Investigation of the Biological Relationship Between of SRA/SRAP and Estrogen Receptor β in Human Breast Cancer

by

Yi Yan

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

Master of Science

Department of Biochemistry and Medical Genetics

University of Manitoba

Winnipeg, Manitoba, Canada

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Abstract

The Steroid receptor RNA activator (SRA) has been implicated in estrogen receptor signaling pathway. Its expression is altered during breast tumorigenesis and its molecular role in underscoring these events has been suggested. The SRA1 gene encodes both functional RNA and protein (SRAP) products, making it a unique member amongst the growing population of steroid receptor co-regulators. We have recently reported that some alternatively spliced SRA transcripts still contain intron-1 sequences. Retention of this intron inhibits the coding potential of these RNAs but does not alter RNAs functions. Using splice-switching-oligonucleotides and real-time PCR, we have observed that increasing intron-1 retention led to an increase in Estrogen receptor β (ERβ) expression in T5 cells. We have also found that the SRAP physically interacts with the ERβ *in vitro* using GST-pull down assay. These preliminary data suggest a putative biological relationship between SRA/SRAP and estrogen receptor β in human breast cancer cells. The goal of this project was to further explore the nature of this relationship.

Three specific aims have been established. First, to characterize whether SRA RNA and/or SRAP regulate the expression of ERβ *in vitro*; second, to confirm an *in vivo* physical interaction between SRAP and ERβ by co-immunoprecipitation experiments; and finally, to establish immunological conditions that will allow for immuohistochemistry studies in human breast tumor tissues.

In order to investigate the respective action of SRA RNA or protein on ERβ expression, we used two different experimental models that would enable the specific overexpression of

either SRA protein or RNA. For the first model, we generated four different SRA constructs through site-directed mutatagenesis (SDM) targeting specific nucleotides that would abolish the respective functions of either SRA RNA or SRAP. In the second model, a new-SRAP construct was generated through artificial gene synthesis. Through the use of degenerate codon sequences, this construct encodes for SRAP but drastically differs (only 35% identity) from the original SRA RNA sequence. By using these two models, we observed that SRA RNA has no impact on the expression of ERβ. However SRAP is able to down-regulate the total ERβ mRNA detected by real-time PCR. In order to investigate the interaction between SRAP and estrogen receptors *in vivo*, we used two cell line models: the first model consisted in using doxycycline-inducible MCF7-β cells which express endogenous SRAP and either recombinant ERβ1 or ERβ 2 proteins. The second model is based on the use of 293 cells co-transfected with epitope-taged SRAP and ERβ (SRAP/Flag; ERβ/V5). Interaction between SRAP and ERβ1 was observed by the co-immunoprecipitation experiments in MCF7-ERβ1 cells but not consistently reproducible. Similarly, interaction has not been consistently found in 293 cells co-transfected with recombinant ERβ1 and SRAP. Immunohistochemical analysis was performed on tissue microarrays (TMAs) from the Manitoba Breast Tumour Bank consisting of 255 ERα negative (ER-ve) and 255 ERα positive (ER+ve) breast cancers. A positive association between SRAP expression and ERβ1 and ERβ2 has been observed (*$P= 0.0219$, $P<0.001$ respectively*). Together, results presented herein demonstrate that SRAP is able to regulate total ERβ mRNA as well as SRAP expression is positively correlated to ERβ1 and ERβ2 expression in breast tumors. This suggests SRAP might potentially be involved in ER signaling in breast cancer.

Acknowledgements:

Firstly, thanks to my supervisor, Dr. Etienne Leygue, for your unselfish and patient guidance and encouragement in scientific field. Most importantly, thank you for teaching me the right attitude for research and how to be a real scientist.

Secondly, it is my pleasure to acknowledge a list of people who contribute to make this thesis possible and who have helped and inspired me during my master study. Dr. Murphy, for all her professional guidance and advises in my ERβ research. In addition, all Murphy lab members were always accessible and willing to help me with my research. As a result, research life became smooth and rewarding for me. I was delighted to interact with Dr. Xie by attending his splicing classes and having him as my committee member. His expertise in alternative splicing help me lots in my project.

I was lucky to have Charlton Cooper, Mohammad Hamedani, Shilpa Chooniedass, Jimin Guo, and Anne Blanchard in the same lab. Charlton, thanks for all the demonstrations and criticisms of goodwill when I embarked on the research. His wide knowledge and logical way of thinking have been of great value for me. Shilpa, an excellent and smart senior graduate student, thanks for sharing expertise and providing all the advice on research. Mohammad, a very experienced teacher and considerate friend, and Jimin, thanks for all the help on my daily life. I would like also to thank Anne, Barb and Dr. Myal, Dr. Amara for all advice and guidance on my project. All colleagues in Department of Biochemistry, Tuntun, Jan, Nadia, Lil..., thanks for helping hands when I was in all different kinds of "trouble".

Finally, I am grateful to my parents and my wife for the mental support. Love brings us together and binds us tight!

Table of contents

List of Figures.

List of the Table:

:

Abbreviations:

AF-1: Activation Function 1
AF-2: Activation Function 2
AR: Androgen Receptor
CHIP: Chromatin Immuprecipitation
DBD: DNA binding domain.
ER: Estrogen receptor
ERE: Estrogen receptor element
GR: Glucocorticoid receptor
HRE: Hormone responsive element
IF: Immunofluorescence
IHC: Immunohistochemistry
LBD: Ligand binding domain
MBD3: (methyl-CpG binding domain protein 3)
MEF's: Mouse embryonic fibroblast cells
NCoR: Nuclear co-repressor
PLAU: urokinase plasminogen activator
PR: Progesterone Receptor
Pus1p: Pseudouridine synthase 1
Pus3p: Pseudouridine syntheses 3
RRM: RNA interacting domain
SERM: Selective Estrogen Receptor Modulators
SDM: Site-directed mutatagenesis
SHARP:SMRT/HDAC1 Associated Repressor Protein
SLIRP: SRA stem-loop interacting RNA binding protein
SRA: Steroid receptor RNA activator
SRA1: Steroid receptor RNA activator gene
SRAP: Steroid receptor RNA activator protein
SR: serine/arginine-rich proteins
SRC-1: Steroid Receptor Co-activator 1
STR: Secondary structural motif
TMA: Tissue microarray
TPPCR: Triple Primer PCR
TR: Thyroid hormone
YB-1: Y-box bindng protein

Introduction

1 Estrogen action in breast cancer

1.1 Breast Cancer in 2008

Breast Cancer remains the second leading cause of cancer deaths of women worldwide. Also breast cancer is one of the most frequently diagnosed cancers with an estimated 1,000,000 new cases detected each year worldwide. Approximately 22,300 Canadian women have been diagnosed with breast cancer in 2008 (1). Due to the early detection of breast cancer, the 5 year survival rate is approximately 89% for women with locally advanced breast cancer (1). However, if the cancer has metastasized, the average survival time drops to less than 2 years. Therefore, it remains of crucial importance to develop new approaches to treat breast cancer patients (2).

1.2 Hormone therapy and breast cancer

One approach widely used to fight breast cancer is hormone therapy. The link between hormones and breast cancer growth and development has indeed been recognized for more than a century (3). Estrogen, an ovarian hormone controlling normal mammary gland growth and development, also participates in breast tumor growth and progression through its mitogenic action on breast epithelial cells. The fact that estrogen is able to induce tumor growth led to the concept that blocking its action would benefit patients. This action is the basis behind endocrine therapy. Endocrine therapy for breast cancer includes blocking the

action of hormones or removing the source of hormones to slow or stop the growth of breast cancers (4). Tamoxifen, which competes with estrogen for binding of their receptors (see below), is considered as (SERM) Selective Estrogen Receptor Modulators and first-line endocrine therapy. This first anti-estrogen had been tested successfully for the treatment of all stages of breast cancer patients for more than 20 years and saved the lives of thousands of women (5). Similarly, Raloxifene, a new generation anti-estrogen for endocrine therapy, have similar efficacy but fewer serious side effects than tamoxifen (6-8). *Schinzinger* first recommended surgical oophorectomy as a treatment for breast cancer in 1889 when he made the observation of a better prognosis of breast cancer in older women than in younger women (9).Currently, aromatase inhibitors are used to treat post-menopausal patients. Indeed aromatase inhibitors could block the activity of aromatase, an enzyme that converts androgen to estrogen, resulting in lower estrogen level (10). Despite significant antineoplastic activities and overall survival benefit of Tamoxifen or Raloxifene achieved in 70% breast cancer patients, most initially responsive breast tumors became resistant (11;12). Aromatase inhibitors are also limited to treat only post-menopausal patients because higher circulating levels of androgens present in pre-menopausal women compete with aromatase inhibitors for the aromatase enzyme complex, resulting in less efficient suppression of estrogen production (13). Therefore, hormone resistance and limitation of aromatase inhibitors raise the hope that more survivors will be spared through the better investigating and understanding of the mechanisms of estrogen action.

1.3 Estrogen receptor and estrogen receptor signaling pathway

1.3.1 Estrogen receptors

Estrogen action is primarily mediated through its two estrogen receptors (3). Over the past 30 years, an enormous amount of research has been dedicated to the study of ER α, largely due to the success of endocrine therapies primarily targeting ER α to treat women with breast cancer (3). Recently, a second estrogen receptor designated as ERβ was identified (14). ER α and ERβ are encoded by 2 genes located on Chromosome 6 and 14, respectively, and belong to the steroid/thyroid/retinoic acid receptors superfamily (15). They possess identical functional and structural organizations: a variable N-terminal region containing a hormone-independent activation domain (AF-1), an identical DNA binding domain and a C-terminal region containing both the ligand binding domain and a hormone-dependent activation domain AF-2 [Fig 1].

In addition to the canonical full-length ER transcripts (which encode all the aforementioned functional domains), there are many different ER variants represented in the transcriptosome that arise due to alternative splicing (16;17). For ER α, the most frequently detected splice variants in breast tumors appear to have exon 7 deleted and/or exon 4 deleted ER α (18). The predicted products of these ER α variant transcripts will be truncated proteins missing some structural/functional domains of the wide-type ER α. For example, the exon 7 deleted ER α variant mRNAs will encode a C-terminally truncated protein in which the ligand-binding domain is missing (19).

For ERβ, several variants sharing the same N-terminal extremity but different in their C-terminal regions have been recently detected in breast tissues (20-22) [Fig 2]. Three major ERβ variants (ERβ 1,2/cx, 5), resulting from alternative splicing events involving exon 7 and 8, have been identified (23) [Fig 2]. ERβ1, 2/cx isoforms are identical in exons 1-7, but differ

in alternative exon 8. ERβ5 variant contains an extended exon-7 and its exon 8 results from the splicing of an intron containing atypical CC and CA donor and acceptor sites (24) [Fig 2].

1.3.2 Estrogen receptor signaling pathway

The estrogen receptors are considered crucial in the mechanism of estrogen mediated growth and differentiation. ERs primarily act as ligand-dependent transcription factors, regulating critical cellular events mainly through mediating gene transcription (25). The classical mechanism of estrogen receptor action has been well established (26). Once bound to the ligands, both ERα and ERβ undergo conformational changes, dissociate from heat shock proteins and translocate into the nucleus [Fig 3]. After dimerization, estrogen receptors specifically recognize cis-acting elements (estrogen responsive elements (ERE)) on the upstream promoters region of target genes. Activated Estrogen receptors, through dynamic interplays with additional proteins, direct the assembly and stabilize the pre-initiation complex that ultimately conducts gene transcription (27-29).

Estrogen receptors can also be activated in a ligand-independent manner by phosphorylation in response to various growth factor signaling pathways, such as epidermal growth factor (30) and insulin-like growth factor type I (31;32). As a result, such as activation by epidermal growth factor receptors (EGFRs) 2 (HER-2/*neu)* may account for the development of tamoxifen resistance in some ER-containing breast cancers (33).

Although ERE-like sequences are required in the two pathways mentioned above, around one third of the genes in human that are regulated by ERs do not contain ERE-like sequence. It has been found that ERs are able to regulate gene expression without binding directly to

DNA (34). ERs can indeed indirectly regulate gene transcription through protein-protein interaction with other transcription factors (35). The interaction of ERs with the activator protein 1 (AP-1) transcription factor complex is a typical example of such ERE-independent genomic actions. ERs therefore crosstalk with many other signaling pathways (26).

Estrogen exerts its effects mainly though the action of ERs on gene expression as mention above, but a number of other effects are so rapid that they cannot depend on the activation of DNA and protein synthesis (36). These actions are known as nongenomic actions and are believed to be mediated through membrane-associated ERs. The actions are frequently associated with activation of various protein-kinase cascades (36). However, nongenomic actions of estrogen might eventually influence gene expression by targeting transcription factors, including AP-1, which is regulated through phosphorylation and might be targets of nongenomic actions of estrogens (26).

Given the many transcription pathways and genes regulated by the estrogen receptors, it is not surprising that the aberrant ER function leads to disease phenotype. The most well studied of these is the relationship between ER and its role in breast cancer and cancer progression.

1.3.3 Relevance of ERs in breast cancer

ERs have been found in about 70% of tumors, where they are believed to potentially regulate cancer cell proliferation and metastasis (37;38). ER α expression is elevated in breast epithelium, whereas ERβ expression appears to decline during breast tumorigenesis (39;40).

This suggests that ER α and ERβ may play different roles in breast cancer tumorigenesis. ER α is a well-established risk factor as well as treatment target (41). However, compared to ER α, the functions of ERβ appear to be more complicated and controversial, because ERβ seems to behave differently in the absence or presence of ER α. Recent progresses have shown that ERβ works as a counter partner of ER α through dominant negative function by heterodimerization with ER α in ERα positive tumor (42). Consequently, ERβ is usually considered as a anti-proliferative factor in ER α positive breast cancer (43). On the other side, ERβ expression had been also detected in 50% of classified ER α negative tumors and ERβ is considered to be the proliferative marker (44).

ER function is also linked to distinct structural domains between different variants likely involved in interaction with a plethora of regulatory proteins. Therefore, associations between different ER variants and breast cancer have been studied. Among them, ERβ2/cx/cx is the most widely investigated but remains functionally controversial. Due to the difference in the last exon, ERβ2/cx lacks the amino acid residues important for ligand binding and AF-2 domain, and thus cannot bind estradiol and lose the ability to activate transcription of estrogen-sensitive gene (45). In addition, ERβ2/cx is able to heterodimerize with ERα with higher affinity than with ERβ, resulting in dominant negative effect on the ligand-dependent transactivation function of ERα which are probably related to its anti-proliferative properties (45). *Speirs's* group found that ERβ2/cx predicted good clinical response to tamoxifen and overall survival (46). However, *Palmieri and Omoto* identified a significantly increased frequency of ERβ/cx positivity in tumors compared to normal/benign tissues (47-49).

Additionally, *Honma et al* examined total ERβ, ERβ1, ERβ2/cx in 442 Japanese breast cancer patients who all received tamoxifen therapy. He showed that ERβ1 was an important independent predictor for overall survival particularly in triple-negative breast cancer (50). Triple negative breast cancer does not express ER α, progesterone receptor or HER2 and therefore remains non-responsive to current targeted therapies. This leads to a higher risk of recurring for breast cancer patients. Recently, by doing immunohistochemistry assay, *Shaaban et al* found that not only the variants of ERβ expressed, but the cellular location of ERβ variants influence prognosis. Nucleus located ERβ2/cx has been shown to correlate with better overall survival in breast cancer. Interestingly, *Leygue* found that increase in ERβ2/cx and β5 RNA variants relative to ERβ1 RNA isoform occurs during breast tumorigenesis (49). As a result, ERβ variants are considered as potentially relevant when assessing factors to be considered in clinical breast cancer trials (46;51).

1.3.4 Estrogen receptors and co-regulators

Over the last few years, further investigations of ER signaling pathway has led to the identification of more molecules potentially controlling estrogen action. In addition to estrogen receptors, the complex mechanism underlying gene transcription also requires transcription co-regulators, which usually form complexes with estrogen receptors through protein-protein interactions followed by dynamical recruitment to specific gene promoters. These complexes regulate the assembly and activity of the transcription initiation complex through chromatin remodeling (27;52). Ever since the characterization of the first co-regulator, the steroid receptor co-activator 1 (SRC-1), the list of these factors grows rapidly.

Based on the outcomes of their regulations, co-regulators could be categorized as co-activators and co-repressors. In general, co-activators are defined as molecules that are directly recruited by ERs to enhance NR-mediated gene expression, usually in a ligand-dependent manner, whereas co-repressors function as counterparts of co-activators to attenuate NR-mediated transcription, primarily through their interaction with unliganded ERs [Fig 3] (53). However, some co-repressors, such as LCoR (ligand-dependent nuclear receptor corepressor), can bind to estrogen receptor in the ligand-dependent manner and compete with co-activators by displacing them (54). Furthermore, some co-regulators, such as the ATP-dependent chromatin remodeling complexes: SWI/SNF, could be involved in regulation of both gene activation and repression (55;56) [Fig 3]. Therefore, it is important to consider that the role of a given co-regulator is context and promoter dependent (57;58) [Fig 3].

Co-regulators are not only crucial mediators of ER action but also play a very important role in the mechanisms of breast tumorigenesis and breast cancer progression (27;59). Therefore many of these co-regulators are believed to behave as oncogenes or tumor suppressor genes depending upon signaling and cell context (60). Alternative to the hormone therapies, co-regulators could provide potential targets to design novel therapeutic target to treat breast cancer. It is indeed perceivable that attenuating the co-activator function or enhancing the co-repressors function in breast cancer may modulate ER activity and ultimately inhibit cancer cell proliferation and invasion. Therefore, it is highly relevant to not only identify new co-regulators but also to characterize their mechanisms of action in regulating ER activity.

2. Steroid Receptor RNA Activator (SRA) and SRAP

2.1 SRA RNA

2.1.1 Discovery of SRA, an RNA Co-activator

In order to identify potential co-regulators interacting with AF-1 domain of the progesterone receptor (PR), *Lanz* screened a human B-lymphocyte library using AF-1 domain as bait in yeast-two-hybrid assay (61). They identified a new clone, they called SRA, for steroid receptor RNA activator. This cDNA was unable to encode a protein, but was required for the growth of the yeast colony. Further experiment confirmed that the potential co-activation role of SRA on PR was mediated through a RNA transcript rather than any protein product.

2.1.2 Core sequence of SRA and predicted functional region

Exon 2-exon 5 of SRA, found to be necessary and sufficient for co-activation function, is called the core sequence (61) [Fig 4A]. Predicted several secondary structural motifs of RNA are distributing throughout the whole core sequence, believed to form a functional structures [Fig 4B]. By doing site-directed mutagenesis experiment, six secondary structural motifs (STR1, 9, 10, 7, 11, 12) have been identified participating independently in PR co-activation (62). It was found that silent mutations in both SRT1 and STR7 of SRA could decrease by more than 80% co-activation function (61) [Fig 4C].

2.1.3 Effect of SRA RNA on ERα and ERβ signaling

Several research groups have now confirmed that SRA is able to increase estradiol induced gene transcription by both full length ERs subtypes (63-66). SRA RNA has been shown to co-activate in a ligand-dependent manner the action of the AF-2 domain of both ERα and ERβ on some but not all ERE- luciferase reporters (64;65). Besides, SRA can also enhance AF-1 domain of ERα but not ERβ in a ligand-independent manner (64;65). Overall, data suggest that the action of the two estrogen receptors are differentially regulated by SRA. Moreover, SRA regulation of a given receptor is also specific of a given ERE sequence (67).

2.1.4 Emerging mechanisms of SRA RNA action

Several studies have been published discussing the mechanism of SRA RNA action (67). It has been previously established that SRA action is not exclusively limited to increasing steroid receptor activity. Indeed it was also confirmed that SRA enhance the activity of other nuclear receptor (68;69) as well as the activity of MyoD, a transcription factor involved in skeletal myogenesis (70;71). Beside, several proteins participating in the formation of complex with SRA have been identified. These include the transcription factors whose activity is enhanced by SRA and proteins acting as positive or negative regulator of ER activity. Firstly, SRA's coactivation function is activated by two pseudouridylases, Pus1p and Pus3p, which have also been characterized as co-activators (72). This modification alters the secondary structure and rigidity of the target SRA RNA molecules to promote proper folding, resulting in synergized co-activation function (73). The other positive regulators include the receptor co-activator 1 (SRC-1) (61) and the RNA helicases P68/72 (74). SRC-1 belongs to p160 family co-activators (SRC1, SRC2/TIF2 and SRC3/AIB1), which can recruit other co-regulators to steroid receptors as well as promote a functional synergy between AF-

1 and AF-2 domains (27;75;76). Using co-immunoprecipitation from an expression system consisting of Xenopus oocytes programmed with in vitro generated RNA, SRA was found to associate with SRC-1 (61). The p72/p68 proteins are DEAD-box RNA binding helicases that can physically interact with p160 family proteins and with ER α.AF-1 region (71). The p72/p68 is able to bind to SRA through a well conserved motif in the DEAD box and synergizes with SRA and SRC2/TIF2 to co-activate ER α activity in the presence of estradiol (77).

On the other side, SRA may also serve as a platform to recruit some negative regulators consisting of the SMRT/HDAC1 associated repressor protein (SHARP) (78) and the SRA stem-loop interacting RNA binding protein (SLIRP) (79). SHARP was found to physically interact with co-repressors through its repression domain (RD) whereas it interacts with SRA through a RNA recognition motif (RRM) (78). Similarly, SLIRP specifically binds to SRA STR-7 and attenuates SRA-mediated transactivation of endogenous ER (79).

The emerging model of SRA action on ER α signaling had been summarized [Fig 5]: Pus1p pseudouridylates specific SRA RNA uridine residues, leading to an optimum configuration of this RNA. The resulting active form of SRA-ψ, could stabilize complex with p68 and SRC-1. Transcription of target genes with suitable ERE will occur. In the other side, regarding inhibition of SRA RNA-mediated ER-α, SLIRP and SHARP act as negative regulators. It has been proposed that they might act by sequestrating SRA by destabilizing the complex SRA/SRC-1 or by recruiting the nuclear receptor corepressor N-CoR at the promoter region of silenced genes (67).

2.1.5 SRA expression and relevance to breast cancer

Different SRA transcripts, detected by Northern blot, have been observed in normal human tissues (61). SRA seems highly expressed in liver, skeletal muscle, adrenal gland and the pituitary gland, whereas intermediate expression levels are seen in the placenta, lung, kidney and pancreas. Interestingly, brain and other typical steroid-responsive tissues such as prostate, breast, uterus and ovary contained low levels of SRA RNA (61)

However, SRA RNA expression, assessed by RT-PCR amplification, is increased during breast and ovarian tumorigenesis (80-82). Interestingly, SRA over-expression might characterize particular subtypes of lesions among different tumors. Indeed, serous ovarian tumors expressed higher levels of SRA than granulosa cell tumors (82).

The involvement of SRA in ER action indicates its possible functions in breast tumor pathology. Indeed, ER-α-positive/PR negative breast tumors expressed more SRA than ER-α-positive/PR-positive breast tumors(81), whereas Tamoxifen-sensitive and resistant breast tumors express similar levels (83).

A possible direct involvement of SRA in the mechanisms underlying breast tumorigenesis and tumor progression has been proposed (80;81). The generation of transgenic mice has however demonstrated that over-expression of the core SRA sequence in the mammary gland led to pre-neoplastic lesions but was not sufficient per se to induce tumorigenesis (80).

This inability of SRA core to lead to a full malignant phenotype by itself suggests that a complex interplay might involve in steroid receptor activities or possibly other SRA–regulated factors need to be further discovered.

2.2 Coding SRA and SRAP

2.2.1 Discovery of SRAP

Kawashima et al. reported in 2003 the cloning of a new rat SRA cDNA mostly identical to the core SRA sequence from exon 2 to exon 5. This cDNA was, however translatable *in vitro* encoding a putative 16KD protein starting at the third methionine codon of the rat SRA cDNA sequence (84). It should be stressed that the existence of a corresponding endogenous 16KD SRAP has never been proved.

In the nucleotide database of the National Center for Biotechnology Information, most human SRA sequences contain an intact core sequence (exon-2 to exon-5) but differ in their 5'-extremity [Fig 6]. Interestingly, some variants having 5' end extention contain two start codons with a large open reading frame potentially encoding a 236/237 amino acid peptide. These cDNAs, as opposed to the original SRA, were translatable *in vitro*, as well as *in vivo*, leading to the production of a protein localized both in the cytoplasm and the nucleus (85). In addition, sequence of SRAP is highly conserved among chordate and the presence of endogenous SRAP had been found in the testes, uterus, ovary and prostate, as well as mammary gland, lung and heart (86). Altogether, accumulated data have demonstrated that SRA1 gene products consist in two characteristic entities: a functional RNA, which through

its core sequence, can co-activate transcription factor and a protein which function remain to be determined.

2.2.2 Function of SRAP

Chooniedass-Kothari et al. reported the existence of putative endogenous human SRA protein in breast cancer cells and differential expression in breast tissues (87). A decreased response to ERα activity observed in stably SRAP transfected MCF-7 cells suggested that this protein might repress estrogen receptor activities (87). This results contrasts with *Kawashima*'s results, who found that the transient transfection of full length rat SRA coding sequence and led to an activation of the response to androgen (88). It should be pointed out that, both coding sequence of SRA used by these two groups also contains the functional core sequence of SRA RNA proven to co-activate ERα. Therefore, it is difficult to draw any conclusions regarding the individual function of SRAP on estrogen receptor activities when functional SRA RNA and SRAP protein are co-expressed.

In order to understand the functional role of SRAP independently of SRA RNA, two different groups have investigated physical protein properties by tandem mass spectrometric analysis of SRAP co-immunoprecipitation samples (89;90). Interestingly, both groups showed that SRAP is able to interact with transcriptional regulators. In *Chooniedass-Kothari's* unpublished results using mass spectrometry, MBD3 (methyl-CpG binding domain protein 3, a member of the nucleosome remodeling and histone deacetylase complex, Nurd), BAF 57 (a core subunit of SWI/SNF chromatin remodeling complex) and YB-1 (Y-box bindng protein, a general transcription factor) have been found to interact with SRAP(89;90).

By using a similar approach, *O'Malley's* group also found that the transcription regulators, such as, BAF 170 (BRG1 associated factor 170, also belonging to SWI/SNF chromatin remodeling complex) and YB-1 are associated with SRAP (89). It is necessary to point out that different cell line models and antibodies were used in these two groups. *O'Malley's* group used Hela cell lines and 743 antibody (commercial available rabbit polyclonal antibody) whereas *Chooniedass-Kothari* used MCF7 stably over-expressed SRAP/V5 cell line and V5 antibody. Interestingly, nobody has confirmed any protein-protein interaction between SRAP and those potential partners by co-immunoprecipitation experiments.

The observation that SRAP forms complex with transcription factors led to investigate its direct association with transcription factors. By using recombinant SRAP and protein arrays, we found that SRAP interact with different transcription factors including ER α and ERβ with different binding affinities (90). To further validate the interaction between ER and SRAP, we perform GST pull down assay and a direct interaction between GST-SRAP and both full length radio-labeled estrogen receptor α and β was observed (90). Interestingly, *Kawashima* showed that SRAP is also able to directly interact with the AF-2 domain of AR *in vitro* by doing GST pull down assay (84).

Therefore, although the exact roles of SRAP on nuclear receptor signaling pathway remain to be fully elucidated, accumulated data suggest an action of SRAP. This action could possibly consist in regulating downstream gene by recruitment of complexes containing ERs in the gene's promoter regions. This putative SRAP protein's action on ERs might be different from that of SRA RNA's action. It is therefore crucial to establish a model to separate SRA RNA and SRA protein function.

3. Alternative RNA splicing of SRA gene

The balance between co-activators and co-repressors may ultimately controls estrogen action in a given tissue (91). A direct participation of this balance during breast tumorigenesis and cancer progression is now suspected, and a search for possible means to control it has started worldwide (57;92). Alternative splicing of SRA gene might control the balance between the coding and non-coding SRA, and ultimately might function as the potential mechanism to regulate the balance between co-activators and co-repressors.

3.1 Coding and non-coding SRA due to alternative splicing

As discussed in section 2.1.1, several SRA transcripts coding and non-coding have been identified. They are respectively fully spliced or alternatively spliced [Fig 6A]. Sequences containing a full or partial retention of intron-1, introducing a frame shift or a premature stop codon to SRAP reading frame, are non-coding [Fig 6A]. These transcripts, however are still able to be co-activator as they contain intact core region. This suggests that accurate splicing of SRA intron-1 is a major event controling the balance between coding and non-coding SRA.

3.2 Coding and non-coding SRA transcripts co-exist in breast cancer

We have now established that both non-coding and coding SRA transcripts co-exist in breast cells (93). Using a previously validated triple-primer PCR (TP-PCR) assay (94), which

allows co-amplification and relative quantification of two transcripts sharing a common region but differing in another, we found that breast cancer cell lines co-expressed normally spliced coding SRA RNA as well as SRA RNA containing intron-1 [Fig 7] (93).

Interestingly, breast cancer cell lines differ in their relative levels of coding/non-coding SRA transcripts. In particular, the three most invasive cell lines (MDA-MB-231, 468, and BT-20) expressed the highest, whereas the "closest to normal" MCF-10A1 breast cells expressed the lowest relative levels of SRA intron-1 RNA. This suggests that a balance changed toward the production of non-coding SRA1 RNA in breast cells might be associated with growth and/or invasion properties (93).

3.3 Increasing the relative ratio of non-coding SRA1 RNAs leads to a change in ERβ expression

Alternative splicing events result from the relative local concentration of RNA binding proteins within the microenvironnement surrounding the nascent pre-mRNA (95). We were recently able to artificially alter the balance between coding and non-coding SRA1 RNAs in T5 breast cancer cells using a previously described strategy (95;96). This approach, which results in an increase in the production of intron retained transcripts, consists in covering the junction between exon and intron with a modified oligonucleotide [Fig 6B]. The binding of this oligonucleotide on exon-intron junction interferes with the binding of SR proteins and hence inhibits the efficient splicing of the intron considered. When introduced in breast cancer cells T5, the oligonucleotide SRA-I1 (designed to cover SRA1 exon-1 and intron-1

junction) increases the relative expression of SRA intron-1 retained RNA (noncoding) and decreases endogenous SRAP expression [Fig 7].

To establish whether this artificial alteration of the balance coding/non coding SRA1 RNAs impacts on gene expression, total RNA from T5 cells transfected with SRA-I1 was analyzed 24 hours post-transfection, by real-time quantitative PCR using a Breast Cancer and Estrogen Receptor Signaling RT2 Profiler™ PCR Array (SuperArray Biosciences USA) and a Biorad I-cycler (Biorad). This technology consists in using 96 wells plates containing each an optimized pair of primers corresponding to the series of genes considered. It provides the fastest way to interrogate the effect of a given treatment on genes historically linked to different pathways or pathologies (97). Interestingly, we observed a significant increase in the expression of the urokinase plasminogen activator (uPA, PLAU), gene intimately linked to invasion mechanisms (98) as well as of ERβ, involved, as highlighted earlier, in breast cancer cell growth (99) [Fig 8]. It is important to stress that those effects could result from the relative increasing amount of SRA RNA or decreasing of fully spliced coding SRA.

