DDX17 (P72), a Sox2 binding partner, regulates Sox2 to sustain tumorigenic and stem-like properties in a phenotypically distinct subset of estrogen positive breast cancer cells

By

Hind Mohammed Alqahtani

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Medical Sciences - Laboratory Medicine and Pathology

University of Alberta

Abstract:

Sox2, an embryonic stem cell marker, is involved in the pathogenesis of breast cancer (BC). Sox2 expression is associated with a poor clinical outcome in BC patients. Based on the differential Sox2 transcriptional activity, we have identified the two phenotypically distinct cell subsets, namely reporter responsive (RR) and reporter unresponsive (RU) cells. RR cells are more tumorigenic and stem-like than RU cells. The goal of this study is to understand the mechanisms of regulating Sox2 transcriptional activity. By using liquid chromatography–mass spectrometry and co-immunoprecipitation, we found that DDX17 is a Sox2 binding partner in ER^+ BC cell lines. The interaction between DDX17 and Sox2 was found to be significantly higher in the RR cell subset than in the RU subset. DDX17 was found to bind to the *Sox2* promoter and regulate its expression in RR cells derived from the MCF7 cell line. Although, the protein level of Sox2 was unaffected in RU and RR cell subsets. Upon siRNA knockdown of DDX17, the transcriptional activity of Sox2 was significantly decreased in RR cells but not in RU cells. Correlating with these findings, siRNA knockdown of DDX17 drastically reduced the tumorigenic and stem-like properties in RR cells, as observed by decreased in colony formation and mammosphere formation efficiency. In conclusion, DDX17 regulates Sox2 to maintain tumorigenic and stem-like properties. The interaction between Sox2 and

DDX17 provides a novel mechanism underlying the functional dichotomy of BC cells, which carries potential therapeutic implications.

Preface:

This thesis is an original work by Hind Alqahtani. All parts of this thesis has been published as:

H. Alqahtani, K. Gopal, N. Gupta, K. Jung, A. Alshareef, X. Ye, F. Wu, L. Li, R. Lai. DDX17 (P72), a Sox2 binding partner, promotes stem-like features conferred by Sox2 in a small cell population in estrogen receptor-positive breast cancer. Cellular Signalling Journal. 2015. Nov 10; 28 (2): 42-50. I was first author of this work.

ACKNOWLEDGEMENTS:

First of all, I am very thankful to my Saudi Arabia government that they gave me the opportunity to earn my master degree and provide funding for my entire program. I also thankful to the Saudi Bureau for their encourage me to do my best during my studies and facilitate obstacles that I faced while I'm living in Canada.

I would like to appreciate my supervisor Dr. Raymond Lai who guided and supported me during my training. Iam also grateful to my committee members Dr. Gilbert Bigras and Dr. Yangxin Fu for their help and suggestions.

I would like to express my gratitude and appreciation to my colleagues for their cooperation.

Dedication to my lovely parents who have been my source of inspiration and for my siblings specially Abdulmohsen for his patience and support during my masters program.

List of Abbreviations

BCA- bicinchoninic acid

BC- breast cancer

ChIP- chromatin immunoprecipitation

Co-IP- co-immunoprecipitation

CSCs- cancer stem cells

DMEM- Dulbecco's Modified Eagle Medium

ECL- chemiluminescent

ER^+ - estrogen receptor positive

ER - estrogen receptor positive

ESCs- embryonic stem cells

ETn- early mouse transponson

FBS-fetal bovine serum

GFP- green fluorescence protein

FGF-4- fibroblast growth factor-4

HER2- Human epidermal growth factor receptor 2

HMG-high mobility group

IHC- immunohistochemical

LC-MS- liquid chromatography–mass spectrometry

NES- nuclear export signals

NIS- nuclear import signals

PR- progesterone receptor

pSp-T^{+}-positive Sox2 promoter activity

pSp-$T^{low/-}$- low Sox2 promoter activity

q-RT-PCR- quantitative revers transcriptase PCR

RR- reporter-responsive

RU- reporter-unresponsive

RIPA- radioimmunoprecipitation assay

SDS-PAGE- sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sox- sex-determining region y-box

Sox2-Sex-determining Region Y-box Protein 2

SRA- steroid receptor RNA activator

SRC-1- steroid receptor co-activator

SRR2- Sox2 regulatory region 2

TBST-Tris-Buffered Saline and Tween 20

TN-Triple negative

1 Table of Contents

List of Tables:

List of Figures:

CHAPTER1: Introduction

Overview:

Breast cancer (BC) constitutes one of the most common malignancies in women worldwide and possesses the second highest mortality rate amongst all cancers (1)**.** BC occurs predominantly in women and rarely in men (2), as only 0.7% of males have been diagnosed with BC (3). In 2015, BC represented one of the highest causes of death in Canadian women, accounting for 13.6% of all cancer deaths (4). One in nine women is expected to develop BC through their lifetime. The Canadian Cancer Society estimates 25,000 new BC cases, which comprises 26% of all cancer cases, in Canadian women in 2015. The incidence rates of BC will increase from 20,110 to 31,255 cases between 2015 and 2030 (4). Compared with other diseases, BC has been intensively studied. The improved understanding of BC pathophysiology has resulted in great improvement in its diagnosis, treatment, and prevention. One of the most commonly studied genes, Sox2, comprises a transcription factor that is expressed in embryonic stem cells. Among other solid tumours, Sox2 is highly expressed in the early stages of BC (5). This work focuses the mechanisms of Sox2 activation and the way in which it contributes to the characteristic phenotypes of BC.

1.1 Molecular subtypes of BC:

BC is a heterogeneous disease in its view of their differential morphological features, clinical outcomes, and responses to treatments (6, 7). Based on its gene expression, BC is classified into the molecular subtypes: estrogen receptor positive (ER^+, including luminal A and luminal B), triple negative (TN, including ER^-, PR^- and $HER2/neu^-$) and human epidermal growth factor receptor 2 ($HER2/neu^+$) (8, 9). All of these molecular subtypes correlate with biological features and clinical outcomes (10).

1.1.1 Estrogen receptor positive (ER^+):

Estrogen receptor positive contains two subtypes: luminal A and luminal B. Researchers distinguish between these subtypes on the basis of Ki67 expression, with luminal A having low Ki67 expression, and luminal B having high Ki67 expression (11).

1.1.1.1 Luminal A:

Luminal A is the most common BC subtype. Immunohistochemical studies have shown that 50–60% of BC are of Luminal A (7, 12). Specifically, the luminal A phenotype is characterized by the expression of ER, a lack of HER2 expression, and a low expression of genes associated with cell proliferation, such as the Ki67 nuclear protein (13). GATA-3 is another marker involved in breast luminal cell differentiation and is a marker of luminal A (14). Patients with luminal A BC have

a fairly high survival rate and significantly lower relapse rate as compared to patients with other BC subtypes (15). The treatment for patients with the luminal A subgroup includes surgery and hormonal therapy, such as tamoxifen (16), and usually does not require chemotherapy.

1.1.1.2 Luminal B:

Luminal B tumors represent 15%-20% of all BC. In addition, these tumors contain a more aggressive phenotype, a higher histological grade, a greater proliferative index (reflected by a higher Ki67 index), and a worse prognosis as compared to the luminal A tumors (17). One of the most relevant features of this tumor type entails the expressions of ER, a lack of HER2 expression and a high expression of Ki67. Moreover, a microarray study has examined 357 BC subtypes and utilized the proliferative index using the Ki67 threshold (14%), which differentiates luminal A (<14%) from luminal B tumors (>14%) (18). In clinical practice, Ki67 is a proliferation marker that is used to differentiate between both luminal subtypes (12). Although patients with luminal B have a worse prognosis than patients with luminal A (19), patients with luminal B tumors respond to neoadjuvant chemotherapy more successfully than those with luminal A BC (20). Moreover, the relapse rate is limited to the first 5 years of follow-up (21).

1.1.1.3 Triple negative BC:

The triple-negative (TN) in subtype represents approximately 15% of all BC. This subtype is characterized by the absence of ER and progesterone receptor (PR) expression as well as the overexpression of HER2; accordingly, there is a lack of targeted therapy currently for this cancer subtype (22). Moreover, TNBC involves the worse prognosis of all the subtypes as well as high rates of relapse and death within the first 3–5 years after diagnosis (23, 24).

1.1.1.4 Human epidermal growth factor receptor2 (HER2)-positive:

Tumors with the HER2 molecular profile constitute between 15% and 20% of all BC subtypes. HER2 is a tyrosine kinase, and it is located in chromosome 17q21 (12). These tumors contain a high expression of HER2 membrane protein, usually secondary to gene amplification, and a low expression of ER and PR genes (25). Moreover, this subtype exhibits an overexpression of genes related to cellular proliferation (7). Accordingly, these tumors show a high degree of proliferation, and 75% of them have a high histological grade (26). Although HER2 tumors have a poor prognosis, two forms of treatment for this subtype currently exist. Specifically, patients with HER2 positive and hormone receptor-positive BC receive anti-HER2 therapy (which is antibody that can bind the HER2 receptor and block its activity) combined with chemotherapy and endocrine therapy while

patients with only HER2 positive receive chemotherapy and anti-HER2 therapy (27).

1.2 Sox2 in Embryonic Stem Cells (ESCs):

Sex-determining Region Y-box (*SOX*) genes encode a family of highly conserved DNA-binding domains known as high mobility group (HMG) transcription factors, which fulfill critical roles in embryonic development. For example, Sox2 is initially expressed in the inner cell mass (ICM); thus, zygotic Sox2 deletion causes early embryonic death as a result of the failure to produce pluripotent epiblasts (28). SOX genes have been investigated in humans and mice through a determination of their DNA-binding domain, which is highly homologous to the SRY box, a sex determining factor located on the Y-chromosome (29, 30). The Sox family contains 20 members divided into 8 groups, ranging from A to H. These subtypes are categorized as the shared HMG box DNA-binding domain (31). Moreover, members within a group usually share more than 80% of the amino acid identities in their HMG-domain, while members of different groups share fewer than 80% of these identities (32). However, all members of the Sox family are involved in different types of cancer (33).

The Sex-determining Region Y-box Protein 2 (Sox2) is a key member of the *Sox* genes, which are highly expressed in ESCs (34). Sox2 binds to target DNA in

specific sequences: C (T/A) TTG (T/A)(T/A)). These sequences identify the applicable HMG domain and regulate the expression of target genes. Sox2 fulfills the necessary function of self-renewal and contains pluripotent ESCs (35, 36). Moreover, Sox2 represents one of the key factors that assist in the generation of induced pluripotent stem (iPS) cells from mouse embryos or adult humans fibroblasts (37). Chromatin immunoprecipitation (ChIP) studies have found that Sox2 binds to the promoters of hundreds of genes in ESCs. Many of these genes contribute to the regulation of cell development and differentiation. However, the mechanisms regulating Sox2 transcription activity remain only partially understood. A recent study has demonstrated that the phosphorylation of Sox2 at threonine 118 (T118) can regulate Sox2 activity in mouse ESCs (38). Moreover, PARP1 has been shown to regulate Sox2 activity in mouse ESCs (39).

In normal adult tissues, Sox2 expression is exclusively restricted to somatic stem cells and arises from fetal $Sox2^+$ progenitors (40). Arnold *et al.* have reported the expression of $Sox2^+$ in many adult epithelial tissues, such as colon, stomach, and cervix, which fulfill a key role in the regeneration and survival of normal tissue (41). In this study, Arnold *et al.* used animal models and knock-in mice with Sox2-GFP reporters to identify the expression of Sox2. In normal human mammary glands, Sox2 mRNA expression occurs exclusively in stem cell populations (42).

1.2.1 Sox2 biological structure:

In 1994, researchers discovered Sox2 in humans by determining its location on chromosome 3q26.3–q27 (43). Sox2 consists of 317 amino acids (43) and contains three primary domains: HMG, the N-terminal, and the transactivation domain, or C-terminal (Figure 1.1) (44).

The HMG domain can regulate Sox2 by mediating nuclear translocation, which involves binding with nuclear import signals (NIS) and nuclear export signals (NES). In addition, this domain can provide potential binding sites for protein partners (45). The C-terminal can stimulate or repress target genes by responding to the promoter binding region (44). The function of the third domain, the N-terminal, involves the activation of transcription (46).

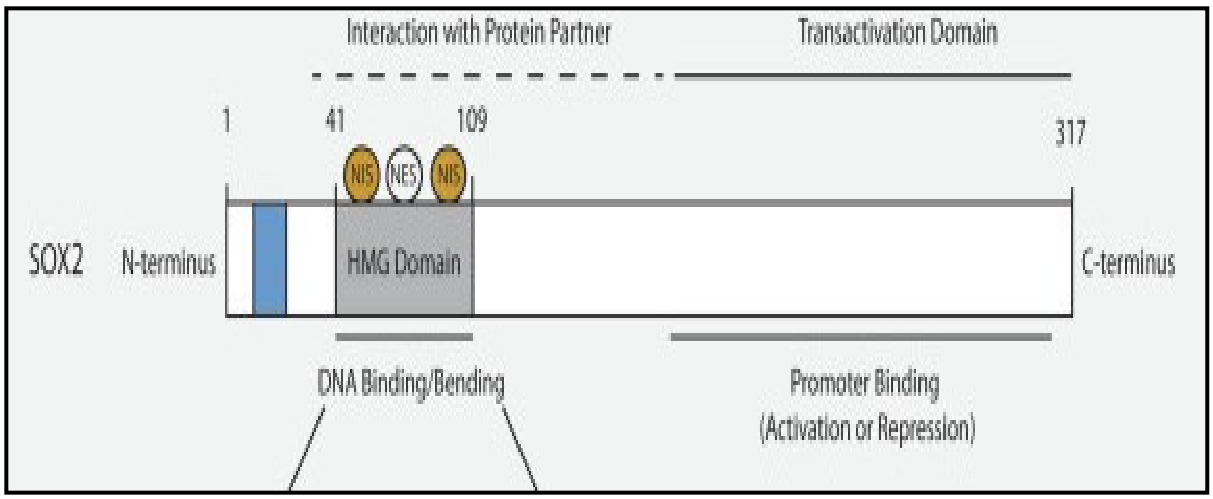

Figure 1.1: Structure of Sox2 protein and its domains. Sox2 has three domains including the HMG domain, the N-terminal and C-terminal domains. The HMG domain is a potential binding region for protein-protein interaction. The N-terminal functions to activate transcriptional activity of Sox2 and the C-terminal functions to activate or repress target genes.

