cancer cells, SRAP, which contains a nuclear localization signal, is detected both in the cytoplasm and the nucleus [11,25,34]. The potential for this protein to localize in the nucleus, further demonstrated by the identification of SRAP and many of its co-immunoprecipitated partners in nuclear extracts from Hela cells [19,28], implies a potential role in this cellular compartment. Altogether, this led us to investigate the possibility of a recruitment of SRAP directly on chromatin.

3.2. SRAP is associated with chromatin

We used a previously described assay consisting in cross-linking DNA and associated proteins, precipitating DNA, and analyzing co-precipitated proteins using Western blot [31]. We found that both exogenous tagged and endogenous SRAP precipitated with DNA in cells stably expressing SRAP-V5 tagged protein (MCF-7-SRAP-V5-High.A cells [6], [Supplementary material, Fig. S1](#)). As anticipated, both long (SP3L) and short (SP3M) transcription factor SP3 isoforms, known to be associated with DNA, were also de-

tected in the DNA bound protein fraction. None of these associations were observed in the absence of cross-linking. On the other hand, the mainly cytoplasmic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was present in the protein lysate but not associated with DNA. The fact that SRAP could indeed be associated with chromatin led us to investigate further whether SRAP might be associated to specific promoter regions.

3.3. SRAP binds to promoter regions enriched in nuclear receptor binding sites

To identify promoter regions potentially recruiting SRAP, we performed Chromatin immunoprecipitation (ChIP)-CHIP arrays. Breast cancer cells stably expressing SRAP-V5 tagged protein [6] were treated, DNA precipitated with anti-V5 antibody, and recognized promoter regions identified as described in Section 2. As a negative control, we used a V5 antibody pre-incubated with 100-fold excess V5 peptide. We were able to identify 2319 regions that were enriched at least by four folds in the SRAP-V5 chromatin immunoprecipitated samples as compared to the negative control ([Supplementary material, S2](#)). The transcription factor binding sites present within these sequences have been sorted according to their over-representation coefficient (Z-score). A list of the name of transcription factor families corresponding to the 40 most over-represented binding sites out of the 176 present is shown [Table 2](#). Binding sites for nuclear receptors (in italics) are highly enriched in

Table 2

Partial list of transcription factor (TF) binding sites families found in 2319 sequences identified following ChIP-CHIP array.

Family of TF	Number of sequences	Number of matches	Z-Score ^a	Transcription factor families full name	Rank/176
V\$NR2F	1652	3300	11.33	<i>Nuclear receptor subfamily 2 factors</i>	1
V\$GREF	1057	1856	9	<i>Glucocorticoid responsive and related factors</i>	2
V\$PERO	920	1218	8.39	<i>Peroxisome proliferator-activated receptor</i>	3
V\$SRFF	886	1583	8.34	Serum response element binding factor	4
V\$STAT	1265	2843	7.13	Signal transducer and activator of transcription	5
V\$CAAT	1163	1766	6.89	CCAAT binding factors	6
V\$AIRE	358	408	6.87	Autoimmune regulatory element binding factors	7
V\$RBPF	688	869	6.76	RBPJ – kappa	8
V\$IKRS	838	1107	6.72	Ikaros zinc finger family	9
V\$HAML	648	792	6.62	Human acute myelogenous leukemia factors	10
V\$GATA	1401	2608	6.51	GATA binding factors	11
V\$EREF	685	936	6.38	<i>Estrogen response elements</i>	12
V\$MOKF	879	1147	6.24	Mouse Krueppel like factor	13
V\$TALE	840	1140	5.98	TALE homeodomain class recognizing TG motifs	14
V\$PRDF	753	994	5.54	Positive regulatory domain I binding factor	15
V\$ZFTR	561	683	5.4	Zinc finger transcriptional repressor	16
V\$YY1F	1109	1618	5.34	Activator/repressor binding to transcription initiation site	17
V\$SORY	1629	3905	5.33	SOX/SRY-sex/testis determining and related HMG box factors	18
V\$SF1F	565	683	5.22	Vertebrate steroidogenic factor	19
V\$NBRE	394	437	5.19	<i>NGFI-B response elements, nur subfamily of nuclear receptors</i>	20
V\$RXRF	1640	3386	5.13	<i>RXR heterodimer binding sites</i>	21
V\$LEFF	801	1043	5.12	LEF1/TCF	22
V\$BTBF	307	320	5.06	BTB/POZ transcription factor	23
V\$RP58	324	371	5.06	RP58 (ZFP238) zinc finger protein	24
V\$CLOX	1099	2055	5.02	CLOX and CLOX homology (CDP) factors	25
V\$EV11	1455	3101	4.98	EV11-myleoid transforming protein	26
V\$TEAF	645	761	4.94	TEA/ATTS DNA binding domain factors	27
V\$HEAT	1142	1937	4.79	Heat shock factors	28
V\$BCDF	953	1510	4.67	Bicoid-like homeodomain transcription factors	29
V\$HAND	1433	2861	4.64	Twist subfamily of class B bHLH transcription factors	30
V\$HMTB	610	763	4.6	Human muscle-specific Mt binding site	31
V\$HOXH	750	1009	4.6	HOX – MEIS1 heterodimers	32
V\$NEUR	891	1223	4.46	NeuroD, Beta2, HLH domain	33
V\$SIXF	543	663	4.39	Sine oculis (SIX) homeodomain factors	34
V\$SMAD	610	777	4.34	Vertebrate SMAD family of transcription factors	35
V\$PBXC	721	936	4.34	PBX1 – MEIS1 complexes	36
V\$GCMF	601	763	4.32	Chorion-specific transcription factors with a GCM DNA bind. domain	37
V\$AP1F	548	872	4.31	AP1, Activating protein 1	38
V\$PAX8	598	728	4.3	PAX-2/5/8 binding sites	39
O\$INRE	636	794	4.29	Core promoter initiator elements	40

^a Distance from the population mean in units of the population standard deviation.

promoter sequences precipitating with SRAP. These include nuclear receptor subfamily two factors binding element, as well glucocorticoid responsive, peroxisome proliferator-activator receptor and estrogen receptor responsive elements. These results suggest that previously seen physical interactions between nuclear receptors such as GR or ESRs and SRAP might result in the targeting of this later protein to specific promoter regions. Interestingly, binding sites for GATA, HAND and AP1 (recognized by FOS) are also enriched (Table 2 in bold). This in turn strongly suggests that SRAP might also be recruited to particular promoter regions through physical interaction with transcription factors such as GATA1, HAND1 or FOS. This is the first time SRAP is found recruited at promoter levels. The overall effect of recruiting SRAP on a given promoter region is likely to be specific of the exact region involved, the existing genomic context, the gene considered, as well as the presence or absence of other factors. Individual studies are therefore obviously needed to decipher the exact role SRAP might have on the transcription of genes controlled by promoters it is located

on. It was however of interest to investigate whether forcing the recruitment of SRAP on a given promoter might result in any transcriptional modification.