Summary and Rationale of project:

Steroid receptor RNA activator (SRA) has been implicated in estrogen receptor signaling pathway. Its expression is altered during breast tumorigenesis and its molecular role in underscoring these events has been suggested. The SRA1 gene encodes both functional RNA and protein (SRAP) products, making it a unique member amongst the growing population of steroid receptor co-regulators. In breast cancer cells, the balance between SRA and SRAP is partially regulated by alternative splicing of SRA intron-1. We have observed that increasing SRA intron-1 retention led to an increase in ERβ expression in T5 cells. Beside, SRAP protein interacts with ERβ protein in vitro by GST-pull down assay. Therefore the potential relationship between SRA/SRAP and ERβ needs to be further explored. The present study was designed to establish models to separate SRA RNA and SRA protein function and to investigate whether SRA RNA and/or SRAP regulate the expression of ERβ. We also aimed at confirming potential interactions between SRAP and ERβ *in vivo*. Beside, in order to investigate in the future potential correlation between SRAP and ERβ expression in breast cancer tissue, we aim at optimizing IHC condition for SRAP antibodies.

Study outline:

Aim1: to characterize whether SRA RNA and/or SRAP regulate the expression of ERβ in vitro, I used two models. **The first Model** is based on a *Lanz et al.* observation that silent mutations modifying SRA secondary structure suppressed the RNA ability to co-activate PR activity (62). Accordingly, we generated 4 constructs with specific outputs: RNA (functional) and/or protein. **The second model** consisted in using a new-SRAP construct generated

through artificial protein synthesis and encoding for SRAP. Compared to the specific site mutations in the first model, this construct is drastically different from the original SRA RNA sequence. Particularly, specific secondary structures of the core, shown to be necessary for the RNA function (62) have been extensively mutated. The constructs established above will be transfected into T5 cells and ERβ mRNA and protein expression will be monitored by real-time PCR and Western blot analysis, respectively.

Aim2: to confirm an in vivo physical interaction between SRAP and ERβ, Co-IP experiments will be performed in MCF7-ERβ1, β2 cells and 293 cells. MCF7-ERβ1, β2 cells express endogenous SRAP and doxycycline-inducible ERβ1/xpress, ERβ2/cx/xpress. Whole cell lysates of MCF7-ERβ1, 2 cells after treatment of doxycycline will be extracted and IP performed using anti-SRAP and anti-xpress antibodies. The interactions between ERβ1, β2 and endogenous SRAP will be checked by Western blot using anti- ERβ (chicken) and SRAP antibodies. To demonstrate whether the structural domains of ERβ potentially interacts with SRAP, ERβ1AF1/DBD, ERβ1AF2/DBD and full length of ERβ1 constructs with V5 tag will be first generated. 293 cells will be co-transfected with SRAP/Flag and ERβ1/V5 constructs. Whole cell lysates will be extracted 24 hours after transfection and IP will be performed by using anti-Flag and anti-V5 antibodies.

to set up immunological IHC conditions for performing future TMA analysis

Aim3: to set up IHC conditions for performing future TMA analysis, different SRAP antibodies have been tested for IHC conditions. (ERβ antibodies conditions for IHC had been well validated by *Dr. Murphy*'s lab). I will pick up one SRAP antibody which is able to detect both mouse SRAP and human SRAP by Western blot and IHC. Specificity of this

antibody should be able to be confirmed by peptides (recombinant protein) competition experiments. Beside, SRAP expression should not be detected in SRAP Knockout MEF's by this antibody.

Materials and Methods

1. Mammalian Cell culture

T5 Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 5% Fatal Bovine Serum (FBS, Hyclone or Cansera), 0.3% D-Glucose (Fisher), 2mM L-Glutamine (GIBCO), 1000 units/ml pencillin and 100 μg/ml streptomycin (GIBCO).

293 (kindly provided by *Dr. Kong's* lab), Hela (ATCC) and MEF's cells (kindly provided by *O'Malley's* lab were maintained in DMEM, supplemented with 5% FBS, 0.3% D-Glucose, 2mM L-Glutamine, 1000 units/ml pencillin and 100 μg/ml streptomycin

293FT (Invitrogen) cells were maintained in DMEM supplemented with 10% FBS, 0.3% D-Glucose, 2mM L-Glutamine, 1000 units/ml pencillin and 100 μg/ml streptomycin, 500 μg/ml G418, 1mM MEM sodium pyruvate, 0.1nM MEM non-essential amino-acid.

MCF7-S151 (stably transfected with SRA1 clone 51) cells were maintained in DMEM, supplemented with 5% FBS, 0.3% D-Glucose, 2mM L-Glutamine, 1000 units/ml penicillin and 100 μg/ml streptomycin and 250 μg/ml G418.

MCF7-β1 (MCF7 doxycycline-inducible ERβ1 (100), clone 91, kindly provided by Dr. Murphy) cells were maintained in DMEM, supplemented with 5% FBS, 0.3% D-Glucose, 2mM L-Glutamine, 1000 units/ml penicillin and 100 μg/ml streptomycin , 100 μg/ml G418

and 100 μg/ml Hygromycin B (GIBCO).

Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in a water-jacketed incubator.

2. Constructs:

2.1 Four pLenti-SRA constructs:

2.1.1 Cloning into pLenti6.2/V5-TOPO vector:

SRA SDM1 and SDM7 mutation in SRA PRO and TTG mutation in SRA RNA constructs were introduced into pCDNA3.1-V5 plasmid using the QuickChange Site-Directed Mutagenesis Kit (Statagene). Subsequently, 4 different SRA cDNAs were amplified using 4 different pCDNA3.1-SRA-V5 plasmids as templates. The plenti-TTGSRAupper (CACCTTGACGCTCTGCCCCGCTG) and SRAupper (CACCATGACGCGCTGCCCCG CTG) and SRAlower primers (TGAAGCCTCCTGGAAGCCTGGT) were used in order to introduce flanking CACC Sites. Following PCR amplification (PCR Conditions: 1. initially denature 98°C 1 min. 2. Denature 98°C.10 sec, annealling 60°C.15 sec, extention 72°C.30 sec. 3 final extention 72°C 8min), the different SRA cDNAs were visualized on the 1% agarose gel under UV light. The corresponding bands on the gel were cut and weighted. After adding same amount of membrane binding solution, the solution containing cDNA were gone through the column and washing with ethanol twice. Final eluted by 50 μl nuclease-free water. By using the 30 μl to check it again by 1.5% agarose gel, 4 μl PCR products (5ng) were inserted into pLenti6/V5 vectors (1 μg). Briefly, 4 μl purified PCR

product was gently mixed with 1 μl salt solution, 1 μl pLenti6.2/V5-TOPO vector and 2 μl ddH_2O. The mixture was incubated for 1 hour on ice. For each cloning, 2 μl of the mixture was gently added 1/2 vial of Stb3 OneShot Chemically Competent E. coli. (Invitrogen). The left half vial of Stb3 cells without adding PCR products went through same following procedures as the negative control. The vial was firstly incubated on ice for 30 min. Following the incubation, a heat-shock at 42°C for 30 second was performed and the vial was immediately placed on ice. 250 μl of room temperature SOC Medium was added into the vial and the vial was incubated at 37°C for 1 hour with vigorous shaking (300rpm). 100-200 μl of the actively growing bacterial culture was spread on a room temperature LB-Agar plate containing Carbenicillin (described in 1.1) and cultured at 37°C for 16-20 hours (overnight).

The presence of plasmids of interests in a colony was checked by PCR (primers used by screening were described in Table 3). A colony was lifted with a sterile tip and re-suspended in 50 μl LB medium. PCR template was prepared by boiling 25 μl of the bacteria suspension for 10 min followed by a short centrifugation. For each plasmid to be sequenced, at least 5 colonies were checked, and one colony presenting a strong signal of correct size was selected. For this colony, the remaining 25 μl bacteria suspension was cultured in 5ml LB medium supplemented with Carbenicillin at 37°C for 16-20 hours with vigorous shaking (300rpm).

2.1.2 Plasmid Preparation

0.5ml bacteria containing the 4 pLenti-SRA constructs were pre-prepared to cryo-cultures. For small amount of plasmid preparation, 5ml Carbenicillin supplemented LB medium were

processed with the Mini-prep Kit (Qiagen) according to the manufacturer's instructions. DNA was dissolved in ddH_2O. For large amount of plasmid preparation, 100 µl primary culture was transferred to 200ml Carbenicillin supplemented LB medium and cultured at 37°C for 16-20 hours with vigorous shaking (250rpm). The cultures were processed with the Maxi-prep Kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was eluted by ddH_2O.

2.1.3 Quantification of Plasmid Yield

Dissolved plasmid DNA was 1:200 diluted by mixing 1 µl plasmid DNA with 199 µl filtered water in a well on a UV transparent 96-well plate (Falcon). Each sample was processed in duplicate to control pipetting accuracy. As a blank, 1 µl ddH_2O was processed in the same way. The Spectra Max 190 spectrophotometer (Molecular Devices) was used to measure optical density at the wavelength of 260nm (OD260). Averages of the OD260 readings (OD260ave) from the duplicated wells were calculated. The concentration of plasmid DNA was calculated by the following equation:

$$\text{Concentration (µg/ul)} = \text{OD260ave X 50 x dil. factor} \;/\; 1000$$

An optical density of 1 is converted to 50 µg/µl for double-stranded plasmid DNA and the dilution factor is 200. Yield of a Maxi-prep was typically >300 µg while yield of a mini-prep was typically >10 µg. Purity of the samples was determined by the ratio of readings at 260nm and 280nm. Typical purities range from 1.5 to 1.7.

2.1.4 Restriction Digest

1 μl plasmid DNA is digested by Hind III or Xho I restriction enzymes (Invitrogen) or together in the compatible buffer React 3 (Invitrogen) to single or double digest the plasmid. Samples were incubated at 37°C for overnight and then migrated on 1.5% agarose gel to check the presence of an insert of correct size. All plasmids had been subjected to sequencing by UC DNA Services (University of Calgary).

2.2 Three pLenti-ERβ constructs:

Full length ERβ cDNA were introduced into pCDNA3.1-V5 plasmid (Kindly provided by Dr. Murphy). Subsequently, AF1/DBD, DBD/AF2 domains of ERβ cDNAs were amplified using pCDNA3.1-ERβ plasmids as templates (Primers sequence see table 6). Following PCR amplification (PCR Conditions: 1. initially denature 98°C 1 min. 2. Denature 98°C 10 sec, annealing 60°C 15 sec, extension 72°C 30 sec. 3 final extension 72°C 8 min), the different ERβ cDNAs were visualized on the 1% agarose gel and purified. 4 μl PCR products (5ng) were inserted into pLenti6/V5 vectors (1 μg) followed by incubation with stb3 cells, a heat-shock at 42°C for 30 sec. The positive colonies had been screened by PCR (Primers sequence see table 5) and restriction enzyme digestion (XhoI and BglII).

2.3 New-SRAP/Flag-eGFP and control vector:

New-SRAP/Flag-eGFP had been synthesized by Genocopia Company. New-SRAP/Flag-eGFP contains the mutated SRAP with flag tag and Ires-eGFP in the FIV-Based vector backbone. An extensive amount of mutations (all silent) have been introduced to drastically

disrupt the secondary structure (and hence the functionality) of the corresponding SRA RNA without altering its ability to encode for SRAP [Fig13a]. pGIPz (GFP control vector) was kindly provided by *Winneta* in *Dr.Murphy's* lab.

2.4 Lentiviral helper vector and control vector:

3 system helper vectors HIV-1 gag and polymerase (pLP1), HIV-1 Rev (pLP2) and VSV-G envelope glycoprotein (pLP-VSVG) were purchased from Invitrogen.

2 system helper vectors psPAX2, pLP-VSVG (addgene) were kindly provide by Dr. Kong's lab (Department of Anatomy, University of Manitoba).

The pLenti/Laz/V5 (viral control vector) was purchased from Invitrogen

2.5 siRNA of ERβ (on-target plus smartpool ESR2)

siRNA of ERβ and control scramble oligonucleotides were purchased from Dharmacon (cat: L-003402-00). Sequences of siRNA are shown in table 9.

2.6 Single-Stranded Oligoribonucleotides of SRA

2'-O-Methyl-oligoribonucleoside phosphorothioate 20-mers anti-sense to the 5'-splice site of SRA intron-1 (SRA-AS, ACCCGGCUUCACGUACAGCU) and to the 5'-splice site of a modified β–globin intron (βgl-AS, ACCUGCCCAGGGCCUCACCA), as well as

fluorophore conjugated versions of the aforementioned oligoribonucleotides were synthesized and purified by Trilink Biotechnologies, Inc. (San Diego, CA).

3. Transduction:

3.1 Determining Blasticidin sensitivity (Killing curve) for T5 cells:

The pLenti expression constructs contain Blasticidin resistance gene (bsd) to allow for Blasticidin selection of mammalian cells that have stably transduced the lentiviral construct. To determine the minimum concentration of Blasticidin which required to kill untransduced mammalian cell line, the kill curve experiments were performed on T5 cells. Typically, T5 cells in 6-well plates (200×10^3 cells/well, 25% confluency) were plated with complete maintenance medium to allow cells to adhere overnight. The next day, replace culture medium with medium containing varying concentrations (0, 2, 4, 6, 8, 10 μg/ml) of Blasticidin (Invitrogen). Replenish the selective media every 3 days and observe the percentage of surviving cells. The concentrations of 6 μg/ml were sufficient to kill all the T5 cells in the plates within 14 days

3.2 Package of viral particles:

For the generation of infectious lentiviral particles, pLenti6/V5 vectors containing 4 mutated SRA constructs were co-transfected with ViraPower™ packaging plasmid mixture: pLP1,

pLP2, and pLP/VSV-G (Invitrogen) or psPAX2, PDM2G (provided by Dr. Kong) into 293FT cells using Lipofectamine 2000 (Invitrogen). In 3 helper system, 9 micrograms of ViraPower mix to 3 micrograms of SRA vector or, in 2 system, 6 micrograms of psPAX2, 3 micrograms pLP-VSVG to 3 micrograms of SRA vectors were used. The T75 flasks were used to produce each batch of lentiviruses [Fig 19]. Virus containing media was collected 48–72 h after transfection, centrifuged at 1000 x *g* for 15 min and filtered through a 0.45 μm filter (Millipore), and viral particles were concentrated as described by *Kung al.* (101) with some modifications. Virus-containing supernatants were carefully loaded in 30-ml conical-bottomed polyallomer centrifuge tubes (Beckman) and centrifuged at 17,000 x *g* for 2.5 hours at 4°C using a swinging bucket rotor SW-28 (Beckman). The medium was carefully removed and discarded. The residual medium containing viruses (around 100 μl each tube) were gently resuspended by adding another 100 μl sterile PBS. The viral supernatant was remaining on ice overnight at 4° C. The next day by gentle vortexed 15s twice, the viral supernatants were aliquoted, and stored at -80 °C until use.

3.3 Titering the lentiviral stock:

Infectious titres of viruses were determined by blasticidine selection method. For this, T5 cells in 6-well plate (3 x 10^5 cells/well) were incubated with serial dilutions (1/10-1/100000) of the viral stock in the presence of 6 μg/ml Polybrene (Invitrogen). Infected cells were selected in medium containing 6 μg/ml blasticidin. The mdeidum was replaced with fresh medium containing antibiotic every 3-4 d. After 10-12 d of selection, there was no live cell in

the mock well. Then, other wells were stained with crystal violet to count the blue-stained colonies. Titer of the lentiviral stock was determined by: Number of clones × Folds of dilution (TU/mL). Our preparations of concentrated lentiviral stocks consistently yielded titres of 2–10 x 10^6 transducing units/ml. Preparations of un-concentrated lentiviral stocks consistently yielded titres of 2–10 x 10^5 transducing units/ml.

3.4 Infect breast cancer cell lines:

T5 and Hela cells were cultured in 12-well plates at 1.5 x 10^5 cells/well were infected by pLenti-SRAP and pLenti-LacZ using different MOI (Multiplicity of Infection) from 0.1, 0.5, 1, 5 in the presence of 10 μg/ml Polybrene. The complete media containing viral particle were replaced by the regular medium containing serum and antibiotics after 24 hours. Protein samples were analyzed after 48-72 hours.

4. Transfection:

4.1. Delivery of Plasmid DNA

Lipofectamine (Invitrogen) was firstly used to transfect plasmid into cultured T5 cells. Optimized 250,000 T5 cells were plated in 6-well dishes 48 hours prior transfection to achieve around 70% confluency in the day of transfection. The cells were incubated in antibiotic-free medium 1 day prior transfection. Transfections were performed according to the manufacturer's instructions at a ratio of 4 μl transfection reagent per 1 μg of DNA in

Opti-MEM reduced serum medium (Invitrogen). 5 hours post-transfection the medium containing lipofactamine was replaced by regular medium containing serum and antibiotics.

Lipofectamine2000 (Invitrogen): Optimized 300,000 T5 cells were plated in 6-well dishes 48 hours prior transfection to achieve around 80% confluency in the day of transfection. The cells were incubated in antibiotic-free medium 1day prior transfection. Transfections were performed at a ratio of 10 μl transfection reagent per 4 μg of DNA in Opti-MEM reduced serum medium (Invitrogen). 4 hours post-transfection the medium containing lipofactamine 2000 was replaced by regular medium containing serum and antibiotics.

Nucleofection (Amaxa): New-SRAP/Flag-eGFP was constructed as described previously. T5 cells were transiently transfected using the nucleofection technology according to the Amaxa Biosystems protocol. Briefly 2 x 10^6 cells were resuspended in 100 μl of Cell Line Nucleofector™ Solution V, and the cell suspension was mixed with 2 μg of pGIPz (negatinve control) and New-SRAP/Flag-eGFP vectors, respectively. The sample was transferred into an electroporation cuvette, and transfection was performed using the program P-20 according to the manufacturer's instructions. Immediately after nucleofection, cells were transferred into pre-warmed complete maintenance medium and were cultured at least 24 hours until proteins were analyzed. Transfection efficiency had been monitored by eGFP and Immunofluence using anti-flag antibody described below.

4.2. Delivery of siRNA of ERβ

DharmaFECT have been used for Delivering siRNA of ERβ: 180,000 T5 cells and MCF7-β1

(control cell line) cells were plated in 6-well dishes 48 hours prior transfection to achieve around 50% confluency in the day of transfection. (During this 48 hours, MCF7-β1 cells were treated with doxycycline (2 μg/μl)). Lipid particles complex were incubated by 4 μl DharmaFECT transfection reagent and 12.5 μg of siRNA of ERβ in Opti-MEM reduced serum medium (Invitrogen) for 30min. Transfections were performed in complete medium containing the DNA-lipid complex for 48 hours at 37°C.

4.3. Delivery of Single-Stranded Oligoribonucleotides of SRA

DMRIE-C (Invitrogen) was used to transfect 2'-O-Methyl oligoribonucleotide (2'-OMe) into cultured mammalian cells. Cells were plated and incubated as described above. Transfections were performed according to the manufacturer's instructions at a ratio of 4 μl transfection reagent per μg DNA in Opti-MEM reduced serum medium (Invitrogen). The medium containing DMRIE-C was replaced by regular medium containing serum and antibiotics 5 hours post-transfection.

5. Western Blot and Immunofluorescence

5.1. Protein Extraction from Cultured Cells and frozen tissue

Cells cultured in 6-well dishes were washed twice with room temperature Phosphate Buffered Saline (PBS) and lysed by 50 μl ice-cold SIB buffer containing 60mM β-glycerophosphate, 1%SDS and a mini-protease inhibitor cocktail tablet (Boehringer Mannheim, Indianapolis, IN, USA) per 10 ml extraction buffer. The lysates were then

sonicated with a Sonicator (Fisher Scientific) for 4 times of 10 sec on ice to sheer genomic DNA. Sonicated lysates were centrifuged at over 10,000g on a personal centrifuge (Eppendorf) to bring down debris to the bottom of the tube. Supernatants were transferred to a new tube and stored at -20°C.

Frozen tumor tissues (from the Manitoba Breast Tumor Bank, MBTB) were lysed and sonicated for 30 min in 100 µl ice-cold SIB buffer. Sonicates were centrifuged at 16,000 g for 20 min at 4°C and supernatant were stored at -20°C until use.

5.2. Protein Concentration Assay

Total protein concentrations in cell lysates were determined using a Micro BCA Protein Assay Kit (Pierce), according to the manufacturer's instructions. Briefly, the cell lysates, alongside with a serial of concentrations of bovine serum albumine (BSA), were 1:500 diluted in sterile water and incubated with Cu^{2+} in an alkaline medium for 60 min at 65°C. A Spectra Max 190 spectrophotometer (Molecular Devices) was used to detect the colorimetric absorbances at 562nm, which indicates the reduction of Cu^{2+} to Cu^{1+} by tripeptides and larger polypeptides.

The SpectraMax software bundled with the spectrophotometer performed a linear regression statistics on the readings of BSA to calculate the equation of the best fit line as a standard (not forced through the origin). The Y-intercept and the slope of the standard line were used to calculate total protein concentrations in the cell lysates.

5.3. SDS-PAGE and Immuno-detection

Cell lysates were thawed on ice. The amounts of lysates containing 100 μg of total protein were transferred to a 1.5ml Eppendorf tube. The lysates were then adjusted to equal volume with SIB. Appropriate amount of 4X sample buffer was adjusted to achieve a final concentration of 1X. Prior to the electrophoresis, samples were boiled for 10 min.

Samples containing 100 μg total protein were migrated on an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN Electrophoresis System (Bio-Rad). Samples were stacked at 80V in 5% polyacrylamide gel for 20 min and then separated at 150V in 15% polyacrylamide gel for 2-2.5 hours.

Following electrophoresis, proteins were transferred to 0.2 μm nitrocellulose membrane (Bio-Rad) in 1X pre-chilled CAPS transfer buffer at 4°C, 80V for 1hour. After baked at 65°C for 10 min, the membranes were blocked in 5% skimmed milk in TRIS buffered saline (TBS) for 1 hour at room temperature.

Membranes were incubated with primary antibodies at 4°C overnight. Primary antibody dilutions used are shown in Table 2. The membranes were washed 3 times for 15 min in 1XTBS containing 0.1% Tween-20 (TBST). Secondary peroxidase-conjugated antibodies compatible with the primary antibody were incubated on the membranes for 1h at room temperature. Then followed by washing with 1XTBSB 3 times for 15 min, the membranes were incubated with Super Signal HRP substrate (Pierce) for 5 min at room temperature. Finally, signal detection and documentation were using the ChemiDoc imaging system (Bio-

Rad).

For ERβ recombinant protein competition experiment, protein lysates of MCF7-β1 (control cell line) cells and T5 cells were analyzed by western blot using anti-ERβ chicken antibody with or without recombinant ERβ incubation. 2 μg recombinant protein had been used for incubation with 2 μg chicken antibody for 3 hour at 4°.

5.4. Immunofluorescence and Fluorescence Microscopy

T5 cells were cultured on cover-slips placed in 6-well dishes and infected with New-SRAP/Flag-eGFP by Lipofectamine2000, nucleofectin or viral particles (as described in Section 3.1-3.3). 48 hours post-transfection, the coverslips were briefly washed with PBS and cells were fixed with 4% formaldehyde (sigma) and 4% sucrose (sigma) in PBS for 15 min at room temperature. The fixed cells were then rinsed with PBS and permeabilized with 0.25% Triton-X100 (sigma) in PBS for 5 minute. After rinsed with PBS twice, cells were blocked by incubation with 10% bovine serum albumin (BSA, sigma) in PBS for 30 min at 37°C. Cells were incubated with monoclonal Anti-Flag M2 antibody diluted at 1/100 in 3% BSA/PBS for 3 hours at 37°C. After washed with PBS 3 times for 15 min, T5 cells were incubated with anti-mouse secondary antibody-Cy3 conjugate (Abcam) at a 1/500 dilution in 3% BSA/PBS for 1 hour at 37°C. Cell nuclei were then stained with 1 μg/ml Hoechst (Invitrogen). Finally, the coverslips were mounted onto microscopy slides with FluorSave™Reagent (Calbiochem). The fluorescent images were captured and visualized with an Eclipse E1000 epifluorescent microscope at wavelength of 510-520nm (GFP), 552-620nm (cy3), 440-450nm (Hoechst) and the ACT-1 software provided with the microscope

(Nikon).

5.5 Anti-SRAP mouse monoclonal antibody

Through the Manitoba Centre for Proteomics and Systems Biology, we obtained ninety hybrydomas isolated from mice immunized with the full recombinant SRAP. The corresponding monoclonal antibodies had been tested by western blot analysis using MCF7S151 (MCF7 overexpressing tagged SRAP-V5, see cell culture) and mouse embryo fibroblasts. 8E4 is one antibody recognizing human SRAP but not mouse SRAP.

In order to purify the antibody, hybrydoma cells were grown in 10% FBS RPMI in four T175 flasks until achieving 0.5 million cell/ml. Cell were spin down and wash with 1X warm RPMI medium without serum followed by replacing with hybrydoma medium (GIBCO). Hybrydoma medium were collected by centrifugation after 10-14 days until cells died. The medium were passed through protein G columns (Amerham) according to the Amerham company protocol. Antibodies were eluted with 0.1 M Glycine HCL (ph 2.7) in 100 µl fractions 3 times followed by neutralizing with 10 µl 1M tris HCL (ph 9). After testing the fractions on western blot, the positive aliquots were frozen down.

5.6 Immunoprecipitation

MCF7-ERβ1 and MCF7-ERβ2/cx cells were seeded in 150 mm dishes (2 X 10^6 cells/dish). 24 hours later medium was changed to medium containing 1 µg/ml doxycycline. After 48 hours doxycycline treatment, the cells were washed twice on the dishes with ice-cold PBS. Cell extracts were prepared after cells were scraped into 1 ml of IP buffer (50 mM Tris–HCl

pH 8, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM PMSF, 10 mM NaF, 1 mM Na3VO4, 25 mM β-glycerophosphate, protease inhibitor cocktail (Roche Diagnostics Indianapolis, IN, USA, 1 tablet per 10 ml IP buffer) and then the cells were passed through a syringe with a 21 gauge needle followed by sonication 3 times (15 s each at 40% output using a Braun-Sonic 1510 sonicator, with the tubes in ice-water). After centrifugation at 16,000 g for 10 min, the supernatant was 20 µl pre-cleared with protein G-sepharose for 30 mins at 4°C, and the supernatant divided into two. The specific primary antibodies (Condition see table 3) was added to one aliquot (20 µl of antibody per 500 µl of lysate protein) and an isotype matched irrelevant antibody was added to the second aliquot as a control for non-specific interactions, and incubated at 4°C with rotation overnight. 50 µl of 50% G-protein Sepharose was then added and incubated for 3 hours at 4°C. Following centrifugation at 16,000 g for 10 sec the supernatant was collected and the pellet washed 3 times with 1 ml of IP buffer. The proteins were extracted from the beads with 50 µl of SDS reducing buffer, boiled for 10 min and then analyzed by western blotting.

293 cells were seeded in 6-well plates (100,000 cells). 24 hours later 293 were co-transfected with 2 µg SRAP/Flag and 2 µg full length ERβ/V5 or ERβ AF1/V5, ERβAF2/V5 domains by using lipofectamine2000. 24 hours after transfection, the cells were washed twice on the dishes with ice-cold PBS. Cell extracts were prepared after cells were scraped into 200 µl of IP buffer, the supernatant was 20 µl pre-cleared with protein G-sepharose for 30 mins at 4°C. The specific primary antibody anti-Flag was added to one aliquot (3 µl of antibody per 200 µl of lysate protein) and an isotype matched irrelevant antibody was added to the second aliquot as a control for non-specific interactions, and incubated at 4°C with rotation overnight. Fifty microlitres of 50% G-protein Sepharose was then added and incubated for 3 hours at 4°C.

Following centrifugation at 16,000 g for 10 sec the supernatant was collected and the pellet washed 3 times with 1 ml of IP buffer. The proteins were extracted from the beads with 50 μl of SDS reducing buffer, boiled for 10 min and then analyzed by western blotting.

6. RNA Extraction and Reverse Transcription

6.1. RNA Extraction

RNA samples were extracted using Trizol reagent (Invitrogen) in most experiments except the experiment studying time course effects of different SRA constructs on the expression and splicing of ERβ.

Cells cultured on 6-well plates were washed twice with room temperature PBS prior extraction. One ml Trizol reagent was added to the entire surface of one well and allowed 5 min for incubation. The slurry was then pippeted up and down for several times and transferred to a DNase/RNase-free 1.5ml Eppendorf tube. The slurry was processed for RNA extraction immediately, or stored at -80°C for no more than 1 week prior extraction.

RNA extractions were carried according to the manufacturer's instructions, with one minor modification. Briefly, 0.2ml chloroform (Fisher Scientific) was added to the eppendorf tube containing the slurry prepared with 1ml Trizol. The tube was inverted vigorously for 15 sec and was set at room temperature for 2-3 min. The sample was then centrifuged at 12,000g for 15 min at 4°C. 400 μl (instead of all) of the aqueous phase was transferred to a new DNase/RNase-free 1.5ml Eppendorf tube and then mixed with 0.5ml Isopropanol (Fisher

Scientific). The mixture was inverted vigorously for 15 sec and incubated at room temperature for 10 min. The sample was then centrifuged at 12,000g for 10 min at 4°C to precipitate RNA. After centrifugation, the supernatant was decanted. The RNA pellet was re-suspended with 1ml 75% ethanol, which replaced the isopropanol, and centrifuged at 120,000g for 5 min at 4°C. Again, the supernatant was decanted. The RNA pellet was air-dried at room temperature. Finally, the pellet was dissolved in 20 to 50 µl ddH_2O, depending on its size, and stored at -80°C.

The minor modification was that, 400 µl solution from the aqueous phase was transferred to a new tube, instead of the whole volume of this layer (usually between 500 to 600 µl). This modification aimed to minimize contaminations from genomic DNA from the intermediate phase or protein from the lower phase.

6.2. Quantification of RNA Yield

RNA stocks were removed from -80°C, placed in a -65°C for 10 min, briefly centrifuged at maximum speed, and placed on ice. In most experiments, the stock RNA was 1:50 diluted by mixing 4 µl RNA with 196 µl filtered water in a well on a UV transparent 96-well plate (Falcon or Costar). Each sample was processed in duplicate to control pipetting accuracy. As a blank, 4 µl ddH_2O was processed in the same way. In some cases when the RNA yield was expected to be small, a dilution factor of 100 was used by mixing 2 µl RNA with 198 µl ddH_2O.

The Spectra Max 190 spectrophotometer (Molecular Devices) was used to measure optical

density at the wavelength of 260nm (OD260). Averages of the two OD260 readings (OD260ave) were calculated. The concentration of plasmid DNA was calculated by the following equation:

$$\text{Concentration (ug/ul)} = \text{OD260ave} \times 40 \times \text{dil. factor} / 1000$$

An optical density of 1 is converted as 40 μg/μl for single-stranded RNA and the dilution factor is 50 (or 200 in some experiments). RNA yield was typically 10-20 μg (per well on 6-well dishes). Purity of the samples was determined by the ratio of readings at 260nm and 280nm. Typical purities range from 1.8 to 2.0.

6.3. Dilution of RNA Stocks for Reverse Transcription

Following quantification, a potion of the RNA stock was transferred to a 200 μl PCR tube and diluted to a concentration of 0.2 μg/μl. Diluted RNA samples were stored at -80°C. Before use, the samples were thawed and centrifuged as described in the previous section.

6.4. DNase Treatment

Genomic DNA in the RNA samples was digested by the RQ1 RNase-free DNase (Promega) according to the manufacturer's instructions with minor modifications. Briefly, in a 200 μl PCR tube, 0.5 to 1.5 μg RNA sample was mixed with 4 μl RT buffer (Invitrogen), 2ul (1U) RQ1 DNase and proper amount of ddH_2O in a total volume of 20 μl. The mixture was placed in a 37°C water bath for 45 min to allow the digestion of DNA. After digestion, 2 μl Stop Solution was added and the mixture was incubated in a 65°C water bath for 10 min to

inactivate DNase. Samples were than placed on ice prior reverse transcription.