1.2.2 Sox2 in cancer biology:

Sox2 is normally expressed in ESCs and somatic stem cells (28, 40). However, aberrant expression of Sox2 has been involved in various cancer types, including lung (47), gastric (48), pancreatic (49), prostate (50), and BC (33, 51, 52). Many studies have reported the importance of Sox2 in cancer biology and development, including cellular proliferation (50, 53) as well as the promotion of invasion and metastasis (54, 55). For example, in pancreatic cancer, Sox2 regulates cellular proliferation. The siRNA knockdown of Sox2 in pancreatic cancer cells resulted in the inhibition of cell growth, (49), thus showing the role of Sox2 in pancreatic cancer. Moreover, Sox2 knockdown led to significant cell death in lung cancer cells and reduced tumor growth in mice models, indicating that Sox2 expression promotes cell proliferation and survival (56). Likewise, in glioma cells, the overexpression of Sox2 induced cancer invasion and migration, while the siRNA knockdown of Sox2 significantly reduced its invasion and migration properties (57). Clinically, several immunohistochemical (IHC) studies have demonstrated that the expression of the Sox2 protein correlated with different clinicopathological parameters, such as the clinical stage and overall outcome of the disease (58, 59).

1.2.3 Sox2 in BC:

Three different IHC studies have detected the aberrant expression of Sox2 in BC. Specifically, these investigations demonstrated that 16-30% of the breast tumors

expressed Sox2 (33, 52, 60), while adult benign breast tissue revealed an absence of Sox2 expression (33). Additionally, research has determined that Sox2 is expressed in all 4 major BC molecular subtypes: luminal A, luminal B, HER2-positive, and triple negative (51).

Our laboratory has previously reported that the aberrant expression of Sox2 occurred in 6 out of 10 BC cell lines, including MCF7, ZR751, T47D (ER^+); BT474 (HER2-positive); MB468 and MB435S (triple-negative). Using western blots, this study found the highest expression of Sox2 in ER^+ MCF7 and ZR751 cell lines (Figure 1.2A). Specifically, Sox2 was localized in both the cytoplasm and nucleus (Figure 1.2B) (61).

Recent studies have reported the biological importance of Sox2 in BC, including its roles in cell proliferation, mammosphere formation, colony formation, and metastasis *in vitro* (33, 51, 61). For instance, Sox2 knockdown prevented mammosphere formation in ER^+ BC cell lines (51), and Sox2 knockdown significantly decreased the colony number of MCF7 cells (33, 61). Research has shown that Sox2 knockdown led to a reduction in cell proliferation for MCF7 and basal-like BC cell lines MDA-MB-231(33).

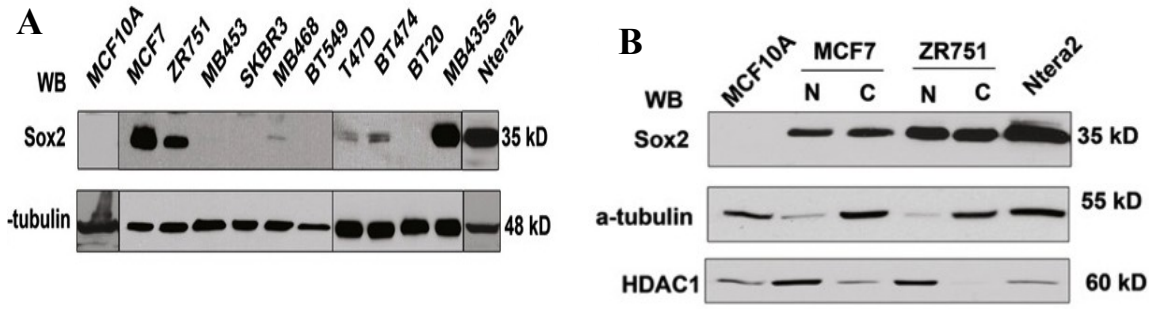

Figure 1.2: Sox2 protein expression and localization in BC cell lines. A) Sox2 expression was determined in BC cell lines including MB435S, BT474, T47D, MB468, ZR751 and MCF7. B) Sox2 expression was detected in nuclear and cytoplasmic fractionations of ER^+ BC cell lines. Ntera2 used as a positive control while MCF10A used as a negative control.

1.2.3.1 Sox2 transcriptional activity in BC:

The activation of Sox2 has been demonstrated in T47D and MCF7 BC cell lines as well as breast tumour initiation and correlated with the efficiency of sphere formation (51). These findings highlight the idea that Sox2 activation comprises part of the malignant progression of cancer (33, 60). From this perspective, previously published work from our laboratory has determined Sox2 transcription activity in BC cell lines. Our lab model used a Sox2 reporter construct, which has three tandems of Sox2 regulatory region 2 (SRR2). Sox2 binds to SRR2 in mouse and human ESCs (62) and transfects the ER^+ MCF7 and ZR751 BC cell lines, which, as previously mentioned, contain the highest Sox2 expression. The expression of green fluorescence protein (GFP) and luciferase represents the read-outs of Sox2 transcription activity. Accordingly, our group identified two distinct populations with different responses to the Sox2 reporter in both MCF7 and ZR751 cells: reporter responsive (RR), which is associated with high Sox2 transcription activity, and reporter unresponsive (RU), which is associated with low Sox2 transcription activity (figure 1.3). We found small RR subsets in both cell lines: 6% for MCF7 and 14% for ZR751 (Figure 1.4). These cells were sorted by using the flow cytometric cell sorter. Importantly, in RR cells, the Sox2 reporter activity depends upon Sox2 expression, as the knockdown of Sox2 abrogated the reporter activity.

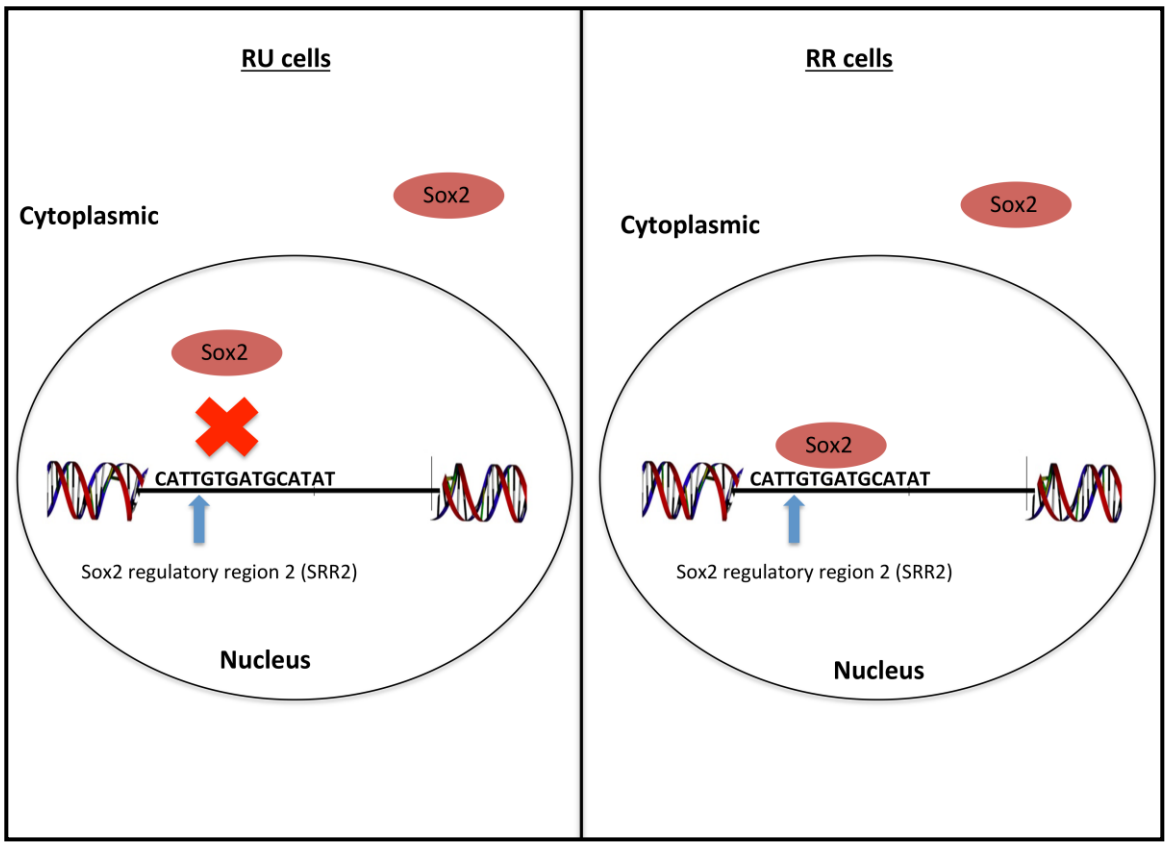

Figure 1.3: A model depicting the Sox2 transcription activity heterogeneity in ER^+ BC cell lines. In RU cells, Sox2 weakly binds to SRR2 and does not regulate the transcriptional activity while in RR cells Sox2 binds to SRR2 and regulates the transcriptional activity.

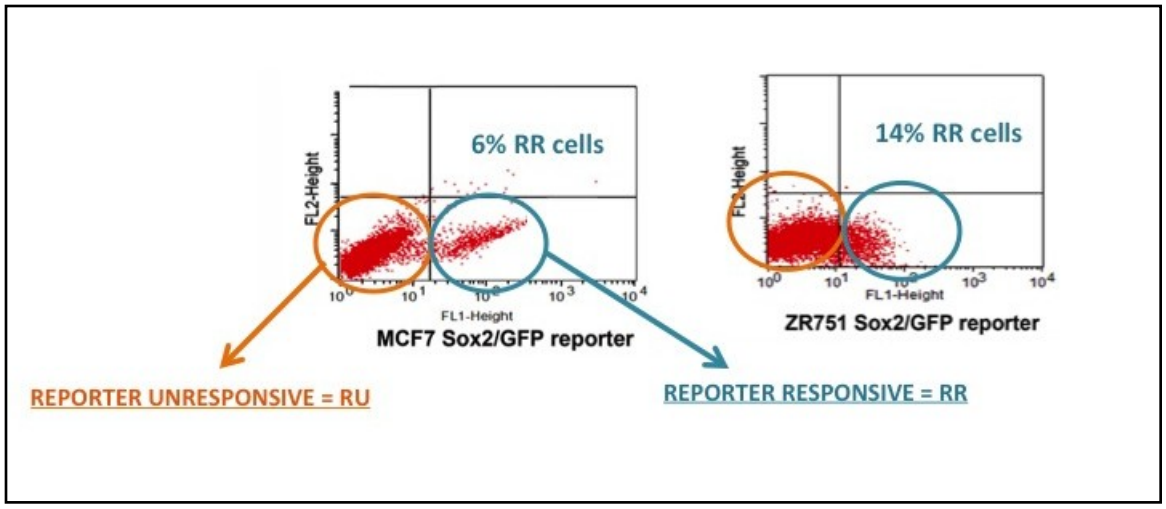

Figure 1.4: Identification of two cell subsets in MCF7 and ZR751 BC cell lines with different responses to the Sox2 reporter.

1.2.3.2 Phenotypic differences between RR and RU cells:

The RR and RU cell subsets show important biological differences. RR cells are associated with greater tumorigenic ability than RU cells, as RR cells form significantly more colonies in methylcellulose agar (61) and soft agar (63) than RU cells. Sox2 fulfills a crucial role in this formation, since the siRNA knockdown of Sox2 in RR cells caused a significant decrease in the colony number, while this process failed to yield any effect in RU cells. In addition, animal studies have found that three mice xenografted with $1x10^6$ MCF7-RR cells developed more tumors than three mice xenografted with $1x10^6$ MCF7-RU cells. Accordingly, our experiments found that RR cells form significantly more mammospheres than RU cells (63, 64). In addition, when we transfected primary BC tumor samples with SRR2 reporter, we found that this dichotomy also exists in patient samples (65). In addition, RR cells were more tumorigenic as compared to RU cells in methylcellulose assay of the patient samples (65). Overall, this study demonstrated that RR cells enhance tumourigenic properties, including the formation of methylcellulose colonies and mammosphere formation in BC cell lines and patient samples.

Two recent publications have confirmed our model study in BC (66, 67). Lgesias et al. recently determined Sox2 activities in BC cell lines, including MCF7. In this study, they constructed Sox2 promoters from an LRT promoter, which originated

from an early mouse transposon (ETn), and four tandem repeats of SRR2. Based on the *Sox2* promoter activities, the researchers isolated and classified the cells into GFP^+ cells (as we named RR cells) and GFP^- cells (as we named RU cells). Xenograft experiments, which purported to determine the tumorigenic properties of these cells, found that in comparison to GFP^- cells, GFP^+ cells contained enhanced tumorigenicity (66).

In addition, other researchers have demonstrated Sox2 activities in BC cells, including, MCF7 and ZR751 (67). In this study, these authors synthesized *Sox2* promoters by selecting two Sox2 promoter regions (-789 to +253) and two regulatory regions (SRR1 and SRR2), which were amplified from the genomic DNA of MCF7 cell lines. These cells were sorted according to Sox2 promoter activities; cells with positive Sox2 promoter activity were labelled pSp-T^+, which corresponds to RR cells, and cells with low Sox2 promoter activity were labelled pSp-$T^{low/-}$, which corresponds to RU cells. The researchers used a sphere formation assay to detect BC stem-like cells in MCF7 cells. Specifically, they showed that pSp-T^+ cells contained a strong sphere formation, especially in comparison to pSp-T^- or parent cells (67).

1.2.3.3 Biochemical differences between RR and RU cells:

Research has demonstrated evidence for biochemical differences between RR and RU cells, which may result in their phenotypic differences.

1.2.3.3.1 Phosphorylation of Sox2 detected in only RR cells:

Sox2 displays biochemical differences between the two cell subsets based on their different responsiveness to the SRR2 reporter. In particular, Sox2 binds to SRR2 in RR cells rather than in RU cells. Interestingly, our research group found that Sox2 phosphorylated at threonine 116 (T116) in RR cells yet failed to phosphorylate in RU cells. These results were confirmed by liquid chromatography-mass spectrometry analysis (LC-MS). Jeong et al. have shown that Sox2 is phosphorylated at threonine 118 (T118), which is homologous to T116, and enhances Sox2 transcription activity in mouse ESCs (38). Based on this finding, our lab group transfected MCF7 RU and RR cells with Sox2T116A, which mutates to alanine, or Sox2T116D, which mutates to aspartic acid, to abrogate phosphorylation at T116. Subsequently, we assessed Sox2 activity by measuring luciferase activity; these findings demonstrated significantly decreased luciferase activity while no change was detected in RU cells. Correlated with these findings, Sox2T116A significantly decreased the colony and mammosphere formation in RR cells. These results indicate that Sox2T116 fulfills important roles in Sox2 transcription activity.