3.4. SRAP has a transcriptional repressive activity sensitive to TSA

In order to determine the generic effect of recruiting SRAP at a given promoter, we used an artificial luciferase reporter system to recruit the hybrid GAL4-SRAP protein in close proximity to the LEXA-VP16 transcriptional activator on the promoter of the GAL4-LEXA-luciferase reporter vector. To exclude any potential influence of SRA-RNA in the effect observed, we used a construct (GAL4-SRAP-SDM), which contains silent mutations (SDM1 and SDM7) shown to alter sub-structures STR1 and 7 and interfere with SRA-RNA co-activation function [24]. We first analyzed the ability of full-length SRAP-SDM to modulate the activity of LexA-VP16 when physically recruited on adjacent promoter sequences (Fig. 2A and B). We found that GAL4-SRAP-SDM decreased the

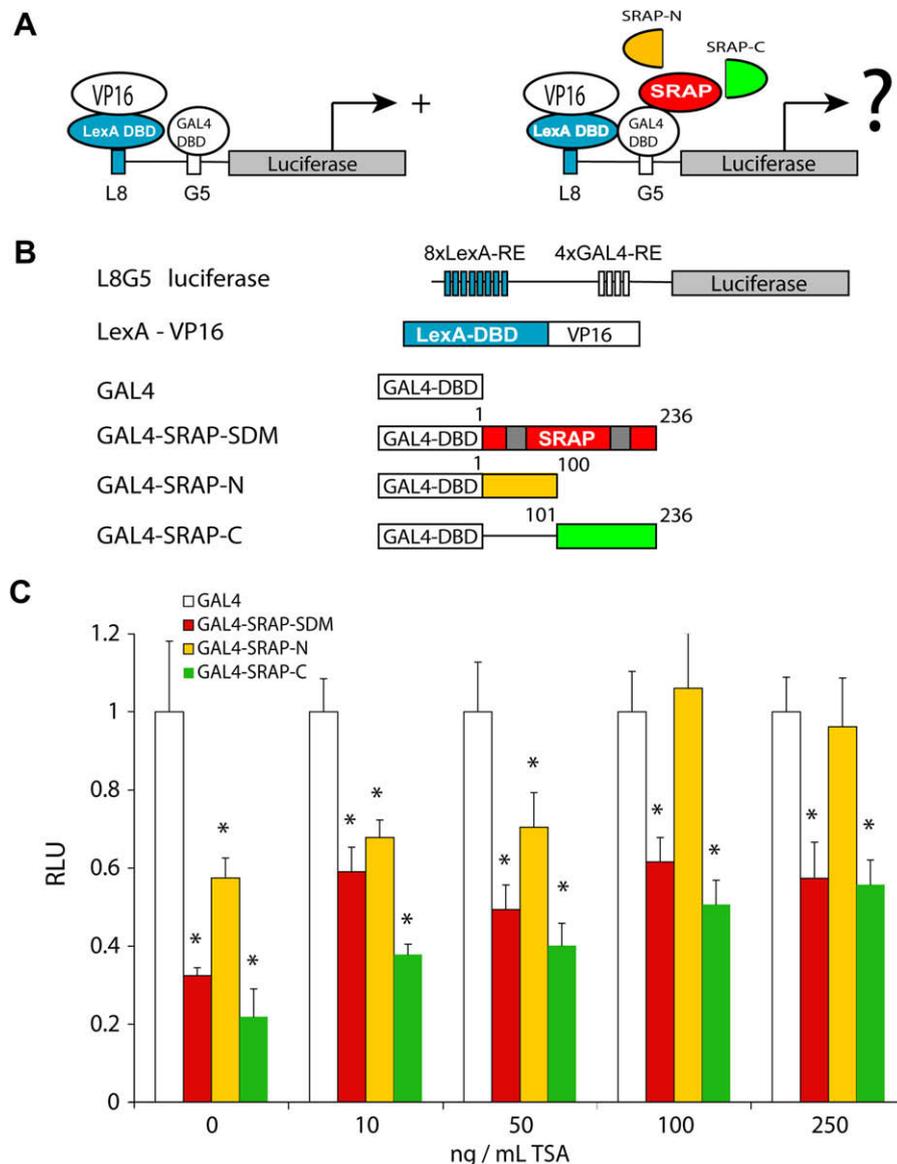


Fig. 2. SRAP and its two conserved domains repress transcription when physically recruited at the promoter level. (A) Schematic of LexA-VP16 assay. (B) Constructs used. (C) HeLa cells were co-transfected with L8G5-luciferase together with GAL-4, GAL4-SRAP-SDM, GAL4-SRAP-N or GAL4-SRAP-C, treated with increasing amount of TSA and luciferase activity assessed as described in Section 2. Bars and stars represent standard deviations ($n = 4$) and significant (Student's t -test, $P < 0.05$) difference with GAL4 control, respectively.

activity of VP16 in this system (Fig. 2C). It should be noted that a construct encoding for SRAP-SDM but exempt of GAL4-DBD (therefore not physically recruited to the promoter) did not have any effects in this system (data not shown). As outlined earlier, SRAP contains two N- and C-terminal phylogenetically conserved domains suspected to participate in SRAP functions. When analyzing the effect of recruiting these domains, we found that the C-terminal domain had by itself an inhibitory effect similar to the full-length molecule (~70–80% inhibition) whereas the N-terminal region, also acting as a repressor, had a weaker impact (40% inhibition). Treatment with TSA (an inhibitor of HDAC activity) fully abolished the repressive activity of the N-terminal domain (0% inhibition of VP16 activity at 100 ng/mL TSA) but only partially inhibited the effect of the full-length protein or its C-terminal domain (~40% inhibition). The differential impact of TSA treatments on the respective repressive action of these domains underlines a possible heterogeneity of the mechanisms involved. It indeed suggests that while the N-terminal mechanism of action mainly involves HDACs, the C-terminal conserved domain might recruit additional inhibitory proteins insensitive to TSA treatment. To further investigate whether SRAP action might potentially involve the recruitment of HDAC activity, we assessed HDAC activity in SRAP-V5-tagged protein co-immunoprecipitated nuclear extracts from previously described breast cancer cells (MCF-7-SRAP-V5-High.A) stably expressing this protein (Fig. 3A). As control of non-specifically immunoprecipitated HDAC activity we treated in parallel extracts from non-expressing cells (MCF-7-SE). We found that 0.2% of total nuclear HDAC activity specifically co-immunoprecipitated with SRAP-V5. To confirm that endogenous SRAP could also form complexes with known molecules harboring HDAC activity, we have performed co-immunoprecipitation experiments using un-

transfected MCF-7 breast cancer cell line nuclear extracts. We found that HDAC-2 was associated with endogenous SRAP in this model (Fig. 3B).

The potential to act through both HDACs and non-HDAC dependent mechanisms has been demonstrated for other transcription repressors. For example, the short heterodimer partner (SHP) and the ligand-dependent nuclear receptor co-repressor LcoR can act through EID1 (EP300 interacting inhibitor of differentiation 1) and CtBP (C-terminal binding protein), respectively [12,17]. The identity of the non-HDAC proteins possibly involved in SRAP mediated transcription repression remains to be determined.