Two modifications were applied to the manufacturer's recommended protocol. First, reverse transcription (RT) buffer was used instead of the original DNase buffer, and second, the digestion of DNA was 45 min instead of 30 min. As the RQ1 DNase was competent in the RT buffer (data not shown), using this buffer for DNase treatment eliminated inconstancy of buffer in the downstream reverse transcription step. A longer incubation time, suggested by the manufacturer as an option, aimed to obtain optimal digestion.

6.5. Reverse Transcription

cDNA was generated from RNA by Moloney Murine Leukemia Virus (MML-V) Reverse Transcriptase (Invitrogen), according to manufacturer's instructions. Briefly, in a 200 µl PCR tube, half of the RNA treated with DNase was mixed with 4 µl RT buffer, 3 µl DTT (Dithiothreitol, 0.1M), 0.3 µl random primer (0.2 µg/ml), 1.5 µl dNTPs (10mM each), 0.3 µl (12U) RNaseOUT Ribonuclease Inhibitor), 1.5 µl (300U) MML-V and appropriate amount of ddH_2O to bring up to a total volume of 30ul. The mixture was incubated at room temperature for 10 min and then in a 37°C water bath for 1 hour. Following incubation, the mixture was transferred to a thermocycler (TECHNE, model number FGEN02TP) in which it was first heated to 99°C for 10 min and then cooled to 4°C. cDNA was stored at -20°C. To control the efficiency of DNase treatment, the other half of the DNase-treated RNA sample was processed through the same procedures, but without the presence of MML-V. For real-time PCR, 0.75 µg RNA was reverse transcribed. The cDNA was then 1:5 diluted by adding 120 µl ddH_2O into the tube.

7. PCR and Electrophoresis

7.1. Regular PCR

7.1.1 PCR amplification

In a 200 µl PCR tube, 1 µl cDNA was mixed with 1.5 µl PCR buffer (10 X), 0.6 µl $MgCl_2$ (50mM), 0.3 µl each primer (0.2 µg/ml), 0.3 µl dNTPs (10mM each), 0.08 µl (0.4U) Platinum TAQ polymerase (Invitrogen), and 11.5 µl ddH_2O. The mixture was then transferred to the TECHNE thermocycler in which it was processed as the following:

1. 1 cycle of 94°C for 4 min;
2. 30 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec;
3. 1 cycle of 4°C hold.

7.1.2 Agarose Gel Electrophoresis

20 µl of the radioactive PCR product was mixed with 5 µl loading blue (4X) briefly spined on a l centrifuge (Eppendorf). 20 µl of the sample was migrated on 1% agarose with 1 X TBE at Room temperature for 1 to 2 hours. Autographs were developed by a Personal Molecular Imager FX (Bio-Rad).

7.1.3. Denaturing Acrylamide Gel Electrophoresis and Quantification

2.5µl of the radioactive PCR product was mixed with 10µl loading blue (80% deionized

formamide, 1mM EDTA, trace xylene cyanol and bromophenol blue), boiled for 5 minutes and briefly spined on a personal centrifuge (Eppendorf). 10μl of the sample was migrated on denaturing polyacrylamide gel (4.8 % acrylamide, 0.16% bis-acrylamide, 42% Urea, 1 X TBE) in a Sequi-Gen or a Sequi-Gen Model S2 apparatus (Bio-Rad) at 50°C for 1.5 to 2 hours. The gel was transferred on a piece of Waterman paper, dried in a Slob Gel Dryer (Savant, model number SDG-4050) and exposed to a Phosphorimager screen (Bio-Rad) for either 2 hours or overnight. Autographs were developed by a Personal Molecular Imager FX (Bio-Rad). The intensity of a band on an autograph represented the radioactivity of a PCR amplicon, which was assumed to indicate the abundance of the amplicon. Quantity1 software (Bio-Rad, version 3.1) was used to quantify the intensity.

7.2. Real Time PCR and Data Analysis

The primers for the ERβ gene (total ERβ and exon 8 alternatively spliced *ERβ1–5* transcripts), the *Gapdh* gene were chosen with Oligo 5.0 software (National Biosciences, Plymouth, MN)(102). Primer nucleotide sequences are shown in Table5. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or on a different exon. The specificity of PCR amplicons was checked by agarose gel electrophoresis.

0.75 μg RNA was reverse transcribed and the cDNA was 1:5 diluted. The final cDNA concentration is 50ng/10 μl. The real-time PCR reaction is consist of 0.56 μl Light Cycler FastSart DNA Master SYBR Green I (1 X final concentration, Roche), 10 μl template cDNA. 2.8 μl PCR buffer (10X), 1.4 μl $MgCl_2$ (50mM), 2.24 μl dNTPs (2.5mM each), 0.7 μl DMSO (Sigma), 0.35 μl each primer (0.2 μg/ml), 0.14 μl (0.7U) Platinum TAQ polymerase

(Invitrogen), 0.28 µl Fluorescin (1 µM, Bio-Rad) and 11.6 µl ddH_2O. The real-time PCRs were accomplished using a iCycler (Bio-Rad) with the following conditions:

1. 95°C for 4 min;

2. 40 cycles of 30 sec at 95°C, 60 sec at 62°C, and 30 sec at 72°C with fluorescence acquisition at the end of each extension.

3. The amplification program was followed immediately by a melt program consisting of 1 minute at 60°C, and a gradual increase to 95°C at a rate of 0.5°C after every 10 sec of hold at the previous temperature. A fluorescence acquisition was performed at the end of each hold.

Quantitative values are obtained from the cycle number at which the increase in fluorescent signal associated with exponential growth of PCR products starts to be detected by the laser detector of the ABI Prism™ 7700 Sequence Detection System (Perkin–Elmer Applied Biosystems, Foster City, CA) using PE Biosystems analysis software according to the manufacturer's manuals.

Firstly, in order to normalize the amount of cDNA of each sample, results of *Ct* value (cycle threshold value) were normalized to the expression of endogenous gene control (Gapdh).

Secondly, the *Ct* value of each sample was normalized by the Ct value of the control sample transfected empty vectors. The ΔCt: 'Normalized mRNA level' of each sample, thus represent the $2^{\Delta Ct}$ fold expression variation compared to the mock transfection which has an artificially normalized mRNA level equal to 1.

8 Tissue microarrays:

The histopathology of all MBTB cases has been assessed and entered into a computerized database to enable selection based on composition of the tissue as well as clinical–pathological parameters. After selection, cases were reviewed on H&E sections by a breast histopathologist. Tissue microarrays (TMAs) from a total cohort of 255 ER α negative (ER α–255TMA), primary invasive ductal breast carcinomas were constructed. Briefly, duplicate core tissue samples (0.6mm diameter), were taken from selected areas of maximum cellularity for each tumour with a tissue arrayer instrument (Beecher Instruments, Silver Spring, MD, USA). Although the TMA consisted of 255 cases of ER-negative tumours as determined by LBA ((ERα +>3 fmol mg protein), 39 of these were subsequently found to be ERα by immunohistochemistry (IHC) and were excluded from the later analysis.

9. Immunohistochemistry:

Serial sections (5 mm) of the ER α–255TMA were cut, mounted on Fisherbrand Superfrost/plus slides (Fisher Scientific, USA) and stained using IHC with commercially available specific antibodies (Table 1). Further details of the specific SRAP antibodies are as follows: 743 (bethyl company, rabbit polyclonal antibody raised to peptide containing amino acids 183-230 at 1: 100 dilution). Briefly, sections were dewaxed in two xylene baths (5 min each), taken through a series of alcohols (100, 95, 70%), rehydrated in distilled water and then submitted to heat-induced antigen retrieval for 8 min in the presence of a citrate buffer (CC1 mild/ standard, Ventana Medical Systems, AZ, USA) using an automated tissue immunostainer (Discovery Staining Module, Ventana edical Systems, AZ, USA). The staining protocol was set to 'Mild and Standard Cell Conditioning' procedure for all antibodies. Primary antibodies were applied for 60 min while secondary antibodies were

incubated for 32 min. Initial dilutions quoted above were diluted further 1: 3 with buffer dispensed onto the slide with the primary antibody. Primary antibodies were omitted for negative controls. The IHC of paraffin-embedded MEF's cell pellets was performed manually; sections were microwaved in the presence of 0.01 M citrate buffer, pH 6.0, for 20 min at full power (Danby, ON, Canada, model DMW 1001 W, 800W maximum output). Sections were blocked and then incubated using a SRAP monoclonal antibody (743, bethyl, USA) at 1 : 100 dilution in a humidified chamber at 41°C overnight, as previously described (103;104) Following incubation with HPR-conjuncted goat anti-rabbit antibody for 60 min at 1: 200 (Jackson ImmunoResearch Laboratories, PA, USA), SRA protein was visualized with 3, 30-diaminobenzidine (DAB, Sigma-Aldrich, ON, Canada). Slides were scored semiquantitatively under a standard light microscope. Images were captured using Polaroid DMC-2 software (version 2.0.1, Polaroid, MA, USA).

Results:

Aim 1. Characterizing whether SRA RNA and/or SRAP regulate the expression of ERβ *in vitro*

1.1. Generation of two models to separate SRA RNA and SRAP protein function.

In order to establish the respective effects of SRA and SRAP on the regulation of known endogenous target gene candidates such as ER-β, we used two models: SDM mutation model and New-SRAP model.

1.1.1 SDM mutation model

As outlined earlier, *Lanz et al.* reported that the introduction of point mutations changing any putative reading frame did not affect the function of SRA RNA (61). They subsequently identified discrete silent mutations that through modifications of the secondary structure of SRA RNA, were suppressing RNA ability to co-activate PR activity [Fig 4] (62). Based upon this finding, we have generated four different constructs that will, once transfected, generate different products [Fig 10]. These 4 constructs consist in:

SRA WT: corresponds to the full wild type SRA sequence, we expect this construct to express both functional RNA and protein.

SRA PRO: also correspond to full SRA WT sequence but with two series of silent mutations SDM1+SDM7 (SDM, site-directed SRA mutants), shown to dramatically decrease SRA RNA activity on PR [Fig 4] (62). The corresponding RNA encodes for SRAP.

SRA RNA: This construct corresponds to the full SRA WT sequence but with the first two AUG codons mutated. The corresponding RNA is therefore not expected to be translated but will be functional at RNA level.

SRA NONE: contains full SRA WT sequence but with the first two AUG codons mutated and with two series of silent mutation SDM1+SDM7. The corresponding RNA is not translated and should not be functional [Fig 4B].

SRA SDM1 and SDM7 mutations in SRA PRO and TTG mutation in SRA RNA constructs have been previously introduced into pCDNA3.1-V5 plasmid using the QuickChange Site-Directed Mutagenesis Kit (Statagene) [Fig 10A]. The resulting four SRA constructs had been integrated following PCR-amplification into pLenti6/v5-D-TOPO vector using the viraPower lentiviral Directional TOPO Expression Kit (Invitrogen) as mentioned in Materials and Methods.

The four different pLenti-SRA constructs were transfected into T5 cells with Lipofectamine. The protein lysates extracted 24 hours after transfection were analyzed by Western blot analysis using anti-SRAP antibodies [Fig 10B top panel] and anti-V5 antibodies [Fig 10B lower panel]. As expected, anti-SRAP detects both endogenous SRAP (29kd) and recombinant SRAP/V5 (33kd) bands, whereas anti-V5 only detect recombinant SRAP/V5 band (33kd).

The RNAs were extracted 24 hour post-transfection. After DNase treatment and reverse transcription, cDNA were analyzed by PCR using specific primers with mutation sites in SDM7 region of SRA NONE and SRA PRO constructs [Fig 10A]. As expected, PCR

products have been amplified in cells transfected with SRANONE and SRA PRO constructs rather than SRA WT and SRA RNA constructs. The relatively equal RNA transcripts among four plenti constructs have been found by using V5 lower primer and SRA upper primer (table 5) targeting un-mutated region of SRA [Fig 10C]. (No PCR products are amplified in untransfected sample.)

1.1.2 New-SRAP artificial protein model

Since SRA PRO construct generated previously might not completely abolish the function of SRA RNA (SRA PRO only mutated in two presumably most important secondary structures (62) [Fig 11B]), we designed a construct (New-SRAP) encoding SRAP but drastically differing from the original SRA RNA sequence. In particular, specific secondary structures of the core, shown to be necessary for the RNA function (62) have been extensively mutated (35% identity to original sequence). This construct together with the corresponding control (empty vector) have been synthesized by GeneCopoeia Inc (MA, USA) [Fig 11C].

New-SRAP constructs were transfected into T5 cells with Lipofectamine. The protein lysates after 24 hours transfection were analyzed by Western blot using anti-SRAP antibodies [Fig 11D top panel] and using anti-Flag antibodies [Fig 11D lower panel]. As expected, anti-SRAP detects both endogenous SRAP (29kd) and recombinant SRAP/Flag (32kd) bands, whereas anti-Flag only detect recombinant SRAP/Flag band (32kd).

The RNAs were extracted 24 hours post-transfection. After DNase treatment and reverse transcription, cDNA were analyzed by PCR using specific primers with mutation region of

New-SRAP [Fig 11C]. As expected, PCR products have been amplified in cells transfected with new-SRAP constructs rather than empty vector [Fig 11E]. Overall these experiments confirmed that our exogenous SRA constructs (model 1 and 2) are properly expressed.

1.2 Differential action of SRA RNA and SRAP on ERβ mRNA and protein expression

1.2.1 SRAP protein rather than SRA RNA downregulates ERβ mRNA expression

As mentioned earlier, the use of alternative acceptor sites leads to the production of ERβ1, β2/cx, and β5 mRNAs. β1,2 variants share a common exon 7 but differ in their alternative exon 8 [Fig 12]. The combined use of alternative donor and acceptor sites produces ERβ5 mRNA, which contains an extended exon 7 and a shorter exon 8. By using those different extremities at splicing sites, we designed specific primer annealing sites specific for each variant [Fig 12A]. For example, forward primer for ERβ1 spread form the end of common exon 7 to the beginning of alternative exon8 of ERβ1 whereas forward primer ERβ2/cx spanned from the end of common exon 7 to truncated exon 8 of ERβ2/cx. Total ERβ primers are located in exon 2 and exon 3 which are common region to each variant. Different PCR products of ERβ variants after amplification with their primer sets were checked by agarose gel [Fig 12B].

Using the first model, four plenti-SRA constructs have been transfected into T5 cells by lipofectamine 2000. RNA samples were extracted after 48 hours. After reverse transcription, cDNA were analyzed by Real-time PCR. Total ERβ expression, as shown in Fig 13 top panel, has been significantly down regulated in cells over-expressing only SRAP after 48

hours transfection (P=0.03). Cells transfected with all 3 other SRA constructs expressed similar level as untransfected sample of total ERβ RNA expression [Fig 13]. To further assess whether the decrease of total ERβ corresponded to a change in the expression of a known ERβ variants, we performed real-time PCR using specific primer sets for ERβ1, 2 and 5. Surprisingly we found that the ERβ5 expression was significantly up regulated in cells transfected with SRA PRO and SRA WT after 48 hours transfection (P=0.04) [Fig 13 lower panel]. Increasing relative ratio of ERβ5 expression was also found in the cells transfected with SRA PRO and SRA WT by using TP-PCR (Table7) [Fig 14]. Both SRA PRO and SRA WT constructs express exogenous SRAP suggesting that SRAP might be involved in regulating ERβ5 mRNA expression. All SRA constructs did not have any effects on ERβ1 and ERβ2/cx mRNA expression.

Using second SRA model, New-SRAP construct and empty vector have been transfected into T5 cells using lipofectamine 2000. RNA samples were extracted after 24 hours, 48 hours and 72 hours. After reverse transcription, cDNA were analyzed by real-time PCR using specific ERβ total as well as ERβ1, ERβ2/cx, ERβ5 primer sets. Total ERβ expression has been significantly down regulated in cells over-expressing new-SRAP 48 hours after transfection (P=0.02) [Fig 15 middle panel]. Interestingly, new-SRAP significantly deceased ERβ5 mRNA expression 24 hours after transfection [Fig15 top panel]. However, neither total ERβ nor ERβ variants expression have been changed by new-SRAP 72 hours after transfection [Fig 15 lower panel].

1.2.2 respective SRA/SRAP modulation of ERβ protein expression

As SRAP appeared in our 2 models to decrease total ERβ expression at the mRNA level, we investigated whether this effect could be observed at the protein level. Using the first model, we transfected four plenti-SRA constructs into T5 cells using lipofectamine 2000. Protein samples were lysed after 48 hours and 72 h and analyzed by Western blot analysis. We monitored the expression of SRAP, ERα, ERβ, and β actin using specific antibodies [Fig 16]. It should be stressed that the anti-ERβ chicken antibody targeting the common N-terminal extremity of ERβ could detect all variants (105). Protein extracts from MCF7-β1 cells were run as the control. Estimated size of ERβ1/Xpress is 62kD whereas estimated size of endogenous ERβ1 is around 60kD. An ERβ-like band with the correct estimated size is detected in my Western blot [Fig16 middle panel]. As expected, over-expression of SRAP decreases the amount of this ERβ-like band after 48 hours transfection as shown by arrows. SRA RNA has no effect on this ERβ-like band [Fig16 middle panel]. ER α expression remains consistent between control and different SRA constructs [Fig16 middle panel].

Using the second model, the new-SRAP construct and empty vector were transfected into T5 cells. Protein samples were lysed after 24 hours, 48 hours and 72 h and analyzed by Western blot analysis [Fig 17]. The expression of SRAP and β-actin were detected using specific antibodies. ERβ-like bands were also detected by using chicken antibody. An apparent band potentially corresponding to ERβ was decreased by SRAP. The signal detected was however very weak. It was crucial to check the identity of this band.

1.2.3 Endogenous ERβ protein expression is not detectable in breast cancer cells

In order to demonstrate that the band detected was indeed the endogenous ERβ, I perform both siRNA experiments and ERβ recombinant protein competition. Indeed, if transfection of ERβ siRNA reduces the ERβ-like band signal, this would confirm that the band recognized by ERβ chicken antibody represent the endogenous ERβ. Similarly, if pre-incubating recombinant ERβ with our primary antibody decreases the ERβ-like band signal, we will be confident that we detect endogenous ERβ.

ERβ siRNA is able to decreases both transfected and endogenous ERβ mRNA checked by real-time PCR using primer set targeting ERβ exon2 and exon3 [Fig 18A]. ERβ siRNA has relative lower down-regulation effects on transfected ERβ1 expression compared to that on endogenous ERβ in T5 cells [Fig 18]. Even though siRNA of ERβ is able to interfere with ERβ mRNA expression, ERβ siRNA only knocks down the exogenous ERβ protein but not the ERβ-like detected protein [Fig 18B]. Identical results have been obtained on recombinant ERβ protein competition experiments [Fig 19]: recombinant ERβ protein can block the recognition of exogenous ERβ band but not that of the ERβ-like band. Overall, even though the identity of the band recognized by the chicken ERβ antibody remains to be determined, it is likely that it is not ERβ.

1.3 The lentiviral expression system used to increase the transfection efficiency.

In order to achieve better transfection efficiency, we try to set up lentiviral transduction system expected to lead to better yields. Lenti-viral transduction systems had been widely used for delivering RNAi or short Oligoribonucleotides into host cells (106). The ViraPower Lentiviral Expression System (Invitrogen) allows certain of a replication-incompetent

lentivirus that is used to deliver and over-express gene of interest. An expression plasmid contains the gene of interest under the control of a specific promoter and elements that allow packing of construct into virons. Three packaging plasmids (pLP1, pLP2 and VSVG) supply the structural and replication protein *in trans* that are required to produce of the lentivirus.

1.3.1 Titer has been determined from 2–10 x 10^5 transducing units/ml

For the generation of infectious lentiviral particles, pLenti6/V5 vectors containing 4 mutated SRA constructs were co-transfected with ViraPower™ packaging plasmid mixture into 293FT cells [Fig 20]. Virus containing media was collected 48–72 hours after transfection and concentrated as described in Materials and Methods. Prior to the titration, I performed killing curve experiments to determine antibiotics (Blasticidin) sensitivity [Fig 21 A]. The final concentration of 6 μg/ml blasticidine was sufficient to kill all the T5 cells in the plates within 14 days. T5 cells in 6-well plate were then incubated with serial dilutions (1/10-1/100000) of the viral stock in the presence of 6 μg/ml blasticidine. The number of transducing units was determined by multiplying the estimated number of colonies by dilution factor [Fig 21 B]. Our preparations of concentrated lentiviral stocks consistently yielded titres of 2–10 x 10^6 transducing units/ml. Preparations of un-concentrated lentiviral stocks consistently yielded titres of 2–10 x 10^5 transducing units/ml [Fig 21].

1.3.2. MOI 1 (Multiplicity of Infection) is optimized MOI for transduction:

T5 cells cultured in 12-well plates were infected by pLenti-SRAP and different MOI from 0.1, 0.5, 1, 5 in the presence of 10 μg/ml Polybrene. Protein samples were analyzed after 48-72 hours by using anti-V5 antibody. Recombinant SRAP with V5 tag can be detected by

using MOI 1 and 5, but not on the MOI below 1 [Fig 22 A]. MOI above 5 (including 5) had been found to severely influence the viability of T5 cells (viability less than 20%). In order to figure out whether viral particle itself or our insert have toxic effects on cells, I tried the control construct pLenti-LacZ which has different insert from pLenti-SRAP. However, toxicity has been found using pLenti-LacZ construct too (data not shown). To investigate whether the toxicity or transduction efficiency are cell line dependent, I tried to use Hela cells. Expression level of recombinant SRAP by transduction was similar when compared to T5 cells. Toxicity of high MOI was also observed on Hela cells. These results suggest that high MOI of virus dramatically affects the viability and does not significantly increase the transduction efficiency.

1.3.3 Transduction efficiency is lower than transfection efficiency in T5 cells.

Plenti-SRAP/V5 and Plenti-lacz/V5 (control construct) had been packaged into lenti-viral particles that were transiently transducted into T5 cells using MOI: 1. Paralleled transfections of the same plenti-constructs had been performed using lipofectamine 2000. 48 hours post transduction/transfection protein sample were extracted and analyzed by Western blot using anti-V5 antibodies [Fig 22 B]. Curiously, expression of recombinant SRAP or LacZ with V5 tag introduced by plasmid are higher than the cell with transduction at MOI 1.

1.3.4 2 helper package system has higher efficiency than 3 helper package system.

Because the yield using ViraPower^{TM} packaging system (3 package system: VSVG, pLP1 and pLP2 from Invitrogen) was not satisfactory, we tried 2 Package systems (PAX2, pLP-

VSVG) instead [Fig 23]. Higher yield (viral titres) can be achieved by using two packages system. I transduced New-SRAP/Flag-GFP viral particles from both 2 and 3 helper systems into T5 cells using MOI 1. As paralleled experiment, New-SRAP/Flag-GFP had been transiently transfected into T5 cells using lipofectamine2000. In order to measure the transfection/transduction efficiency, IRIS/GFP was analyzed by fluorescence microscope and SRAP/Flag were analyzed by Immunofluorescence using anti-flag antibody 48 hours post transfection. Protein samples were extracted after 48 hours transduction and analyzed by Western blot using anti-SRAP antibodies. Viral particles packaged by 2 helper systems have higher transduction efficiency than virus packaged by 3 helper vectors based on Western blot result [Fig 23 B top panel]. However, transfected New-SRAP/Flag can be detected by neither IRIS/GFP nor Immunofluorescence using anti-flag antibody [Fig 23 B lower panel]. I have to stress that even though the recombinant New-SRAP had higher expression in 2 helper package system, recombinant protein expression is still much lower when compared to transfection by lipofectamine 2000.

Based on the above results, the lentiviral expression system (viralpower expression system, Invitrogen) did not increase the transfection/transduction efficiency in our hands.

2. Confirming an *in vivo* physical interaction between SRAP and ERβ by Co-IP.

As outlined in the introduction, we have shown by GST-pull down that the SRAP associates with ER α and ERβ in vitro (90). *Kawashima* also showed that recombinant SRAP is able to directly interact with the AF-2 domain of AR *in vitro* (84). In order to establish whether SRAP and estrogen receptors β indeed interact with each other *in vivo*, I used two cell lines

models for Co-immunoprecipitation. The first model used consists in MCF7-ERβ1 or ER 2 cells which express endogenous SRAP and inducible ERβ1, ERβ2/cx proteins. The second model used consists in 293 cells co-transfected with SRAP/Flag and ERβ/V5.

2.1 Model 1: MCF7-ERβ1, 2 cells

I performed Co-immunoprecipitation experiments in MCF7-ERβ1 and MCF7-ERβ2/cx cells (MCF7 cells expressing inducible ERβ1 and ERβ2/cx). After 48 hours doxycycline treatment, the cells were processed for standard IP analysis as described in the Methods section using 8E4 (anti-SRAP mouse monoclonal antibody) as well the appropriate irrelevant isotype matched antibody (V5 antibody) as a non-specific control (NS). Anti-ERβ chicken antibody was used for Western blotting [Fig 25A]. As expected, we have successfully immunoprecipitated endogenous SRAP whereas non-specific mouse IgG does not immunoprecipitate SRAP [Fig 25]. In the same experiment, a weak ERβ1 band could be pulled down by SRAP antibody but not control mouse IgG, suggesting a potential interaction between SRAP and ERβ1 [Fig 25A left panel]. Interestingly, ERβ2/cx had not been pulled down by SRAP antibody [Fig 25 right panel]. These Co-Immunoprecipitation experiments have been repeated by using 743 (anti-SRAP rabbit antibody), no interaction has been found (data not shown).

Co-immunoprecipitation experiments in MCF7-ERβ1 cells have been also performed the reverse way by using AP1A (anti-ERβ1 rabbit polyclonal antibody), anti-Xpress (mouse monoclonal antibody) with appropriate irrelevant isotype matched antibody (rabbit, mouse IgG) as a non-specific control (NS). Anti-ERβ chicken antibody and Anti-SRAP were used

for Western blotting [Fig 25B]. As expected, we have successfully immunoprecipitated exogenous ERβ1 by both AP1A and Xpress antibodies whereas non-specific mouse IgG does not immunoprecipitate ERβ [Fig 25B]. In few experiment, a weak SRAP band has been pulled down by ERβ1 antibody [Fig 25B left panel] and anti-Xpress antibody [Fig 25B right panel] but not control rabbit or mouse IgG. However, there results are not reproducible. Overall, using this model, we have not confirmed "SRAP and ERβ" *in vivo* interaction.

2.2 Model 2: 293 cells.

ERβ1 and ERβ2/cx share the same N-terminal AF1 domain but differ in AF2/LBD. *Kawashima* showed that SRAP is able to directly interact with the AF-2 domain of AR *in vitro* (84). These suggests that AF-2 domain of ERβ might also specifically interact with SRAP. Therefore, I cloned AF1/DBD, AF2/DBD domains and full length ERβ into Plenti vectors (see Materials and Methods). The resulting V5 tagged Full length ERβ, AF1/DBD, AF2/DBD domains of ERβ were co-transfected with Flag tagged SRAP into 293 cells. The whole cell lysate was first checked by Western blot analysis using anti-V5 and anti-Flag antibody [Fig 26A]. All constructs are properly expressed. The cells were next processed for standard IP analysis 24 hours after transfection, as described in the Materials and Methods section using anti-Flag mouse monoclonal antibody as well the appropriate irrelevant isotype matched antibody (α-GST antibody) as a non-specific control (NS) [Fig 26 B]. As expected, Flag tagged SRAP were successfully immunoprecipitated by Flag antibody in all cells transfected with SRAP/Flag construct but not in un-transfected cells [Fig 26 B left panel]. However, none of V5 tagged ERβ constructs had been pulled down by Flag antibody.

3. Correlation of expression between ERβ and SRAP by using TMA analysis

We have previously shown that SRA recombinant protein was mainly located in the nucleus of tranfected cells (87). However, no information are available regarding the localization of endogenous SRAP protein or the cell type (epithelial, fibroblastic) responsible for the observed variable SRAP expression detected by Western blot in some tumors. No data is available for the expression pattern of SRAP in breast tumor tissue and its potential correlation with the other biomarker such as ERβ. Such information are critical to establish the potential relevance of SRAP as a prognostic or predictive factors. Therefore, there is an urgent need to set up optimized experiments conditions to assess SRAP expression in breast cancer tissues by IHC.

3.1 validation of SRAP antibody for Immunohistochemistry

We wanted to characterize an anti-SRAP antibody, able to detect both mouse SRAP and human SRAP by Western blot and IHC. The advantage of recognizing mouse SRAP is that specificity of antibody could be checked in SRA knockout MEF's cells. Beside, specific signal detected by antibody should also be blocked by peptides competition.

In order to find this antibodies, several SRAP antibodies were screened and tested further suitability in studies by Western blot, immunofluorenscence and Immunohistochemistry, respectively (See Methods and Materials). 7H1G1 mouse monoclonal antibody (abcam) was raised to full length human SRAP. 743, 742 (bethyl) rabbit polyclonal antibody were raised

to an N-terminal 50-100aa, C-terminal 180-237aa of SRAP, respectively. 8E4 and 10F4 (in house) mouse monoclonal antibody were raised by full length human SRAP (table1).

All five SRAP antibodies are able to detect human SRAP of breast cancer cells by Western blot and immunofluorescence [data not shown]. Among them, 743, 742 and 10F4 antibodies are able to detect mouse SRAP by Western blot and immunofluorescence in wild type mouse embryonic fibroblast cells. Specificity of 743 has been checked by both peptide competition and SRA Knockout MEF's cells (where SRA gene had been knocked down, and SRAP should not expressed) [Fig 27]. Firstly, using Western blot, mouse SRAP band (31kb) detected by 743 antibody in wide type MEF's could be successfully neutralized by 743 blocking peptides [Fig 27 A]. Unspecific band at the apparent size of 30 kD is not affected by 743 blocking peptides. Using immunofluorescence, Mouse SRAP expression has been detected in both nuclear and cytoplasm of wide type MEF's cells [Fig 27B top panel]. SRAP Signal could be neutralized by 743 blocking peptides [Fig 27B lower panel]. Interestingly, heterozygous and homozygous SRA knockout MEF's express less or no SRAP compared to wide type MEF's, respectively [Fig 27B lower panel].

I have further validated the 743 antibody in manual IHC condition by using agar embedded MEF's cell pellets (see Methods and Material) [Fig 28]. WT MEF's cells express higher SRAP level when compared to heterozygous Knockout MEF's cells. The SRAP expression of SRA homozygous knockout MEF's cells is not detectable. Beside, blocking peptide for 743 could partially neutralize the signal of SRAP of WT MEF's cells.

All five SRAP antibodies have also been tested by Western blot using frozen human breast tumor samples in the presence or absence of blocking peptides. Positive Western blot signals are successfully detected by 10F4, 8E4, 742 and 743 (743 is showed in Fig 29A, the others data not shown). Level of SRAP expression varies in different tumor sample [Fig 29A]. However, only signal given by 743 are able to be neutralized by blocking peptide [Fig 29B]. In paralleled, IHC condition for SRAP antibodies have been also tested by using the paraffin embedded human breast tumor samples in the presence or absence of blocking peptides. Positive IHC signals are detected by 10F4, 8E4, 7H1G1 and 743. Both nuclear and cytoplasm staining of SRAP have been showed by using 743 antibody [Fig 30]. I have to stress that the frozen human breast tumor samples and paraffin embedded human breast tumor samples are the same series of samples from same tumor bank. Interestingly, tumors expressing low SRAP by Western blot have been also detected low score by IHC tumors [Fig 30 A], whereas expressing high level of SRAP by Western blot have high score by IHC [Fig 30 B]. The signal recognized by 743 is able to be neutralized by blocking peptide [Fig 30 C, D]. Above all, 743 antibody is able to detect SRAP in breast cancer tissue and specificity of this antibody has been demonstrated by peptide competition as well as on knockout MEF's cells. Therefore 743 is suitable to future TMA analysis for SRAP in breast cancer tumors.