1.2.3.3.2 The target genes of Sox2 in RR and RU cells are mutually exclusive:

Sox2 regulates many genes, including Cyclin D1 (*CCND1*) (33), *MUC15*, and *CD133* (65). Based on our model, Cyclin D1 comprises one of the Sox2

downstream targets in MCF7 RR cells; by using a chromatin immunoprecipitation (ChIP) assay, we found that Sox2 bound to the *Cyclin D1* promoter in RR cells but not in RU cells (61). A previous study from our laboratory performed a ChIP-on-chip study and determined 463 gene targets in RR cells; among these targets, 94% of them are not found in RU cells. On the other hand, the same study discovered 1866 target genes in RU cells, with 98% of these targets not occurring in RR cells (65). In addition, we identified 15 genes that have been associated with cancer stem cells, including *CD133* (68, 69) and *MUC15*. More specifically, we have identified *MUC15* as a novel target of Sox2 in RR cells; by either knockdown or overexpression, Sox2 significantly modulated the expression of MUC15 (65). In conjunction, these results suggest that Sox2 binds to other gene promoters and exerts transcription regulation differently in the two cell subsets of RR and RU cells (65).

Since RR cells provide stem-like features, we will focus on genes that have been identified as Sox2 downstream targets and involved in oncogenesis in human ESCs. In this regard, La et al. used genome scale location analysis, which involves chromatin immunoprecipitation, to demonstrate that Sox2 binds to the promoter region of its target genes in human H9 ESCs (70). The results show that Sox2 participates in pluripotency and self-renewal by activating or repressing its downstream target genes, which are essential to differentiation. However, this

study does not provide any functional findings. Consequently, we performed ChIP-on-chip in MCF7 RU and RR cells and found 15 genes in MCF7 RR cells that overlapped in H9 ESCs.

1.3 Sox2 Protein-binding partner:

As previously discussed, Sox2 protein binds to DNA in specific sequences: C(T/A) TTG (T/A)(T/A)). This pattern indicates that the HMG domains function as transcription factors to regulate the expression of targeted genes by the activation or repression of their expression (45, 71). In fact, the Sox protein family lacks the ability to independently bind to DNA because this family possesses low DNA binding affinity; hence, the Sox proteins require other protein partners for forming a stable Sox transcription factor complex (33). For instance, Sox2 constitutes one of the most common Sox proteins in the regulation of the targeted genes. A well-recognized target of Sox2, *δ-crystallin* gene minimal enhancer in mouse ESCs (DC5), causes a lens-specific gene expression. Sox2 binds to the DC5 sequence at the 5′ half site to trigger the enhancer in the lens cells. Also, Sox2 affects the DNA sequence at the 3′ half of the DC5. *In vivo*, the 3′ site of lens cells is foot-printed and might represent the effect site of δEF3: the Sox2 partner factor (72). These studies used a ChIP assay to confirm that Sox2 and δEF3 bind to the DC5 enhancer (74).

Moreover, Sox2 interacts with another partner, the Oct-3 transcription factor. This complex activates the Fgf4 enhancer, which is stimulated in ESCs and embryonic carcinoma cells (ECCs) (73, 74). The collaboration between Sox2 and Oct-3 is also represented in the expression of the *UTF1* gene, which constitutes a co-activator in

ES-cells (75). The UTF1 enhancer depends on the interaction between Sox2 and Oct-3; this concept was confirmed through a mutation at the site of either Sox2 or Oct-3. This manipulation resulted in a significant decrease in UTF1 enhancer activation (75). Overall, members of the Sox family have a specific functionality for each tissue. The functionality is determined through the diverse partnership of Sox proteins with other proteins (33, 45).

1.4 DDX17 structure and function:

The DEAD box contains the amino acid sequence D-E-A-D (Asp-Glu-Ala-Asp), which accounts for the name of the family. This protein group comprises a subfamily of RNA helicases (76). The DEAD box family contains 37 members in humans (77). These proteins have been involved in RNA metabolism, including transcription, translation, and mRNA degradation, which constitutes pre-mRNA splicing, ribosome biogenesis, RNA turnover, and mRNA export as well as the translation and modulation of complex RNA structures (78, 79). *DEAD* box genes can be mutated, resulting in the deregulated expression of several proteins, such as DDX17, which has been linked to cancer development (80).

DDX17 mRNA can undergo translation into two isoforms: p72 and p82. Between these two isoforms, p82 is larger than p72 because alternative translation initiates the codons in a non-AUG fashion, while the translation site of p72 starts at the AUG codon (81, 82). Whether these two isoforms have different functions unknown; however, most studies investigating p72 (DDX17) do not differentiate the two isoforms.

Like other DEAD box members, DDX17 contains nine conserved motifs, including motif II, also known as Walker B, which consists of the Asp-Glu-Ala-Asp motif (83, 84). Notably, DDX17 has a remarkable homology to DDX5, with a

90% amino acid sequence across the conserved core of the protein; however, the N and C-termini contain significant differences (81) (Figure 1.5).

In addition, another function of DDX17 relates to the unwinding of RNA. Research has shown that DDX17 binding to double-stranded and single-stranded RNA cases stimulates their ATPase activity, which supplies energy to unwind RNA duplexes in both the 3’→ 5’ and 5’→ 3’ and direction (85). Moreover, DDX17 performs annealing actions in addition to RNA helicase activity, which, in combination, can stimulate the rearrangement of RNA secondary structures (86). DDX17 also affects the alternative splicing of exons containing AC-rich exon enhancer elements. An increase in the concentration of DDX17 in transient transfections augmented the enhancer-containing CD44 alternative exons, v4 and v5, while the mutation of DDX17 in either the ATP-binding site or in the deletion of the carboxy-terminal region of the protein decreased the capacity of the transfected protein to impact CD44 variable exon splicing. These findings highlight the importance of DDX17 as a regulatory factor for alternative splicing (87).

Moreover, DDX17 functions as a co-regulator of many transcription factors of myogenesis, such as MyoD, which is a master regulator of muscle differentiation. The knockdown of DDX17 and its close relative, DDX5, inhibited the expression of MyoD and its target genes, Myog and Mef2c, the master regulators of

myogenesis (88). DDX17 also serves as a co-regulator of SMAD, which mediates the transforming growth factor β (TGF- β); this factor induces the epithelial to mesenchymal transition (EMT). The knockdown of both DDX17 and DDX5 caused a reduction of SMAD 2/3 and its target genes SNAI1 and SNAI2, the master regulators of EMT. In conjunction, these results highlighted the importance of DDX17 in cell differentiation as transcriptional co-regulators of the MyoD and SMAD transcription factors (88).

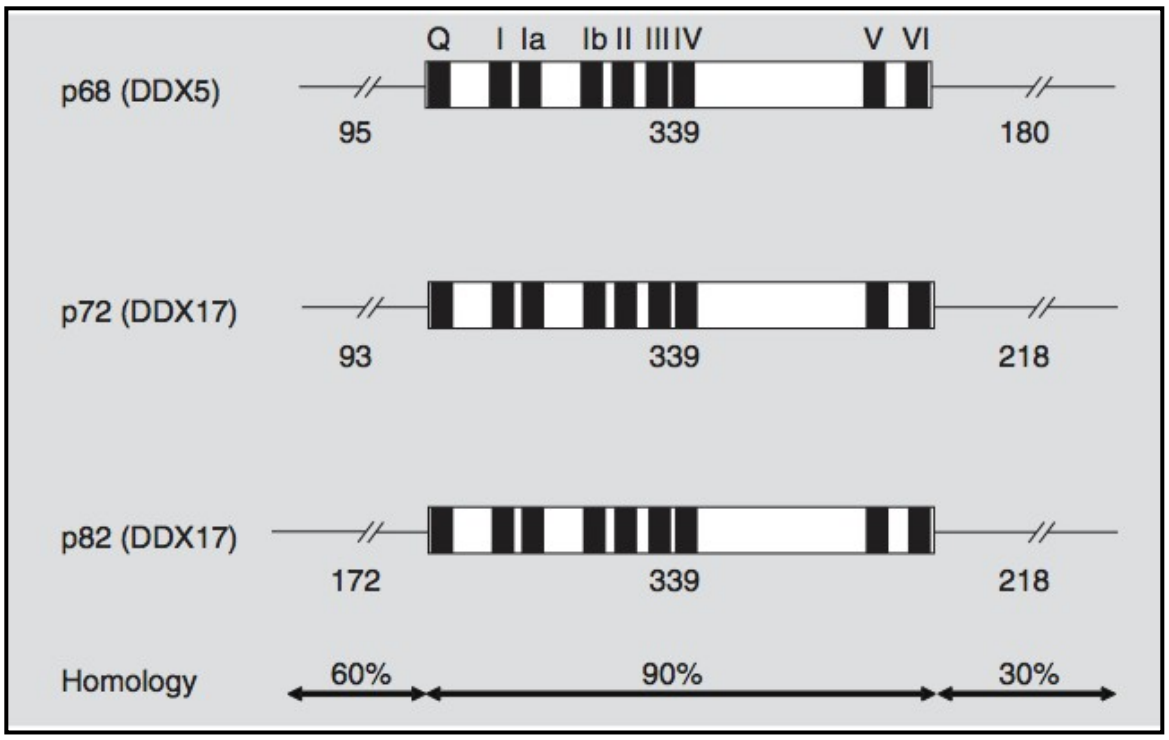

Figure 1.5: DDX17 and DDX5 protein domains. DDX17 shares 90% identity with DDX5 in the conserved core, which includes the nine conserved motifs (Q, I, Ia, Ib, II, III, IV, V and VI) while they are different in the N and C-termini. The numbers in the N-terminal, C-terminal and conserved core represent amino acids while the percentages show the similarity of these domains in P72 (DDX17), p82 (DDX17) and DDX5.

1.4.1 DDX17 in cancer:

Studies have shown that DDX17 is overexpressed in 72-76% of BC (89). ER-α is positively expressed in approximately 70% of breast tumors (90). In BC, DDX17 interacts with ER-α through co-immunoprecipitation experiments and co-activates ER-α in luciferase reporter assays. These two experiments provide evidence of the way in which DDX17 functions in breast carcinogenesis (89). Moreover, DDX17 formed a complex with the steroid receptor co-activator (SRC-1) and the steroid receptor RNA activator (SRA), thus suggesting that DDX17 may stimulate ER-α activity by acting as a link between ER-α and SRA/SRC1 to enhance the activation (89). Unlike the case in wild type DDX17, mutations in the helicase domain of DDX17 possess the ability to stimulate estrogen-dependent transcription, demonstrating that DDX17 lacks the need for RNA helicases to stimulate ER-dependent transcription. The knockdown of DDX17 significantly decreased the expression of estrogen–regulated genes, including pS2 and cathepsin D, at both the protein and mRNA levels (90). Furthermore, the knockdown of DDX17 resulted in significantly reduced cell growth in both MCF7 and ZR751 (89). In conjunction, these findings show the necessity of DDX17 for the estrogen-dependent transcription of ER-responsive genes and estrogen-dependent cell growth (89). On the other hand, DDX17 lacked the ability to affect the transcriptional activity of the p53 tumor suppressor from responsive promoters in luciferase reporter assays (93).

However, DDX17, in comparison with its close relative, DDX5 (p68), displays a decreased interaction with p53. Moreover, the siRNA knockdown of DDX17 fails to affect the p53 response to DNA damage in MCF7 BC cell lines, hence indicating that DDX17 lacks involvement in tumor suppressor roles (91).

DDX17 has been reported to coactivate NFAT5, which is a transcription factor that controls cell migration and coimmunoprecipitate with NFAT5 in MDA-MB-231 BC cells. The overexpression of DDX17 and the related DDX5 protein increased the transcriptional activity of NFAT5 by measuring luciferase reporter genes driven by the NFAT5-responsive promoter. Moreover, the knockdown of DDX17 resulted in the inhabitation of cell migration through decreased NFAT5 expression levels. The knockdown of both DDX17 and DDX5 resulted in the decrease of the target genes of NFAT5, which is involved in BC progression. This study identified DDX17 as a co-activator of the NFAT5 transcription factor and its role in cell migration.

In colon cancer, DDX17 is overexpressed; an immunohistochemistry study detected DDX17 in 30-90% and 90-100% of adenomas and adenocarcinomas respectively, indicating the association between the level of its expression and the progression of the adenoma-carcinoma sequence (93). Importantly, the degree of

DDX17 overexpression was highly correlated with increased β-catenin expression in colon cancer cells. Moreover, DDX17 and its close relative, DDX5 (p68), form complexes with β-catenin and function as transcriptional co-activators through their interaction with gene promoters of the β-catenin target genes, including cyclin D1, c-MYC, c-Jun, and Fra-1 (93). In addition, the knockdown of DDX17 and DDX5 resulted in the decreased expression of β-catenin regulated genes and inhibited colon cancer cell proliferation as well as preventing the formation of tumors *in vivo* (92). However, the individual roles of DDX17 in various cancers remain unclear (93).

1.5 Rationale and objectives of this study:

The normal expression of Sox2 is found exclusively in ESCs and somatic stem cells. However, research shows that Sox2 was aberrantly expressed during tumor progression. In this study, our main objective seeks to investigate the mechanism that regulates Sox2 transcriptional activity in ER^+ BC cells. To achieve this goal, we focused on Sox2 downstream targets in human ESCs and tumor cells. Nonetheless, the function of Sox2 in regulating ESC genes in the stemness of cancer cells remains largely unknown. Therefore, **this work hypothesizes that the Sox2 transcription factor can regulate ESC genes in ER^+ BC cell lines.** There are two aims for testing this hypothesis:

- To detect the expression of ESC genes in BC and subsequently compare their expression in RU and RR cells.
- To determine the regulatory role of Sox2 downstream ESC genes in RU and RR by knocking down Sox2.