The present observation that SRAP has an intrinsic repressive ability contrasts with previous results presenting SRAP as an activator of transcriptional activity [1,7,20,22]. It should however be noted that transcriptional co-regulators are known to behave differentially in different cellular and promoter contexts. For example, SRA differentially activates estrogen receptor controlled transcription of reporter genes driven by different estrogen receptor elements [21]. Similarly, molecules such as the co-activator independent of AF-2 function (CIA) or the zinc-finger gene involved in apoptosis and cell-cycle control (ZAC1), have the ability to act either as co-activator or co-repressor [18,29]. Further studies are urgently needed to establish what are the exact effects of SRAP on the transcription of specific genes controlled by a particular transcription factor in a given context.

The binding of a given transcription factor on a specific promoter results in the sequential recruitment of multiple co-regulatory molecules. Using ChIP-re-ChIP experiments, Metivier et al. have elegantly illustrated the complexity of the dynamic events that occur following the initial recruitment of the estrogen receptor alpha on the promoter of the estrogen dependent pS2 gene [26]. Similar experiments could be performed to characterize the co-recruitment of the estrogen receptor and SRAP on a given estrogen regulated promoter region. The two faces of the products of the SRA1 gene, a functional RNA and a protein, however make the choice of specific targets to be analyzed potentially challenging. Indeed, even though both SRA-RNA and SRAP are believed to regulate estrogen receptor activity [1,7,21,23], silencing SRA1 gene does not affect the induction of pS2 gene by estrogen [4]. More recently, Foulds et al. even reported that knocking down the expression of both RNA and protein only affected a very small subset of direct estrogen receptor target genes in MCF-7 breast cancer cells [14]. This emphasizes the need to first identify endogenous target genes specifically regulated by SRAP in order to further establish the biological significance of the binding on promoter sites of this protein, alone or associated with specific transcription factors. This could potentially be achieved through the use of specific models allowing the distinction between SRA-RNA and SRAP respective functions.

Interestingly, Foulds's study also demonstrated that even though silencing SRA modified the expression of a common subset of genes in Hela uterus and MCF-7 breast cancer cell lines, some other genes were differentially affected in the two cell types [14]. This highlights cell type differences in SRA/SRAP potential functions, potentially resulting from the different relative amount of transcription factors and co-regulators interacting with these two regulatory molecules. For a given gene, SRAP effects on transcription might therefore be positive or negative potentially as a direct result of the balance between multiple transcription factors present in specific cell types.

SRA is a very peculiar example of a bi-faceted system consisting of a functional RNA and its corresponding protein. Our results show for the first time that SRAP can physically interact with multiple transcription factors and is recruited by promoter regions. Altogether our data suggest that SRAP, as its RNA, has the potential to be involved in many critical pathways and putatively directly participates to the regulation of gene expression. Interestingly both

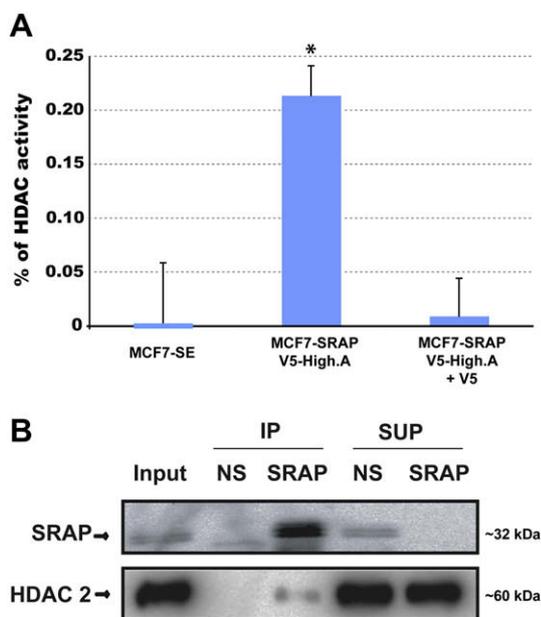


Fig. 3. Specific co-immunoprecipitation of SRAP-V5 and HDAC activity. (A) Nuclear extracts from MCF-7-SE (control) and MCF-7-SRAP-V5-High.A cells were immunoprecipitated with anti-V5 antibodies and HDAC activity measured as detailed in Section 2. MCF-7-SRAP-V5-High.A extract was also treated in parallel with competitive V5 peptide. Standard errors ($n=4$) and significant (Student's t -test, $P<0.05$) difference with MCF-7-SE (control) are indicated by bars and stars, respectively. (B) Nuclear extract from MCF-7 cells were divided into two pools and subsequently immunoprecipitated with rabbit polyclonal anti-SRAP antibodies (SRAP) or with non-specific rabbit polyclonal antibodies (NS) as described in Section 2. Immunoprecipitated fractions (IP) or supernatant (Sup) were checked by Western blot using anti-SRAP and anti-HDAC-2 antibodies. Nuclear extracts (Input) was used as positive control.

SRA-RNA and protein might be implicated in similar signaling pathways. Specific studies deciphering SRAP and SRA exact mechanisms of action in the context of particular transcription factors are warranted. Identifying SRA/SRAP target genes is crucial for a comprehensive understanding of the bi-faceted system represented by the products of this peculiar gene.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2010.04.022](https://doi.org/10.1016/j.febslet.2010.04.022).

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Chapter 6: Discussion

6.1 Summary of the SRAP study presented in this thesis

Herein we have unequivocally demonstrated that the once thought non-coding SRA is also able to encode for a SRA protein (SRAP) that is well conserved among chordates (see figure 6). We further found that several SRA peptides of distinct sizes are expressed in breast cancer tissues. Interestingly, we demonstrated that an SRA peptide with an apparent molecular size of 30 kDa is differentially expressed among breast cancer tumors. The expression of this particular peptide correlates with a better survival in breast cancer patients with estrogen receptor positive breast tumors treated with tamoxifen. This observation lead us to hypothesize that SRAP could be involved in subduing estrogen action. Indeed, MCF-7 cells stably expressing SRAP have lower estrogen responsiveness than the parental MCF-7 cells suggesting that SRAP can potentially possess transcriptional repressive function. However, transient SRAP expression in HeLa cells demonstrated that SRAP co-activates estrogen receptor alpha activity as monitored by three separate ERE luciferase constructs. The bifaceted aspect of the SRA gene presents a challenge in separating the SRA RNA and protein function. Therefore, in order to unequivocally confirm that the ESR1 co-activation effect is attributed to SRAP rather than SRA RNA, we designed an artificial SRA sequence by incorporating extensive silent mutations. This artificial SRA sequence is highly unlikely to function as an RNA molecule due to widespread alterations in its secondary structure. The transient expression of the artificial SRA sequence results in SRAP expression and ESR1 co-activation. These results thus confirm that SRAP, independently of its RNA counterpart is able to co-activate ESR1. In addition, we have shown that both endogenous and over-

expressed SRAP are able to associate to DNA. We have found that SRAP binds to the promoter regions of an array of genes. We have also demonstrated that SRAP can interact with various different transcription factors with different affinities. The wide variety of transcription factors interacting with SRAP suggest its broad role in transcription regulation likely affecting multiple pathways and controlling the expression of a multitude of genes. Overall these observations strongly suggest the importance of SRAP in regulating transcription.