3.2 TMA analysis of SRAP expression and correlation between SRAP and ERβ expression

We have investigated SRAP expression in TMAs corresponding to a large cohort of breast cancer cases with different established clinical parameters. Staining and scoring has been performed as described in the Materials and Methods section. SRAP staining varies greatly from one sample to another, with H-scores ranging from 0 to 215 (n = 372, median = 75,

average = 80). SRAP expression, ER / PR / node status, Nottingham grade, size of the tumor, patient age at surgery and clinical follow-up were available for 343 patients.

Significant (Mann-Whitney rank sum test, two-sided, $P<0.0001$) SRAP H-Score values are higher in ERβ2/cx high than in ERβ2/cx/cx low tumors (ERβ2/cx high, n = 285, median = 85 versus ERβ2/cx low, n = 58, median = 55). Similarly, SRAP staining is stronger ERβ1 high than in ERβ1 low cases even though it is not significant. ($P=0.1485$, ERβ1 high, n = 283, median = 70 versus ERβ1 low, n = 60, median = 55).

To further investigate potential non-random distributions of SRAP H-Score staining in ERβ sub-groups, we have arbitrarily divided the cohort in Low (Low SRAP, H-score lower or equal to 80, n = 157) and High (High SRAP, H-Scores higher than 80, n = 186) SRAP expressors. Contingency table analyses showed that Low SRAP cases were significantly (Fisher's exact test) over-represented in ERβ1 low and ERβ2/cx low compared to ERβ1 high and ERβ2/cx/cx high, respectively (Table 10).

Discussion

1. SRAP decreases the total ERβ mRNA after 48 h but not ERβ variants (ERβ1, 2/cx, 5).

Herein, we have used two models to investigate the respective functions of SRA RNA and protein on ERβ expression. The first model showed that total ERβ mRNA expression 48 hours after transfection is decreased by over-expression of SRA PRO construct, which consists of a discretely mutated RNA encoding SRAP [Fig13 top panel]. Over-expression of SRA RNA alone (wild-type or mutated) has no effect on ERβ expression. However, when both wild-type SRA RNA and protein are co-expressed by SRA WT construct, the down-regulatory effects of SRAP on total ERβ mRNA expression disappear [Fig13 top panel]. This suggests that wild-type SRA RNA has the ability to inhibit the SRAP-mediated down-regulation of total ERβ expression. This action of wild-type RNA is dependent upon the integrity of STR1 and STR-7 regions, hence potentially involving SLIRP and SHARP. It should be stressed that when SRAP is expressed from a drastically mutated RNA (in our second model), the same negative effect of the protein on ERβ mRNA expression is observed.

Based upon our preliminary data mentioned earlier, increasing the relative proportion of non-coding SRA compared to coding SRA led to an increase in ERβ expression in T5 cells [Fig 8]. This up-regulation could result from:

-(1) The increased expression of non-coding SRA RNA

or/and

-(2) The decrease in SRAP level.

Herein, we found that increasing non-coding SRA RNA (Wild-type or discretely mutated in our first model) does not change the expression of ERβ [Fig 13 top panel]. However, we found that constructs expressing SRAP decrease ERβ expression. This fits with the scenario - 2, where decreasing SRAP level might remove the SRAP-mediated inhibition of ERβ expression, therefore leading to an over-expression of this gene.

Both models support the hypothesis that SRAP down-regulates total ERβ expression but does not alter ERβ 1 nor ERβ 2 expression. They however lead to different results when measuring the effect of SRAP on ERβ 5. In the first model, ERβ 5 expression is significantly up-regulated by SRA WT and SRA PRO, constructs which both lead to SRAP but from a wild-type or a discretely (STR-1+STR-7) mutated SRA RNA, respectively [Fig 13 lower panel]. In the second model, ERβ 5 mRNA expression is not altered by our New-SRAP construct [Fig 15 middle panel]. I have to stress that all three constructs (SRA WT, SRA PRO and New-SRAP) express SRAP at similar levels (Fig 16 and Fig 17). This excludes a potential differential effect resulting from differences in SRAP levels. It should however be stressed that in the second model, the RNA has been so extensively modified, that we believe that the resulting protein is behaving independently of any overexpressed functional RNA entity. The absence of effect of SRAP therefore suggests that the overexpression of the protein alone does not alter ERβ 5 expression. Similarly, over-expression of non-coding RNA (wild-type or discretely mutated) alone has no effect [Fig 13 lower panel]. Interestingly, using SRA WT and SRA PRO, i.e overexpressing SRAP in a context of overexpressed wild-type or discretely mutated RNA, ERβ 5 expression is increased. This suggests that SRAP and non-coding SRA, unable to act separately, synergize to modulate

ERβ 5 expression. This effect is not depending on STR-1 nor STR-7 structures but could involve other functional structures cited by *Lanz* (62).

Theoretically, total ERβ expression is determined by the sum of all different ERβ variants. We therefore expect changes in total ERβ expression to be paralleled by changes in ERβ variant expression. The decrease in total ERβ expression observed at 48 hours is, in our first model, surprisingly is associated with an increase in ERβ 5 and no changes in two other variants ERβ1 and ERβ2/cx. Similarly, in our second model, this decrease in total ERβ expression is not accompanied by a modification of ERβ1, ERβ2/cx or ERβ1 and ERβ5 levels. This possibly results from the fact that we are not measuring all known ERβ variant mRNAs, including ERβ 3 or ERβ 4, in our assays. Although ERβ 3 and ERβ 4, two C-terminal splice variants of full length ERβ found by *Moore* (23), are not believed to be expressed in breast cancer cell lines (23), several exon(s) deleted ERβ variants are found in breast cancer and could contribute to the total ERβ expression change observed here, but were individually not measured (107). Therefore, in order to address this question, all possible ERβ variants' expression need to be checked. Especially, we could also take other unknown ERβ variants into consideration and perform analyses using long-range PCR (18). This approach which consists of using a primer set in exon 1 and in exon 7 would indeed give critical information on the ERβ exon-deleted variant population.

The specific mechanisms behind SRAP apparent down-regulation of ERβ expression are unknown to date. It is not currently possible to draw any conclusion whether this regulation is direct or indirect, or if it occurs at the transcriptional or post-transcriptional levels. To address this question, nuclear run-off transcription assays could be done to identify the

quantity of nascent mRNA transcripts' changes (108). More importantly, based upon *Chooniedass-Kothari's* recent unpublished results, SRAP can bind to an intron 8 fragment of ERβ gene, as determined by Chip-on-Chip analysis. This raises the hypothesis that ERβ gene could be directly regulated by protein complexes containing SRAP.

As ERβ is believed to be a potential good prognostic marker and a critical player in the ERα positive breast cancer (109), the fact that ERβ expression is down-regulated by SRAP raises the hypothesis that SRAP is likely associated with poor clinical outcome in the ERα positive breast cancer. This hypothesis and SRAP potential effect on breast cancer could be confirmed by using TMA analysis based on large cohort. Indeed, it might be considered as a potential target pathway to treat breast cancer patients in the future.

2. Can endogenous ERβ protein expression be detected in breast cancer cell lines?

In my experiments, I tried to monitor the expression of endogenous ERβ by using chicken anti-ERβ antibody targeting on N-terminal of ERβ (105). I detected a weak ERβ-like band of 57-59kD in T5 cells which migrates faster than the control tagged ERβ1 band. Even though this weak band migrated at the correct estimated endogenous ERβ1 size, I wanted to confirm the identity of this ERβ-like band recognized by this chicken antibody. siRNA experiment s were performed to block ERβ expression in both ERβ-inducible MCF7 cells and non-inducible cells. The mechanism of siRNA mediated protein down-regulation is through incorporating into RNA-induced silencing complexes (RISCs) to target the mRNA, where they cleave and destroy the cognate RNA. In my experiments, ERβ siRNA is able to reduce both transfected and endogenous ERβ mRNA checked by real-time PCR [Fig 18A]. The

reason why ERβ siRNA only decreases exogenous ERβ mRNA by 20% compared to 50% of endogenous ERβ in T5 cells is putatively because the amount of exogenous ERβ transcripts in inducible cells is much more than endogenous transcripts. Transcriptional down-regulation efficiency by the same amount of siRNA in the two cell lines could therefore differ.

The other interesting finding is that ERβ siRNA can drastically knockdown the inducible ERβ protein but have much less effect on its corresponding ERβ mRNA [Fig 18 A and B left panel]. This could result from the fact that ERβ mRNA and protein have only been measured at one time point (48 hours after transfection). Indeed the time taken for RNA to be decreased might not parallel the time needed for the protein to disappear. In other words, the dramatic decrease in protein might result from a decrease in RNA that is no longer maintained. To address this issue, time course experiments will be done. Alternatively, ERβ siRNA might incorporate into non-cleaving RISCs rather than cleaving RISCs. Non-cleaving RISCs can indeed target the mRNA only for translational repression but not cleavage (110). Therefore, siRNA-mediated translational repression can lead to the down-regulation of protein level whereas ERβ mRNA steady state is not affected.

We have concluded that this ERβ-like band detected by the chicken antibody was likely not ERβ. The ability to detect endogenous ERβ protein by Western blot in breast cancer cell lines is controversial (105;111;112). *Speirs's* group found a single band of 55kD detected in MCF7 cells by using a N-terminal recognizing ERβ antibody (111). *Younes's* group also published that expression of ERβ and its variants ERβ1, 2, 5 could be detected in MCF7 cell line by AP1A, AP2A and AP5A antibodies, respectively (all recognizing the C-terminal) (112). The size of ERβ1 found by *Younes's* group is around 52 kD. However, *Murphy's* group reported that no specific detection of endogenous ERβ protein in breast cancer cell

lines was found by Western blot using any antibodies available (including ab14021 AP1A, Ab288, 14C8, Sc8974/H150) (105). Interestingly, the antibody used by the first group targets the N-terminal extremeity of ERβ, and therefore the antibody can recognize all known ERβ variants. However, the author did not mention which variants represent the signal recognized by their antibody. Neither 55kD nor 52kD showed by the first two groups corresponds to the expected size for ERβ1. The predicted size for an endogenous ERβ1 is indeed supposed to be around 59 kD [Fig 1]. Beside, both *Speirs's* and *Younes's* group failed to provide any control experiments to fully demonstrate that the band recognized by their antibodies is indeed an endogenous ERβ. Therefore, it is difficult to draw any conclusion from their results.

Above all, the expression of endogenous ERβ protein in many circumstances appears to be too low to be detected by Western blot analysis. However, the existence of total ERβ and specific variants ERβ1, 2/cx and ERβ 5 proteins in breast tumor detected by IHC has been reported in different groups (46;103;111). The specificity of ERβ antibodies on IHC has been well validated with controls (103). Therefore, in order to monitor a potential decrease of ERβ protein expression by over-expression of SRAP, we will try to use IHC analysis instead of Western blot analysis. Ideally, by transfecting our GFP tagged SRAP construct into breast cancer cells, we should be able to detected potentially decreased ERβ protein expression in those cells in which GFP is expressed.

3. Different transfection/transduction methods have been compared.

In order to achieve better transfection efficiency, we tried a lenti-virus transduction system (from Invitrogen). Lentivirus expression systems based on HIV-1 offer many advantages over both traditional retroviruses and adenoviruses (113). Indeed, lentiviruses can be used for either transient or stable expression, and they can infect both replicative and quiescent cells (113). The package efficiency depends on the size of insert, the transfection reagents as well as the helper vectors (101;114). The 3 package helper vectors include pLP1, pLP2 and VSVG, supplying the structural and replication protein *in trans* that are required for replication, regulation of viral protein synthesis and viral packaging, respectively. The resulting overall transduction efficiency is known to potentially depend on cell lines, size of vector and viral titres (114). By following all the protocols from Invitrogen, I could only achieve titres of 100,000 UI (Units of Infection) for un-concentrated virus (10 times more for concentrated virus). I transduced them into different cell lines. However, the transduction efficiency always remained very low. This led me to try different methods to optimize conditions for this lentiviral system.

3.1. Optimizing the ratio of packaging plasmids. As mentioned earlier, the component of helper vectors are crucial to the packaging efficiency. Therefore, further optimization of the ratio between the 3 helper vectors was tried. Even though Invitrogen company supplies them as a mixture, the 3 helper vectors were successfully separated by digestion with EcoRI and grew up individually. 3 helper vectors with ratios 1:1:1, 2:1:1 and 1:1:2 have been tested. Unfortunately, there doesn't seem to be one ideal ratio. The use of double vector plasmids that Invitrogen company recommend was tried and had slightly higher titres, however the 293FT cells used to generate viral particles looked more stressed. Overall, different ratio of packaging plasmids did not dramatically increase the package efficiency.

3.2 Two packaging system Versus three packaging system: Switching to a 2 plasmid system by using psPAX2 together with PDM (available from Addgene) to package improved titre by 5 fold. This might result from the higher chance to get 3 plasmids (including the gene of interest) rather than 4 plasmids into one 293FT cell. The packaging efficiency of viral particles really depends on how many 293FT cells get all the helper vectors and target vector at same time. Therefore, the 2 package system has a higher efficiency than 3 package system.

3.3. Another issue I still need to address is putting specific sequences missing in the Invitrogen vector backbones, such as WPRE and cPPT into the backbone of the lentivector in order to get better titre and transgene expression. The WPRE (Woodchuck Posttranscriptional Regulatory Element) from the woodchuck hepatitis virus is placed directly downstream of the gene of interest, allowing for increased transgene expression (115), with more cells expressing the gene of interest. cPPT (Polypurine Tract) from the HIV-1 integrase gene, increases the copy number of lentivirus integrating into the host genome and allows for a two-fold increase in viral titer (116). Both WPRE and cPPT together, produce at least a four-fold increase in protein expression in most cell types, compared to other vectors that do not contain these elements. Interestingly, Invitrogen has now created a new version of their Virapower system called "HiPerform" which contains the cPPT and WPRE in the lentivector backbone [Fig 24]. I could also try to remove some optional components, such as antibiotic selective gene, from the backbone to increase the efficiency. Because it has been proven that smaller vectors have much higher transduction efficiency (117) [Fig 24].

Since I could not achieve high transduction efficiency using the lentiviral system, I also tried to optimize my yield using electroporation. It has been shown that exposing eukaryotic cells to high voltage electrical fields can induce the uptake of exogenous DNA, presumably through the transient formation of micropores in the cell membrane (118). The magnitude and characteristics of the generated electrical field are critical for successful electroporation (119). These techniques may allow the transfection of a variety of host cells which cannot be easily transformed by other methods (120). 60% of cells were expressing New-SRAP/GFP using this approach [data not shown]. Therefore, I concluded that transfection efficiency Amaxa (electroporation) > Lipofectamine 2000> viralpower system (Invitrogen). However, T5 cells look stressed and multi-nucleolus cells existed after electroporation both in control (empty vector transfected) and New-SRAP transfected cells, suggesting that electroporation transfection method is limited by viability and toxicity for some cells.

4. The interaction between ERβ and SRAP needs to be further investigated.

We have previously showed by GST-pull down that SRAP associates with ER α and ERβ *in vitro*. In order to further investigate potential interaction between SRAP and ERβ *in vivo*, I performed co-immunoprecipitation experiments in two cell line models: inducible MCF7-ERβ cells and transfected 293 cells. As described in the results section, a weak interaction between SRAP and ERβ was sometimes found, however this result was never consistently obtained. Possibly, the interaction might exist but was too weak to be reproducibly detected. Indeed, our results support in breast cancer cells or tumors majority of SRAP is located in the cytoplasm whereas ERβ is mainly expressed in the nucleus (103). Different subcellular localization of SRAP and ERβ could result in the limited interaction population of these two

molecules. Theoretically, interaction between SRAP and ERβ could be enhanced by using nuclear extract in the co-immunoprecipitation experiments. Alternatively, complex formation between SRAP and ERβ might depend on a specific cell context and treatments that needs to be identified. The other possibility is that the binding of antibodies used competes with sites needed for SRAP/ ERβ interactions. If that scenario is true, we have to test more antibodies with different epitopes.

Interestingly, *Kawashima et al.* showed that SRAP is able to directly interact with the AF-2 domain of AR by GST-pull down assay (84). By doing mammalian two-hybrid assays, this author also showed that the DBD-AF2 domain of AR associates with the SRAP in a ligand-dependent manner (84). This led me to test whether AF-2 domain of ERβ interacted with SRAP *in vivo*. In my Co-IP experiment neither AF-1 nor AF-2 domains of ERβ was found to interact with SRAP. The reason might be similar to the reasons mentioned earlier. One might also hypothesize that Native buffer extraction conditions still need to be optimized. Once native buffer conditions for SRAP are optimized, I could also identify more candidate proteins interacting with SRAP. Ideally, proteins embedded in the different SRAP complexes in both nuclear and cytoplasm extracts could be separated by the two-dimensional blue native/SDS gel electrophoresis (2D/BN/SDS). 2D/BN/SDS consists of a first dimension non-denaturating separation on a gradient gel based on size and charge (Coomassie blue G-250 brings negative charges to complexes without changing their composition) of different complexes. The second dimension, performed on classical denaturing SDS-PAGE, resolves each complex into its individual protein components followed by isolation from gel and sequencing (121). The effect of estrogen and anti-estrogen on the formation of these complexes could also be investigated in the future. Understanding how SRAP containing

complexes are generated and behave under SERM treatment will help us to better clarify SRAP potential relevance in breast cancer.

5. Localization of SRAP in breast tumor by IHC and correlation of SRAP and ERβ expression.

Several SRAP antibodies were tested under IHC conditions for their future suitability in TMA analyses. I have ultimately selected the rabbit polyclonal 743 SRAP antibody which is able to detect both mouse SRAP and human SRAP by IHC. Specificity of this antibody was confirmed by excess peptide competition and using SRA gene knockout MEF's cells as mentioned earlier in my results section. Both nuclear and cytoplasm staining could be observed with 743 SRAP antibodies in epithelial cells in series of breast cancer tissues [Fig 30]. This is first time the cellular localization of endogenous SRAP expression detected by IHC has been reported in breast cancer tissues. It has been suggested that SRAP is able to interact with different transcription factors in the nucleus (89;90). Also, SRAP has been found to be recruited by chromatin and able to bind specific genome regions by CHIP analysis (our unpublished data). The nuclear localization of SRAP supports the possibility of such events occurring, and presumably a nuclear function of SRAP in regulating gene expression. However, cytoplasmic function of SRAP has not been reported to date and our results support that the majority of SRAP is located in the cytoplasm of breast tumor in *vivo*. Indeed, to find more cytoplasm proteins interacting with SRAP through two-dimensional blue native/SDS gel electrophoresis (2D/BN/SDS) as I mentioned earlier might help us to understand SRAP cytoplasm function. I would like to further explore the specific mechanism controlling the subcellular localization of SRAP.

By using several TMAs representing different breast cancer cohort (including ER α negative and positive) which have been extensively characterized by *Skliris* (103), we are able to correlate SRAP to ERβ1 and ERβ2/cx expression in the breast tumor *in vivo*. Positive correlation between SRAP and ERβ expression could explain the potential interaction of two molecules in breast tumorigensis even though this positive correlation appears not to support the fact that ERβ expression is down-regulated by SRAP in our cell line models. Additionally, in a large breast cancer cohort, whether levels of this protein could be associated with outcome or established clinical parameters could be assessed using TMA data. In corroboration with *Dr. Murphy's* lab, we will be able to correlate SRAP expression not only to ERβ but also to a number of prognostic and proliferative markers, such as ER α, Ki67, CK5/6, Her2/neu, EGFR, c-Jun *et al*. Overall, TMA analysis of SRAP will provide us some valuable clinic relevance by which we could further understand the biological relation between SRAP and breast cancer.

6. Summary and conclusion

Above all, I have successfully shown that SRAP rather then SRA RNA could significantly decrease the expression of total ERβ mRNA. Whether or not ERβ protein expression was been changed by SRAP needs to be further determined. The apparent low expression of ERβ protein might however make this task extremely difficult. Even though I tried several alternatives, I was unable to set up lentiviral transduction experimental conditions to deliver our SRA constructs. Beside, interaction between ERβ and SRAP was been observed by co-immunoprecipitation in our cell models. Positive correlation between SRAP and ERβ expression in breast tumors was observed by TMA analysis.

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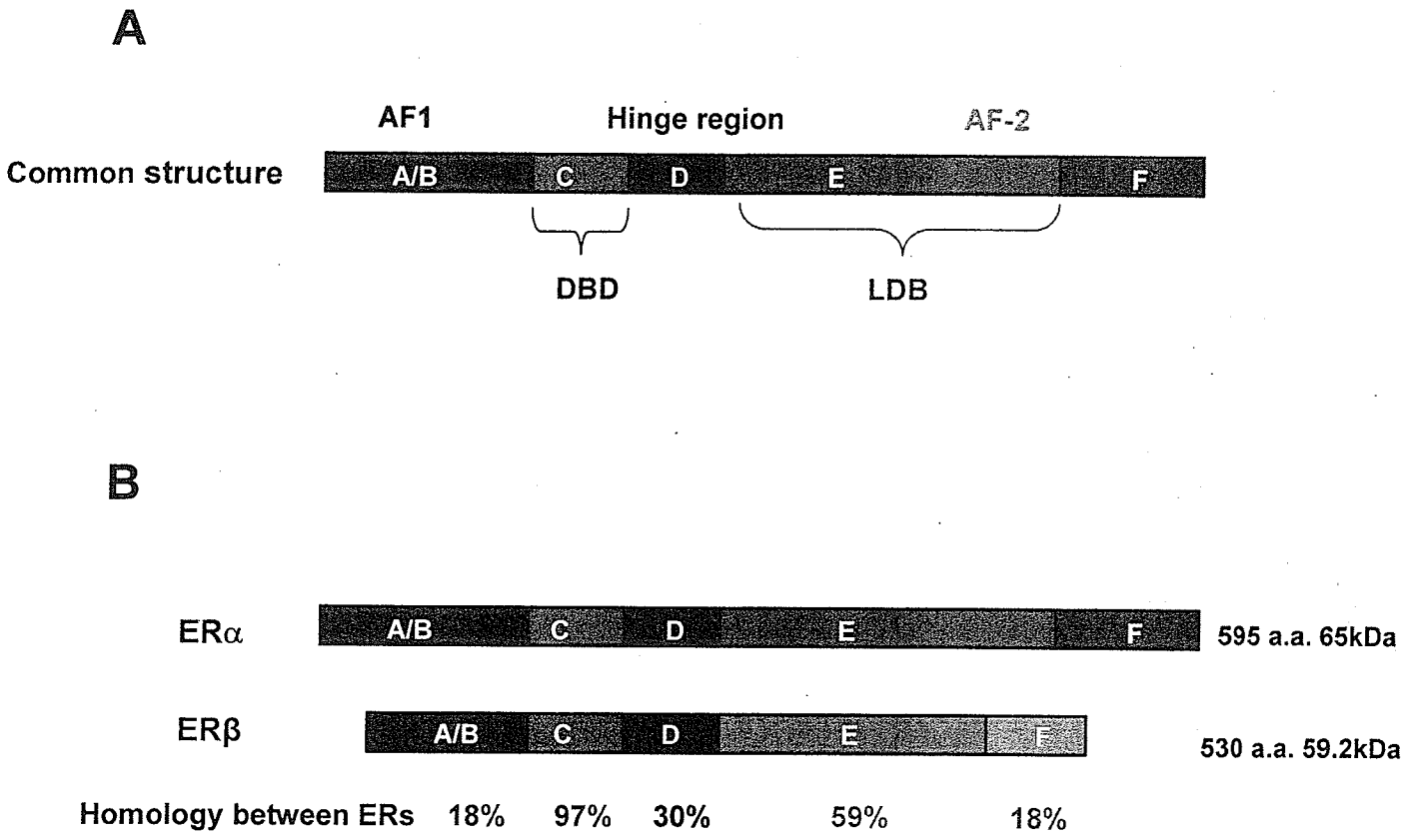


Figure 1. Schematic diagram of structural organization of human ER α protein, human ER β variants proteins. A) Structure of the ER protein and functional domains: structural organization consists of six functional regions (A-F). The A and B domains, on the N-terminal, contain the AF-1 segment. The C domain is the DNA-binding domain, or the DBD. The D-domain, or the "hinge region" has the nuclear localization signal. The E and F domain helps with dimerization, ligand binding, and has several other functions. **B)** Homology of different functional domains between ER α and ER β protein.

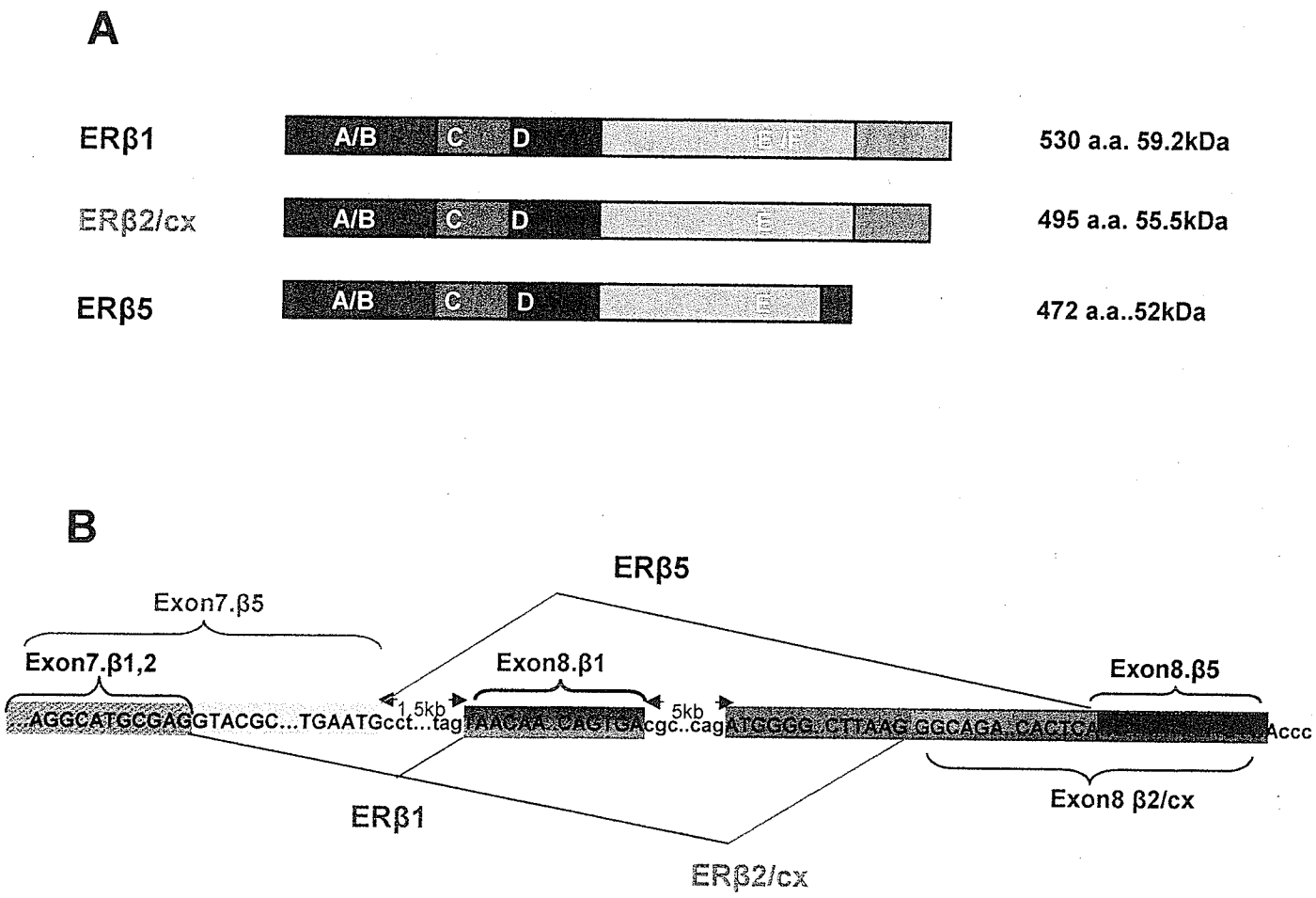


Figure 2. Schematic diagram of structural organization of human ER β variants proteins. A) Structure of the ERβ protein isoforms: The two isoforms share 47% homology but the conservation is 94% and 57% in the DBD and LBD respectively. The differences among ERβ variants are mainly on C-terminal Ligand binding domain. **B)** Schematic representation of alternative splicing events resulting in the production of the different ERβ isoforms. The use of alternative acceptor sites leads to the production of ER β1, β2 mRNAs. Indeed, these isoforms share a common exon 7 (Exon 7 β1,2, blue) but differ in their alternative exon 8 (purple, pink). The combined use of alternative donor and acceptor sites produces ER β5 mRNA, which contains an extended exon 7 (light blue) and a shorter exon 8 (red).