Sox proteins lack the ability to independently bind to promoters, hence requiring the presence of a partner that can achieve stable DNA binding (71). Thus, the functional specificity of the Sox proteins depends on the partnership of other proteins (73). For instance, a study has identified that in embryonic development, PARP1 functions as one of the Sox2 binding proteins that regulates the transcriptional activity of Sox2 mouse embryonic stem cells (39). In addition, our previous study of BC has shown that β-catenin constitutes one of the Sox2 binding

partners. This protein negatively regulates Sox2 transcription activity, which was evident by an increase in the luciferase activity of Sox2. As well, Sox2 increased the expression of target genes, such as Cyclin D1, in MCF7 RR cells after the knockdown of β-catenin (64). This finding supports the notion that Sox2-binding proteins can also regulate the transcriptional activity of Sox2 in cancer cells. To the best of my knowledge, existing studies have not yet identified protein partners for Sox2 that could activate Sox2 transcription activity in BC. Accordingly, our lab has established a novel model to study the transcriptional activity of Sox2 in BC cell lines. Our aims include the following:

- To identify Sox2 protein partners in ER^+ RU and RR BC cell lines.
- To determine the localization of binding between Sox2 and DDX17 in RU and RR cells.
- To examine how DDX17 regulates the transcription activity of Sox2 in ER^+ RU and RR BC cell lines.
- To investigate the role of DDX17 in regulating the tumorigenic properties in RR cells as compared to RU cells.

CHAPTER2: Material and Methods

2.1 Cell lines

The MCF7 and ZR751 BC cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Both cell lines, sorted into RR and RU cells, were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY) and supplemented with 10% fetal bovine serum (FBS) (Sigma, Oakville, ON, Canada), puromycin, and streptomycin under conditions of 5% CO_2 at 37 °C.

2.2 Short interfering RNA transfections

ER^+ BC cell lines (MCF7 and ZR751) were transfected with SMARTpool short interfering RNA (siRNA) designed against Sox2 (Thermo Scientific Company, Rockford, U.S.A) or DDX17 (Fisher Scientific). The use of scrambled non-targeting siRNA (Thermo Scientific) served as the negative control. For each reaction, 40 pmol of siRNA (20 nM final concentration) and 5 µL of Lipofectamine RNAiMAX (Life Technologies) were added to 0.5 mL of OptiMEM media (Life Technologies) and reverse transfected to $1X10^6$ cells/ well in a normal culture medium with a 6-well plate format. Subsequently, cells were incubated with siRNAs for 48 hours before harvesting.

2.3 Plasmid transfections

MCF7 and ZR751 cells were transfected with 18 μg of pMXs Flag-tagged Sox2 (Addgene, MA, USA), pDEST MYC-tagged DDX17 (Addgene, MA, USA) or an empty vector with 30 μL of Lipofectamine 2000 (Life Technologies) in 3 mL of OptiMEM media (Life Technologies) to 5 million cells in a 100 mm tissue culture dish. Then, cells were incubated for 48 hours before harvesting.

2.4 Nuclear cytoplasmic fractionation

MCF7 cells were extracted for nuclear and cytoplasmic proteins by using the NE-PER Protein Extraction Kit (Thermo Scientific) according to the manufacturer's protocol. Briefly, after harvesting and washing the cells, 1 ml of CER I reagent was added and vortexed on the highest setting for 15 seconds. The samples were 55 ,Then .minutes 10on ice for dncubateiμL of CER II reagent was added and the samples were The tube was ,seconds on the highest setting 5for edvortex 5for edvortex the samples were ,subsequently ;minute 1on ice for dncubatei minutes at maximum speed in a microcentrifuge 5for dseconds and centrifuge ,Finally .(g × 16,500) the samples were the ,the supernatant from transfered s were tthe pelle ,nuclear extraction To perform .to a clean tube ,cytoplasmic extract extract before cytoplamic the of erremaind the washed with RIPA buffer to remove 500μL of NER reagent was added. The samples were seconds on 15for edvortex d fourrepeate s step wasThi .minutes 10on ice for dncubatei the highest setting and

the minutes at maximum speed in 10for were centrifuged samples the and times ,Finally .(g × 16,500)microcentrifuge the nuclear extracts were clean transfered to .C°80- at and stored stubeFor western blotting analyses, α-tubulin and histone deacetylase 1 (HDAC1) were used as cytoplasmic and nuclear loading controls respectively.

2.5 Western blotting:

2.5.1 Cell lysate preparation:

Cells were grown until they reached a stage of approximately 95% confluency. Then, the cells were washed with PBS and trypsinized before undergoing transfer to 15 mL conical tubes. The cells were harvested by centrifugation at a rate of 300 xg for 5 min at 4°C. Pelleted cells were washed with cold PBS and lysed by a radioimmunoprecipitation assay (RIPA); the protease and phosphatase inhibitors as well as the samples were kept on ice at all times. Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Scientific). Samples were heated with Laemmli 4X buffer dye to 95°C for 5 min and then loaded into the gel.

Next, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separated the protein sample. SDS-PAGE consists of two layers: a top layer known as the stacking gel and a lower layer referred to as the separating or resolving gel.

Ten percent separating gel was prepared by adding a 7.9 ml of H_2O with 6.7 ml of 30% Acrylamide Mix, 5 ml of 1.5 M Tris (pH 8.8), 200 µl of both 10% SDS, APS and 8 µl of TEMED. The stacking gel was prepared by adding 4.1 ml of H_2O, 1 ml of 30% Acrylamide Mix, 750 µl of 1.0M Tris (pH 6.8), 60 µl of both 10% SDS and APS, and 6 µl of TEMED.

Samples were loaded in the gel with a running buffer at 100 V for 2 hours. Then, the proteins were transferred from the gel to the nitrocellulose membrane with a transfer buffer at 100 V for 2 hours. The membrane was blocked with 5% milk for 1 hour at room temperature. Proteins on the membrane were incubated overnight with the following antibodies: SOX2, α-tubulin, and β-actin from Cell Signaling Technologies; DDX17 from Abcam; and HDAC1 from Santa Cruz Biotechnology; the dilution ratio for these antibodies was 1:1000. The membrane was washed in Tris-buffered saline and Tween 20 (TBST) for ten minutes. This procedure occurred three times, for a total of 30 minutes, removing the unbound antibody and incubating with the secondary antibody for one hour at room temperature; then, this step was repeated. Proteins were detected by using chemiluminescent (ECL) substrate and exposed to Kodak X-ray film (Fujifilm Corporation Company, Duesseldorf, Germany).

2.6 Co-immunoprecipitation (Co-IP)

After transfecting the cells with flag SOX2 or Myc-DDX17 according to the previous steps, the cells were harvested and lysated with Cellytic M (Sigma) as well as protease and phosphatase inhibitors. Then, 1 mg of the protein lysates were combined with 40 µL of Anti-FLAG M2 Affinity gel beads (Sigma) and rotated for 2 hours at 4 °C. A negative control, which involved a similar amount of protein lysate incubated with a non-specific IgG antibody, was used. After, the samples wereat minute 1d for centrifuge washed the beads were and ,xg 5000with CelLytic M and d threeateerep this step was ;xg 5000minute at 1for dcentrifuge ,Finally .timesthe samples were eluted with Laemmli 2X buffer dye to 95°C for 5 min.

2.7 RNA extraction

The total RNA was extracted from the MCF7 and ZR751 cell lines by using the Qiagen RNeasy Kit (Qiagen Canada) according to the manufacturer's protocol. First, 350 µl of RLT buffer and 350 µl of 70% ethanol were mixed with the pellet and transferred to an RNeasy spin column. Then, the mixture was centrifuged for 15s at 17,900 xg, and the flow-through was discarded after each centrifugation. The samples were washed by adding 700 µl of RW1 buffer, centrifuging the mixture, adding 500 µl of RPE buffer, and centrifuging. Finally, 30 µl of RNase-free water was added to elute the RNA and the mixture was centrifuged. The

quality of the RNA samples was assessed by a spectrophotometer.

2.7.1 Reverse Transcription

cDNA synthesis was performed using SuperScript II Reverse Transcriptase (RT) (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. 1 ug of total RNA, 1 µl of Oligo (Invitrogen), 1 µl of dNTP (Invitrogen), and 12 µl of ultra pure water were added to a nuclease-free microcentrifuge tube. Using PCR, the mixture was heated to 65°C for 5 min. Then, 4 µl of 5X First-Strand buffer, 2 µl of 0.1 M DTT and 1 µl of ultra pure water were added and incubated at 42°C for 2 min. Finally, 1 µl of SuperScript II RT was added and incubated at 42°C for 50 min followed by heating at 70°C for 15 min.

2.7.2 Quantitative reverse transcriptase PCR (q-RT-PCR):

Following reverse transcription, the expression level of 15 ESC genes and the target genes of SOX2 were detected by a real time PCR reaction (qRT-PCR) according to the SYBR Green assays protocol. This detection occurred under the following cycling parameters: hold at 95 °C for 10 min followed by 40 cycles of denaturing at 95°C for 15 s and an annealing or final extension at 60°C for 30 s. All genes were amplified with specific primer sequences, as shown in Table 1, and normalized to the GAPDH expression level.

Gene name	Forward strand	Reverse strand
KCNJ1	CATCTTCGGAAATGGGTCGTC	ATTGCCAAATTCTATGTTGCACC
SLC25A18	CTCATCAATGGAGGTGTAGCAG	GAGCCGTCTTCATCAGGCA
TSC22D1	CGGCAATGTTCCCTCGAAG	GGCGGAAAATCCTCGGAAGA
DHDDS	TGCCGAAACACATTGCATTCA	GCGTAGACTGTCACCTCTAGGAT
BUB1B	AAATGACCCTCTGGATGTTTGG	GCATAAACGCCCTAATTTAAGCC
NR4A2	GTTCAGGCGCAGTATGGGTC	CTCCCGAAGAGTGGTAACTGT
NBR1	AGATGGCAGTTAAACAGGGAAAC	GTGGGGCTTCATCAACGACA
TBX5	GTACCTGCCGACGATCACAG	CACGATGTGTAATCTAGGCTGG
PITX2	CGGCAGCGGACTCACTTTA	GTTGGTCCACACAGCGATTT
PPP2RIB	CTTGTGTCAGTATTGCCCAGT	TGCTGCTTGTCGAAGTGTAGG

DDX17	GATGTTTGTCCTAAACCCGTGT	CCAACGGAAATCCCTGGCA
NEBL	AGAGGCTTTACTCCCGTCGT	ACCCCTTTATAGGCAGCATCG
REST	GCCGCACCTCAGCTTATTATG	CCGGCATCAGTTCTGCCAT
FZD2	GTGCCATCCTATCTCAGCTACA	CTGCATGTCTACCAAGTACGTG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
SOX2	GCTACAGCATGATGCAGGACCA	TCTGCGAGCTGGTCATGGAGTT
Cyclin D1	GCTGCGAAGTGGAAACCATC	CTCCTTCTGCACACATTTGAA
MUC15	TATTCACTTCTATCGGGGAGCC	GGGAATGACTCGCCTTGAGAT

Table 2.1: Primer sequences for RT-PCR.

2.8 Mammosphere assay

The cells were counted and passed through a 40 μm cell strainer (BD, Franklin Lakes, New Jersey). Then, the cells were seeded at about 10,000 cells per well and plated into ultra-low adherent plates (Corning) in Mammocult media (StemCell Technologies, Vancouver, BC, Canada). The Mammocult media was provided with frozen supplements, Heparin solution, and hydrocortisone solution. Mammospheres were counted after 7-10 days of seeding.

2.9 Colony formation assay

The cells were counted and seeded at about 1000 cells per well. Then, the cells were plated in six-well plates and incubated for two weeks at 37°C in 5% humidified CO_2. After incubation, the cells were fixed with 4% buffered formalin for 15 min and then stained with 1% crystal violet (Sigma Aldrich) for 30 min. The plates were washed with PBS and dried. Finally, visible colonies, which consisted of at least 50 cells, were counted.

2.10 Chromatin immunoprecipitation (ChIP):

The EZ ChIP chromatin immunoprecipitation kit (Millipore catalog #17-371) was used according to the manufacturer's protocol. Briefly, cells were transfected with scrambled siRNA or DDX17 knockdown for 48 hours and treated with 1% formaldehyde to cross-link proteins to DNA. Cells were lysed with protease inhibitors, sonicated to shear DNA into fragments (average size 200-1000pb) and incubated with antibody against Sox2 (Santa Crus #20088) or anti-rabbit IgG (negative control) overnight. The purified DNA and input genomic DNA was analyzed by real time PCR. The primers were used in this experiment are as following, promoter sequences for SRR2-1 are:

Forward: 5'- ACATTGTACTGGGAAGGACA-3', Reverse:5' AGCAAGAACTGGCGAATGTG-3'. Promoter sequences for SRR2-2 are: Forward: 5'- GGATAACATTGTACTGGGAAGGGAAGGG-3', Reverse: 5'-GTGAGCAAGAACTGGCGAAT-3'. Promoter sequences for LGR5 are: Forward:5'-GCGCTGGGACACTTAAGATG-3'Reverse:5'-CTTCCTATCTCTTGCGGGGT-3'. Promoter sequences for MUC15 are: Forward: 5'-GTCCTGCCCAATCATGTTCA-3', Reverse:5'-AAGGCCCCTTCAGAGTTTGA-3'.

2.11 Statistical analyses:

The data were analyzed using Student's t-test to determine statistically significant differences. All graphs represent at least two independent experiments with triplicates. Using error bars to represent the standard error of the mean.

CHAPTER 3: Results

3.1 Embryonic stem cell genes differentially express in two different cell subsets of RR and RU cells:

As mentioned before, we detected 15 ESC genes in MCF7 RR that overlap with H9 human ESCs including, *REST, NBR1, DDX17, BUB1B, NR4A2, DHDDS, PITX2, KCNJ1, SLC25A18, FZD2, NEBL, FGFR1, TBX5, TSC22D1* and *PPP2R1B*. By using RT-PCR, we validated their expression in MCF7 RU and RR cells. As shown in Figure 3.1, all 15 of the ESC genes were expressed higher in RR cells as compared to RU cells.