In order to determine the effect of SRAP on transcription, SRAP was artificially recruited to the promoter of a luciferase reporter vector. SRAP recruitment lead to lower luciferase activity further accentuating SRAP's role in transcription regulation and more precisely transcription repression. Interestingly, SRAP mediated repression is partially alleviated by trichostatin A (TSA) treatment suggesting a potential role of HDACs in SRA mechanism of action. TSA treatment is able to fully alleviate the repressive action of SRAP's N-terminal domain while only partially alleviating the repressive action of full length and C-terminal domain. These results suggest that SRAP possess different mechanisms to mediate its repressive action. One such mechanism involves HDAC activity.

Overall, we thus suspect that SRAP is a co-regulator that modulates gene expression by associating with transcription factors and co-regulatory proteins. But is SRAP truly a *bona fide* transcriptional co-regulator?



Figure 6: Schematic diagram representing the human SRAP. The N and C terminal conserved domains are illustrated as CD1 (pink box) and CD2 (yellow box) respectively. The predicted nuclear localization domain is shown in blue.

6.2 Is SRAP a bone fide nuclear receptor co-regulator?

The term transcriptional co-regulator refers to a molecule that associates with transcription factors directly or indirectly and regulates target gene expression. Taking in account this definition, in order for SRAP to be considered a *bona fide* transcriptional co-regulator, it has to fulfill two conditions. Evidence must prove that first SRAP associates directly or indirectly with transcription factors and that SRAP alters their transcriptional activity.

Several reports highlight the interaction between SRAP and steroid receptors. Indeed, a partial rat SRAP has been shown to directly interact with the AF2 domain of the androgen receptor (Kawashima et al. 2003). We have also shown through an *in vitro* assay that SRAP interacts directly with transcription factors with different binding affinities (Chooniedass-Kothari et al. 2010a). It is interesting to note that SRAP interacts with co-regulatory molecules that themselves have been associated with steroid receptors. These include p68, polybromo 1 and ARID (Leygue 2007). The association with such proteins strengthens the notion that SRAP is present in complexes involved in transcription regulation and therefore likely directly associated with steroid receptors. We

can therefore conclude that SRAP fulfills the first condition of being a *bone fide* co-regulator.

Several reports including our studies have also demonstrated SRAP ability to modulate steroid receptors transcription activity. Kawashima et al have shown that a short rat SRAP, likely inactive at the RNA level, co-activates the transcriptional activity of AR, GR as well as PPARgamma (Kawashima et al. 2003). We have also shown that SRAP independently of its RNA counterpart is able to co-activate ESR1 transcriptional activity (Chooniedass-Kothari et al. 2010b). In addition, a recent report has further underlined the ability of SRAP to regulate ESR1 activity by demonstrating that a chlamydial protease CT441 sequesters SRAP to cytoplasm and consequently partially alleviates ESR1 co-activation in mammalian cells (Borth et al. 2010).

Overall, the current data on SRAP thus suggest that SRAP fulfills the two criteria for it to be considered as a *bona fide* transcriptional co-regulator.

6.3 Is SRAP a coactivator or a co-repressor?

The preceding section has underlined the evidence strongly indicating that SRAP is a co-regulator. This appreciation of its function however raises the next question. Where does SRAP fit among its peers? Is SRAP a co-activator or a co-repressor?

Co-activators are defined as factors directly or indirectly binding to nuclear receptors and enhancing their transcriptional activity. On the other hand, co-repressors are factors binding to nuclear receptors and inhibiting their transcriptional activity. With these definitions in mind, one can question whether SRAP is a co-activator or co-repressor. SRAP has apparently opposite effects on ESR1 transcriptional activity. On one hand, we

have observed that MCF-7 cells stably expressing SRAP have a lower estradiol responsiveness than parental cells (Chooniedass-Kothari et al. 2006). These results prompted us to speculate that SRAP was perhaps repressing ESR1 action. On the other hand, transient SRAP transfection in HeLa cells results in up-regulation of ligand dependent ESR1 activity monitored on three independent ERE reporter vectors (Chooniedass-Kothari *et al.* 2010b). These later data therefore suggested that SRAP acts as an estrogen receptor co-activator.

One possible explanation behind the opposing and apparently contradictory co-activating versus co-repressing effects observed could be rooted in the difference in cell lines used in the two studies. While MCF-7 cells were used in the stable transfection study, HeLa cells were used in the transient transfection study. The difference in cell lines used can alter the outcome as illustrated by a recent study by Foulds *et al.* The authors show that the knock down of SRA RNA and protein in MCF-7 and HeLa cells leads to a change in distinct sets of genes in addition to a common set of genes. These results suggest that the two cell lines possess some common as well as distinct mechanisms/ players affecting SRA RNA and protein pathways (Foulds et al. 2010). It has been long demonstrated that co-regulatory molecules are not equally expressed across all cell lines. The differential expression of co-regulators has been shown to affect the action of estrogen receptors differently. Indeed the disparity in co-regulators expression is thought to be at least partially responsible for the opposite action of tamoxifen in breast (antagonist) versus uterine (agonist) tissues (Jordan 2007; Lonard et al. 2007; McKenna et al. 1999).

Another possible source for the apparent contradicting results is also the nature of the transfections used in the two studies. MCF-7 cells were stably overexpressing SRAP

while HeLa cells were subjected to transient SRAP expression. The stable overexpression of SRAP in MCF-7 cells could have therefore reprogrammed among others the ESR1 signaling pathway. It is indeed possible that SRAP overexpression in these cells lead to the change in expression of a protein or group of proteins that are then ultimately responsible for a lower estrogen responsiveness detected. In support of this hypothesis, we have observed that increasing intron 1 retention in T5 breast cancer cells leads to a change in gene expression. Furthermore, an increase in SRA intron-1 level changes T5 cells responsiveness to estradiol treatment. Significant differences in mRNA expression levels were observed for non metastatic cells 1 (NME1), keratin 18 (KRT18), FAS, beta catenin (CTNNB1) and fibronectin leucine rich transmembrane protein 1 (FLIRT1) in T5 cells treated with estradiol with increased intron-1 retention as compared to cells where the coding/ noncoding ratio were undisturbed. Interestingly, several of the affected genes have been implicated in cell growth, migration and invasion. SRA intron-1 retention leads to a change in gene expression that translates to decreased cell growth/viability of T5 cells. These results suggest that a change in coding/ non-coding SRA ratio leads to altered gene expression and a disruption of estrogen signaling (Cooper et al. 2009). In the MCF-7 cells stably expressing SRAP used in our study, the coding/non-coding SRA ratio is clearly modified due to the stable over-expression of the SRA coding cDNA. This change in SRA coding/ non-coding transcript ratio could have thus significantly altered the estrogen signaling pathway in these cells.

It is interesting to note that when recruited artificially to the promoter of a luciferase gene, SRAP lead to lower luciferase activity further accentuating its role in transcription repression. Interestingly, this SRAP mediated repression is partially alleviated by

trichostatin A (TSA) treatment suggesting a potential role of HDACs in SRA mechanism of action. TSA is a HDAC inhibitor that affects class 1 and 2 HDACs. HDACs have been shown to play a role in transcription repression as they remove acetyl groups from histone tails. This change in histone post translational modification results in a condensed chromatin structure that is not favorable for transcription.