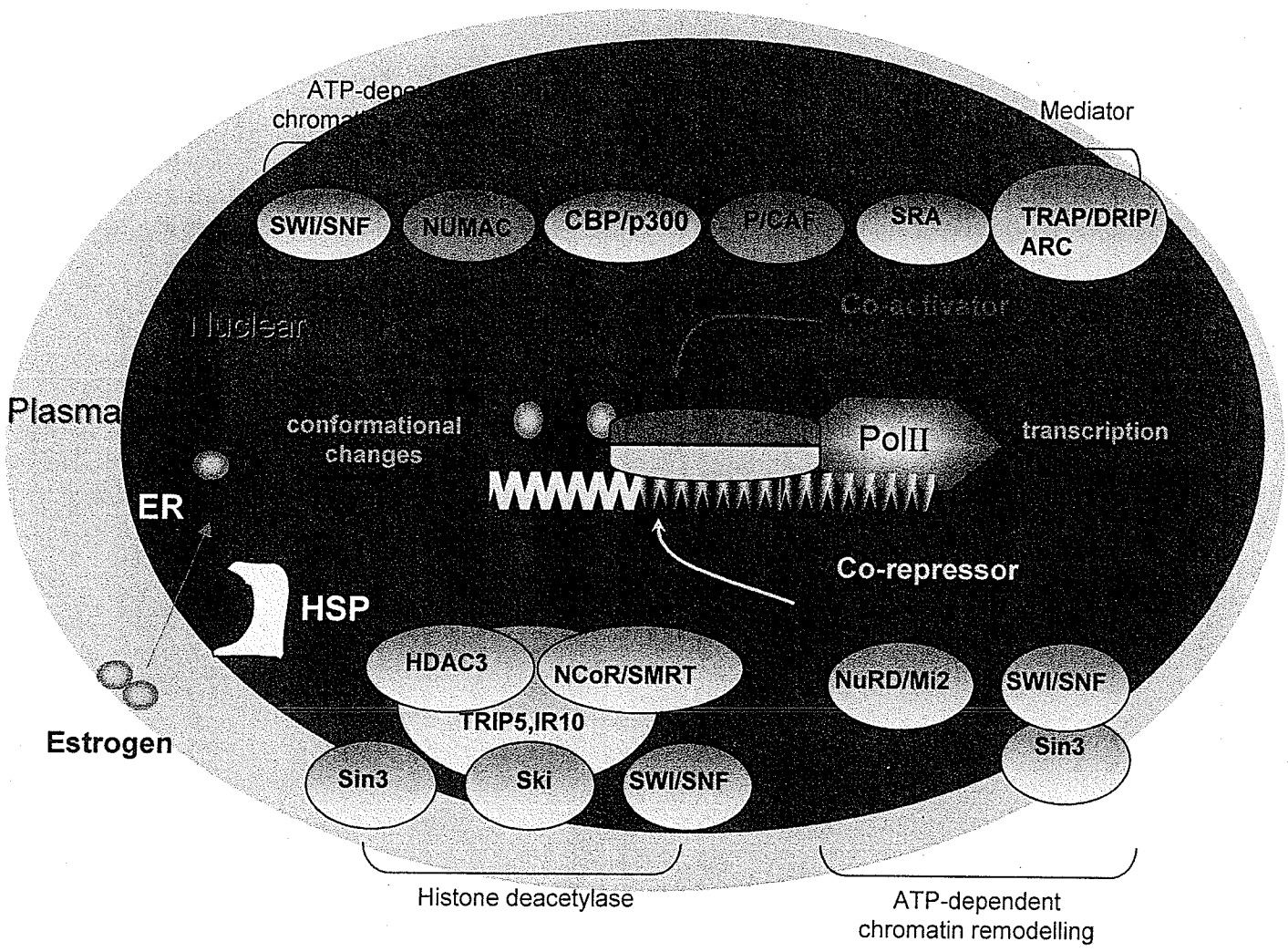


Figure 3. Estrogen signaling pathway and co-regulators. Once bound to the ligands, ER undergo conformational changes, dissociate from heat shock proteins (HSP) and translocate into the nucleus, dimerize, and then specifically recognize hormone responsive elements (ERE). Activated estrogen receptors, through dynamic interplays with additional co-regulators, direct the assembly and stabilize a pre-initiation complex that ultimately conducts gene transcription. (Co-activator complexes and their mechanisms of action are shown on the top; co-repressor complexs are shown on the bottom. Complexes cited here are briefly described: **CBP/P300** :CREB binding protein/E1A binding protein p300 are two closely related proteins able to co-activate various transcription factors. **pCAF**: p300/CBP associated factor binds to p300 and CBP. It has histone acetyltransferase activitiy. **NCoR/SMRT**: nuclear receptor co-repressor/silencing mediator for retinoid and thyroid-hormone receptors in a co-repressor complex that physically interacts with ER and can recruit histone deacetylase (HDAC) activity. **NuRD/Mi2**:The Mi-2/nucleosome remodeling and deacetylase (NuRD) complex is unique in that it couples histone deacetylation and chromatin remodeling ATPase activities in the same complex. **SWI/SNF**: is a yeast nucleosome remodeling complex composed of several proteins products of the SWI and SNF genes (SWI1, SWI2/SNF2, SWI3, SWI5, SWI6). It possesses a DNA-stimulated ATPase activity and can destabilize histone-DNA interactions in reconstituted nucleosomes in an ATP-dependent manner.

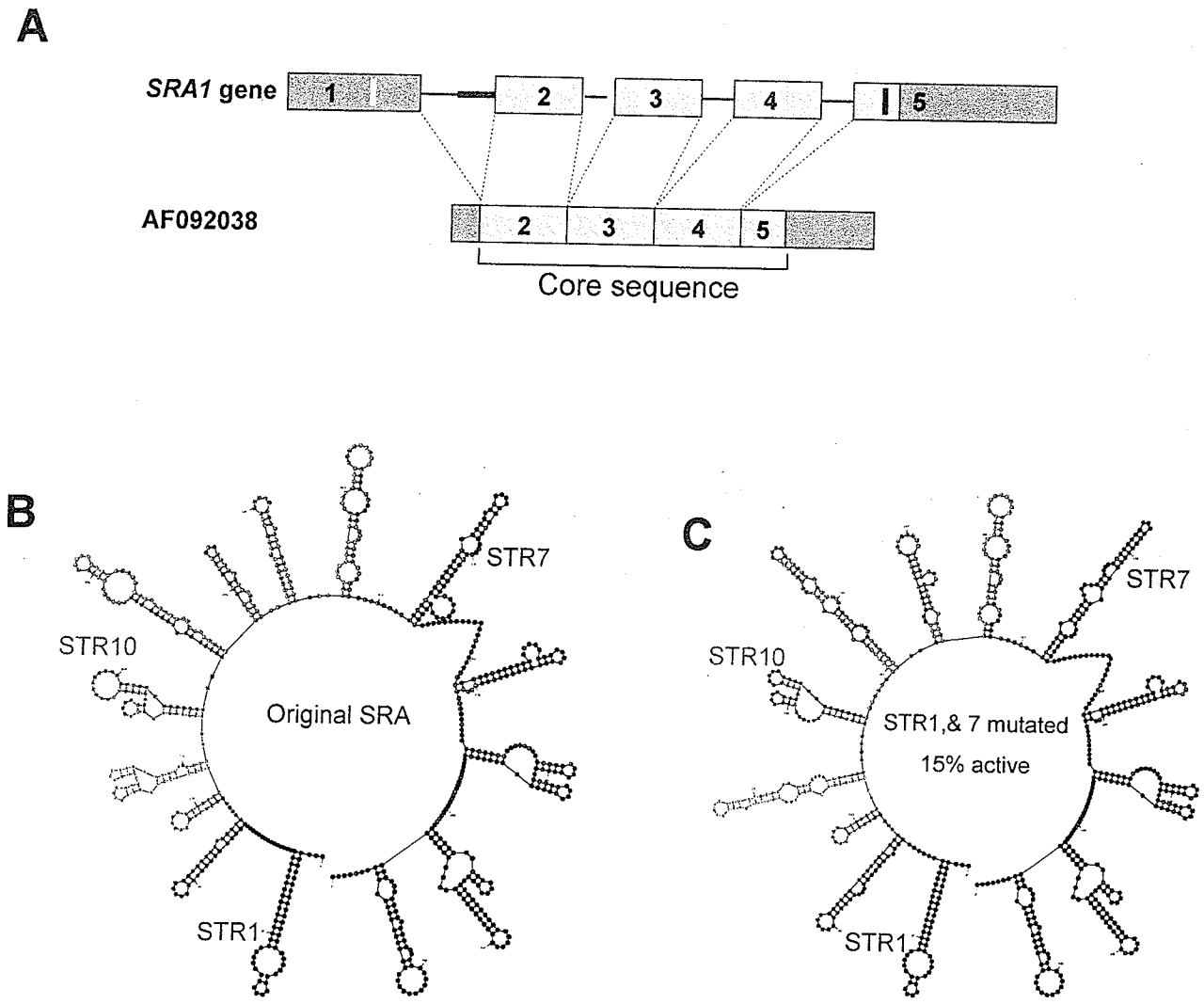


Figure 4. SRA1 genomic structure and core sequence. A) SRA sequences were originally described, differing in their 5' and 3' extremities, but sharing a central core sequence depicted in light blue [Lanz et al., 1999]. One sequence has been registered with the NCBI nucleotide database (AF092038). Alignment with chromosome 5q31.3 genomic sequence is provided. Introns and exons are represented by black lines and blue boxes, respectively. **B)** Schematic profile of the predicted secondary structure of human core SRA RNA. The secondary structure profile of SRA core sequence has been modeled using Mfold software [Zuker, 2003]. Detailed structure of STR1, 10, 7 [Lanz et al., 2002] is provided. **C)** By doing site-directed mutagenesis experiment, six secondary structural motifs (STR1, 9, 10, 7, 11, 12) have been identified to participate in co-activation respectively. Especially, silent mutations in both SRT1 and STR7 of SRA could nullify above 80% SRA co-activation function [Lanz et al., 2002].

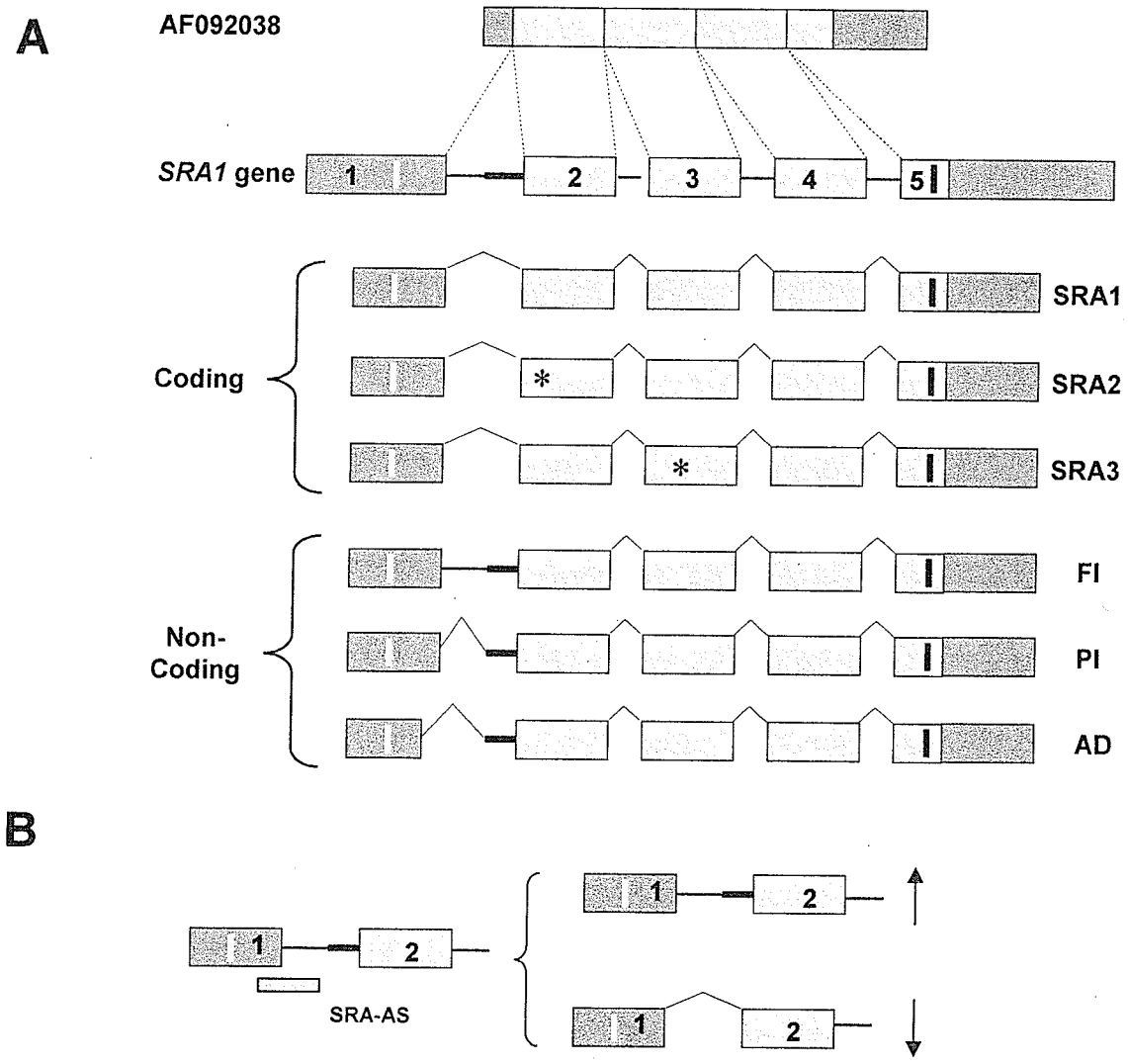


Figure 6: Coding and non-coding SRA transcripts in human breast cancer cells and Model for SRA-AS (antisense SRA oligonucleotides). . A) *SRA1* gene, located on chromosome 5q31.3, consists of 5 exons (boxes) and 4 introns (plain lines). The originally described SRA sequence (AF092038) contains a core sequence (light gray), necessary and sufficient for SRA RNAs to act as co-activators (Lanz et al., 1999). Three coding isoforms have now been identified (SRA1, SRA2, SRA3), which mainly differ from AF092038 by an extended 5'-extremity containing AUG initiating codons (vertical white bar in exon 1). The stop codon of the resulting open reading frame is depicted by a black vertical bar in exon5. Black stars in exon 2 and 3 correspond to a point mutation (position 98 of the core: U to C) and a point mutation followed by a full codon (position 271 of the core: G to CGAC), respectively. Three non-coding SRA isoforms containing a differentially-spliced intron-1 have been characterized: FI, full intron-1 retention; PI, partial intron-1 retention; AD, alternative 5' donor and partial intron retention. Thick straight line, 60 bp of intron 1 retained in PI; triangulated lines represent splicing events. **B)** Model for SRA-AS (antisense SRA oligonucleotides). SRA-AS is located in the junction of intron1 and exon 1. It will effect the normal intron 1 splicing by inhibiting the splicing factors binding. As a result, intron 1 retention non-coding RNA would be expected increased while the intron 1 fully spliced fragment would be expected to decrease after transfection.

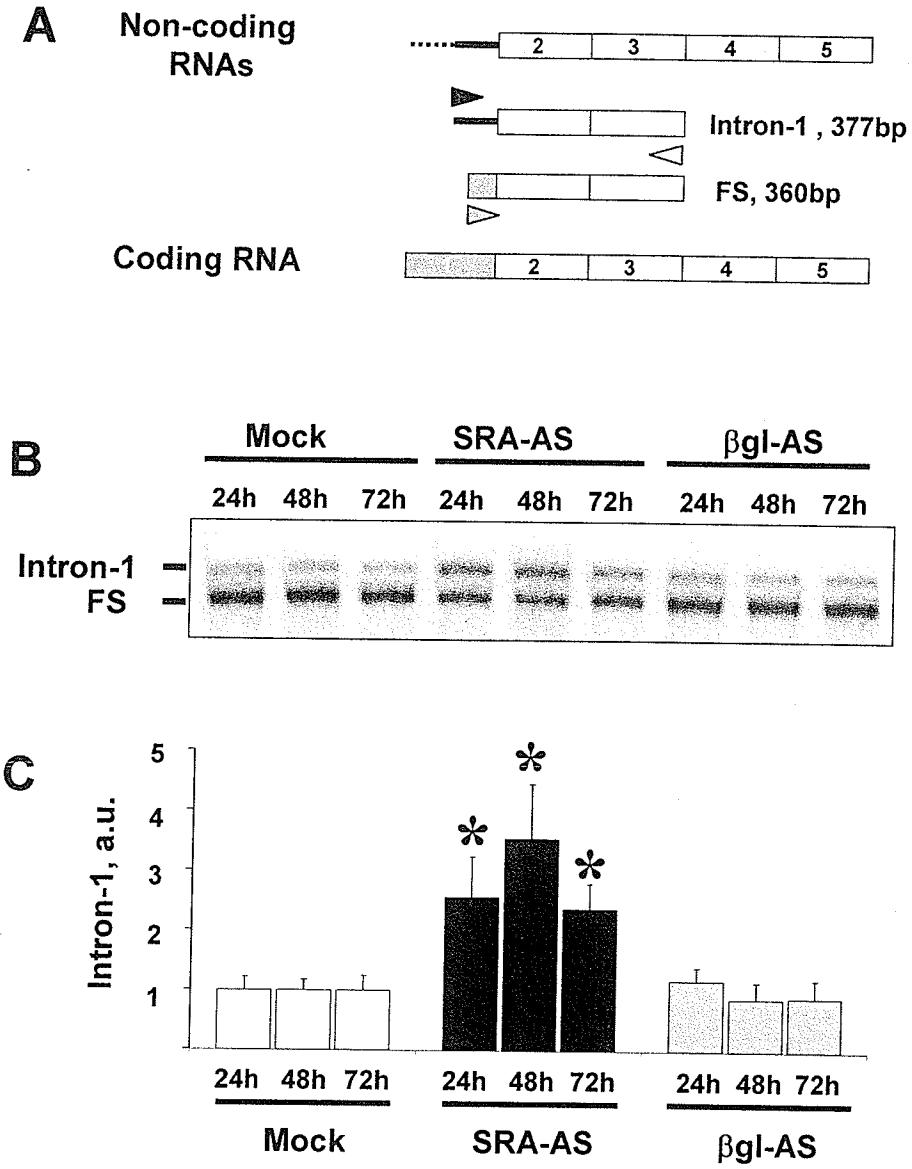


Figure 7: SRA-AS oligos increase the relative amount of endogenous intron-1 retaining SRA transcripts. **A)** Principle of TP-PCR amplification used to assess the relative proportion of coding and non-coding SRA mRNAs. Three primers are used during the PCR reaction: a lower primer recognizing a shared region in exon-3 and two upper primers, specific for exon-1 and intron-1 sequences, respectively. one has been chosen to recognize all intron-1 alternative splicing events, generating a single PCR product of 377 bp corresponding to non-coding SRAs (intron-1), whereas the other will participate to the amplification of a 360 bp fragment corresponding to fully spliced coding SRA (FS). **B)** Lasting effect of SRA-AS on the relative proportion of intron-1 retaining SRA transcripts in T5 cells. T5 cells were treated with no oligos (Mock), SRA-AS or bgl-AS oligos. At t: 24h, 48h and 72h, total RNA was extracted, reverse-transcribed and amplified by TP-PCR. Samples were separated on PAGE gel and visualized using a Molecular ImagerTM-FX. **C)** Signals corresponding to intron-1 and FS were quantified and the proportion of intron-1 retention expressed in arbitrary unit (a.u). Bars represent the average value of 3 independent experiments normalized to values obtained for mock transfection at a given time point. Error bars represent standard deviation. * corresponds to p values lower than 0.05 (Student's t-test).

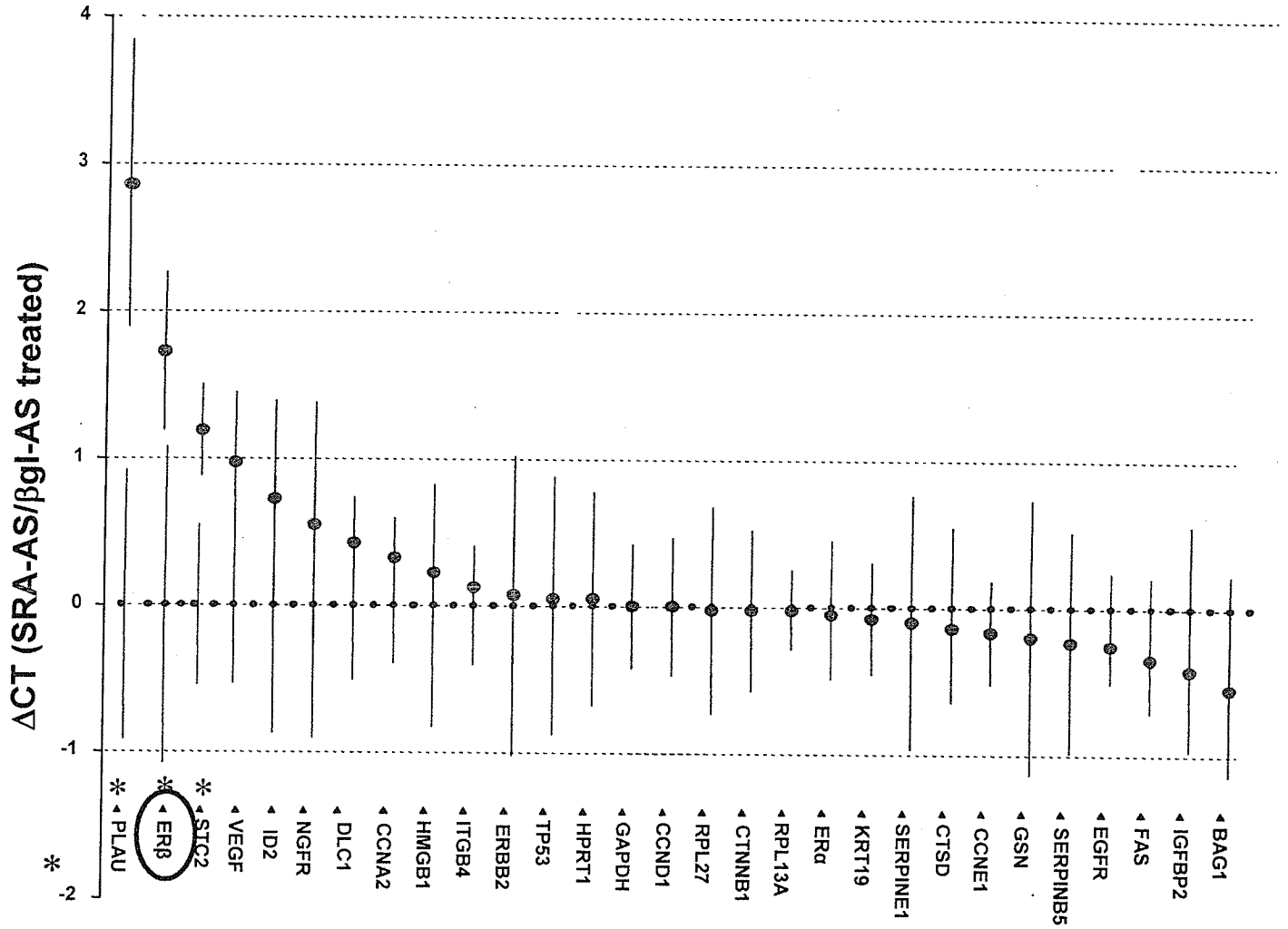
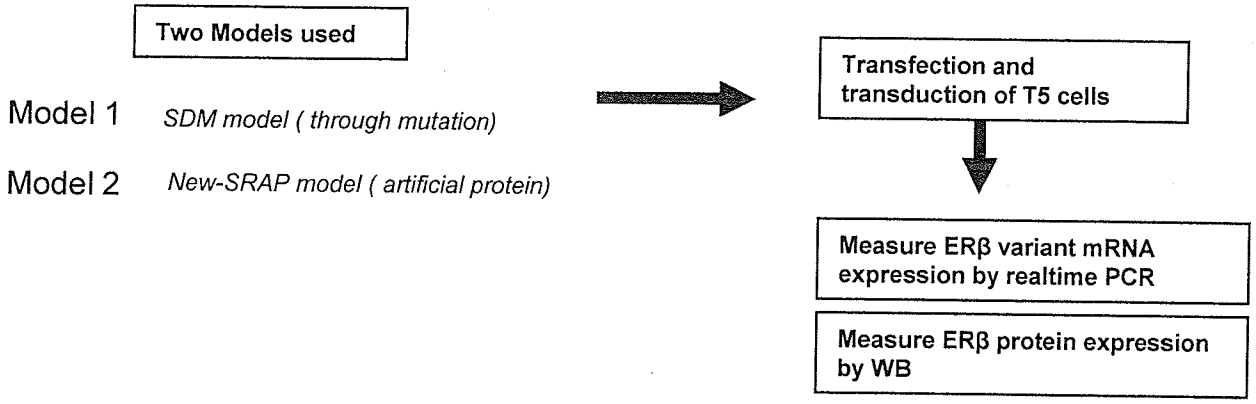


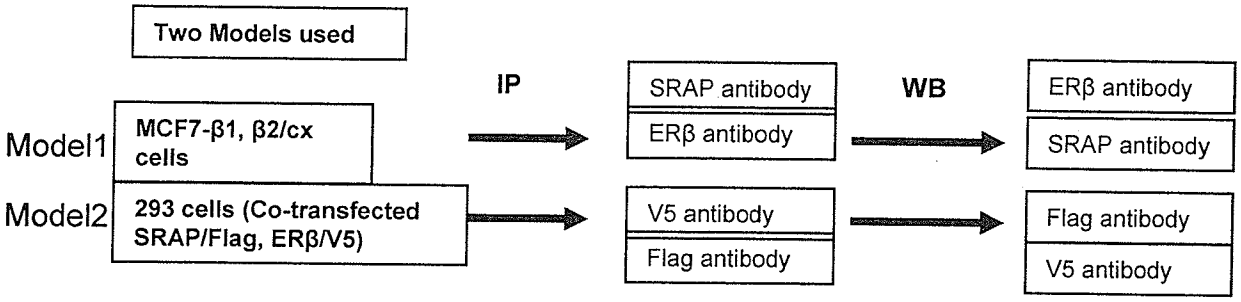
Figure 8: Change in ERβ gene expression in T5 after increase the relative amount of endogenous intron-1 retaining SRA transcripts. T5 cells were treated with 0.5 μM of SRA-AS or 0.5 μM bgl-AS oligos. At t: 24h, RNA was extracted, reverse-transcribed, checked by TP-PCR for modification of intron-1 retention and used to assess, by real-time PCR, the expression of a series of 56 genes historically linked to breast cancer, as described in the Materials and Methods section. Four independent experiments were performed. Blue dots represent, for each gene, the normalized expression upon bgl-AS oligos treatment. Orange dots represent the average DCT increase or decrease in gene expression upon SRA-AS treatment. Bars represent the standard deviation for each gene and treatment. Genes whose expression is significantly modified ($p<0.05$, Student's-t-test, two sided) upon SRA-AS treatment are indicated by orange boxes.

Study Outlines:

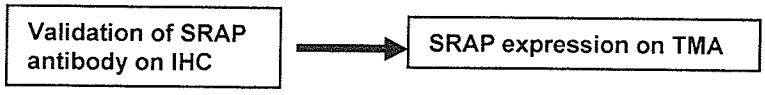
Aim1: investigate respective SRA RNA and SRA protein function on ERβ expression



Aim2: investigate potential interaction between SRA protein and ERβ by Co-IP



Aim3: Set up IHC conditions for performing future TMA analysis



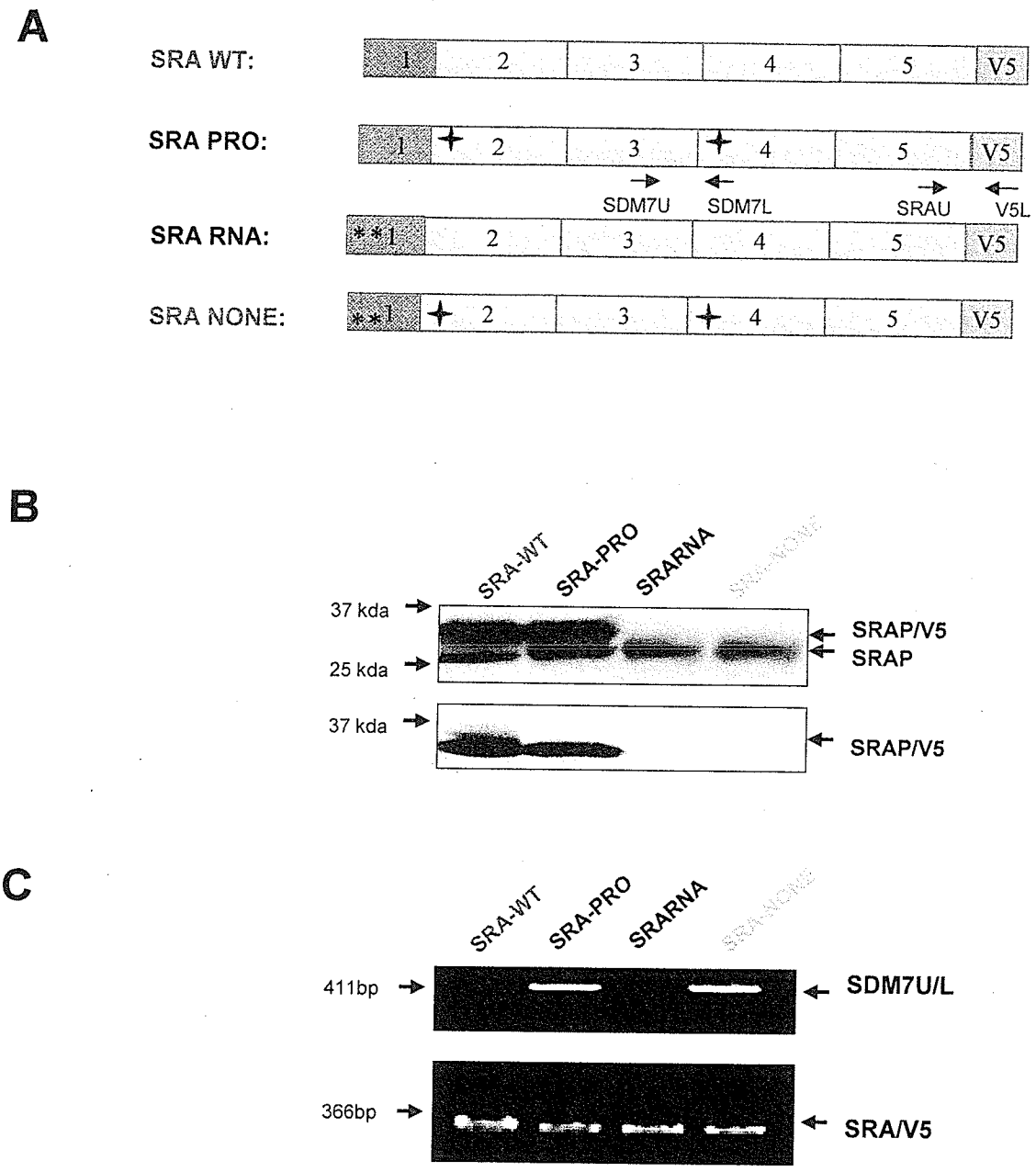


Figure 10: First model of SRA constructs. A) Four SRA structures shown in the above figure(exon1-exon5). First two mutated AUG codons have been shown as stars and silent mutations SDM1, SDM7 have been shown as crosses in the exon 2 and 4, respectively. **B)** four pLenti-SRA constructs were transfected into T5 cells with Lipofectamine2000. The protein lysates after 24h transfection were analyzed by Western blot analysis. Expressions of SRAP were detected by using anti-SRAP antibodies and transfected SRAP/V5 were detected by using anti-V5 antibodies. **C)** The RNAs were extracted 24h post-transfection. After DNase treatment and reverse transcription, cDNA were analyzed by PCR using specific primers (SDM7U/L) with mutation sites in SDM7 region (location shown in A). The relatively equal RNA transcripts among four plenti constructs have been checked by using V5L and SRAU (table 5).