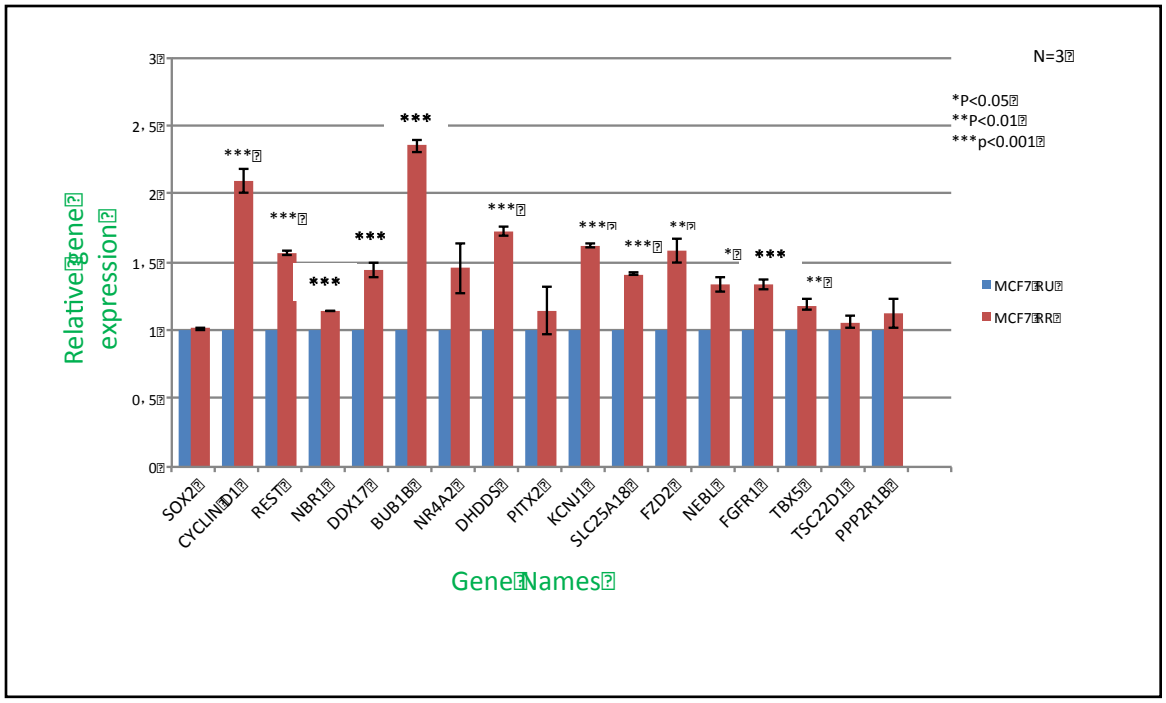

Figure 3.1: The mRNA levels of 15 ESC genes in MCF7 RU and RR cells. The experiment was performed by RT-PCR.

3.2 Sox2 down-regulated the ESC genes expressions in MCF7 RU and RR cells:

This part of the investigation seeks to identify the regulatory roles of Sox2 in 15 ESC gene candidates. Eight genes demonstrate significant expressions in RR cells (P :(0.001≥*REST, NBR1, DDX17, BUB1B, DHDDS, KCNJ1, SLC25A18* and *FGFR1*. A siRNA knockdown of Sox2 in MCF7 RU and RR cells revealed that Sox2 positively regulated 4 out of 8 genes, including *REST, NBR1, DDX17* and *BUB1B* (Figure 3.2).

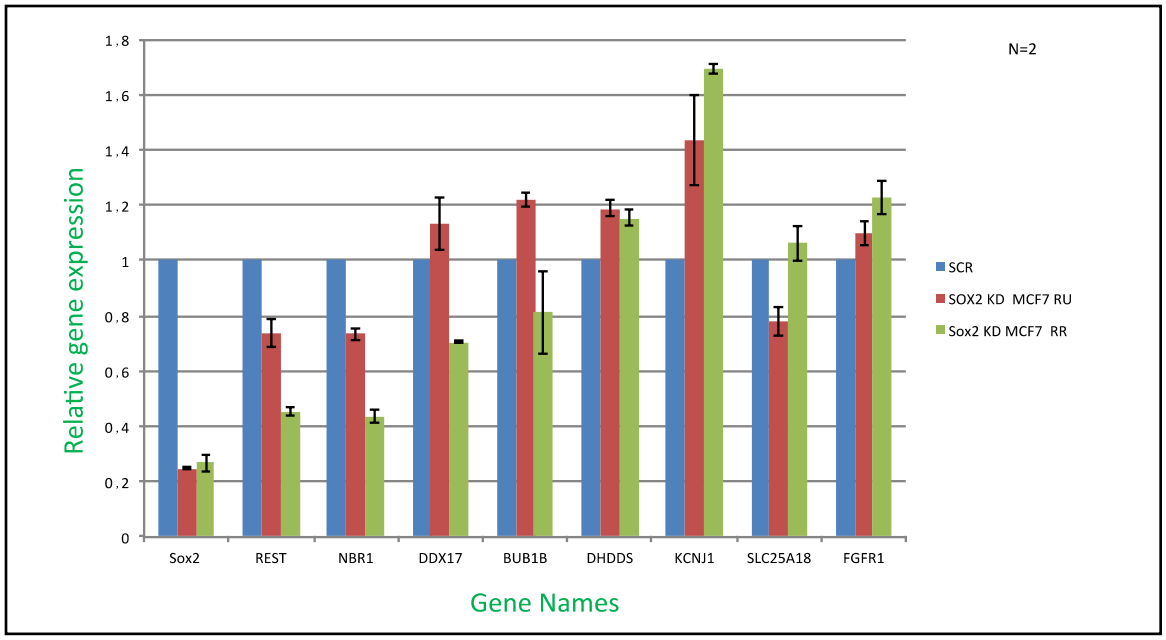

Figure 3.2: siRNA inhibition of Sox2 differentially regulates the ESC genes expression in MCF7 RU and RR. The experiment was performed by RT-PCR.

3.3 DDX17 is a Sox2 binding protein:

Among the 15 genes, DDX17 showed the most significant decrease after the knockdown of Sox2 in RR cells. In addition, our group found that DDX17 interacted with Sox2 by using chromatography-mass spectrometry. We validated the binding between Sox2 and DDX17 in MCF7 RU and RR cells by the overexpression of FLAG-Sox2 followed by co-immunoprecipition. As shown in Figure 3.3A, the anti-FLAG antibody pulled down a similar amount of Sox2 protein between RR and RU cells while the amount of co-immunoprecipitated DDX17 protein was higher in RR cells as compared to RU cells. Concurrently, by using Myc tagged-DDX17 overexpression and Myc pulldown, we found co-immunoprecipitated Sox2 in only RR cells (Figure 3.3B). Notably, β-catenin, a known SOX2 binding protein (64), was used as a positive control in these experiments. The same results were found in ZR751 RU and RR cells (Figure 3.3C).

A previous study reported that DDX17 localized in the nuclear fraction during colon cancer. As shown in Figure 3.4A, we performed a co-immunoprecipition experiment and detected the binding of DDX17 and Sox2 in the nuclear fraction of only RR cells. However, we did not observe that DDX17 binds to Sox2 in the cytoplasmic fraction of both RR and RU cells. Moreover, reverse co-

immunoprecipitation experiments were performed (Figure 3.4B). Overall, DDX17 expression was observed equally in the nuclear fractions of RR and RU cells while in the cytoplasmic fraction, DDX17 expression was higher in RU cells as compared to RR cells.

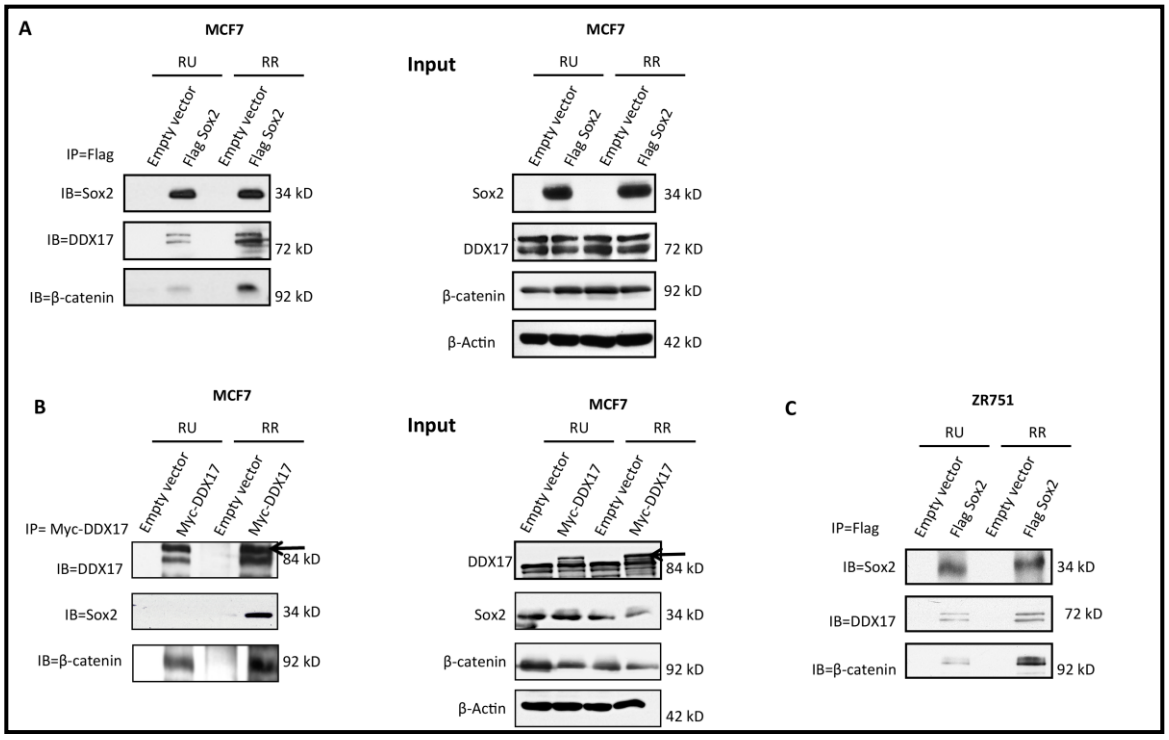

Figure 3.3: Identification of DDX17 as a Sox2 binding protein. (A) The binding between DDX17 and Sox2 was confirmed by co-immunoprecipitation and western blotting. While the anti-FLAG affinity beads pulled down a similar amount of Sox2 between RR and RU cells, the amount of DDX17 protein co-immunoprecipitated was higher in RR cells than in RU cells. (B) Reverse co-immunoprecipitation experiment using anti-c-Myc magnetic beads to pull down a similar amount of DDX17 between RR and RU cells and the amount of Sox2 protein co-immunoprecipitated was found only in RR cells. The input for transfection by either Sox2 and/ or DDX17 was confirmed by western blotting and β-Actin was used as a loading control. (C) Co-immunoprecipitation and western blotting were performed in ZR751 RU and RR cells.

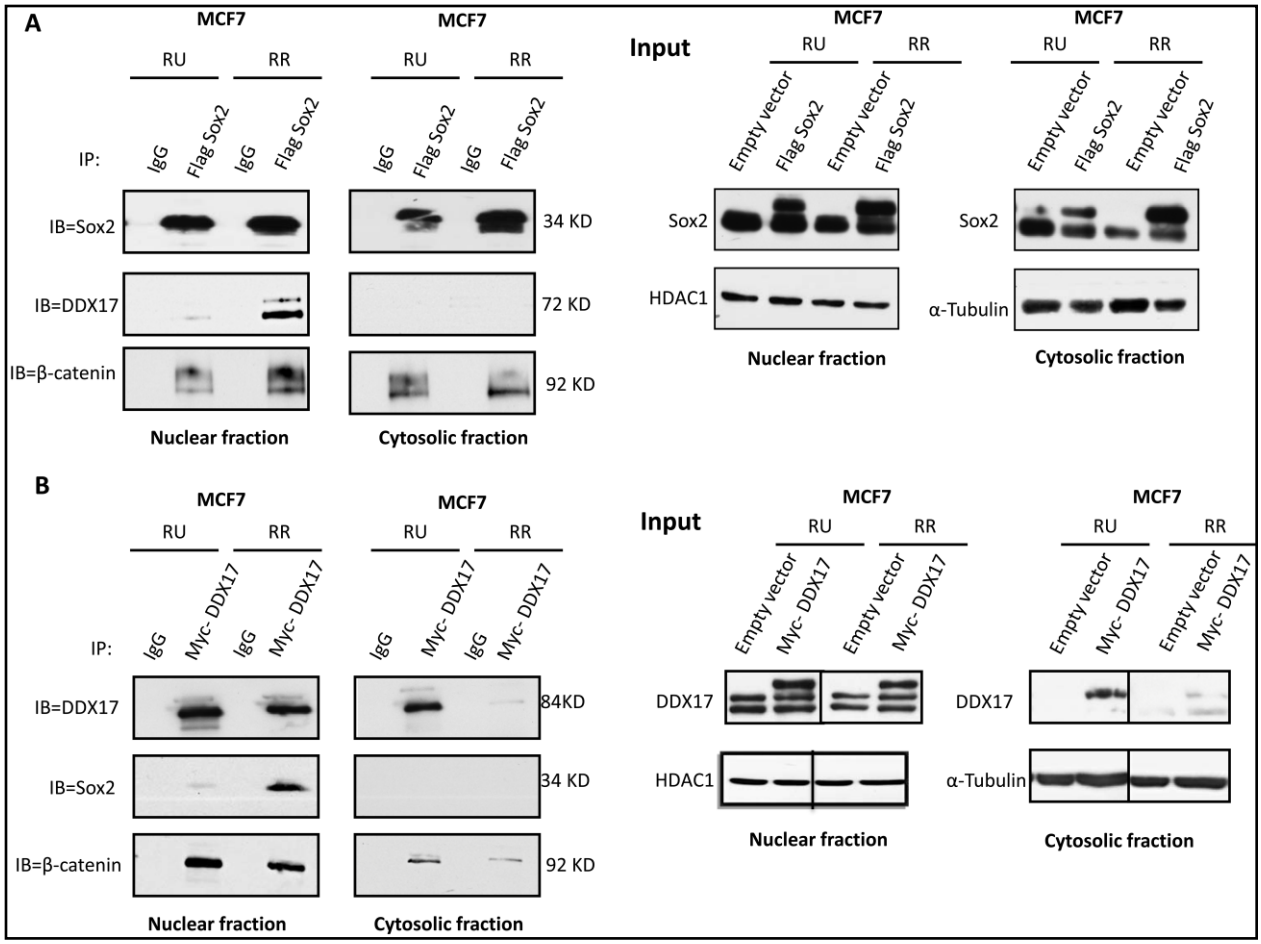

Figure 3.4: The binding between Sox2 and DDX17 in the nuclear fraction was detected only in RR cells. (A) The co-immunoprecipitation of flag-Sox2 pulled down SOX2 in the nucleus and cytoplasm of both RR and RU cells. In contrast, DDX17 occurred only in the nuclear fraction of RR cells. Reverse co-immunoprecipitation experiments using anti-DDX17antibodies for pull down showed similar findings. The input for transfection by either Sox2 and/or DDX17 was confirmed by western blotting; α-Tubulin and HDAC1 were used as loading controls in the cytoplasmic and nuclear fractionation.