In our transient transfection assay, the luciferase reporter vector is unlikely to form a chromatin structure similar to the one described for genomic DNA. The observed TSA mediated relief in SRA transcription repression is thus unlikely due to a reorganization of chromatin structure upon histone deacetylation. Alternatively, transcription repression is likely mediated via other mechanisms. In fact, HDACs are often part of protein complexes that contain other members regulating transcription such as chromatin remodeler and transcription repressors. TSA binding is likely to induce a conformational change in HDAC. Such change might influence protein-protein interactions and thus might alter the stability of protein complexes containing HDACs. It is therefore likely that the relief in SRAP mediated transcriptional repression upon TSA treatment is the result of a similar effect via a change in protein complex stability rather than change in the luciferase reporter vector chromatin structure.

In addition to the arguments presented above, it is nonetheless important to note that the opposite co-activating versus co-repressive actions observed are not a unique feature to SRAP. Several other co-regulators have been shown to function as either co-activator or repressor depending on the cell line and promoter contexts. For example the co-activator independent of AF2 function (CIA) possesses both co-activating and co-repressing

functions (Sauve et al. 2001). The apparent opposing effect of SRA on ESR1 transcriptional activity is therefore perhaps not as contradictory as it appears.

6.4 SRAP possible model of action

Although SRAP likely affects the transcriptional activity of other steroid receptors and an array of transcription factors, I have chosen to focus this section on the possible SRAP model of action with respect to the estrogen receptor.

The exact mechanism by which SRAP modulates ESR1 activity remains to be deciphered. SRAP could mediate ESR1 action either by directly binding to the receptor and acting as a *bona fide* co-regulator or through indirect mechanisms. It is indeed possible that SRAP indirectly influences ESR1 activity by first regulating the expression of a factor that is in turn responsible for the change in ESR1 activity observed. However, considering the fact that SRAP has been shown to directly physically interact with AR, it is therefore likely that SRAP also mediates ESR1 action via direct interaction with ESR1 (Kawashima et al. 2003). In support of this hypothesis, we have in fact detected a direct interaction between SRAP and ESR1 and ESR2 *in vitro* (Chooniedass-Kothari et al. 2010a). Nonetheless more investigations need to be conducted to decipher the exact nature of the possible *in vivo* interaction between SRAP and ESR1.

It is interesting to note that several if not the majority of co-activators and corepressors possess the signature LXXLL motif (where L is a leucine and X is any amino acid) or ϕ XX $\phi\phi$ motifs (where ϕ is a hydrophobic residue and X is any amino acid) respectively. These motifs mediate the direct interaction of co-regulators and steroid receptors. SRAP does not possess such a motif but rather an interestingly similar LXXAL motif. This exact sequence is also present on coactivator NcoA62 and mediates the direct interaction

between the co-activator and ESR1 (Baudino et al. 1998). The significance of SRAP's LXXAL motif remains unknown and is subject to further studies. Its apparent conservation in multiple genes is perhaps indicative of its important role. In addition, a sequence consisting of overlapping LXXLL ϕ XX $\phi\phi$ motifs is also present in SRAP. Such a sequence has been found in the bifunctional co-regulator Co-activator Independent of AF2 function (CIA), and has been demonstrated to be critical for CIA-ESR1 interaction (Sauve et al. 2001). The significance of this motif in SRAP is once again unknown and therefore subject to further investigation.

Today, we do not know precisely how SRAP increases ESR1 activity. One could speculate that SRAP could act as a classical co-activator and thereby participate in ESR1 co-activation through multi protein complexes containing other co-activators. However, we have shown that SRAP co-immunoprecipitates with HDAC activity suggesting its association with proteins containing co-repressive action. This observation could therefore potentially indicate that SRAP perhaps removes co-repressors away from ESR1 and thereby allows its transactivation. By the same token, SRAP could possibly in a cell and promoter context dependent manner act as a repressor recruiting HDAC activity to specific promoters and thereby repressing transcription. Alternatively, SRAP could sequester co-activators from estrogen receptor and thereby inhibit transactivation. These four hypothetical models of action are summarized in figure 7.

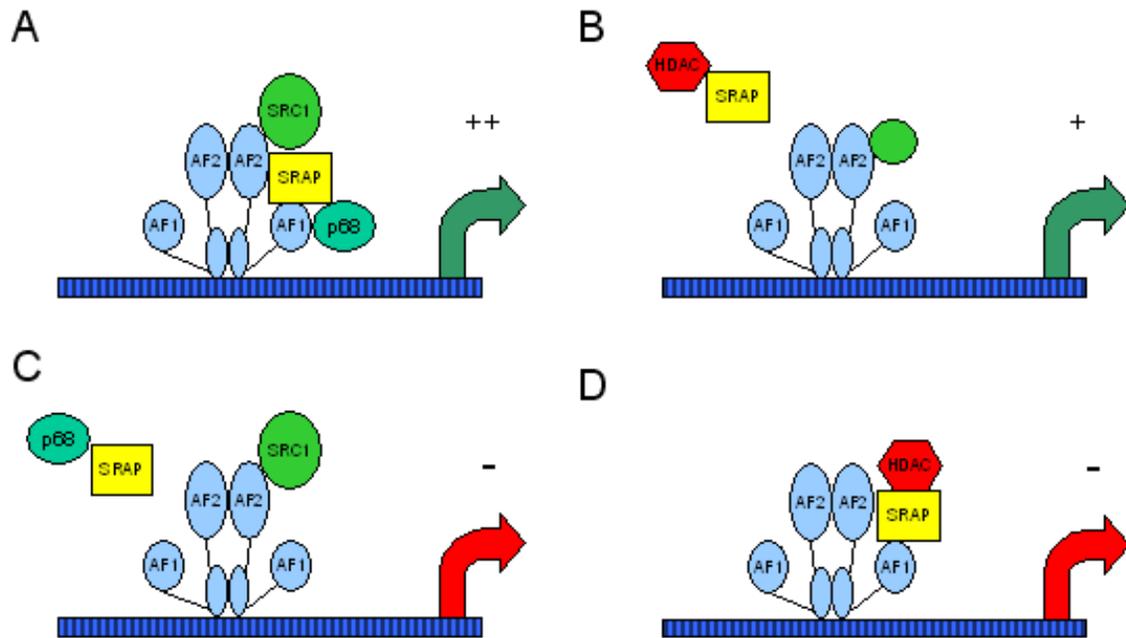


Figure 7: Possible modes of action of SRAP in transcription modulation. A. SRAP could activate transcription by participating within co-activator protein complexes. B. SRAP could permit transcription by removing transcriptional repressive proteins away. C. SRAP could repress transcription by sequestering co-activators away from estrogen receptors. D. SRAP could repress transcription by recruiting HDAC activity to specific promoters.