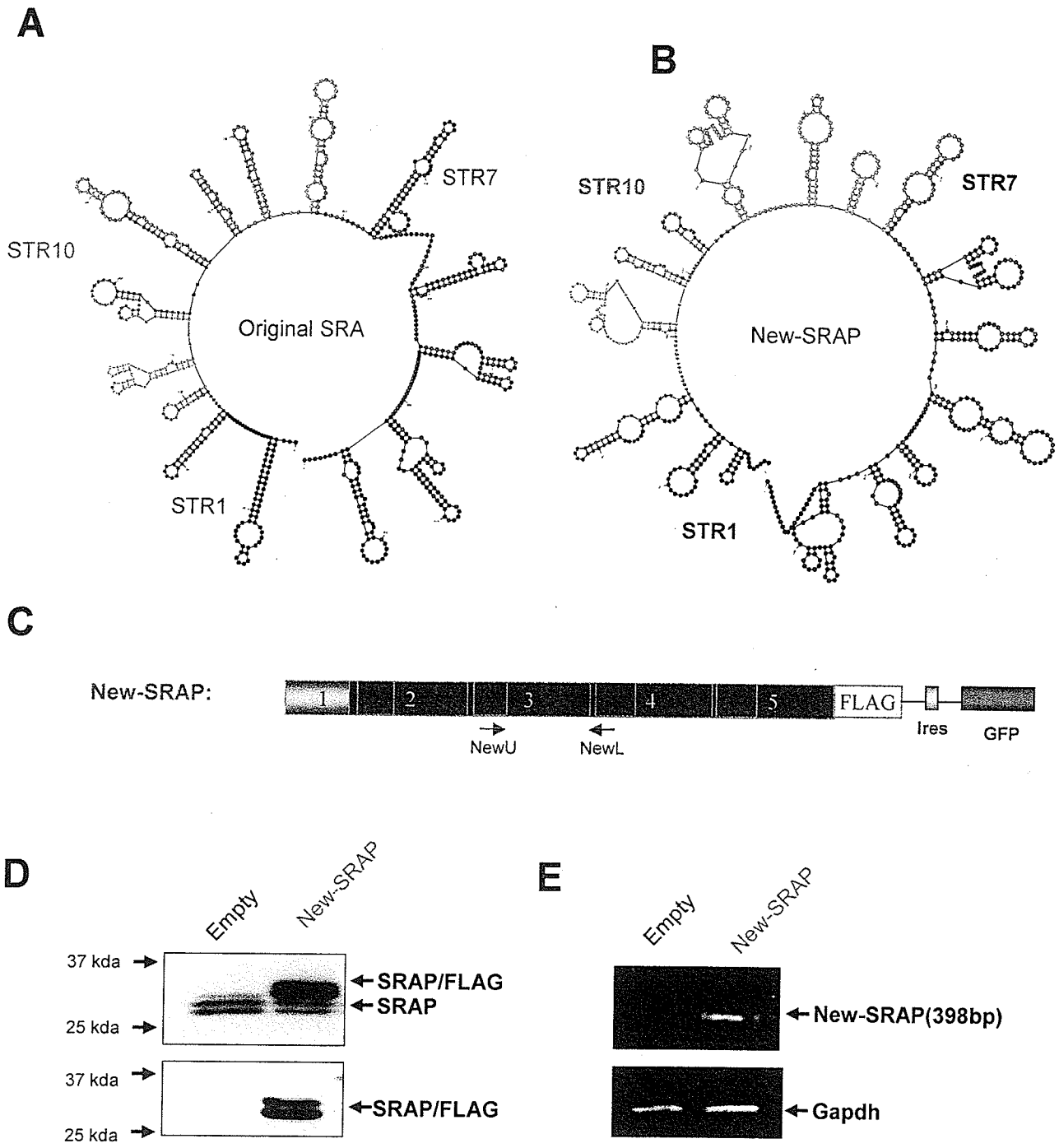


Figure 11: Designing Second SRA Model, a coding artificial sequence (new-SRAP), not functional at the RNA level. A) SRA has a secondary structure described by a series of Stem loops (STRs). **B)** We have designed an artificial SRA sequence (New-SRAP) herein aligned to the original SRA sequence. An extensive amount of mutations (all silent) have been introduced to drastically disrupt the secondary structure of the corresponding SRA RNA without altering its ability to encode for SRAP. Predicted secondary structure of new-SRAP RNA (established using Zuker's algorythms as detailed ref). **C)** The construct of New-SRAP lentivirus vectors expressing SRA genes are schematically shown. Green bars distribute to exon1-5 represent the extensively silent mutated region. New-SRAP construct follow by flag tag and IresGFP structures. **D)** New-SRAP (N) was transfected into T5 cells with empty vector (E). The protein lysates were analyzed by Western blot analysis. Expressions of SRAP were detected by using anti-SRAP antibodies and transfected SRAP/FLAG were detected by using anti-Flag antibodies. **E)** The RNAs were extracted 24h post-transfection. After DNase treatment and reverse transcription, cDNA were analyzed by PCR using specific primers (NewU,NewL) with New-SRAP mutated region (location shown in C). Gapdh PCR products are showing the loading.

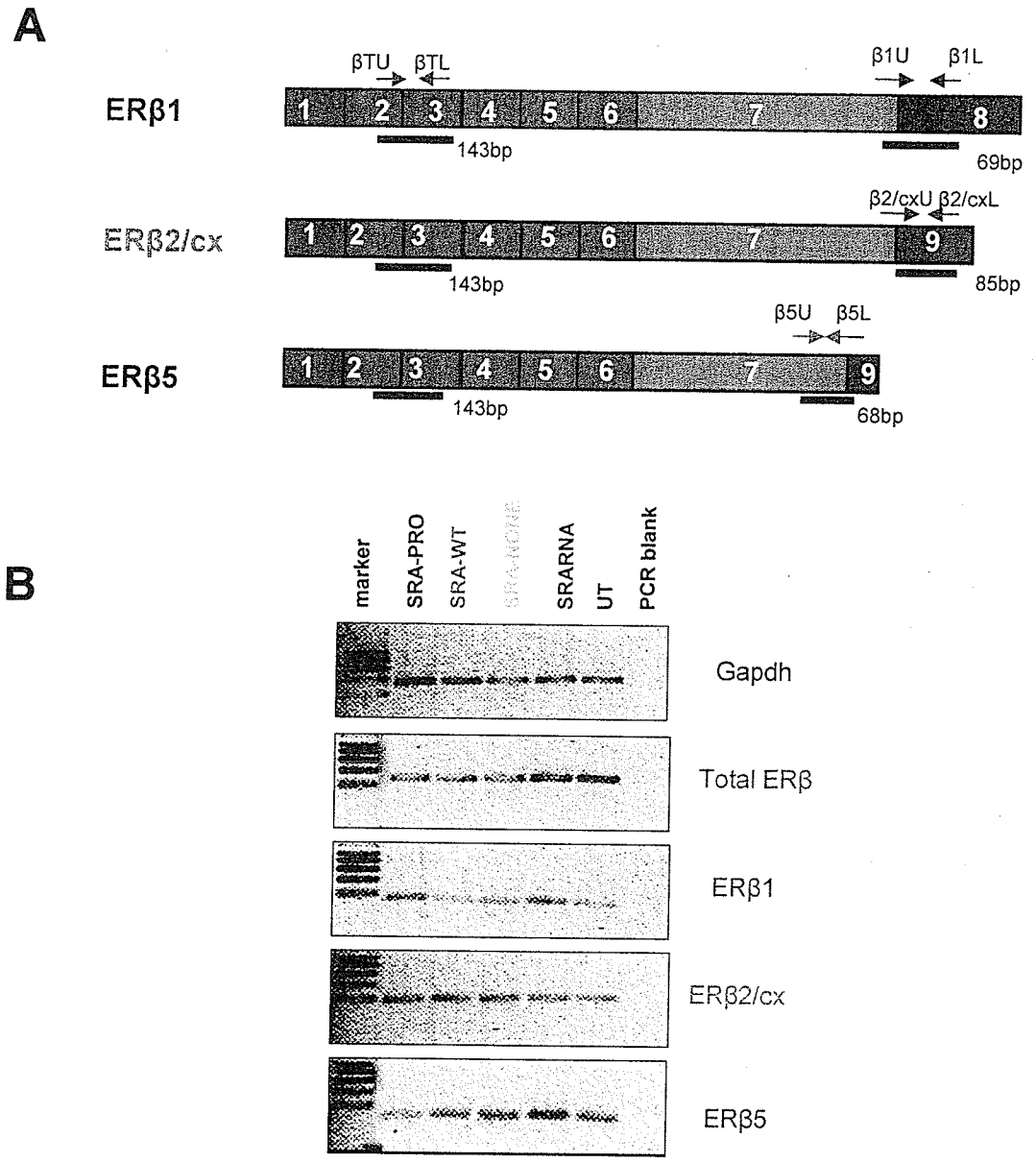


Figure 12: Designing the specific ERβ variants primer sets to investigate the mRNA expression change after over-expression SRA. A) Schematic representation of primers location of different ERβ isoforms. The use of alternative acceptor sites leads to the production of ER β1, β 2/cx, and β 5 mRNAs. β1,2/cx isoforms share a common exon 7 (dark blue) but ER β 2/cx has an alternative exon 9 (orange). The combined use of alternative donor and acceptor sites produces ER β 5 mRNA, which contains an extended exon 7 (light blue) and a shorter exon 9 (red). Primer annealing sites specific for each isoforms are also presented. Total ERβ primer are located in exon2 and exon3. **B)** Four plenti-SRA constructs have been transfected into T5 cells by lipofectamine 2000 (UT: untransfected, transfection efficiency is around 10-15%) . RNA samples were extracted after 48h. After reverse transcription, cDNA were analyzed by PCR using specific primers for ER β variants showed A. The specificity of PCR products have been checked on the agarose gel.

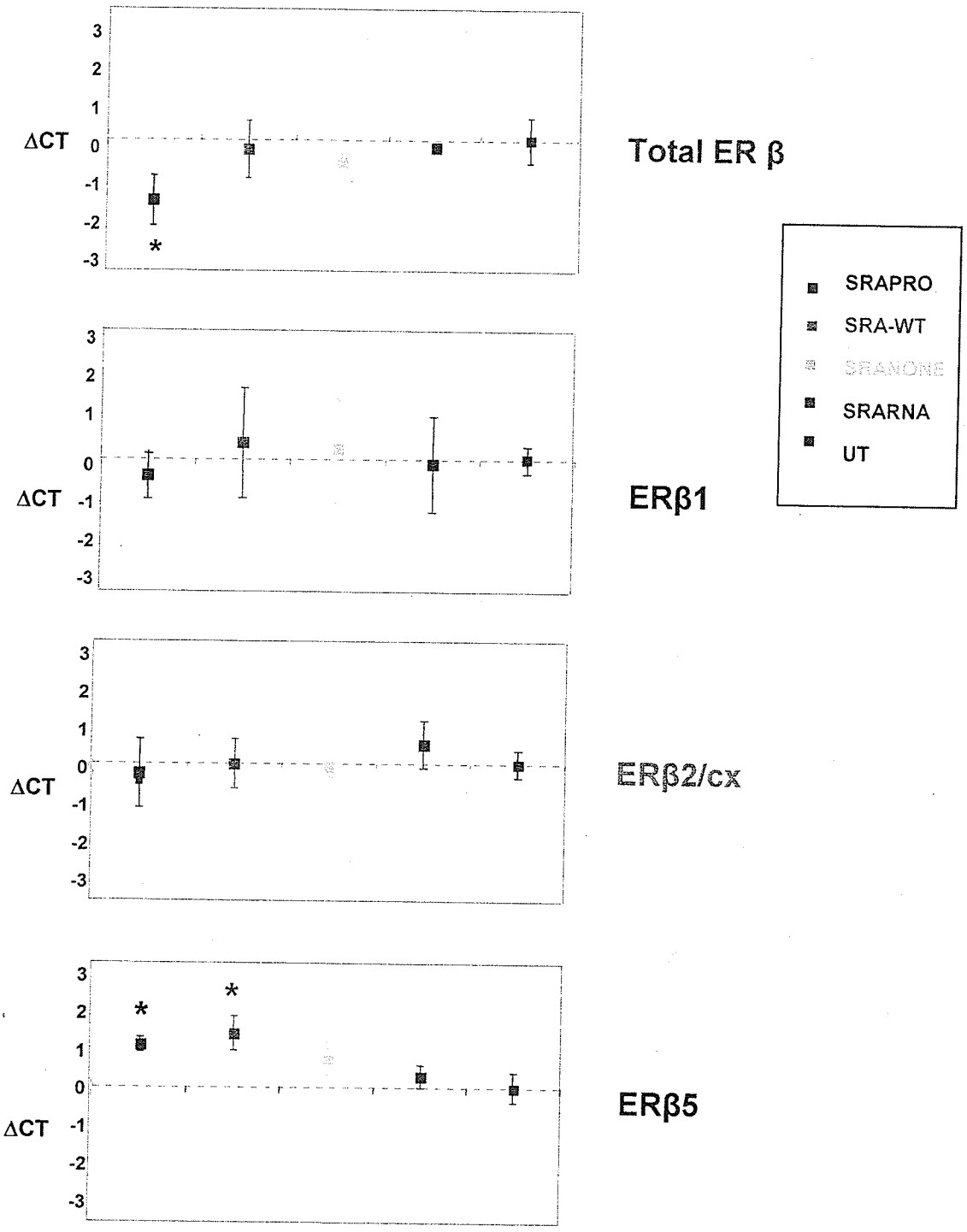


Figure 13: SRAP effects the mRNA expression of ERβ variants .
Four plenti-SRA constructs have been transfected into T5 cells by lipofectamine 2000 (transfection efficiency is around 10-15%). RNA samples were extracted after 48h. After reverse transcription, cDNA were analyzed by Real-time PCR by using specific primers for ER β variants showed in Figure 9. The total ERβ expression normalized by Gapdh had been significantly down regulated in the cells over-experssing only SRAP after 48h transfection (P=0.03). The ERβ5 expression had been significantly up regulated in the cells over-experssing SRAP and SRA RNA after 48h transfection (P=0.04) . The expression of ERβ1 and ERβ2/cx has no change in both cells over-expressing SRAP or SRA RNA when comparing to the untransfected cells.

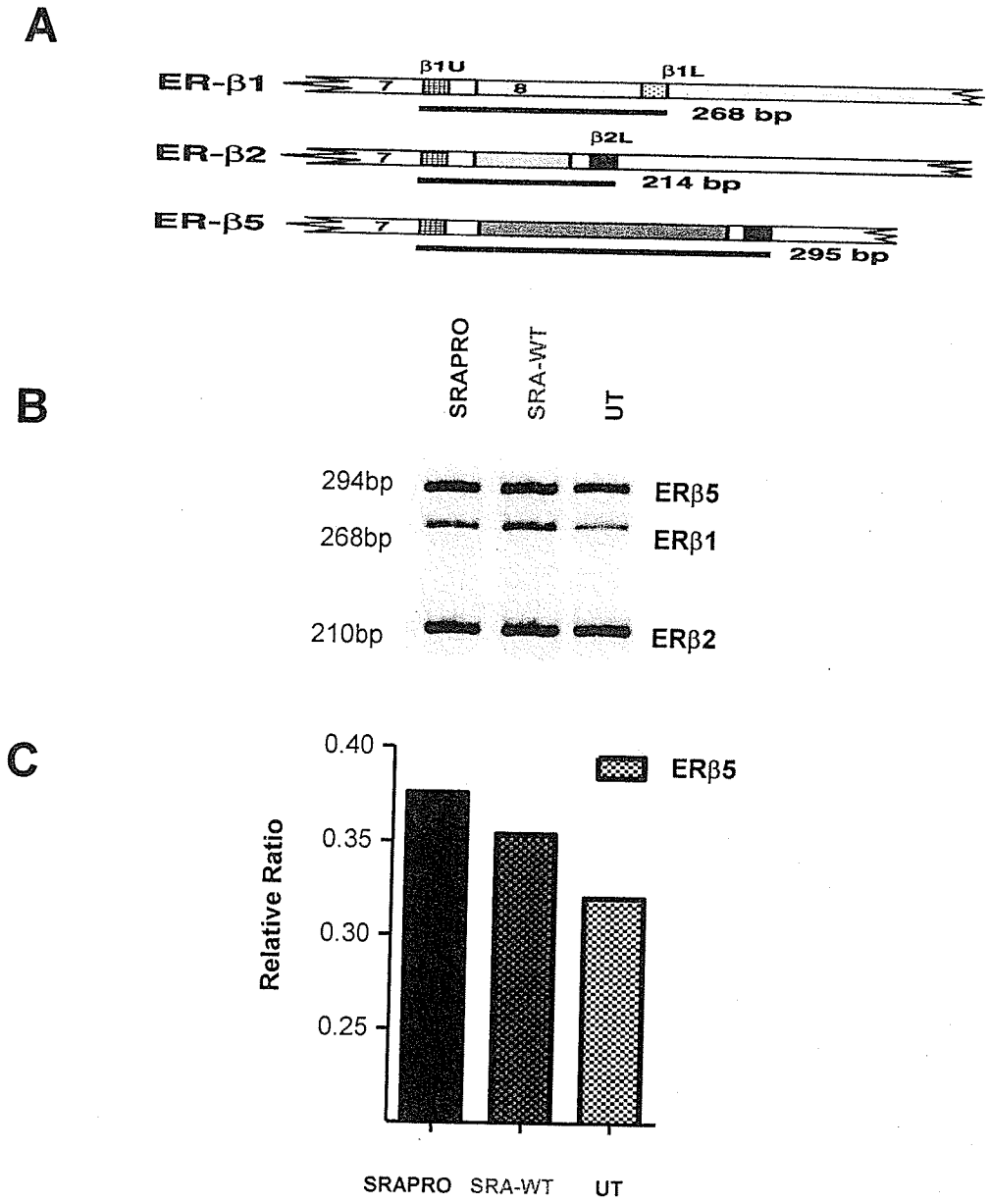


Figure 14: SRAP effects the relative ERβ5 variants mRNA expression.
A) N-terminal alternative splicing events result in different ERβ variants. Common sequence (white region) and specific sequences (grey region) are depicted in exon 7 and 8 for each CDNA (β1,β2/cx, and β5). ERβ1U, ERβ1L and ERβ2l primer annealing sites are also represented. The sizes of possible PCR products obtained after TP-PCR are indicated. B) plenti-SRA constructs have been transfected into T5 cells. RNA samples were extracted after 48h. After reverse transcription, cDNA were analyzed by TP-PCR. Quantification of relatively ratio between ERβ variants have been showed below.

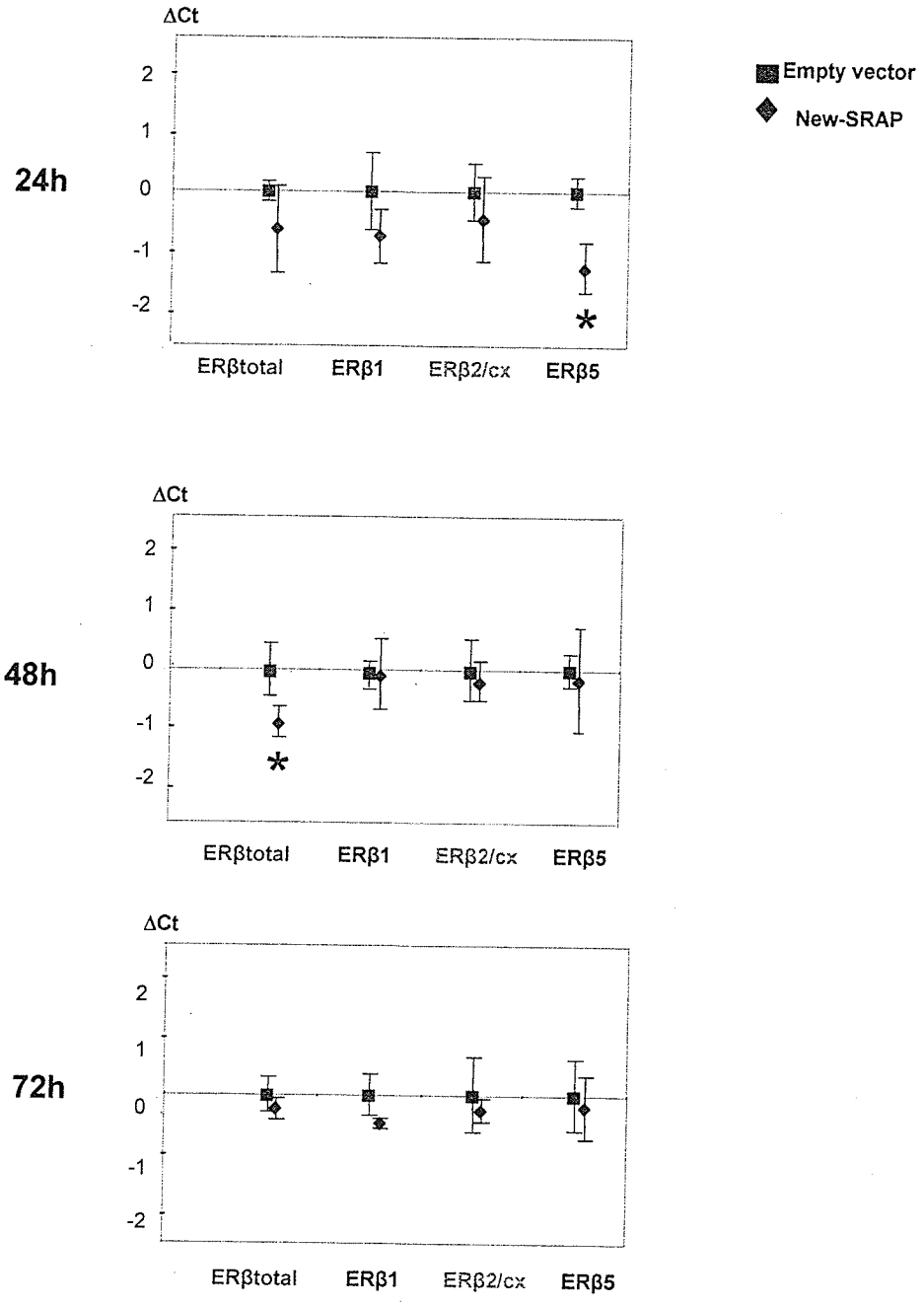


Figure 15: SRAP down regulates the ERβ mRNA expression using New-SRAP constructs.
New-SRAP construct has been transfected into T5 cells with lipofectamine2000 (transfection efficiency is around 10-15%). **A)** Protein samples were lysed after 24h, 48h and 72 h and analyzed by Western blot analysis. The expressions of SRAP, ERβ, and beta actin were detected using specific antibodies. SRAP decreases the expression of ERβ after 48, 72 hours transfection as shown by arrows. **B)** RNA samples were extracted after 24h, 48h and 72h. After reverse transcription, cDNA were analyzed by real-time PCR using specific ERβ total as well as ERβ1, ERβ2/cx, ERβ5 primer sets. The total ERβ expressions had been significantly down regulated in the cells over-expressing new-SRAP after 48h transfection (P=0.02).

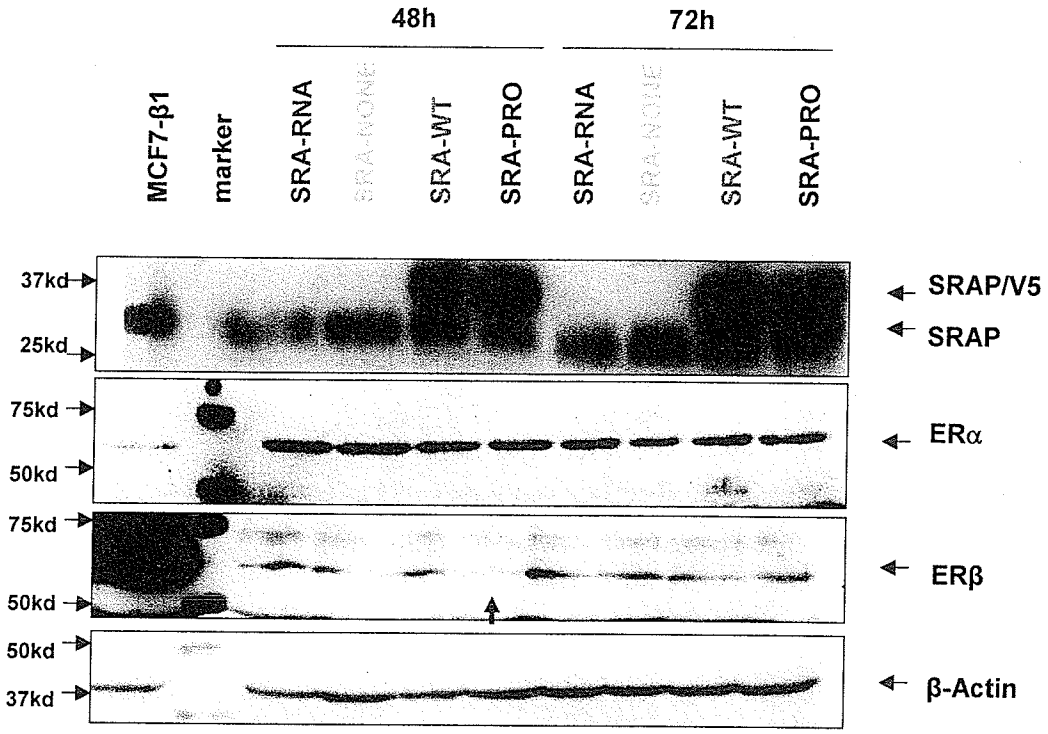


Figure 16: SRAP down regulates the ERβ-like protein signal. A) Four plenti-SRA constructs have been transfected into T5 cells by lipofectamine (transfection efficiency is around 10-15%). Protein samples were extracted after 48h and 72 h and analyzed by Western blot analysis. We monitored the expressions of SRAP, ERα, ERβ (total), and beta actin using specific antibodies. SRAP decreases the expression of ERβ-like protein after 48 hours transfection as shown by arrows (First lane in ERβ is the positive control with cells over-expressed of ERβ1). SRA RNA has not effect on ERβ-kike expression. The experiments had been repeated twice.

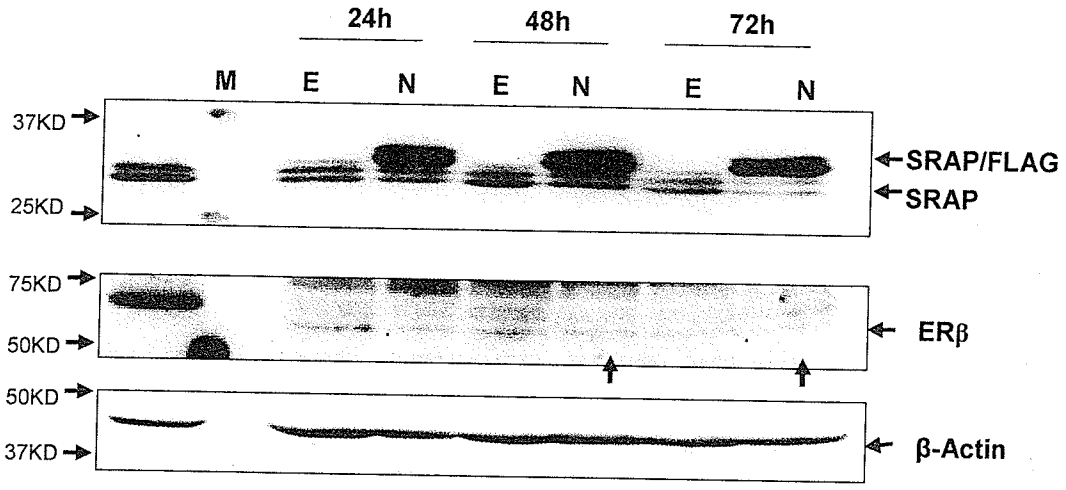


Figure 17: SRAP down regulates the ERβ-like protein signal. New-SRAP (N) construct has been transfected into T5 cells with empty vector (E) (transfection efficiency is around 10-15%). Protein samples were extracted after 24h, 48h and 72 h and analyzed by Western blot analysis. The expressions of SRAP, ERβ, and beta actin were detected using specific antibodies. SRAP decreases the expression of ERβ after 48, 72 hours transfection as shown by arrows. The experiments had been repeated twice.

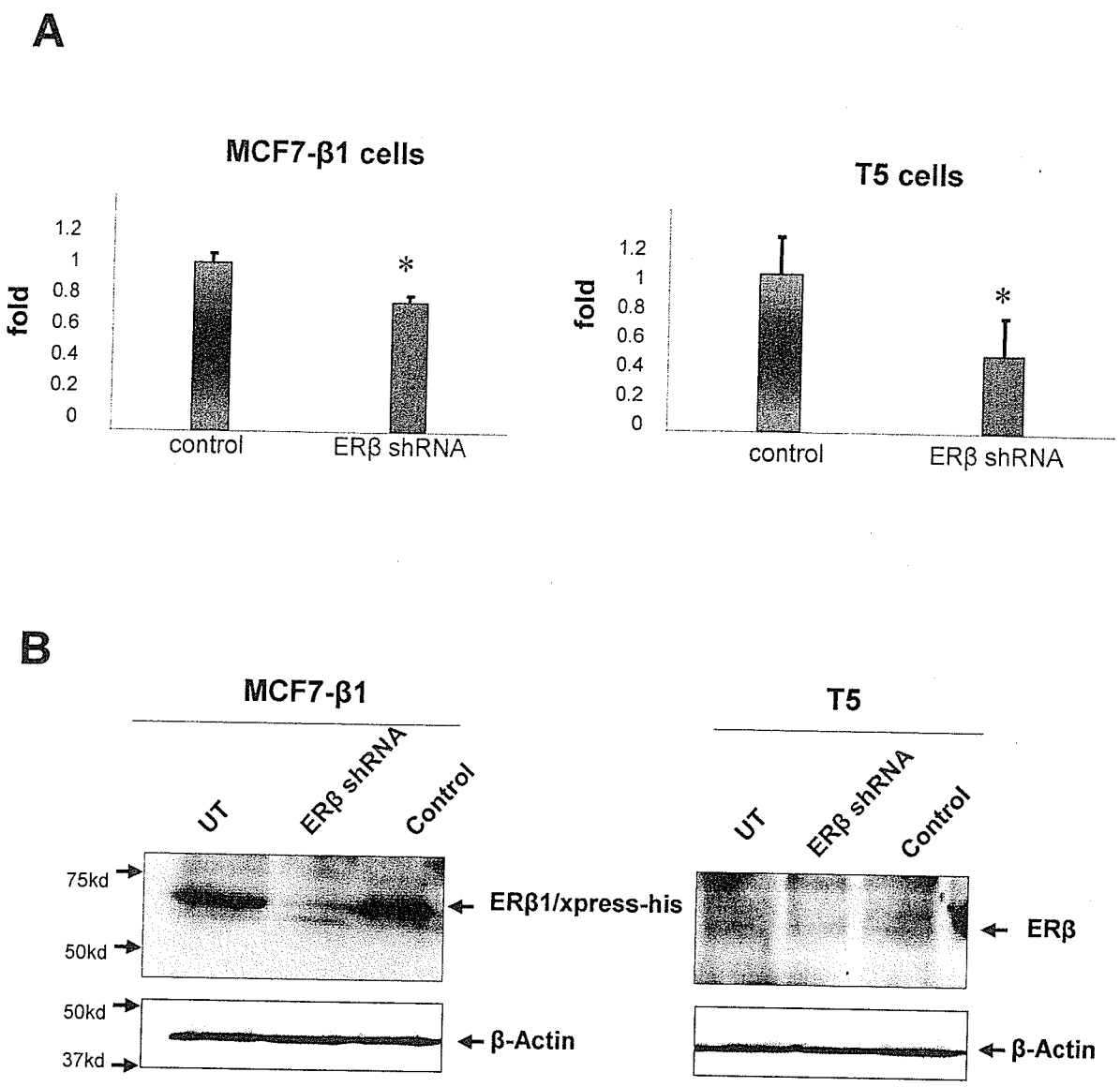


Figure 18: Block the ERβ-like protein signal by delivering of siRNA of ER β. T5 cells and MCF7-β1 (control cell line) cells were plated in 6-well dishes 48h prior transfection. (During this 48h, MCF7-β1 cells were treated with doxycycline (2 μg/μl)). Transfections were performed in complete medium containing the DNA-lipid complex for 48 hours. After 48h, cells were lysed and analyzed by western blot using anti-ERβ chicken antibody and RNA was extracted in paralleled experiments followed by reverse transcription and real-time PCR using total ER β primers. Scramble siRNA and untransfected cells as the negative control. Inducible recombinant ERβ1 (full length) rather than endogenous ERβ signal could be blocked. ER β mRNA of both T5 and MCF7-β1 could be significantly down-regulated by transfecting ERβ siRNA. (The experiment had been repeated at lease 3 times)