3.4 DDX17 regulates the transcriptional activity of Sox2 in RR cells:

Research studies have shown that DDX17 functions as a co-activator of various transcription factors, such as estrogen (e.g. receptor-α (ERα)). We speculate that DDX17 may activate the transcriptional activity of Sox2 in BC cells. Moreover, we also want to determine if Sox2 protein expression can be regulated by DDX17. As shown in Figures 3.5A and 3B, we found that upon siRNA knockdown of DDX17, the total protein level of Sox2 did not change in the RR and RU cell subsets of MCF7 and ZR751.

Subsequently, we assessed the transcriptional activity of Sox2 by a luciferase assay. As shown in Figure 3.5A, we found that the siRNA knockdown of DDX17 resulted in a significant decrease in the transcriptional activity of Sox2 in RR cells while no significant change was detected in the RU subset. Similar results were determined in ZR751 (Figure 3.5B).

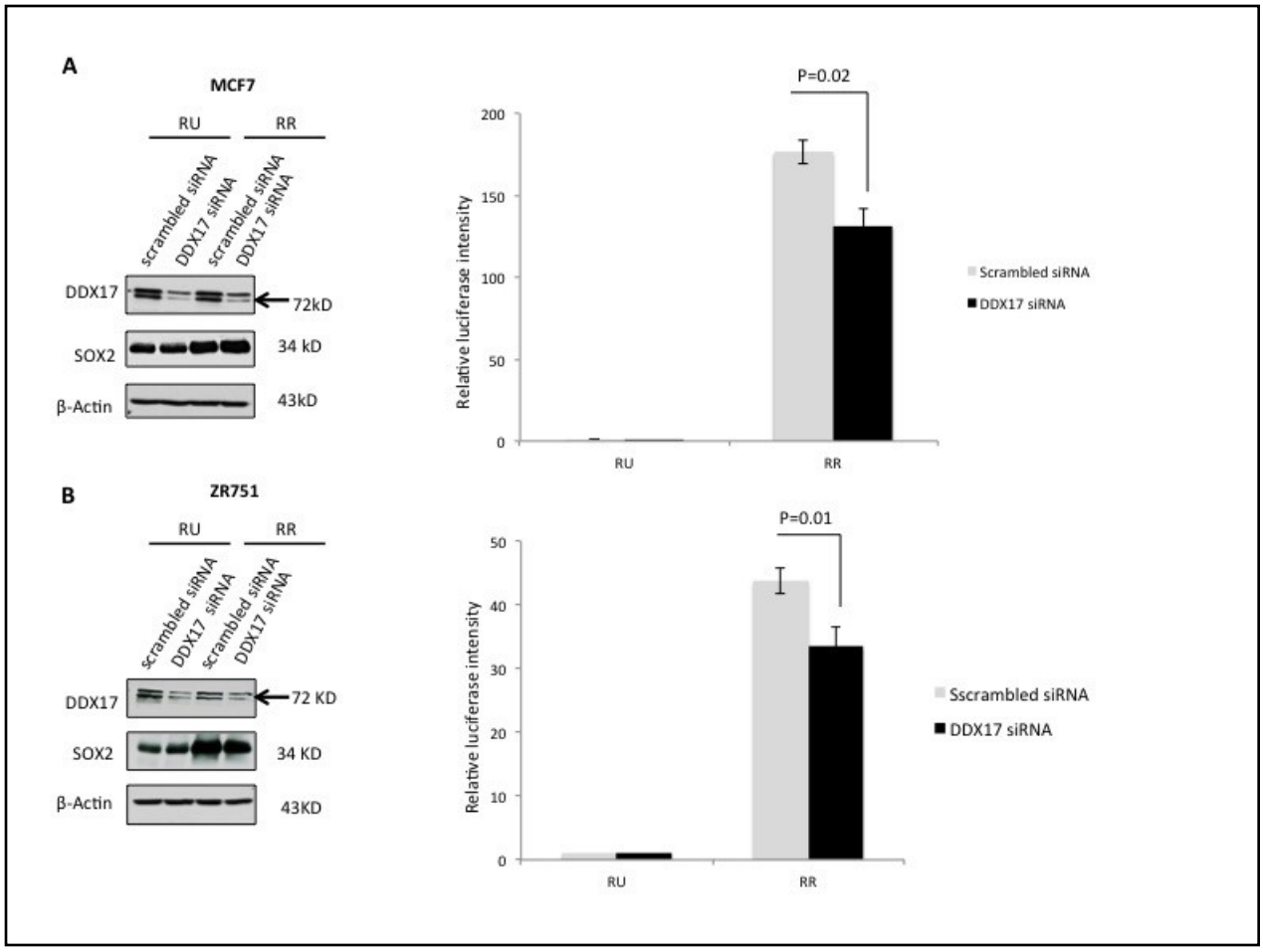

Figure 3.5: DDX17 activated the transcriptional activity of Sox2 in only RR cells. (A) RR and RU cells derived from MCF7 were treated with a negative control, either DDX17 siRNA or scrambled siRNA, for 48 h. β-Actin was used as a loading control. Despite the decrease in the protein level of DDX17, the protein level of Sox2 remained unchanged. The siRNA knockdown of DDX17 resulted in an approximately 30% decrease in the luciferase activity of RR cells ($p = 0.05$) while causing no significant change in the luciferase activity of RU cells. (B) The same experiment was performed in the RR and RU cells of ZR751. All of the experiments were performed in triplicates.

3.5 DDX17 regulates the DNA binding property of Sox2 in the RR cell subset:

To further support the fact that DDX17 regulates Sox2 transcription activity in only RR cells, we used a biotinylated SRR2 probe carrying the Sox2 consensus sequence to evaluate the DNA binding ability of Sox2. By using streptavidin-conjugated beads to pull down SRR2 and bound proteins, we found that Sox2 as well as DDX17 was bound to SRR2 only in the nuclear fraction of RR cells. Of note, we used an SRR2 mutation to negate the binding of both DDX17 and Sox2 to the SRR2 probe (Figure 3.6A). As shown in Figure 3.6B, we found that the siRNA knockdown of DDX17 induced an observable decrease in the binding of Sox2 to the SRR2 probe. To validate these results, we performed ChIP-qPCR analysis and used an SRR2 promoter; consequently, we found a significant decrease in Sox2 binding to the SRR2 promoter in RR cells (Figure 3.6C). To further support our ChIP-qPCR results, we also used two promoters of Sox2 target genes, Muc15 and Lgr5, and found that these genes were significantly decreased binding to their promoters in RR cells as compared to RU cells (Figure 3.6C).

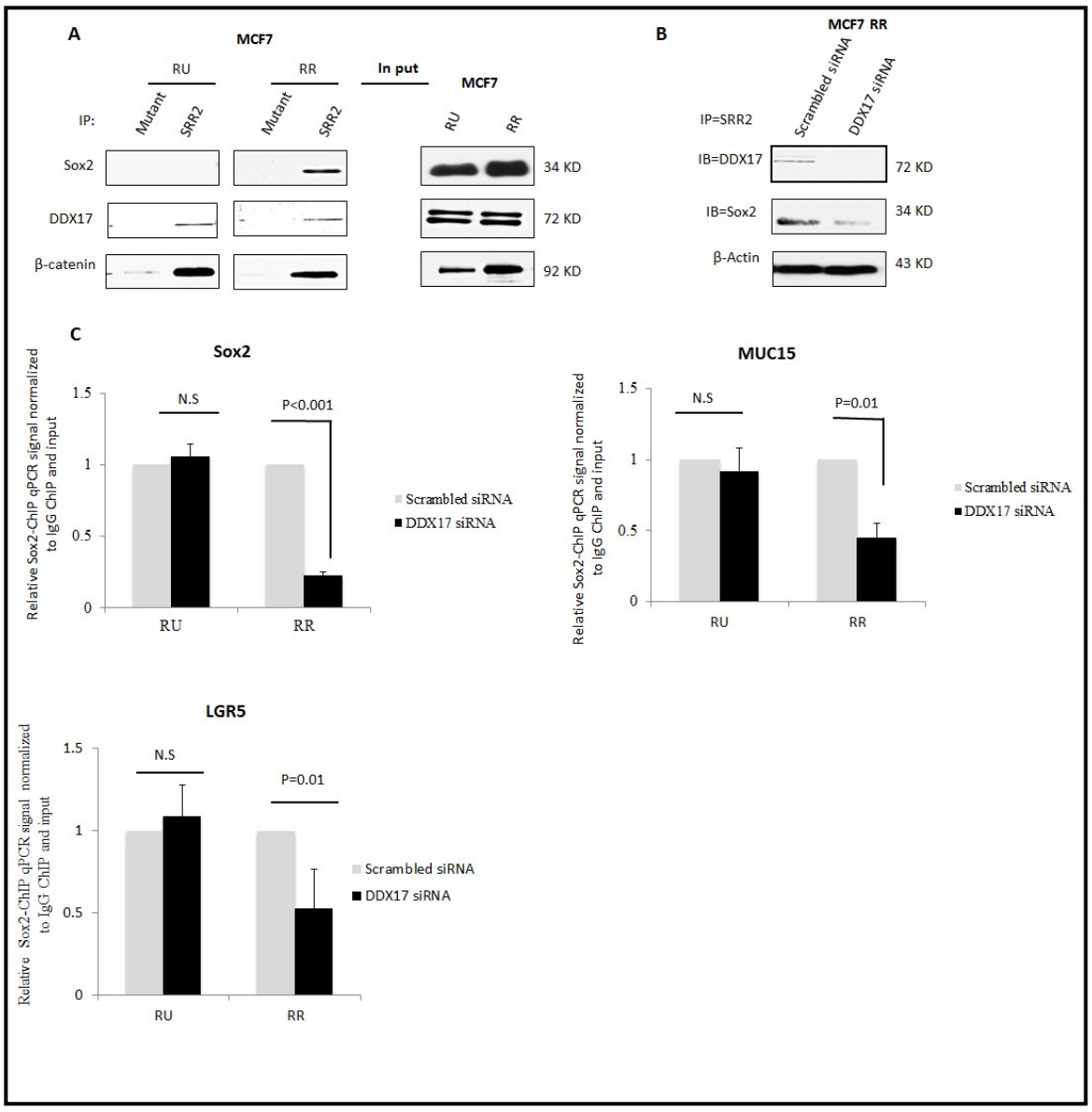

Figure 3.6: DDX17 bound to the Sox2 promoter and regulated Sox2 transcription activity. (A) The binding between DDX17 and SRR2 probe was performed by co-immunoprecipitation in the nuclear fraction of RR and RU cells. DDX17 was detected in both RU and RR cells while Sox2 was detected only in RR cells. (B) The knockdown of DDX17 decreased the binding of Sox2 to the SRR2 probe in RR cells. (C) A ChIP-qPCR experiment was performed after the siRNA knockdown of DDX17 and used promoter-specific primers for Sox2, Muc15 and Lgr5. The results were normalized to the IgG signal as well as to the RU and RR input signal.

3.6 DDX17 knockdown results in different gene expression patterns in RU and RR cells:

Since we determined that DDX17 increases the transcriptional activity of Sox2 in only RR cells, we expected that the modulation of DDX17 would affect the downstream target of Sox2 differently in the two cell subsets. In performing qRT-PCR analysis, we selected genes that are up-regulated by Sox2, including CCND1 (Cyclin D1) (33), MUC15, and CD133 (65), Consequently, we found that DDX17 knockdown significantly decreased the expression of these genes in RR cells. In addition, the same experimental manipulation slightly increased the expression of these genes in RU cells (Figure 3.7A). To further confirm our qRT-PCR findings, we used western blot analysis to validate the protein levels of these genes, and found a reduction of their expression in RR cells (Figure 3.7B).

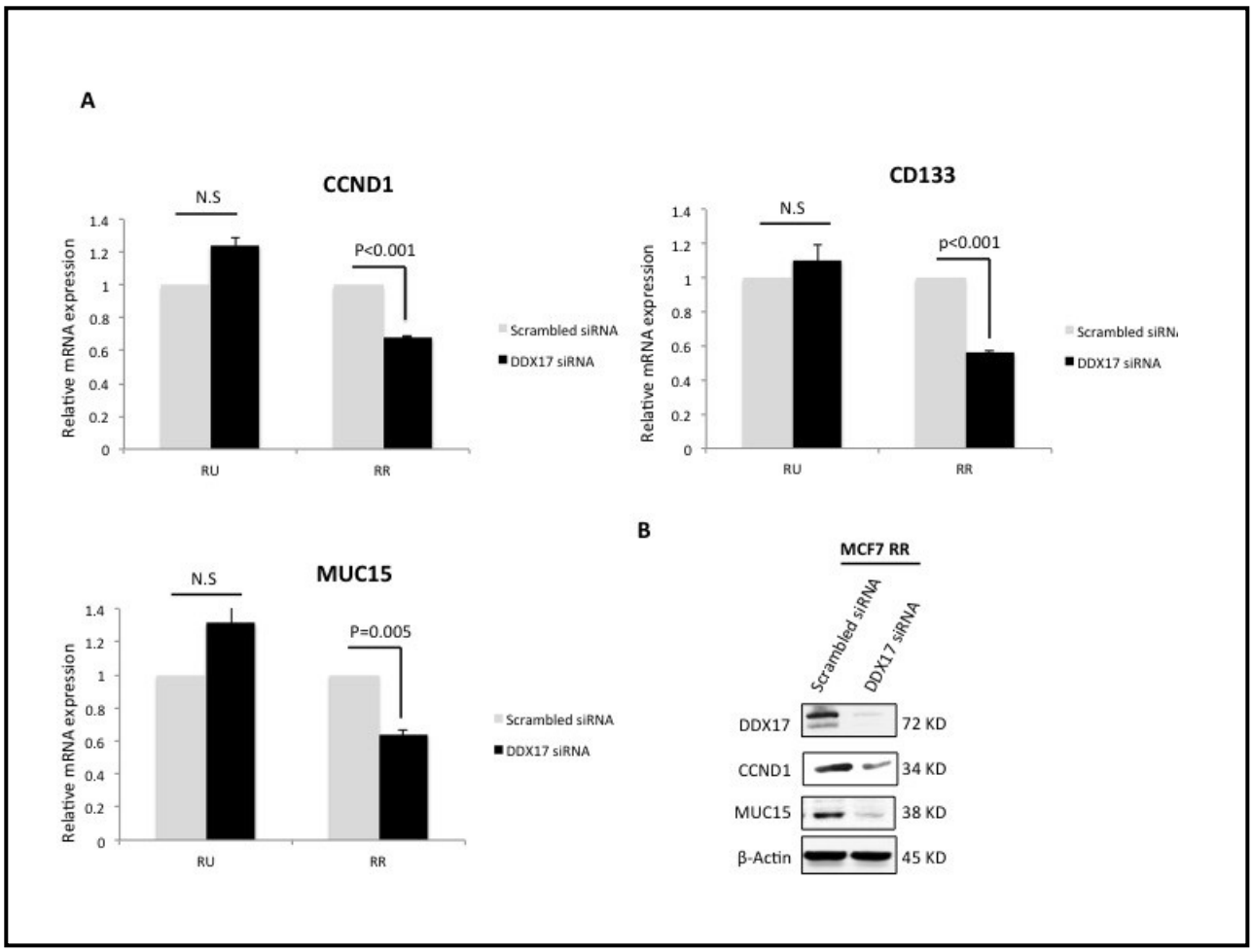

Figure 3.7: DDX17 knockdown results in different gene expression patterns in RR and RU cells. (A) The siRNA knockdown of DDX17 was performed in RR and RU cells derived from MCF7 for 48 h. The mRNA expression levels of Sox2, CyclinD1, and CD133 were measured by RT-PCR. (B) The same experiment was performed in ZR751 RU and RR cells.