ESR1 possess two activation domains that are either ligand dependent (AF2) or independent (AF1). Co-regulators have been shown to influence either of these domains. P160 family of proteins has been shown to co-activate the ligand dependent AF2 domain. On the other hand, P68 has been shown to enhance ESR1 activity through its ligand independent AF1 region. Kawashima *et al* have demonstrated that SRAP interacts with AR AF2 thus suggesting that SRAP mediates its action via AR AF2 domain (Kawashima

et al. 2003). On the other hand, Borth *et al* have shown that SRAP is capable of co-activating ESR1 AF1 domain in HEK-293 cells (Borth et al. 2010). The observation that SRAP has also the ability to activate the AF-1 ligand independent functional domain fits with our results indicating that SRAP can increase the agonist effects of tamoxifen (Chooniedass-Kothari et al. 2010b). Indeed, tamoxifen has been shown to act as a partial agonist by potentiating the activity of ESR1 AF1 domain (Berry et al. 1990). Further studies will determine the exact mechanism of SRAP action on ESR1 transcriptional activity.

6.4 What are SRAP target genes?

Our CHIP assay identified promoter regions associated with SRAP and thus offers a list of possible SRAP target genes. In order to identify SRAP target genes, a combined analysis of SRAP CHIP assay and expression array by either down regulating or overexpressing SRAP would be necessary. Fould *et al* have recently published a study describing the global change in gene expression upon SRA RNA and protein knock down (Foulds et al. 2010). The authors recognized that the change in gene expression could be attributed to either decreased levels of SRA RNA or protein. In order to clarify this issue, the authors monitored the effect of coding versus not coding SRA expression on transcriptional activity of three target gene promoters. In all three cases, both coding and noncoding SRA were able to increase the transcriptional activity leading the authors to conclude that the SRA as opposed to SRAP is responsible for the activation. The authors used a previously generated antibody to ascertain that the SRA non-coding vector (pSCT SRA) was indeed unable to encode for a SRA protein (Lanz et al. 1999). No SRA proteins were detected in the lysates of cells transiently transfected with pSCT SRA

(Foulds et al. 2010). In contrast to these results, we have found that the transient transfection in HeLa cells of pSCT SRA and pSCT SRA SDM1/7 vectors results in the expression of smaller 30 kDa, 25 kDa (doublet) and 17 kDa SRAP peptides recognized by the commercially available antibody A300-743A anti-SRAP (bethyl laboratory) (Figure 8). Interestingly, only the 30 kDa peptide is recognized by the A300-742A anti SRAP antibodies. As shown in figure 8 panel C, the two anti-SRAP antibodies used here recognize different epitopes within the SRAP sequence. These results suggest the 25 kDa doublet and 17 kDa peptides that are not recognized by the A300-742 antibodies are likely truncated proteins products of an alternative use of the downstream methionines 3 and 4 as starting codons. Since the SRA sequence within the pSCT SRA and pSCT SRA SDM1/7 vectors does not contain methionine 1 or 2, we were surprised to detect a 30 kDa peptide specifically in the lysates of cells transfected with these vectors. Furthermore this particular peptide was recognized by both antibodies used. We suspect that this peptide is a trans-splicing product of the SRA transcripts generated from both the pSCT-SRA or pSCT-SRA SDM1/7 vectors and endogenous SRA transcripts. Indeed, HeLa cells possess SRA isoform 1 while the SRA sequence encoded by the pSCT vectors is that of the isoform 3. We have previously demonstrated that the protein resulting from SRA isoform 3 translation contains an extra amino acid but migrates faster than its counterpart protein resulting from SRA isoform 1 or 2 translation.

Overall we have demonstrated that the pSCT-SRA vectors lacking the first exon and thus the first two methionines are nonetheless capable of producing truncated SRAP peptides. We suspect that in the study by Foulds *et al*, the inability to detect truncated SRA proteins might reside in the reduced sensitivity of the antibody used. Indeed in their

initial study, the same antibody was not able to recognize endogenous SRAP in cell lines later shown to possess SRAP (Lanz et al. 1999).

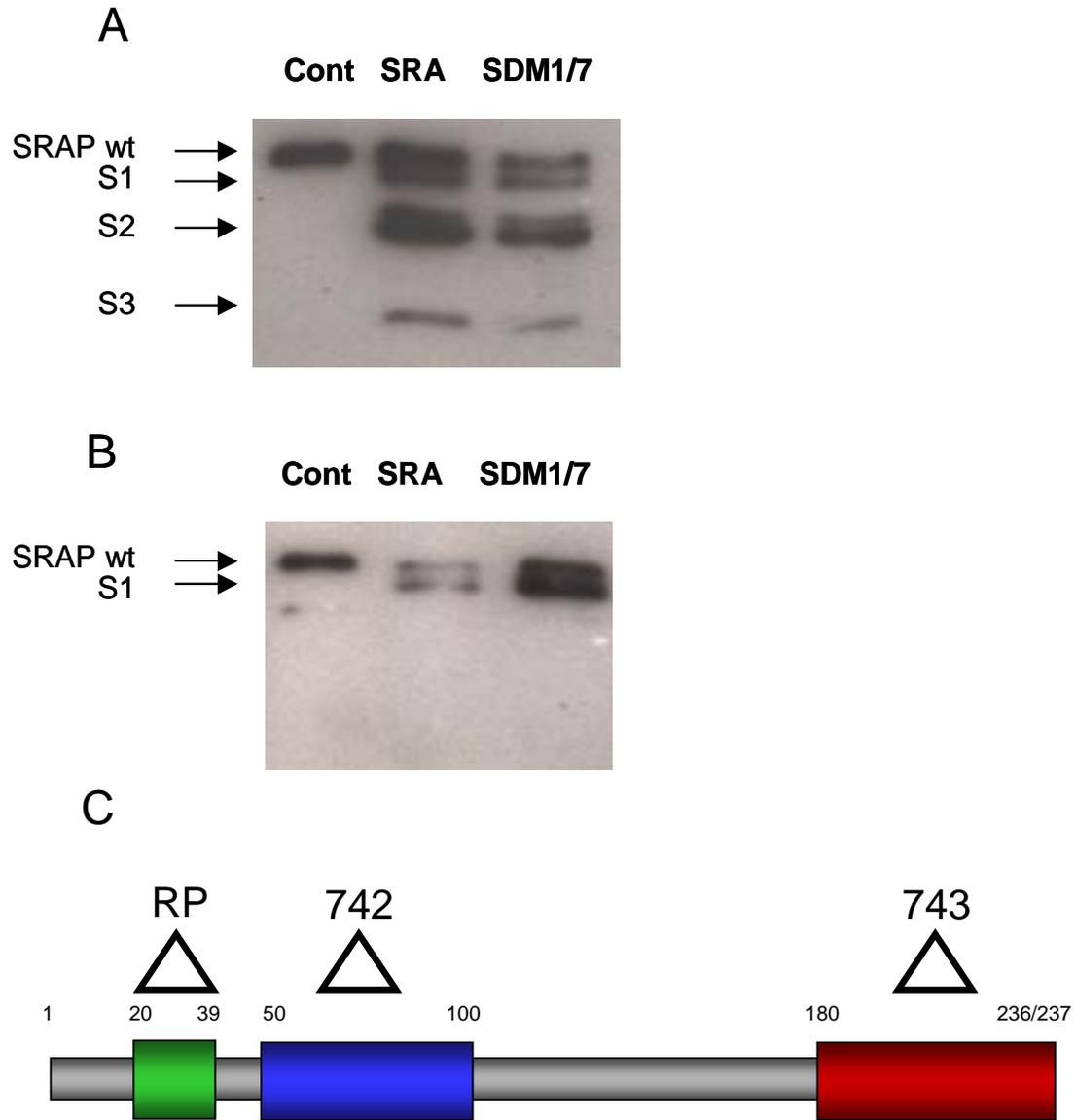


Figure 8. The “non-coding” pSCT SRA vector is capable of expressing truncated SRA peptides. **A.** Western blot Analysis of HeLa cells transfected with pSCT SRA (SRA) and pSCT SRA SDM1/7 (SRA1/7). Cont refers to control cells subjected a mock transfection. The anti-SRAP antibodies A300-743A and A300-742 (Bethyl Laboratories) were used for immuno detection in panels A and B respectively. A 30 kDa (S1), 25 kDa doublet (S2) and 17 kDa peptides are detected in the lysates of