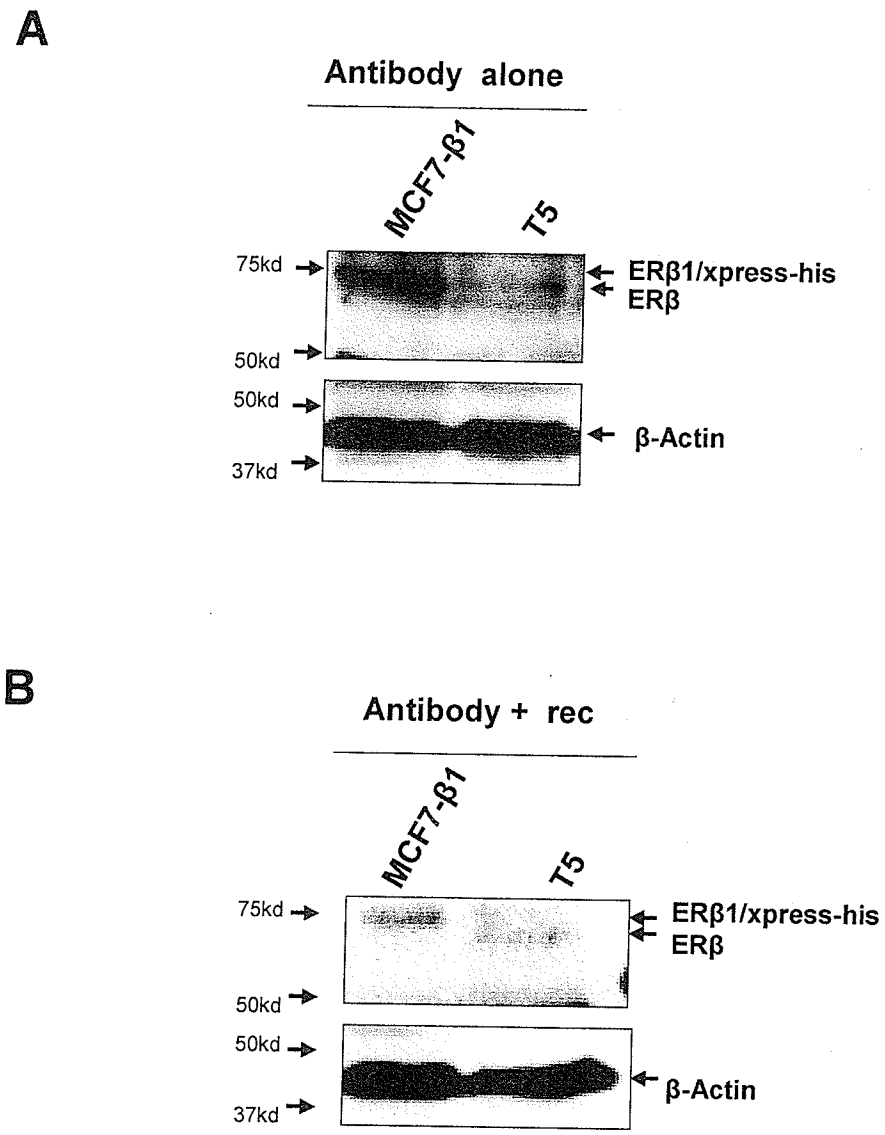


Figure 19: Block the ERβ-like protein signal by pre-incubating recombinant ERβ protein with antibody. Protein lysates of MCF7-β1 (control cell line) cells and T5 cells were analyzed by western blot using anti-ERβ chicken antibody with (**A**) or without (**B**) recombinant ERβ incubation. 2μg recombinant protein had been used for incubation with 2μg chicken antibody for 3 hour at 4 degree. Inducible recombinant ERβ1 (full length) signal could be blocked but not endogenous ERβ signal)

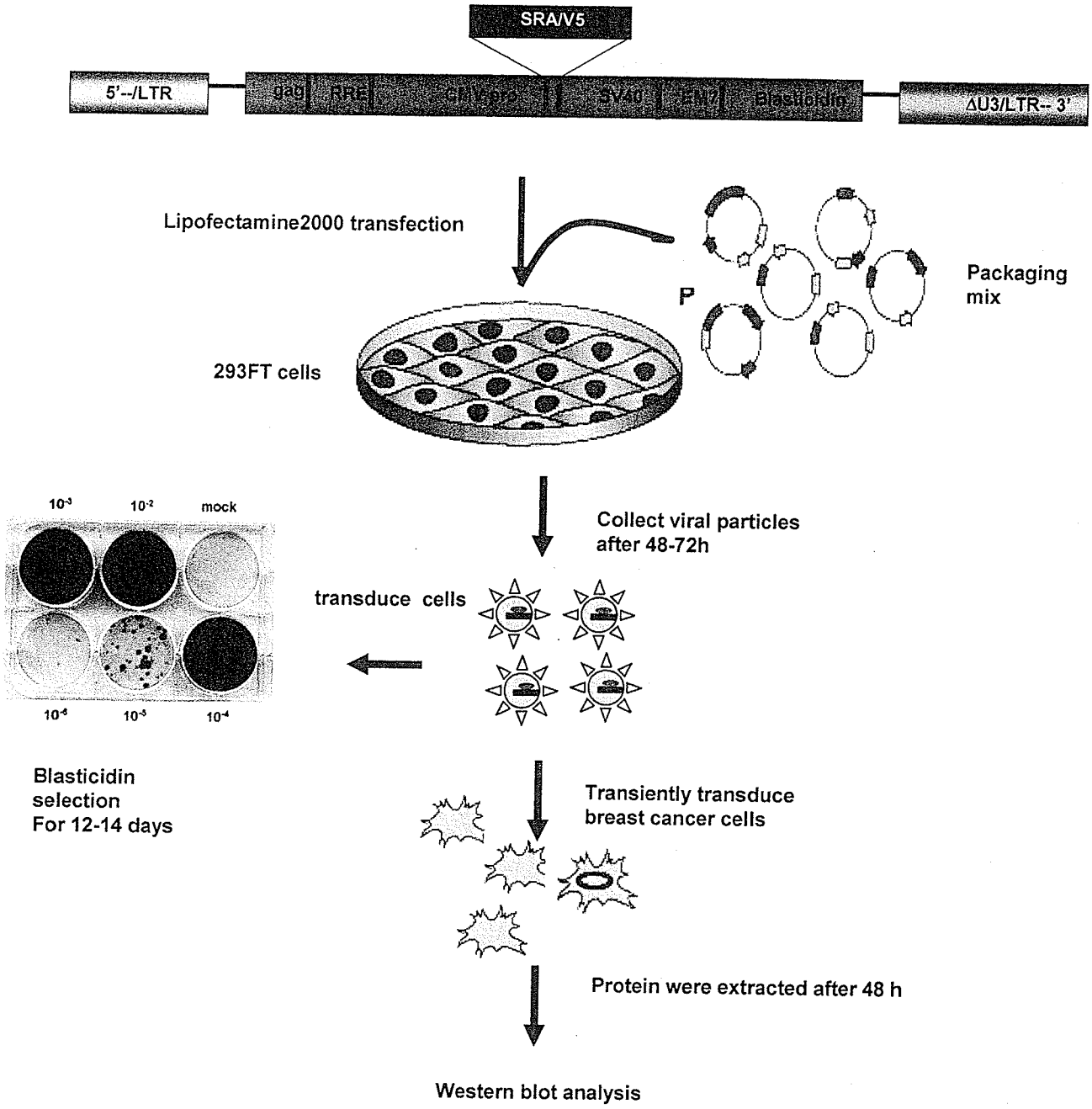


Figure 20: **Lentiviral Packaging System:** the expression plasmids were co-transfected along with packaging plasmid mixture which including three packaging vectors (pLP1, pLP2 and pLP/VSVG) in the 293FT cell line. After harvesting the virus-containing supernatant 48-72 hours post-transfection, the concentration of titers of lentiviral yields could be determined by staining and counting the number of Blasticidin-resistant colonies in proper dilution. Lentiviral suspensions containing Lenti-SRARNA were transiently transduced in T5 cells at different MOI (0, 0.5, 1, 2, 5 and 10). Protein samples were analyzed by western blot after 48h.

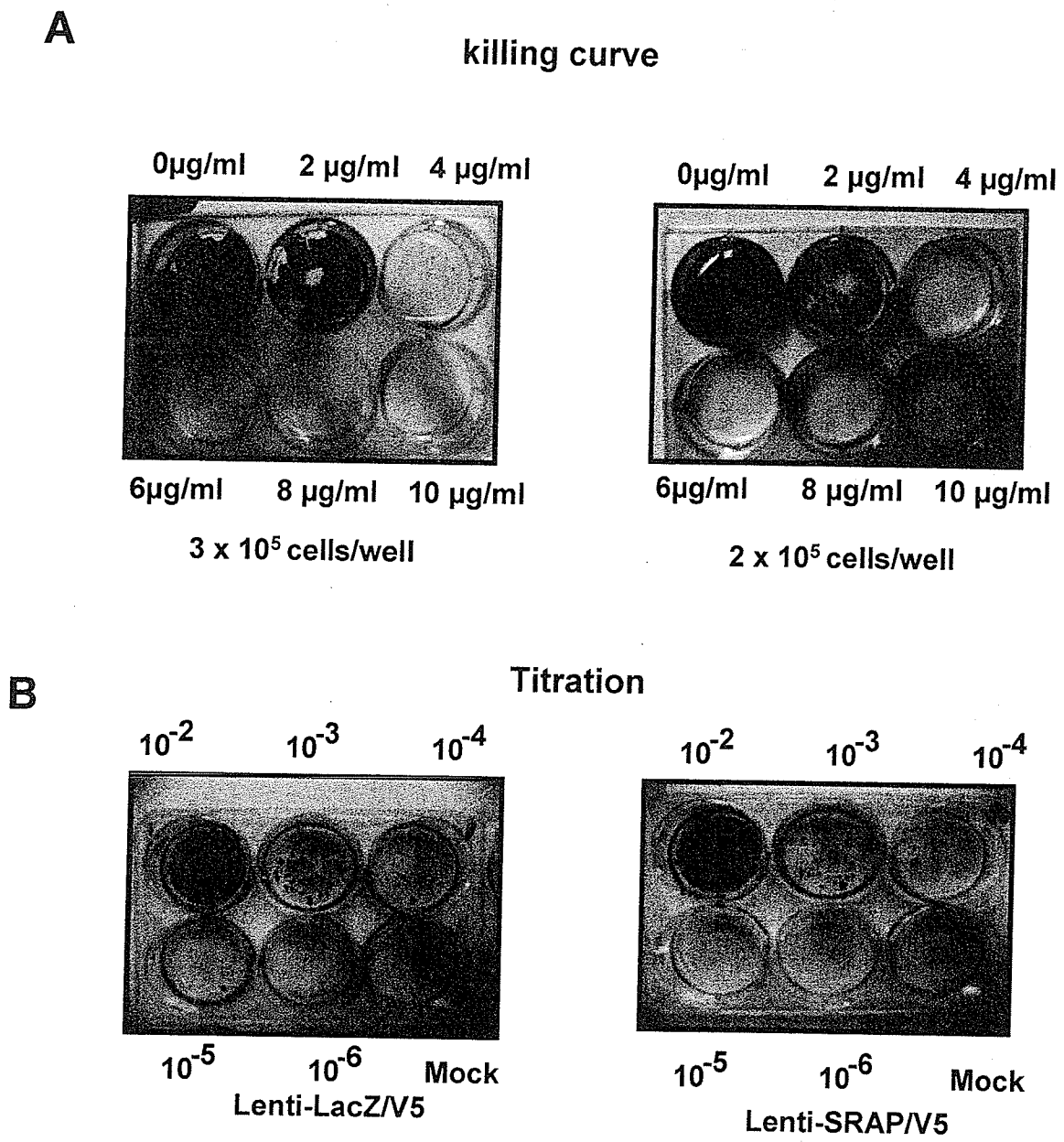


Figure 21: Optimization of Lentiviral transfection system:
A) Determining Blasticidin sensitivity: T5 cells in 6-well plates (200 or 300 x 103 cells/well, 25% or 40% confluency, respectively) were plated with complete medium followed by replacing with medium containing varying concentrations (0, 2, 4, 6, 8, 10 µg/ml) of Blasticidin. The concentrations of 6 µg/ml were sufficient to kill all the T5 cells within 14 days (cells had been stained with crystal violet). **B)** Titering the lentiviral stock: T5 cells in 6-well plate were incubated with serial dilutions (1/10-1/100000) of the viral stock in the presence of 6 µg/ml Polybrene. Infected cells were selected in medium containing 6 µg/ml blasticidin. The number of transducing units was determined by multiplying the estimated number of colonies by dilution factor. Our preparations of un-concentrated lentiviral stocks consistently yielded titers of 2–10 x 10^5 transducing units/ml.

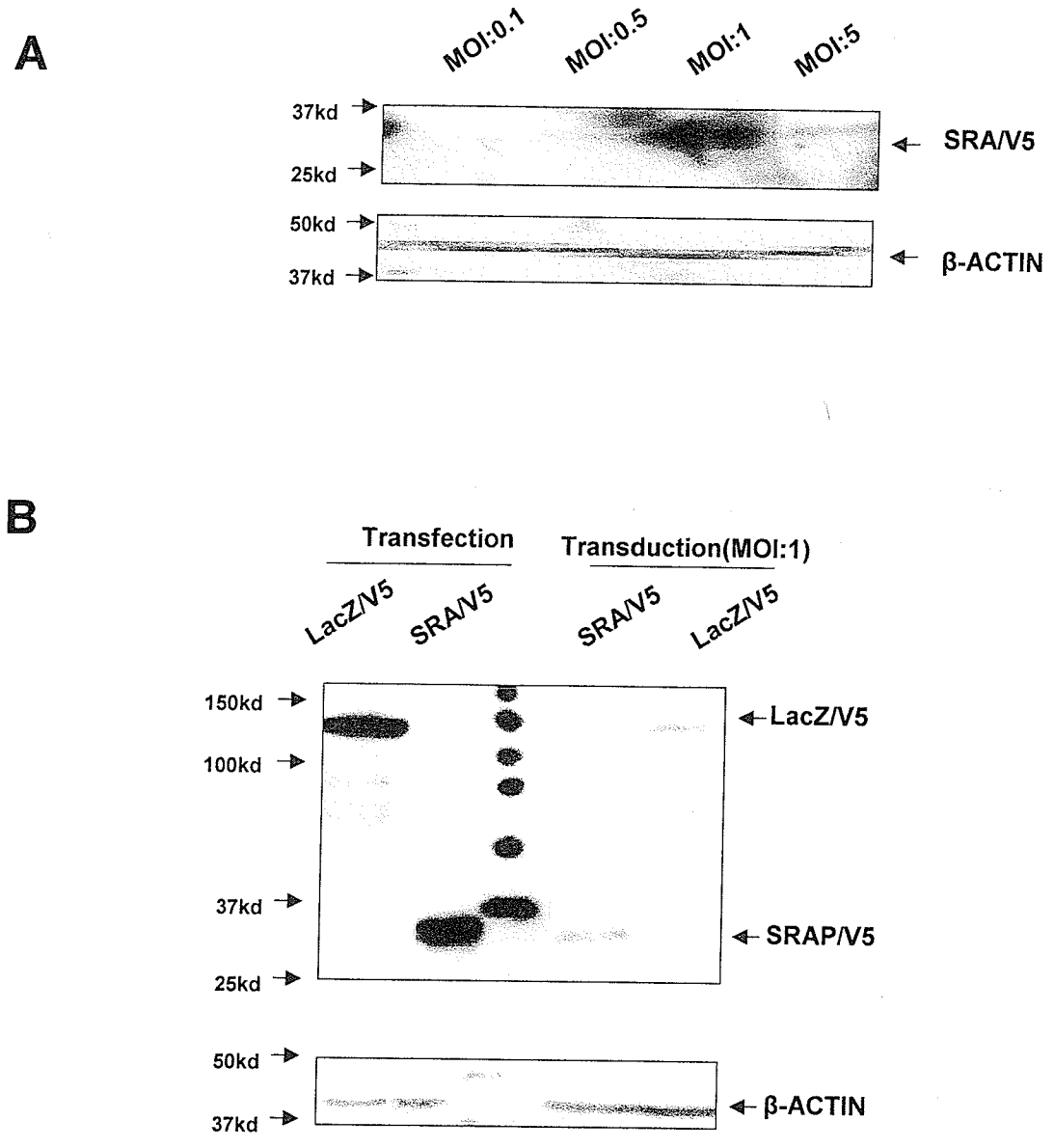


Figure 22: Optimization of Lentiviral transfection system:
A) Infect the T5 cells with different MOI: T5 cells were cultured in 12-well plates at 1.5 x 105 cells/well were infected by pLenti-SRAP at different MOI (Multiplicity of Infection) from 0.1, 0.5, 1, 5 in the presence of 10 μg/ml Polybrene. The complete media containing viral particle were replaced by the regular medium containing serum and antibiotics after 24h. Protein samples were be analyzed after 48-72h by western blot using anti-V5 antibody. **B)** transfection efficiency versus transduction efficiency in T5 cells: Plenti-laz/V5 AND Plenti-SRAP/V5 had been packaged into lenti-viral particles that were transiently transducted into T5 cells using MOI:1. Paralleled transfections by lipofectamine2000 using same plenti-constructs had been performed. 48h post transduction/transfection. Protein samples were extracted and analyzed by western blot using anti-V5 antibodies.

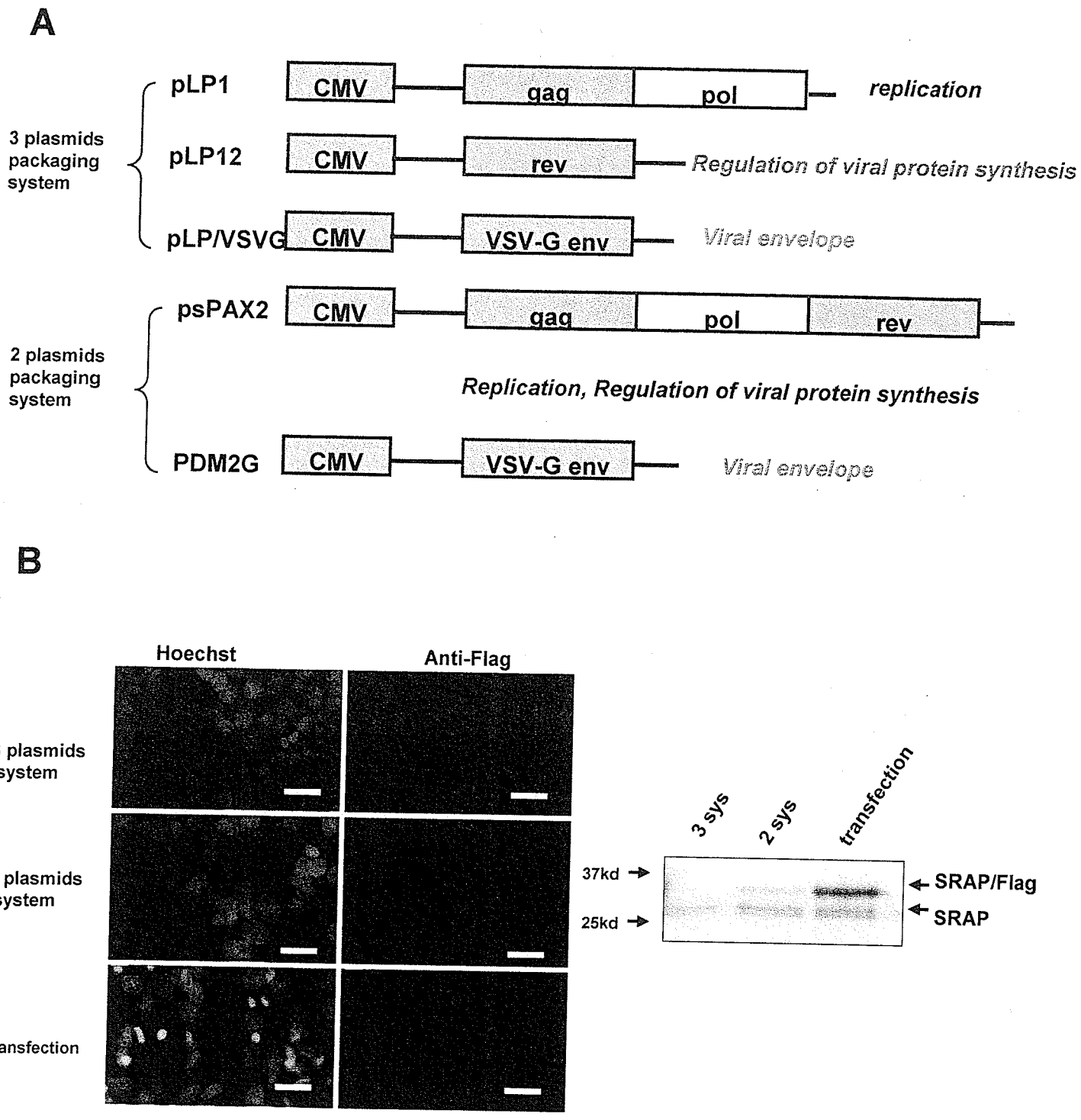


Figure 23: Optimization of Lentiviral transfection system: A) 3 plasmids packaging and 2 plasmid packaging systems are schematically shown. **B)** By using 2 helpers or 3 helpers system, new-SRAP/GFP have been packaged into lenti-viral particles that were transiently transducted into T5 cells (paralleled transfection as control) . 48h post transduction New-SRAP has been detected by anti-flag antibody. Paralleled protein sample were extracted after 48h transduction and analyzed by western blot using anti-SRAP antibodies. Scale bar=20μM.

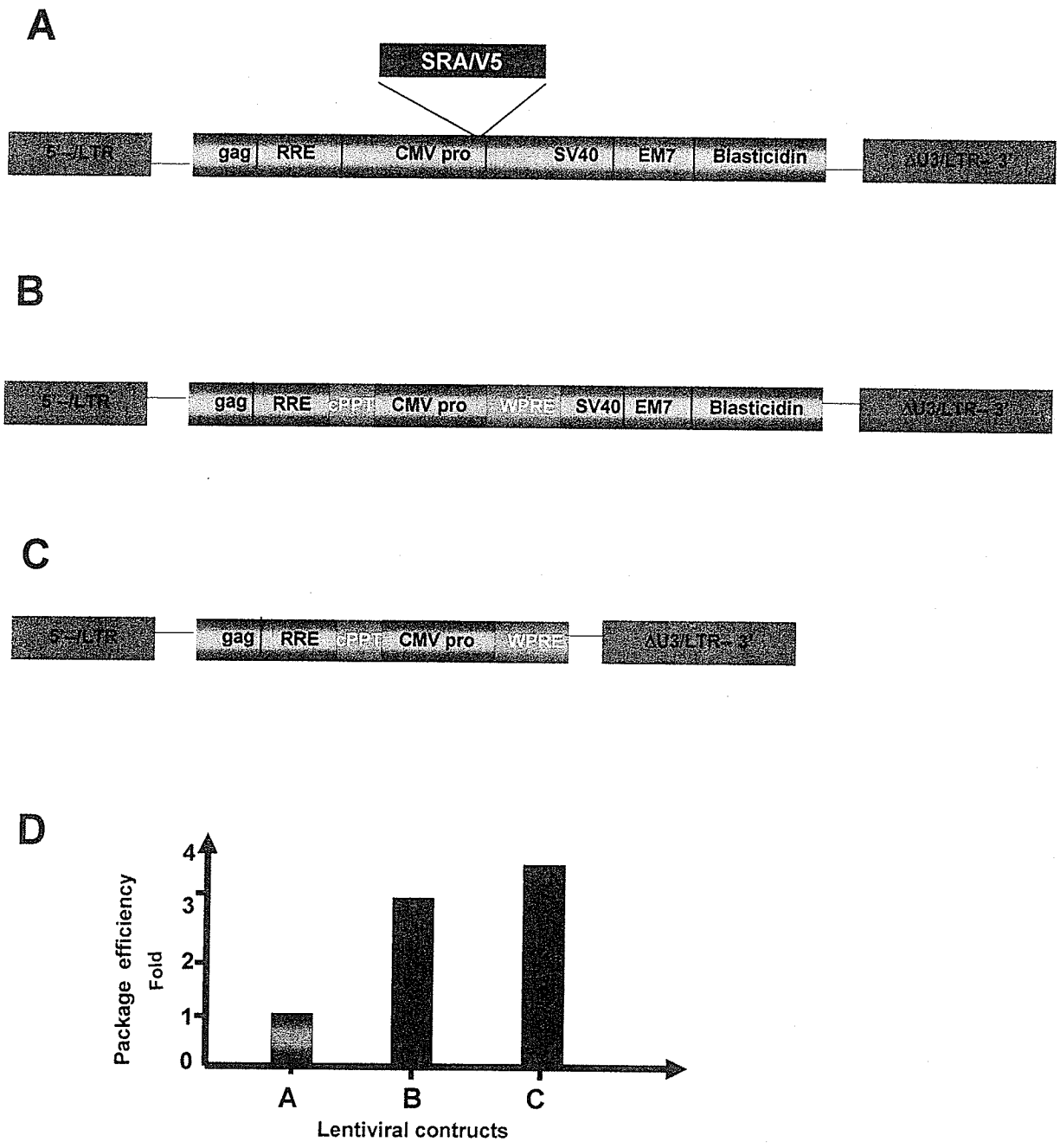


Figure 24: The potential ways to solve the problem of low transduction efficiency. A) The schematic structures of Plenti6.2/SRA/V5 from orginal virapower system (Invitrogen) have been showed. **B)** new version of their Virapower system called "HiPerform" which contains the cPPT and WPRE in the lentivector backbone. The WPRE (Woodchuck Posttranscriptional Regulatory Element) is placed directly downstream of the gene of interest, allowing for increased transgene expression, with more cells expressing your gene of interest. cPPT (Polypurine Tract) increases the copy number of lentivirus integrating into the host genome and allows for a two-fold increase in viral titer. Both WPRE and cPPT together, produce at least a four-fold increase in protein expression compared to other vectors that do not contain these elements. **C)** After removing downstream SV-40, EM7 and Blasticidin from the backbone would reduce the size of vector, therefore smaller vectors have much higher transduction efficiency. **D)** Estimated package efficiency expected for 3 lentiviral constructs showed in A, B, C.

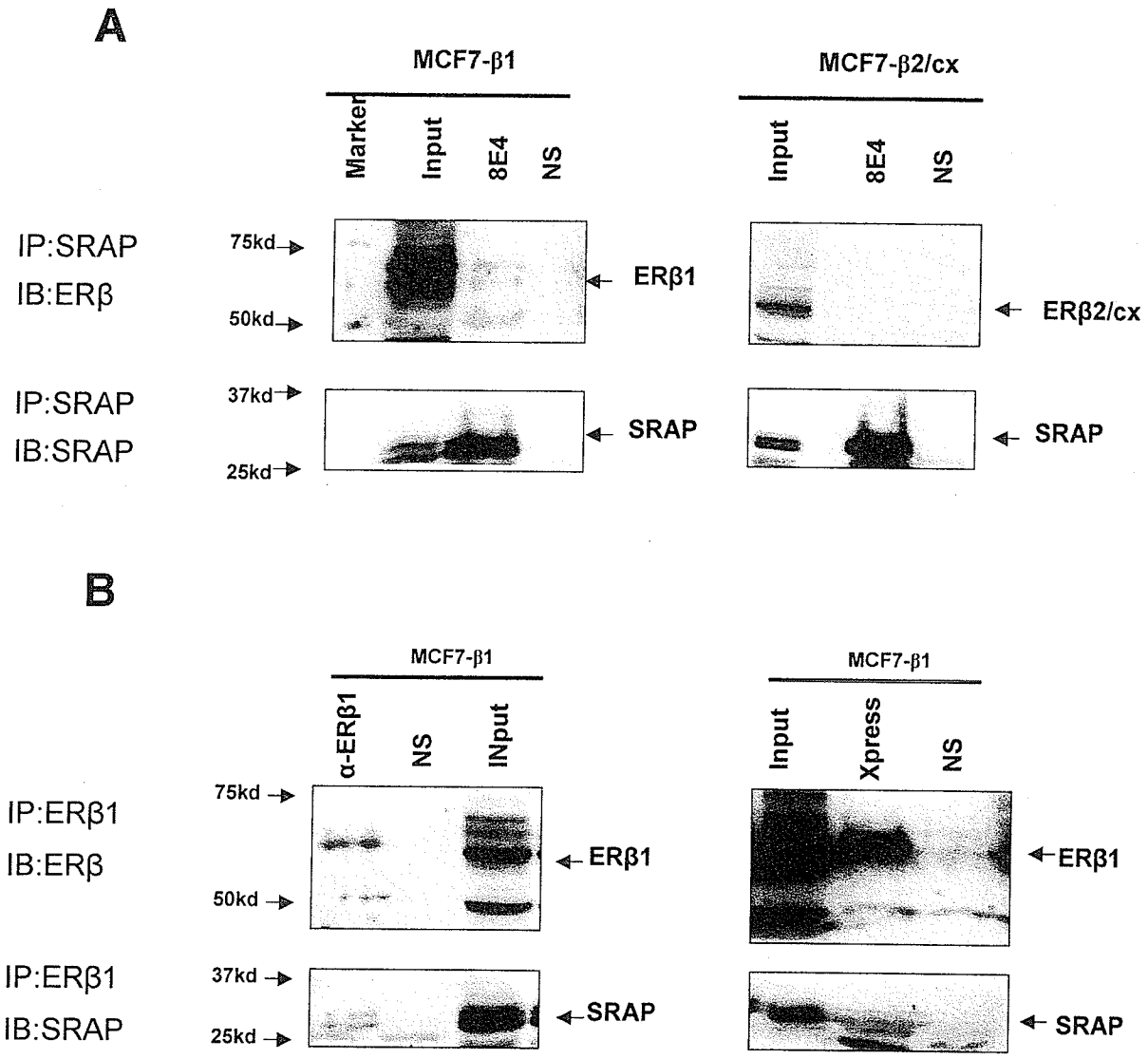


Figure 25: Interaction between SRAP and ERβ1 in inducible MCF7-ER β1 cells was observed but not consistent.

A: MCF7-ER β1 and MCF7-ER β2/cx cells were incubated with medium containing 1μg/ml doxycycline for 48h. The cells were processed for standard IP analysis as described in the Methods section using 8E4 (anti-SRAP mouse monoclonal antibody) as well the appropriate irrelevant isotype matched antibody as a non-specific control (NS). The IP protein=Beads. Inputs were whole cell lysate before IP. Anti-ER β chicken antibody was used for western blotting. **B:** MCF7-ER β1 and MCF7-ER β2/cx cells were incubated with medium containing 1μg/ml doxycycline for 48h. The cells were processed for standard IP analysis as described in the Methods section using AP1A (anti-ER β1 rabbit polyclonal antibody), anti-Xpress (mouse monoclonal antibody) as well the appropriate irrelevant isotype matched antibody (rabbit, mouse IgG) as a non-specific control (NS). The IP protein=Beads. Inputs were whole cell lysate before IP. Anti-ER β chicken antibody and Anti-SRAP was used for western blotting. All experiments have been repeated 3 times, the results are not consistent.