3.7 DDX17 inhibition differentially regulates tumorigenic properties in RR and RU cells:

Based on our observation that DDX17 can regulates Sox2, we expected that the inhibition of DDX17 would differentially regulate the phenotypic differences in RR and RU cells, such as mammosphere formation and colony formation. As shown in Figure 3.8A, we performed a clonogenic assay and found that DDX17 knockdown significantly reduced the number of colonies in MCF7 RR cells while no significant change occurred in RU cells. Moreover, as shown in Figure 3.8B, we performed a mammosphere formation and found that DDX17 knockdown significantly downregulated mammosphere formation in MCF7 RR cells while no significant change occurred in RU cells.

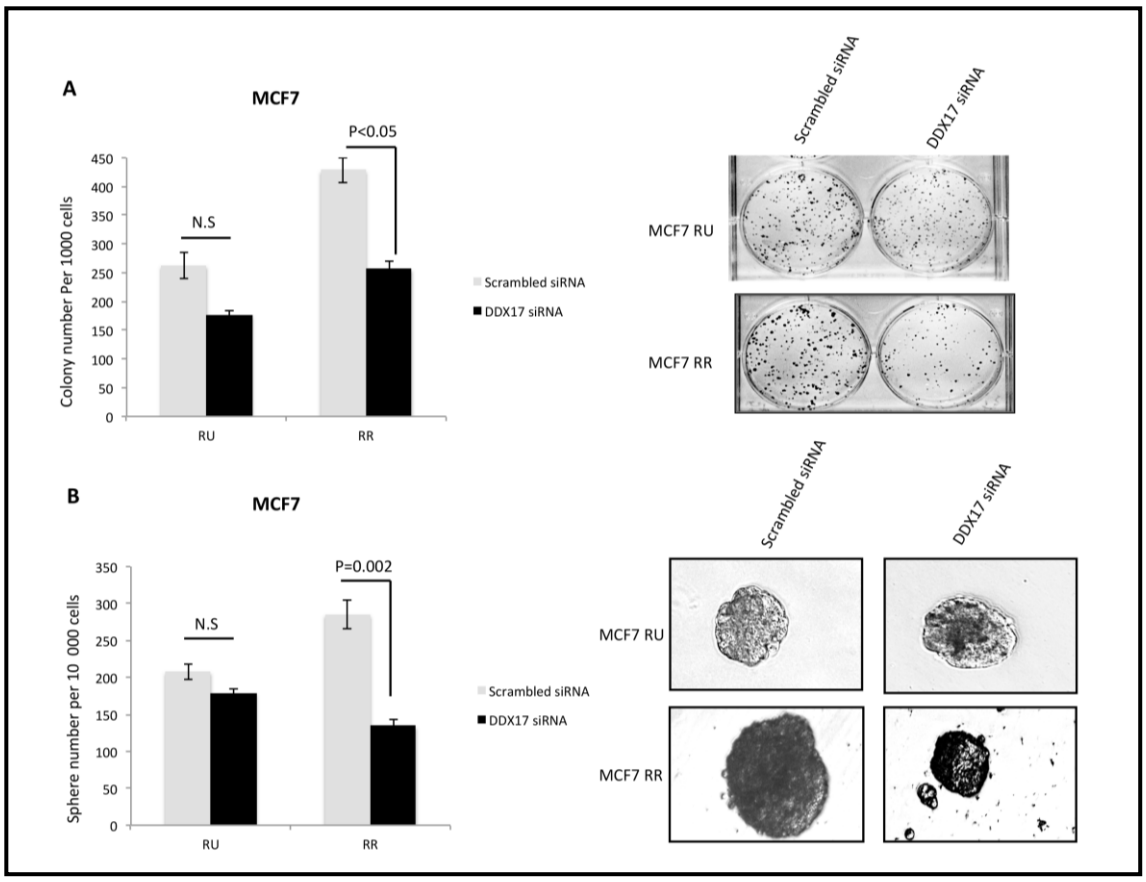

Figure 3.8: DDX17 knockdown causes different changes in the tumorigenic properties of RR and RU cells. (A, B) A clonogenic assay and mammosphere were performed after RR and RU cells were treated to either scrambled siRNA or DDX17 siRNA for 48 h.

CHAPTER 4: Discussion

4.1 Discussion:

Sox2 functions as an ESC marker, which comprises one of the essential transcription factors that fulfills an important role in maintaining pluripotency and self-renewal (35, 36) as well as the generation of inducible pluripotent stem cells (37). Moreover, the inactivation of Sox2 leads to embryonic lethality, which highlights the importance of Sox2 in embryogenesis. On the other hand, Sox2 is expressed in several solid tumors, including lung (47), gastric (48), pancreatic (49) prostate (50) and BC (33, 51, 52). However, few studies have explored the role of Sox2 in cancer development. Clinically, Sox2 expression is associated with poor outcomes in BC patients (52).

Cancer stem cells, also named cancer stem-like cells or tumor-initiating cells, have the ability to rapidly proliferate and invade tissues. Sox2 expression is reactivated during tumor generation (51). As previously mentioned, research has confirmed that Sox2 promotes cellular proliferation (50, 53), invasion, and metastasis in different types of cancers (54, 55). The biological roles of Sox2 in cancer remain only partially understood. In this study, I explored whether the mechanism of Sox2 in regulating ESC genes resembles that in BC. To this end, a previous study from our laboratory reported that Sox2 transcription activity demonstrated phenotypic heterogeneity in ER^+ BC cell lines, MCF7 and ZR751, despite their homogeneous

Sox2 expression (61). By using a lentiviral Sox2 transcription activity reporter, we detected a phenotypically distinct, tumorigenic, and stem-like cell subset with high Sox2 transcription activity, known as the RR cells (61). In contrast, the rest of the cells, known as the RU cells, demonstrated low or absent levels of Sox2 transcription activity. In conjunction, these results highlighted the biological importance of Sox2 transcription activity in the development of breast tumors.

In this study, we found that DDX17 binds with Sox2 in only MCF7 RR cells by using liquid chromatography-mass spectrometry and confirmed their binding through a co-immunoprecipitation experiment. This result corresponds with a previous study in which Sox2 binds with DDX17 in LN1299, a glioblastoma cell line, by using IP-mass spectrometry (93). Interestingly, the intracellular localization between Sox2 and DDX17 occurred only in the nuclear fraction of RR cells. This finding is consistent with a previous study that reported the localization of DDX17 in the nucleus of colon cancer (92). In addition, DDX17 is a nuclear protein (81).

As a co-activator of transcription factors, DDX17 has been involved in the pathogenesis of cancer, including colon cancer (93) and BC (90). Specifically, a previous study has revealed the role of DDX17 as a transcriptional co-activator for ER-α in MCF7 and ZR751 BC cells (89). Furthermore, inhibition of DDX17

significantly reduces the expression of estrogen-regulated genes, *pS2*, and *cathepsin D*, in the level of mRNA (90). This thesis shows that DDX17 regulates Sox2 transcription activity in MCF7 and ZR751 RR cells. In order to support the finding that DDX17 also regulates Sox2 target genes, I identified that the knockdown of DDX17 significantly decreases the expression of the Sox2 target genes, *Cyclin D1, CD133* and *MUC15*, in RR cells. In contrast, the expression of these two genes slightly increased in RU cells, which entailed a rather unexpected finding. One possible explanation for this result may involve the fact that other transcriptional factors can regulate the expression of these genes.

Prior to this investigation, existing research had not examined the biological function of DDX17 binding to Sox2 in BC. These findings show that DDX17 regulates Sox2 transcription activity in MCF7 RR cells. In particular, the knockdown of DDX17 resulted in decreased luciferase expression in only RR cells while exhibiting a lack of change in the Sox2 protein level. A previous study has shown that the siRNA knockdown of both DDX5 and DDX17 inhibits cell proliferation and decreases the cell's ability to form tumors in colon cancer cells *(92)*. In the present study, I have shown that the siRNA knockdown of DDX17 resulted in decreased colony numbers and reduced mammosphere formation in MCF7 RR cells. The unique contribution of this study demonstrated, for the first

time, that the transcriptional activity of Sox2 is positively regulated by DDX17 in ER^+ BC cell lines.

4.2 Future directions and conclusion:

Future studies are required to investigate the individual roles of DDX17 (P72) and its highly related isoform p82, since no existing studies differentiate between them. Furthermore, additional investigations are needed to confirm that DDX17 promotes tumorigenesis. To this end, *in vivo* studies of knockout mice can be used to examine if a knockdown of DDX17 can suppress tumorigenesis. In addition, investigations with knock-in mice could examine the relationship between overexpression and tumorigenesis.

In conclusion, the data of this study has identified DDX17 as a Sox2 binding partner in ER^+ BC cells. This work represents a novel study showing that DDX17 activates the transcriptional activity of Sox2 in a small subset of BC cells (Figure 4.1).

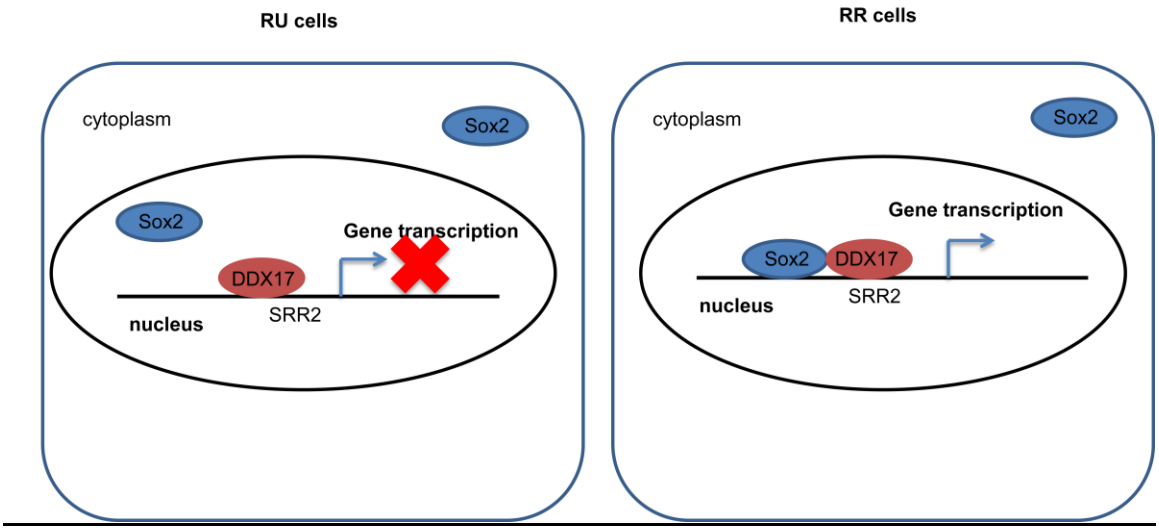

Figure 4.1. A model depicting the relationship between DDX17 and Sox2 in ER+ BC cell lines. In RU cells, DDX17 did not bind Sox2 and led to be Sox2 transcriptional inactive while in RR cells DDx17 did bind to Sox2 and led to be Sox2 transcriptional active.

.

Reference:

1. Xu Y-C, Wang H-X, Tang L, Ma Y, Zhang F-C. A Systematic Review of Vinorelbine for the Treatment of Breast Cancer. The Breast Journal. 2013:n/a-n/a.
2. Karmakar S, Chaudhuri T, Nath A, Neyaz Z. Male breast cancer presenting with bilateral pleural effusion. North American Journal of Medical Sciences. 2013;5(1):72-4.
3. Nahleh ZA, Srikantiah R, Safa M, Jazieh A-R, Muhleman A, Komrokji R. Male breast cancer in the veterans affairs population. Cancer. 2007;109(8):1471-7.
4. Canadian Cancer Statistics 2015 Toronto, ON: Canadian Cancer Society; 2015 [updated June 2015; cited 2015].
5. Lengerke C, Fehm T, Kurth R, Neubauer H, Scheble V, Müller F, et al. Expression of the embryonic stem cell marker SOX2 in early-stage breast carcinoma. BMC Cancer. 2011;11:42-.
6. Viale G. The current state of breast cancer classification. Annals of Oncology. 2012;23(suppl 10):x207-x10.
7. Eroles P, Bosch A, Alejandro Pérez-Fidalgo J, Lluch A. Molecular biology in breast cancer: Intrinsic subtypes and signaling pathways. Cancer Treatment Reviews. 2012;38(6):698-707.
8. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-52.
9. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proceedings of the National Academy of Sciences. 2001;98(19):10869-74.
10. Zhao X, Rodland E, Tibshirani R, Plevritis S. Molecular subtyping for clinically defined breast cancer subgroups. Breast Cancer Research. 2015;17(1):29.
11. Inic Z, Zegarac M, Inic M, Markovic I, Kozomara Z, Djurisic I, et al. Difference between Luminal A and Luminal B Subtypes According to Ki-67, Tumor Size, and Progesterone Receptor Negativity Providing Prognostic Information. Clinical Medicine Insights Oncology. 2014;8:107-11.
12. Yersal O, Barutca S. Biological subtypes of breast cancer: Prognostic and therapeutic implications. World Journal of Clinical Oncology. 2014;5(3):412-24.
13. Carey LA, Perou CM, Livasy CA, et al. RAce, breast cancer subtypes, and survival in the carolina breast cancer study. JAMA. 2006;295(21):2492-502.
14. Cimino-Mathews A, Subhawong AP, Illei PB, Sharma R, Halushka MK, Vang R, et al. GATA3 expression in breast carcinoma: utility in triple-negative, sarcomatoid, and metastatic carcinomas(,). Human pathology. 2013;44(7):1341-9.
15. Kennecke H, Yerushalmi R, Woods R, Cheang MCU, Voduc D, Speers CH, et al. Metastatic Behavior of Breast Cancer Subtypes. Journal of Clinical Oncology. 2010;28(20):3271-7.
16. Guarneri V, Conte P. Metastatic Breast Cancer: Therapeutic Options According to Molecular Subtypes and Prior Adjuvant Therapy. The Oncologist. 2009;14(7):645-56.
17. Creighton CJ. The molecular profile of luminal B breast cancer. Biologics : Targets & Therapy. 2012;6:289-97.