cells transfected with pSCT SRA and pSCT SRA SDM1/7 with the A 300-743A antibodies. Only the S1 peptide is detected in the corresponding lysates with the A 300-742A antibodies. C. Schematic diagram illustrating SRA epitopes recognized by the “in house” anti SRAP rabbit polyclonal antibody (RP), the commercially available anti-SRAP rabbit polyclonal antibodies from Bethyl Laboratories A300-742A (742) and A300-743A (743).

Overall we suspect that the change in gene expression presented by Foulds *et al* could result from the combined or independent action of SRA and/or SRAP. Interestingly, we have detected SRAP bound to the promoter of several genes whose expression has been altered upon SRA (RNA and protein) knock down. These genes include acyl-Coenzyme A oxidase 2 (ACOX2), leukemia inhibitory factor receptor alpha (LIFR) and ATP-binding cassette, sub-family A member 1 (ABCA1) to name a few. Further studies are needed to determine whether the change in gene expression is attributed to SRA RNA or protein function or a combined action of both molecules.

6.4 What is the significance of SRAP expression in breast cancer?

As of today, only two studies have investigated SRAP expression in breast tumor tissues and have reported contradictory outcomes. Both these studies were conducted in our laboratory. As described in chapter 3, Western blot analysis of SRAP expression in ER positive breast tumor tissues suggests that the presence of a 30 kDa SRAP correlates with increased overall survival in patients with ER positive tumors treated with tamoxifen. However tumor micro array (TMA) immunohistochemical analysis suggest that higher SRAP levels correlate with a poor prognosis in breast cancer patients younger than 64 years, with ER positive tumors and node negative tumors (Yan et al. 2009).

One intriguing possibility responsible for the contradicting results lies in the nature of SRAP peptide detected. Western blot analysis of the tumor samples presented in chapter 3 detected the presence of several peptides (30 kDa and 25 kDa) recognized by N terminal targeted anti-SRAP antibody. The expression of the 25 kDa SRA peptide did not correlate with incidence of breast cancer recurrence or overall survival. On the other hand, the expression of the 30 kDa SRA peptide in breast tumor tissues correlated with a better overall survival of patients treated with tamoxifen.

Interestingly, the commercially available A300-743A anti-SRAP antibody used to perform the TMA analysis also detects several SRAP peptides migrating at 40 kDa, 30 kDa and 25 kDa by Western blot analysis of tumor tissue protein samples. However the immunohistochemical analysis does not permit the distinction in the detection of these SRA peptides. The level of SRAP expression recorded during TMA analysis is therefore the collective expression of all SRAP peptides. We therefore suspect that all SRAP peptides do not have an equal prediction value. In addition there is a huge difference in the level of sensitivity and subsequent method of quantification between Western blot analysis and TMA assays. Western blot analysis only permitted the classification of breast tumors into two categories defined as either SRAP positive or SRAP negative, depending upon detection or lack thereof in our experimental conditions. TMA assays allow a more continuous quantification based SRAP expression levels.

Another difference between the two studies that could account for the contradictory results is the cohort size. While the study presented in chapter 3 had a small cohort size with only 74 samples, the TMA analysis was performed with a significantly larger cohort

of 271 ER positive tumor tissues. In addition, different median ER levels (45.5 fmol/mg versus 37 fmol/mg) were observed in the two studies.

Despite the apparent contradictory results, the observation that SRAP expression associates with a clinical outcome is sufficient in itself for further investigation into the biological role of the protein. It would also be interesting to further dissect the potential for distinct prognostic values of the different SRA peptides.

6.5 SRA a bifaceted gene with multifaceted challenges

Up to recently, RNA molecules were categorized into well defined categories of either coding RNAs (mRNA) or non-coding RNAs (tRNA, rRNA, Sno RNA). However, emerging studies have uncovered the existence of bifaceted RNA that possess both properties. Examples of such molecules include Oskar RNA and protein involved in *Drosophila* oogenesis and Tveg RNA and protein involved in *Xenopus* oocyte development (Mercer et al. 2009; Dinger et al. 2008). It is interesting to note that for both of these genes, the role of the protein was initially determined while the translational independent role of the RNA was subsequently deciphered. Nonetheless, the account of such RNAs with an inherent duplicity remains limited.

A question therefore arises inquiring into whether the limited account of such functional RNA encoding a protein is founded biologically or is it due to a current dogma of RNA defined in distinct categories and consequent inadequacy in our ability to identify them (Dinger et al. 2008). Indeed, examination of the molecular protocols used today suggest that most assays are designed to examine either RNA or protein function and thus posing a challenge when examining bi-functional RNAs.

Several studies employ SRA knock down assays to analyze SRA RNA function. The knock down has been mediated via the use of RNAi technology or antisense oligonucleotides that target RNAs for degradation. In both cases, the decrease in SRA RNAs levels will invariably lead to decreased protein levels. The functional consequence in gene expression and/or change in steroid receptor activity could therefore be the direct consequence of the alteration of the SRA RNA or protein signaling. Assays affecting both RNA and protein levels are therefore not ideal in unraveling the distinct function of the two faces of the SRA gene.

Overexpression is another widely used method for functional analysis. However transient or stable over-expression of wild-type coding SRA would invariably lead to the over-expression of both coding and functional RNA. Indeed SRA core sequence expanding from exon 2 to exon 5 has been demonstrated to be sufficient and necessary for the non-coding RNA function. Constructs that are coding for the wild-type SRA contain the core sequence and therefore have the full potential to be active at the RNA level. Over-expression assays of this kind are thus also failing in dissecting the role of SRA and SRAP.

Another possible way to analyze only SRA RNA function is through the study of an SRA expression construct missing the first two ATG start codons present in the first exon. One of such construct that is widely used to analyze the RNA function is the pSCT SRA vector (Lanz et al. 1999; Watanabe et al. 2001; Lanz et al. 2002; Deblois and Giguere 2003; Coleman et al. 2004; Hatchell et al. 2006; Caretti et al. 2006; Xu et al. 2009a; Foulds et al. 2010). This construct contains a portion of SRA intron 1 followed by the core sequence and does not contain exon 1 therefore lacking the first two ATG codons.