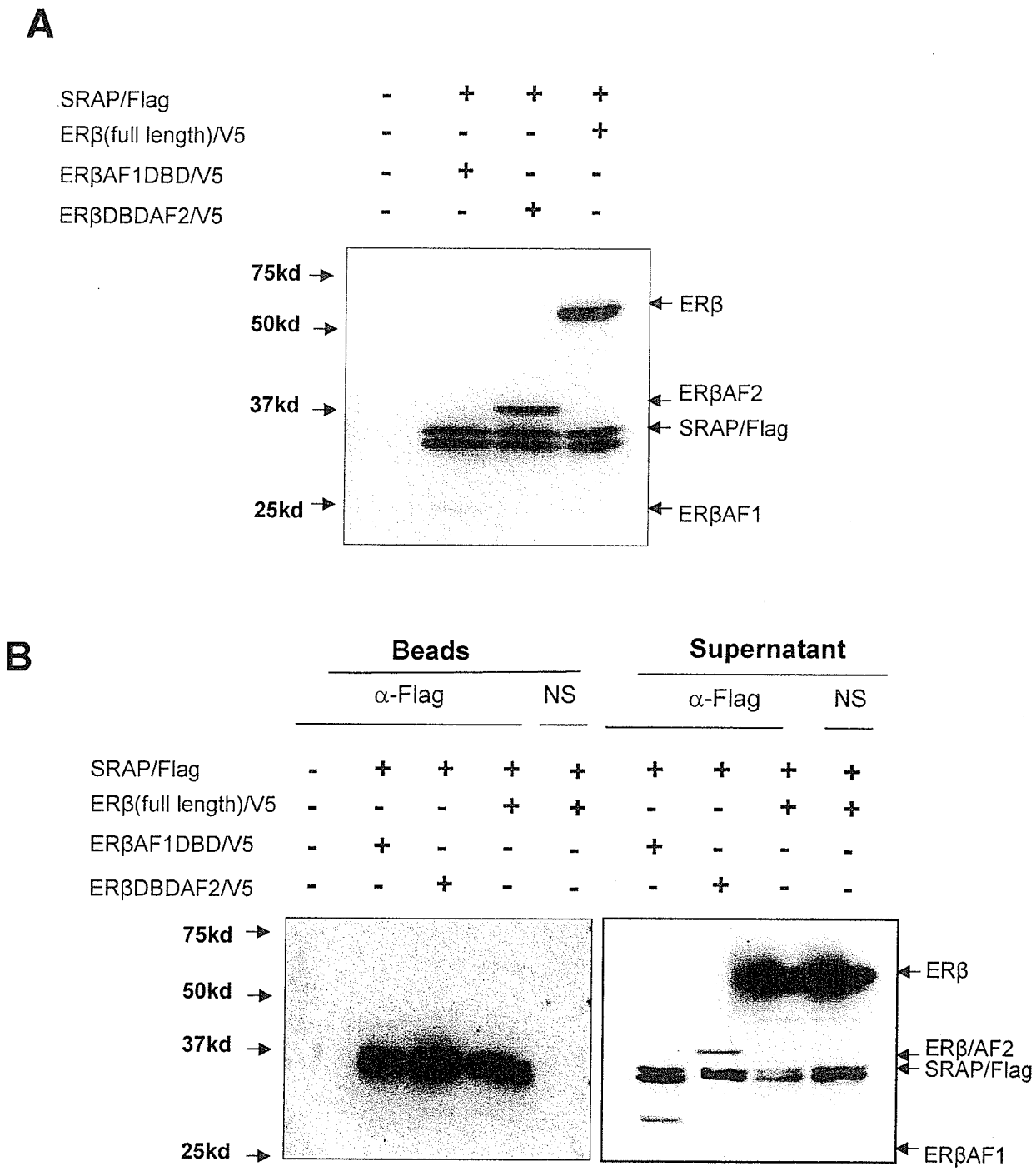


Figure 26: The interaction between Recombinant SRAP and ERβ in HEK 293 cell was observed. but not consistent.

HEK293 cells were seeded in 6-well dishes (2-10^5 cells/dish). 24h later co-transfection with SRAP/Flag and ERβ/V5 (full length), ERβAF1DBD/V5, ERβDBDAF2/V5, respectively by using lipofectamine 2000. After 24h transfection, the cells were processed for standard IP analysis as described in the Methods section using anti-Flag mouse monoclonal antibody as well the appropriate irrelevant isotype matched antibody (α-GST antibody) as a non-specific control (NS). **A)** 10% inputs of whole cell lysate before IP were loaded and detected by western blot using both anti-V5 and anti-Flag antibodies. The sizes of constructs are indicated. **B)** The IP protein=Beads. Aliquots of supernatant after IP were also western blotted. Anti-Flag and anti-V5 antibody were used for western blotting. The experiments have been repeated 3 times, the results are not consistent.

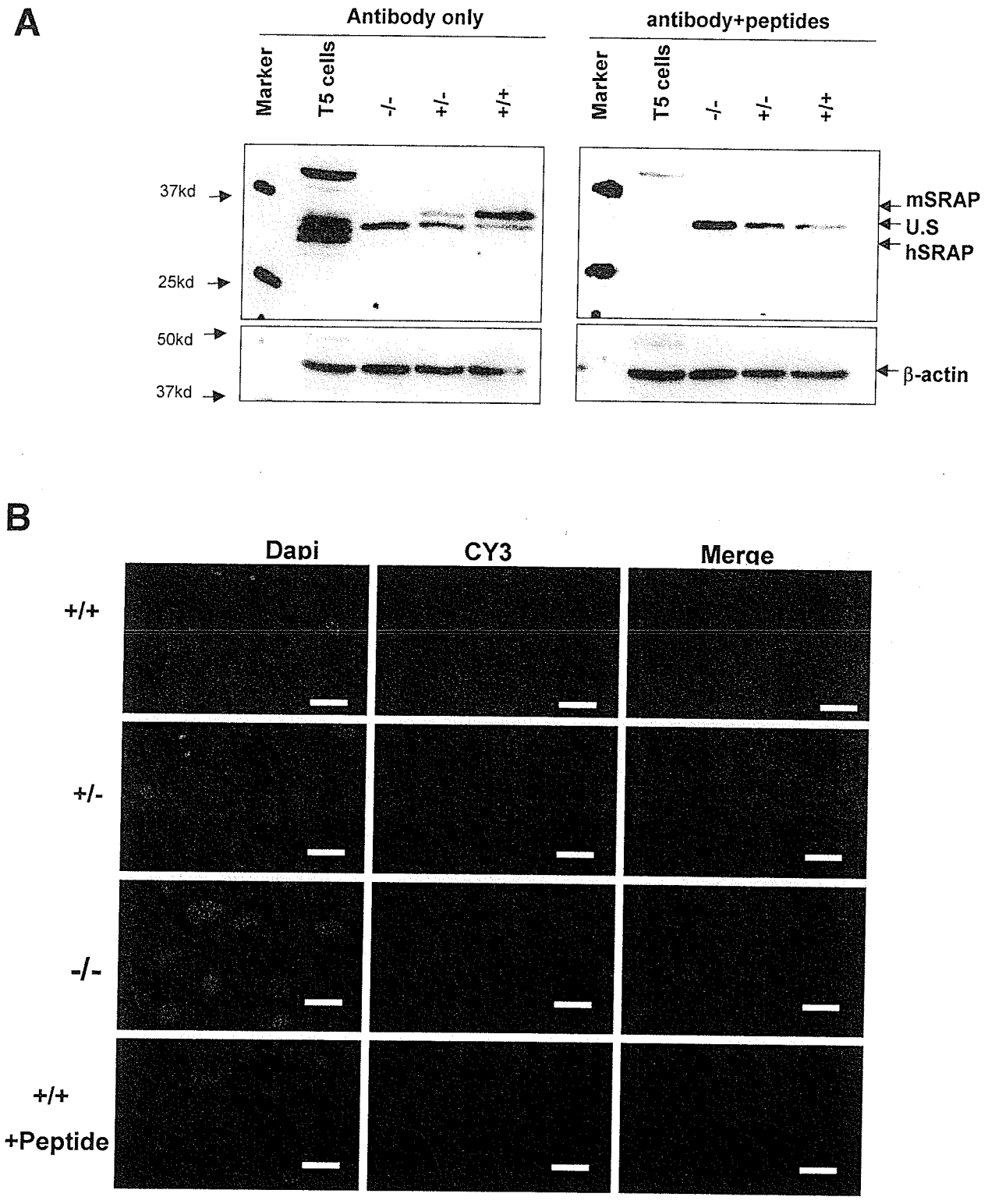


Figure 27: Validation of anti-SRAP rabbit antibodies for IHC.

A) Protein extracts of moue embryonic fibroblast (MEF'S) from +/+(WT), +/- and -/- (heterozygous and homozygous KO) have been analyzed by western blot using 743 anti-SRAP antibody.

B) MEF'S from WT, KO (heterozygous and homozygous) have been analyzed by Immuno-fluorescence using 743 anti-SRAP antibody with and without blocking peptides. Scale bar=20μM.

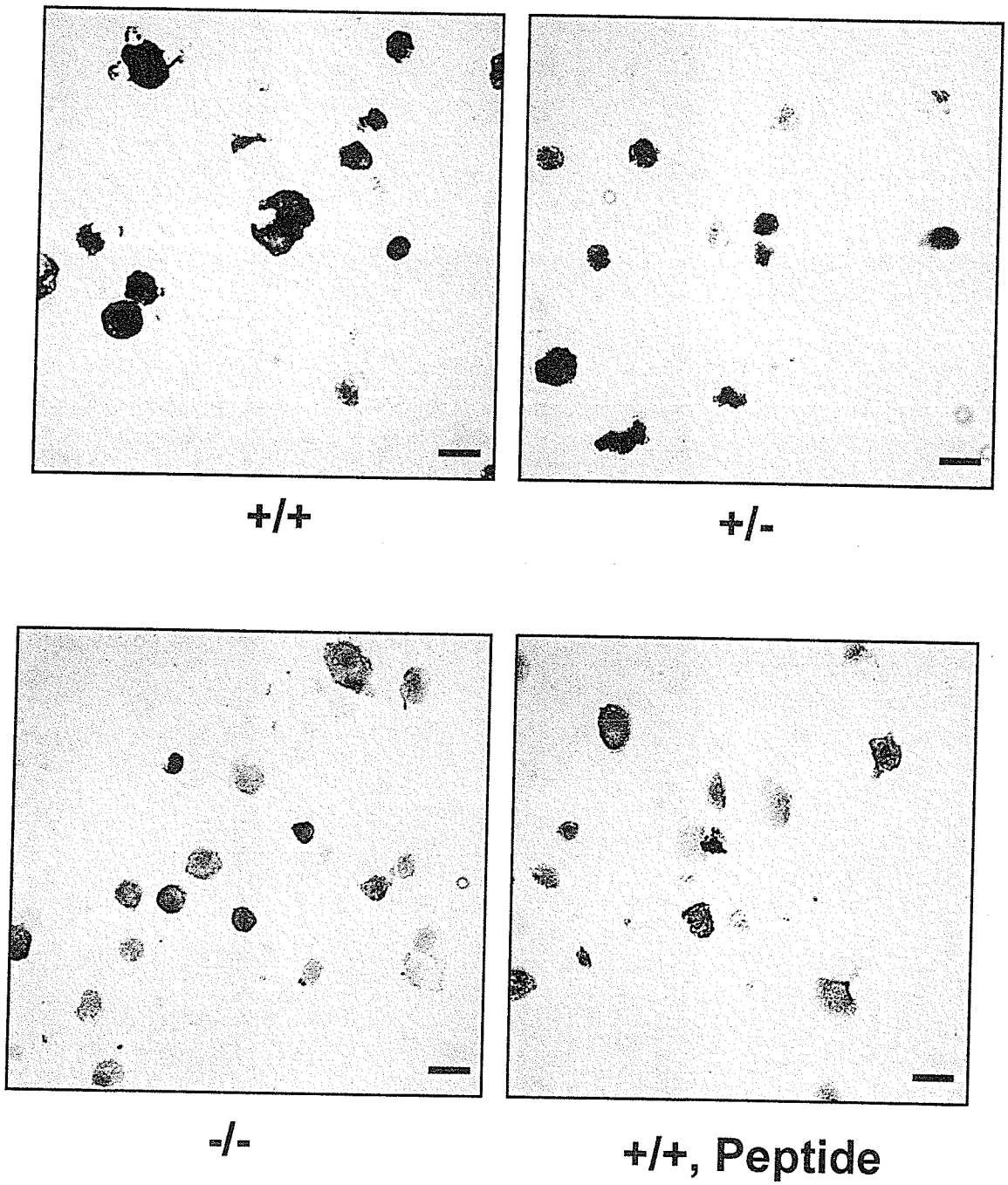


Figure 28: Validation of anti-SRAP rabbit antibodies for IHC. MEF'S from WT, KO (heterozygous and homozygous) have been embedded in paraffin and performed by IHC analysis using 743 anti-SRAP antibody with and without blocking peptides. Scale bar=20μM.

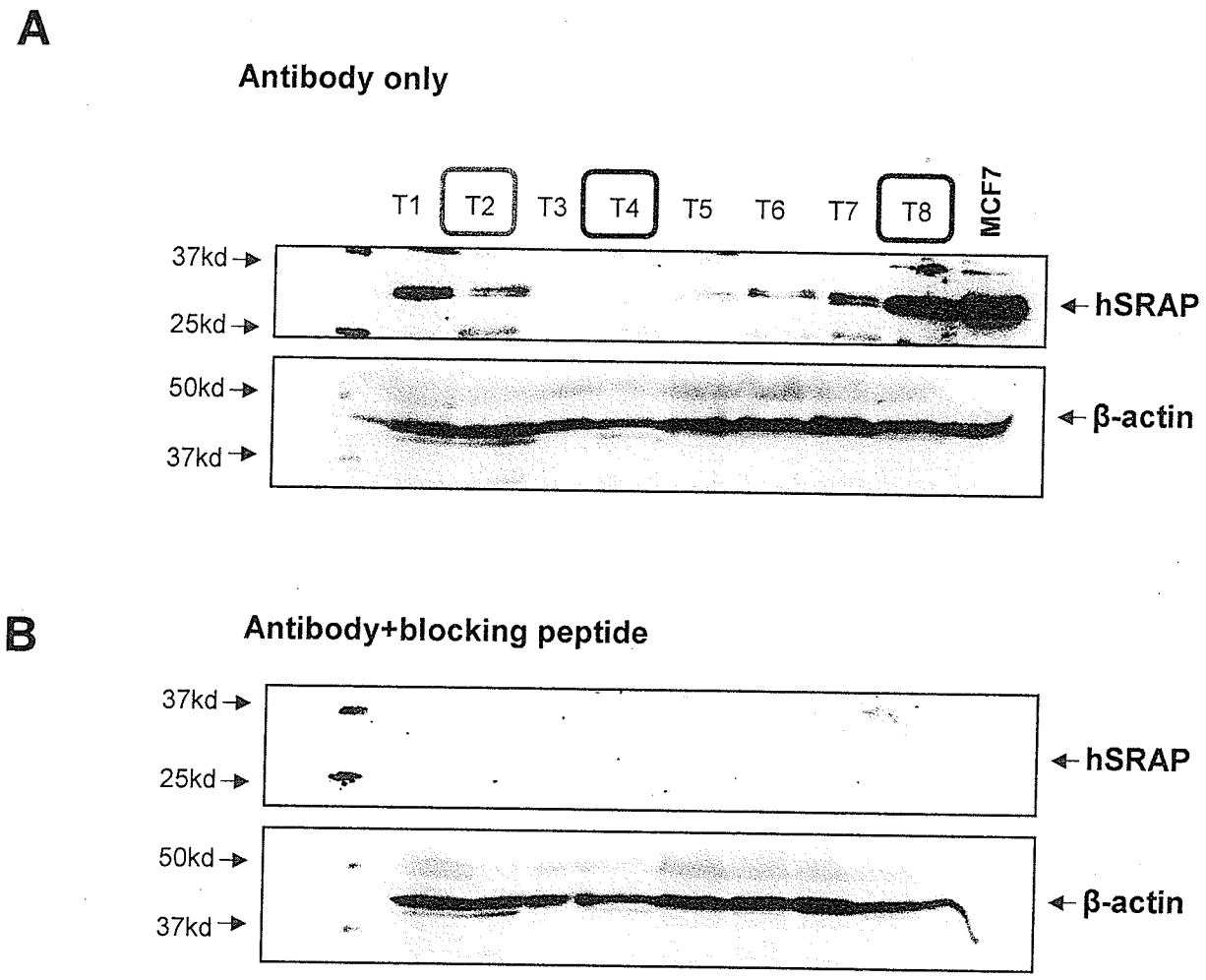


Figure 29: Validation of anti-SRAP antibodies for western blot on human tissue. A,B) WB 743 on human tumor: Protein extracts from Human tumor have been analyzed by western blot using 743 anti-SRAP antibody with and without blocking peptide.

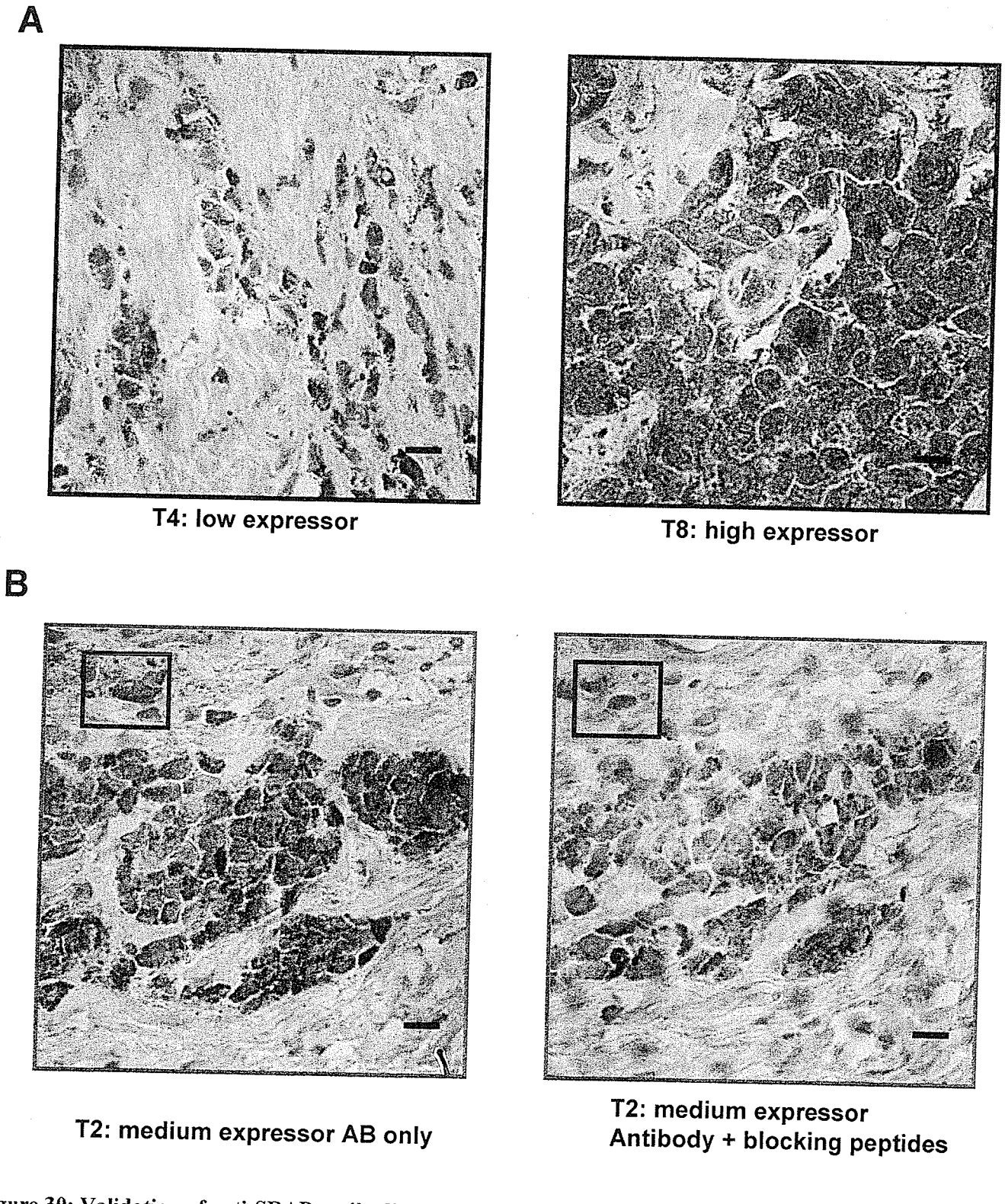


Figure 30: Validation of anti-SRAP antibodies for IHC on human tissue.
A) IHC analysis of Paraffin embedded tumors with high (T8) and low (T4) SRAP expressing, using 743 anti-SRAP antibody with the Ventana system as describe in Materials and methods.
B) IHC analysis of Paraffin embedded tumors (T2), using 743 anti-SRAP antibody in the presence of control Peptide (left) or SRAP specific peptide (right). Red box showed both nuclear and cytoplasm staining of SRAP. Scale bar=20μM.

Table1 Names, characteristics and sources of antibody

Name	Species	Epitope	Supplier
8E4	Mouse monoclonal (IgG)	Full length (SRAP)	In house
743	Rabbit polyclonal (IgG)	180-237 a.a (SRAP)	Bethyl
6F11	Mouse monoclonal (IgG1)	Full length (ERα)	Novocastra.
ab14021	Chicken polyclonal (IgY)	1-150a.a (ERβ)	Genway
ab8226	Mouse monoclonal(IgG1)	β-actin	Abcam Inc
Anti-V5	Mouse monoclonal (IgG2a)	N-gly-lys-pro-ile-pro-asn-pro-leu-leu-gly-leu-asp-ser-thr-C (V5)	invitrogen
F1804	Mouse monoclonal(IgG1)	N-asp-tyr-lys-asp-asp-asp-asp-lys-C (Flag)	Sigma
AP1A	Rabbit polyclonal	513-530aa (ER β1)	Dr.Murphy
Xpress	Mouse monoclonal(IgG1)	N-Asp- Leu-Tyr-Asp-Asp-Asp-Asp-Lys-C (Xpress)	Invitrogen

Table2 conditions for Western Blot and immunofluences

Name	Species	1°dilution	Incubation details	2° dilution
8E4(SRAP)	Mouse monoclonal (IgG)	1/100	4°C O/N or 1h RT	RaM1/3000
743(SRAP)	Rabbit polyclonal (IgG)	1/1000	4°C O/N or 1h RT	GaR1/3000
6F11 (ERα)	Mouse monoclonal (IgG1)	1/1000	1h RT	RaM1/3000
ab14021 (ERβ)	Chicken polyclonal (IgY)	1/2000	4°C O/N	RaC1/3000
ab8226 (β-actin)	Mouse monoclonal(IgG1)	1/10000	45min RT	RaM1/3000
Anti-V5	Mouse monoclonal (IgG2a)	1/5000	4°C O/N or 1h RT	RaM1/3000
F1804 (Flag)	Mouse monoclonal(IgG)	1/100	3h 37°C	GaM1/3000

Table3 conditions for Immunoprecipitation

Name	Species	Amount	Incubation details
8E4(SRAP)	Mouse monoclonal (IgG2a)	10ug	4°C O/N
743(SRAP)	Rabbit polyclonal (IgG)	7ug	4°C O/N
6F11 (ERα)	Mouse monoclonal (IgG1)	2ug	1h RT
AP1A(ERβ1)	Rabit polyclonal (IgY)	2ug	4°C O/N
Xpress	Mouse monoclonal(IgG1)	3ug	4°C O/N
Anti-V5	Mouse monoclonal (IgG2a)	3ug	4°C O/N or 1h RT
F1804 (Flag)	Mouse monoclonal(IgG)	2ug	4°C O/N

Table 4: pLenti-SRA Primers sequences

lentiSRAU : 5'-CACCATGACGCGCTGCCCCGCTG-3'
lentiSRAL: 5'-TGAAGCCTGCTGGAAGCCTGGT-3'
LENDSRAU: 5'-CACCTTGACGCTCTGCCCCGCTG-3'
Primer sequences using in the sequencing:
COREU:5'-AGGAACGCGGCTGAACGA-3'
LASL: 5'-GACGTCTTCCAATGCCTGTT-3'
Primer sequences for checking 4 SRA constructs by PCR:
Prot U: 5'-GCCAAGCGGAAGTGGAGAT-3'
LASL: 5'-GACGTCTTCCAATGCCTGTT-3'
SDML: 5'-GGAGCAGCAGTGCGAGGCGTCGGGA-3'

Table 5: Primer sequences for real-time PCR:

Primer names	sequence	size of fragment (bp)
ER β Total Upper:	5'-AGA GTC CCT GGT GTG AAG CAA G-3'	
ER β Total lower:	5'-GAC AGC GCA GAA GTG AGC ATC-3'	143
ER β 1 Upper:	5'-CAC GTC AGG CAT GCG AGT AAC A-3'	
ER β 1 Lower:	5'-TGG GAC CAC ATT TTT GCA CTT CA-3'	69
ER β 2 Upper:	5'-CAG GCA TGC GAG GGC AGA-3'	
ER β 2 Lower:	5'-CAT CGT TGC TTC AGG CAA AAG AGT-3'	85
ER β 5 Upper:	5'-GGT GAA GTG ATT TGG GAA AAG TGT C-3'	
ER β 5 Lower:	5'-CCA TCT TCA TTC CAA ATG AGG CA-3'	
Gapdh upper:	5'-ACCACAGTCCATGCCATCAC-3'	68
Gapdh lower:	5'-TCCACCACCCTGTTGCTGTA -3'	
V5 Lower:	5'-CGTAGAATCGAGACCGAGGAGAGGTTGAGGGATAGGCTTACC-3'	
SRA Upper:	5'-CCGCTCCCTCATGGTTGA-3'	176
		127

Table 6: pLenti-ERβ Primer for cloning:

Primer names	sequence	size of fragment (bp)
ERβAF1U	5'-CACCATGGATATAAAAAACTCACC-3'	
ERβAF1L	5'-CTTCACCATTCCCACTTCGTAAC-3'	
ERβAF2U	5'-CACCATGCGCTGTCTGCAGCGATTA-3'	648
ERβAF2L	5'-ACTCGCATGCCTGACGTGGGAC-3'	
ERβU	5'-CACCATGGATATAAAAAACTCACC-3'	963
ERβL	5'-CTGAGACTGTGGGTTCTGGGAG-3'	
		1590

Table 7:Triple-primer PCR for fragment analysis

Primer names	sequence	size of fragment (bp)
ERβ1 Upper primer Uβ	5'-TGA ACG CCG TGA CCG ATG CT-3'	
Lower primer Lβ1	5'-GCC CTC TTT GCT TTT ACT GTC-3'	281
ERβ2/cx Upper primer Uβ	5'-TGA ACG CCG TGA CCG ATG CT-3' 1	
Lower primer Lβ2/cx/5	5'-TCT CCA TCT TCA TTC CAA ATG AGG-3'	68
ERβ5 Upper primer Uβ	5'-TGA ACG CCG TGA CCG ATG CT-3'	
Lower primer Lβ2/cx/5	5'-TCT CCA TCT TCA TTC CAA ATG AGG-3'	259

Table 8:Multi-primer PCR for ERα and ER β ratio analysis

Primer names	sequence	size of fragment (bp)
ERβ-U	5'-GTCCATCGCCAGTTATCACATC-3'(130-151)	
ERβ-L	5'-GCCTTACATCCTTCACACGA-3' (371-352)	
ER-α-U	5'-TGTGCAATGACTATGCTTCA-3' (792-811)	233
ER-α-L	5'-GCTCTTCCTCCTGTTTTTA-3' (940-922)	
		149

Table 9: Target sequence of ER β siRNA (ON-TARGETplus SMARTpool L-003402-00-0005, Human ESR2, NM_001437)

AUGGAUAUAAAAAACUCACCAUCUAGCCUUAAUUCUCCUUCCUCCUACAACUGCAGUCAAU
CCAUCUUACCCCUGGAGCACGGCUCCAUAUACAUACCUUCCUCCUAUGUAGACAGCCACCA
UGAAUAUCCAGCCAUGACAUUCUAUAGCCCUGCUGUGAUGAAUUACAGCAUUCCCAGCAAU
GUCACUAACUUGGAAGGUGGGCCUGGUCGGCAGACCACAAGCCCAAAUGUGUUGUGGCCA
ACACCUGGGCACCUUUCUCCUUUAGUGGUCCAUCGCCAGUUAUCACAUCUGUAUGCGGAAC
CUCAAAAGAGUCCCUGGUGUGAAGCAAGAUCGCUAGAACACACCUUACCUGUAAACAGAG
AGACACUGAAAAGGAAGGUUAGUGGGAACCGUUGCGCCAGCCCUGUUACUGGUCCAGGUU
CAAAGAGGGAUGCUCACUUCUGCGCUGUCUGCAGCGAUUACGCAUCGGGAUAUCACUAUG
GAGUCUGGUCGUGUGAAGGAUGUAAGGCCUUUUUUUAAAAGAAGCAUUCAAGGACAUAAUG
AUUAUAUUUGUCCAGCUACAAAUCAGUGUACAAUCGAUAAAAACCGGCGCAAGAGCUGCC
AGGCCUGCCGACUUCGGAAGUGUUACGAAGUGGGAAUGGUGAAGUGUGGCUCCCGGAGAG
AGAGAUGUGGGUACCGCCUUGUGCGGAGACAGAGAAGUGCCGACGAGCAGCUGCACUGUG
CCGGCAAGGCCAAGAGAAGUGGCGGCCACGCGCCCCGAGUGCGGGAGCUGCUGCUGGACGC
CCUGAGCCCCGAGCAGCUAGUGCUCACCCUCCUGGAGGCUGAGCCGCCCCAUGUGCUGAUC
AGCCGCCCCAGUGCGCCCUUCACCGAGGCCUCCAUGAUGAUGUCCCUGACCAAGUUGGCCG
ACAAGGAGUUGGUACACAUGAUCAGCUGGGCCAAGAAGAUUCCCGGCUUUGUGGAGCUCA
GCCUGUUCGACCAAGUGCGGCUCUUGGAGAGCUGUUGGAUGGAGGUGUUAAUGAUGGGGC
UGAUGUGGCGCUCAAUUGACCACCCCGGCAAGCUCAUCUUUGCUCCAGAUCUUGUUCUGGA
CAGGGAUGAGGGGAAAUGCGUAGAAGGAAUUCUGGAAAUCUUUGACAUGCUCCUGGCAAC
UACUUCAAGGUUUCGAGAGUUAAAACUCCAACACAAAGAAUAUCUCUGUGUCAAGGCCAU
GAUCCUGCUCAAUUCCAGUAUGUACCCUCUGGUCACAGCGACCCAGGAUGCUGACAGCAGC
CGGAAGCUGGCUCACUUGCUGAACGCCGUGACCGAUGCUUUGGUUUGGGUGAUUGCCAAG
AGCGGCAUCUCCUCCCAGCAGCAAUCCAUGCGCCUGGCUAACCUCCUGAUGCUCCUGUCCC
ACGUCAGGCAUGCGAGAACAAGGGCAUGGAACAUCUGCUCAACAUGAAGUGCAAAAAUG
UGGUCCCAGUGUAUGACCUGCUGCUGGAGAUGCUGAAUGCCCACGUGCUUCGCGGGUGCAA
GUCCUCCAUCACGGGGUCCGAGUGCAGCCCGGCAGAGGACAGUAAAAGCAAAGAGGGCUCC
CAGAACCCACAGUCUCAGUGA

ON-TARGETplus SMARTpool siRNA J-003402-13, ESR2
GGAAAUGCGUAGAAGGAAU

ON-TARGETplus SMARTpool siRNA J-003402-14, ESR2
UUCAAGGUUUCGAGAGUUA

ON-TARGETplus SMARTpool siRNA J-003402-15, ESR2
GCACGGCUCCAUAUACAUA

ON-TARGETplus SMARTpool siRNA J-003402-16, ESR2
GAACCCACAGUCUCAGUGA

Table10 : Distribution of Low and High SRAP cases in ERβ sub-groups.
P-values correspond to Fisher's exact test

	SRAP high (%)	SRAP low (%)	P-value
ERβ1high	138 (87.9)	145 (77.9)	P=0.0219
ERβ1low	19 (12.1)	41 (22.1)	
ERβ2high	147 (93.6)	138 (74.2)	P<0.0001
ERβ2low	10 (7.4)	48 (25.8)	
Total	157	186	343