The pSCT SRA vector is therefore believed to encode solely for a non-coding SRA RNA. Unfortunately, we have seen that the transient transfection of this construct in HeLa cells results in the expression of truncated SRA proteins likely starting at downstream methionines 3 and 4 (figure 8). In addition, we have demonstrated that these truncated proteins are able to coactivate ESR1 (Chooniedass-Kothari et al. 2010b). Similarly, a truncated rat SRAP lacking the first functional domain is also able to co-activate AR transcriptional activity. Therefore one cannot dismiss the possibility that the functional consequences in any assay employing this construct could be attributed to the action of truncated SRA peptides rather than or in addition to the non-coding SRA function. Interestingly, while we observed truncated SRA peptides arising from the pSCT SRA vector, we did not observe these smaller peptides from the SRA RNA construct employed in our study described in chapter 3. There is a significant difference in the SRA 5' sequence between the two types of SRA vectors. While the pSCT SRA vector contains a portion of the SRA intron 1 sequence, our construct does not contain an intronic sequence but has an additional exon 1 sequence with the first two ATG mutated to TTG. It has previously been demonstrated that the 5' UTR plays an influential role in the mechanisms regulating translation. Different 5'UTR have been observed among the different SRA transcripts. Additional studies are thus needed to determine the importance of these sequences in SRAP expression.

Silent mutations are an avenue to alter SRA RNA action without affecting the primary protein sequence. Lanz et al used this approach to emphasize the importance of SRA RNA structure for its function (Lanz et al. 1999). We also used the same approach to its extreme and generated a SRAP coding construct that would be devoided of its RNA

activity. While theoretically sound, this approach has its own downfalls as the secondary structure of a messenger RNA has been shown to influence translation and/or the folding of the resulting protein (Welch 2004; Shabalina et al. 2006).

The extensively mutated SRA construct (described in chapter 5) was translatable and has demonstrated an effect on ESR1 signaling pathway. Nonetheless Western blot analysis of cells transfected with this artificial SRA construct also demonstrated an alteration in the mechanisms regulating its translation. Indeed, unlike the wild type SRA sequence, this new sequence is more permissive to the initiation of translation from downstream ATG codons. Therefore, the putative implications of such extensive mutations on the consequent SRA protein folding and perhaps function cannot be dismissed.

In summary, as discussed here, it is apparent that no model or assay is ideal in dissecting the separate functions of SRA RNA and protein. It is therefore important to recognize the down falls of the current molecular protocols in examining the functions of bifunctional genes such as *SRA*.

6.6 Biological relevance of a bifaceted molecule

In the light of recently emerging studies identifying functional RNAs able to encode for a protein, the following question naturally arises: What is the biological relevance of such bifaceted molecules?

From an evolutionary perspective, one can ponder whether the presence of such molecules is in fact a remnant from an ancient “RNA world”. The RNA world theory proposes that life based solely on RNA molecules preceded the current world where life is based on DNA, RNA and protein. In this context, RNA molecules were one of the

major if not sole players regulating all cellular activities. This theory is founded on the functional versatility of RNA molecules able to both pass on genetic information and perform enzymatic reactions (Forterre 2005). The ancient RNA world nonetheless evolved into the current DNA, RNA, protein world. It is thought that the increased stability of DNA compared to RNA and the greater enzymatic flexibility of proteins might have encouraged this change (Meli et al. 2001; Forterre 2005). Considering this evolutionary perspective, RNA molecules whose functions could not be replaced entirely by proteins are thus considered as potential remnants from the ancient RNA world (Jeffares et al. 1998). In this regard, could SRA be considered as an RNA world remnant whose function is perhaps evolving and being overtaken by its protein partner?

Another possibility is that RNAs such as SRA capable of performing molecular functions in addition to their ability to encode for a protein are in fact widespread. Such molecules are undiscovered and are perhaps essential in performing complex biological roles. The discovery of SRA was “fortuitous” as stated by Lanz et al. The accidental character of this finding therefore perhaps only underlines our current limited perception of RNAs functionality. Indeed it is possible that more mRNAs might have non-coding functions. However, current studies have not undertaken the analysis of the non-coding functional potential of mRNAs with well -established protein products.

Inspection of the few examples of bifunctional genes suggests that both members are usually functionally intertwined affecting the same biological pathways. For example, Oskar RNA and protein products are both involved in *Drosophila* oocyte development (Mercer et al. 2009). The VegT RNA and protein are both involved in the mechanisms regulating primary germ layers development in *Xenopus* (Dinger et al. 2008). Finally

SRA RNA and protein are also both involved in regulating gene expression. What would be the biological significance of such redundancy? One can speculate that involvement of two molecules arising from the same gene would assure an inherent functional safety. It is however also possible that the bifaceted aspect offers qualities that would go beyond functional redundancy. The interplay between the RNA and protein from the same gene would indeed present a higher level of temporal and spatial flexibility in regulating and coordinating signaling pathways. Studies examining the individual functions of SRA RNA and protein as well as studies deciphering the mechanisms regulating the levels of these two faces will unravel the exact nature of the SRA gene.

6.7 Conclusion and future directions

We have demonstrated that the once thought non-coding SRA RNA is indeed able to encode for a protein that is also involved in modulating transcription. Today, among the long list of nuclear receptor co-activators and co-repressors, SRA holds a unique status. SRA is indeed the only known co-regulator to function not only as an RNA but also as a protein. While most studies are devoted to deciphering SRA RNA function and mechanisms of action, little is known about SRAP. Herein we have taken a significant step in deciphering the role of SRAP. However additional studies will be crucial towards a better understanding of SRAP functions. The following future directions are therefore proposed.

6.7.1 Identification of SRAP target genes.

The ChIP results, described in chapter 5, outline a long list of putative SRAP target genes. In addition, as discussed in section 6.3, several candidate genes have altered

expression upon the knock down of SRA RNA and protein and could therefore be targets of either SRA RNA or protein. In order to identify *bone fide* SRAP target genes from this list of proteins the following experimental approach could be used. First luciferase reporter vectors under the control of the promoter region of candidate genes could be used to establish the role of SRA RNA and protein in their transcriptional regulation with an approach using distinct SRA constructs separating SRA RNA and protein functions as previously described (Chooniedass-Kothari et al. 2010a). In addition, the protein and RNA levels of selected target genes could be monitored upon the overexpression of specific SRA constructs separating RNA and protein function. To ascertain SRAP role, an artificial protein containing the chlamedia CT441 PDZ domain can be transfected into cells to sequester endogenous SRAP to the cytoplasm and monitor the effect to transcriptional regulation of candidate genes.

6.7.2 Investigation of SRAP mechanisms involved in ESR1 regulation

We have established that SRAP is involved in the modulation of ESR1 activity. Further studies are needed to dissect its exact mechanisms of action. SRAP has been shown to associate with the AR AF2 domain. Interaction studies using separate ESR1 domains and SRAP domains could be performed to identify the ESR1 and SRAP domains involved in ESR1/ SRAP interaction.

Analysis of SRAP effect on ESR1 AF1 and AF2 in the presence and absence of ligand could be established by using different constructs encoding for ESR1 AF1 and AF2 domains and distinct ERE-responsive luciferase vector.

6.7.3 Investigation of the role of SRAP in regulating ESR2

Studies similar to the one described for ESR1 can be performed with ESR2 in order to determine SRAP's role in modulating the activity of this steroid receptor.

Chapter 7: References

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