18. Cheang MCU, Chia SK, Voduc D, Gao D, Leung S, Snider J, et al. Ki67 Index, HER2 Status, and Prognosis of Patients With Luminal B Breast Cancer. Journal of the National Cancer Institute. 2009;101(10):736-50.
19. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A Multigene Assay to Predict Recurrence of Tamoxifen-Treated, Node-Negative Breast Cancer. New England Journal of Medicine. 2004;351(27):2817-26.
20. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, et al. Breast Cancer Molecular Subtypes Respond Differently to Preoperative Chemotherapy. Clinical Cancer Research. 2005;11(16):5678-85.
21. Ignatiadis M, Bedard P, Haibe-Kains B, Haibe-Kains B, Singhal S, Loi S, et al. A Meta-Analysis of Gene Expression Profiling Studies Identifies Clinically Relevant Oncogenic Pathways in Basal-Like Breast Cancer. Cancer Research. 2009;69(24 Supplement):106.
22. Oakman C, Viale G, Di Leo A. Management of triple negative breast cancer. The Breast. 2010;19(5):312-21.
23. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-Negative Breast Cancer: Clinical Features and Patterns of Recurrence. Clinical Cancer Research. 2007;13(15):4429-34.
24. Haffty BG, Yang Q, Reiss M, Kearney T, Higgins SA, Weidhaas J, et al. Locoregional Relapse and Distant Metastasis in Conservatively Managed Triple Negative Early-Stage Breast Cancer. Journal of Clinical Oncology. 2006;24(36):5652-7.
25. Prat A, Carey LA, Adamo B, Vidal M, Tabernero J, Cortés J, et al. Molecular Features and Survival Outcomes of the Intrinsic Subtypes Within HER2-Positive Breast Cancer. Journal of the National Cancer Institute. 2014;106(8).
26. Tsutsui S, Ohno S, Murakami S, Kataoka A, Kinoshita J, Hachitanda Y. Prognostic significance of the coexpression of p53 protein and c-erbB2 in breast cancer. The American Journal of Surgery. 2003;185(2):165-7.
27. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, et al. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Annals of Oncology. 2013;24(9):2206-23.
28. Sarkar A, Hochedlinger K. The Sox Family of Transcription Factors: Versatile Regulators of Stem and Progenitor Cell Fate. Cell Stem Cell. 2013;12(1):15-30.
29. Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, et al. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature. 1990;346(6281):240-4.
30. Gubbay J, Koopman P, Collignon J, Burgoyne P, Lovell-Badge R. Development. 1990;109:647-53.
31. Schepers GE, Teasdale RD, Koopman P. Twenty Pairs of Sox: Extent, Homology, and Nomenclature of the Mouse and Human Sox Transcription Factor Gene Families. Developmental Cell. 2002;3(2):167-70.
32. Wegner M. From head to toes: The multiple facets of Sox proteins. Nucleic Acids Research. 1999;27(6):1409-20.
33. Chen Y, Shi L, Zhang L, Li R, Liang J, Yu W, et al. The Molecular Mechanism Governing the Oncogenic Potential of SOX2 in Breast Cancer. Journal of Biological Chemistry. 2008;283(26):17969-78.

34. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes & Development. 2003;17(1):126-40.
35. Adachi K, Suemori H, Yasuda SY, Nakatsuji N, Kawase E. Role of SOX2 in maintaining pluripotency of human embryonic stem cells. Genes Cells. 2010;15(5):455-70.
36. Li Y-Q. Master Stem Cell Transcription Factors and Signaling Regulation. Cellular Reprogramming. 2010;12(1):3-13.
37. Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell. 2006;126(4):663-76.
38. Jeong C-H, Cho Y-Y, Kim M-O, Kim S-H, Cho E-J, Lee S-Y, et al. Phosphorylation of Sox2 Cooperates in Reprogramming to Pluripotent Stem Cells. STEM CELLS. 2010;28(12):2141-50.
39. Lai Y-S, Chang C-W, Pawlik KM, Zhou D, Renfrow MB, Townes TM. SRY (sex determining region Y)-box2 (Sox2)/poly ADP-ribose polymerase 1 (Parp1) complexes regulate pluripotency. Proceedings of the National Academy of Sciences. 2012;109(10):3772-7.
40. Arnold K, Sarkar A, Yram Mary A, Polo Jose M, Bronson R, Sengupta S, et al. Sox2+ Adult Stem and Progenitor Cells Are Important for Tissue Regeneration and Survival of Mice. Cell Stem Cell. 2011;9(4):317-29.
41. Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, et al. Sox2(+) adult stem/progenitor cells are important for tissue regeneration and survival of mice. Cell stem cell. 2011;9(4):317-29.
42. Simões B, Piva M, Iriondo O, Comaills V, López-Ruiz J, Zabalza I, et al. Effects of estrogen on the proportion of stem cells in the breast. Breast Cancer Res Treat. 2011;129(1):23-35.
43. Stevanovic M, Zuffardi O, Collignon J, Lovell-Badge R, Goodfellow P. The cDNA sequence and chromosomal location of the human SOX2 gene. Mammalian Genome. 1994;5(10):640-2.
44. Weina K, Utikal J. SOX2 and cancer: current research and its implications in the clinic. Clinical and Translational Medicine. 2014;3(1):19.
45. Wilson M, Koopman P. Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators. Current Opinion in Genetics & Development. 2002;12(4):441-6.
46. Wiebe MS, Nowling TK, Rizzino A. Identification of Novel Domains within Sox-2 and Sox-11 Involved in Autoinhibition of DNA Binding and Partnership Specificity. Journal of Biological Chemistry. 2003;278(20):17901-11.
47. Rudin CM, Durinck S, Stawiski EW, Poirier JT, Modrusan Z, Shames DS, et al. Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. Nat Genet. 2012;44(10):1111-6.
48. Hütz K, Mejías-Luque R, Farsakova K, Ogris M, Krebs S, Anton M, et al. The stem cell factor SOX2 regulates the tumorigenic potential in human gastric cancer cells. Carcinogenesis. 2014;35(4):942-50.
49. Herreros-Villanueva M, Zhang JS, Koenig A, Abel EV, Smyrk TC, Bamlet WR, et al. SOX2 promotes dedifferentiation and imparts stem cell-like features to pancreatic cancer cells. Oncogenesis. 2013;2:e61.

50. Jia X, Li X, Xu Y, Zhang S, Mou W, Liu Y, et al. SOX2 promotes tumorigenesis and increases the anti-apoptotic property of human prostate cancer cell. Journal of Molecular Cell Biology. 2011;3(4):230-8.
51. Leis O, Eguiara A, Lopez-Arribillaga E, Alberdi MJ, Hernandez-Garcia S, Elorriaga K, et al. Sox2 expression in breast tumours and activation in breast cancer stem cells. Oncogene. 2012;31(11):1354-65.
52. Lengerke C, Fehm T, Kurth R, Neubauer H, Scheble V, Muller F, et al. Expression of the embryonic stem cell marker SOX2 in early-stage breast carcinoma. BMC Cancer. 2011;11(1):42.
53. Stolzenburg S, Rots MG, Beltran AS, Rivenbark AG, Yuan X, Qian H, et al. Targeted silencing of the oncogenic transcription factor SOX2 in breast cancer. Nucleic Acids Research. 2012;40(14):6725-40.
54. Sun C, Sun L, Li Y, Kang X, Zhang S, Liu Y. Sox2 expression predicts poor survival of hepatocellular carcinoma patients and it promotes liver cancer cell invasion by activating Slug. Med Oncol. 2013;30(2):1-10.
55. Han X, Fang X, Lou X, Hua D, Ding W, Foltz G, et al. Silencing SOX2 Induced Mesenchymal-Epithelial Transition and Its Expression Predicts Liver and Lymph Node Metastasis of CRC Patients. PLoS ONE. 2012;7(8):e41335.
56. Chou Y-T, Lee C-C, Hsiao S-H, Lin S-E, Lin S-C, Chung C-H, et al. The Emerging Role of SOX2 in Cell Proliferation and Survival and Its Crosstalk with Oncogenic Signaling in Lung Cancer. STEM CELLS. 2013;31(12):2607-19.
57. Alonso MM, Diez-Valle R, Manterola L, Rubio A, Liu D, Cortes-Santiago N, et al. Genetic and epigenetic modifications of Sox2 contribute to the invasive phenotype of malignant gliomas. PloS one. 2011;6(11).
58. Sholl LM, Barletta JA, Yeap BY, Chirieac LR, Hornick JL. SOX2 PROTEIN EXPRESSION IS AN INDEPENDENT POOR PROGNOSTIC INDICATOR IN STAGE I LUNG ADENOCARCINOMA. The American journal of surgical pathology. 2010;34(8):1193-8.
59. WANG Q, HE W, LU C, WANG Z, WANG J, GIERCKSKY KE, et al. Oct3/4 and Sox2 Are Significantly Associated with an Unfavorable Clinical Outcome in Human Esophageal Squamous Cell Carcinoma. Anticancer Research. 2009;29(4):1233-41.
60. Rodriguez-Pinilla SM, Sarrio D, Moreno-Bueno G, Rodriguez-Gil Y, Martinez MA, Hernandez L, et al. Sox2: a possible driver of the basal-like phenotype in sporadic breast cancer. Mod Pathol. 2007;20(4):474-81.
61. Wu F, Zhang J, Wang P, Ye X, Jung K, Bone KM, et al. Identification of two novel phenotypically distinct breast cancer cell subsets based on Sox2 transcription activity. Cellular Signalling. 2012;24(11):1989-98.
62. Tomioka M, Nishimoto M, Miyagi S, Katayanagi T, Fukui N, Niwa H, et al. Identification of Sox‐2 regulatory region which is under the control of Oct‐3/4‐Sox‐2 complex. Nucleic Acids Research. 2002;30(14):3202-13.
63. Jung K, Wu F, Wang P, Ye X, Abdulkarim BS, Lai R. YB-1 regulates Sox2 to coordinately sustain stemness and tumorigenic properties in a phenotypically distinct subset of breast cancer cells. BMC Cancer. 2014;14:328-.
64. Ye X, Wu F, Wu C, Wang P, Jung K, Gopal K, et al. β-Catenin, a Sox2 binding partner, regulates the DNA binding and transcriptional activity of Sox2 in breast cancer cells. Cellular Signalling. 2014;26(3):492-501.

65. Jung K, Wang P, Gupta N, Gopal K, Wu F, Ye X, et al. Profiling gene promoter occupancy of Sox2 in two phenotypically distinct breast cancer cell subsets using chromatin immunoprecipitation and genome-wide promoter microarrays. Breast Cancer Research : BCR. 2014;16(6):470.
66. Iglesias JM, Leis Esnaola O, Perez Ruiz E, Gumuzio Barrie J, Garcia Garcia F, Aduriz A, et al. The activation of the Sox2 RR2 pluripotency transcriptional reporter in human breast cancer cell lines is dynamic and labels cells with higher tumorigenic potential. Frontiers in Oncology. 2014;4.
67. Liang S, Furuhashi M, Nakane R, Nakazawa S, Goudarzi H, Hamada J-i, et al. Isolation and characterization of human breast cancer cells with SOX2 promoter activity. Biochemical and Biophysical Research Communications. 2013;437(2):205-11.
68. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a Cancer Stem Cell in Human Brain Tumors. Cancer Research. 2003;63(18):5821-8.
69. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature. 2007;445(7123):111-5.
70. Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, et al. Core Transcriptional Regulatory Circuitry in Human Embryonic Stem Cells. Cell. 2005;122(6):947-56.
71. Kamachi Y, Uchikawa M, Kondoh H. Pairing SOX off: with partners in the regulation of embryonic development. Trends in Genetics. 2000;16(4):182-7.
72. Kamachi Y, Sockanathan S, Liu Q, Breitman M, Lovell-Badge R, Kondoh H. Involvement of SOX proteins in lens-specific activation of crystallin genes. The EMBO Journal. 1995;14(14):3510-9.
73. Ambrosetti DC, Basilico C, Dailey L. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. Molecular and Cellular Biology. 1997;17(11):6321-9.
74. Yuan H, Corbi N, Basilico C, Dailey L. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes & Development. 1995;9(21):2635-45.
75. Nishimoto M, Fukushima A, Okuda A, Muramatsu M. The Gene for the Embryonic Stem Cell Coactivator UTF1 Carries a Regulatory Element Which Selectively Interacts with a Complex Composed of Oct-3/4 and Sox-2. Molecular and Cellular Biology. 1999;19(8):5453-65.
76. Linder P, Lasko PF, Ashburner M, Leroy P, Nielsen PJ, Nishi K, et al. Birth of the D-E-A-D box. Nature. 1989;337(6203):121-2.
77. Fairman-Williams ME, Guenther U-P, Jankowsky E. SF1 and SF2 helicases: family matters. Current Opinion in Structural Biology. 2010;20(3):313-24.
78. Fuller-Pace Frances V, Ali S. The DEAD box RNA helicases p68 (Ddx5) and p72 (Ddx17): novel transcriptional co-regulators. Biochemical Society Transactions. 2008;36(4):609-12.
79. Fuller-Pace FV. DExD/H box RNA helicases: multifunctional proteins with important roles in transcriptional regulation. Nucleic Acids Research. 2006;34(15):4206-15.
80. Abdelhaleem M. Do human RNA helicases have a role in cancer? Biochimica et Biophysica Acta (BBA) - Reviews on Cancer. 2004;1704(1):37-46.

81. Lamm GM, Nicol SM, Fuller-Pace FV, Lamond AI. p72: A Human Nuclear DEAD Box Protein Highly Related to p68. Nucleic Acids Research. 1996;24(19):3739-47.
82. Uhlmann-Schiffler H, Rössler OG, Stahl H. The mRNA of DEAD Box Protein p72 Is Alternatively Translated into an 82-kDa RNA Helicase. Journal of Biological Chemistry. 2002;277(2):1066-75.
83. Bleichert F, Baserga SJ. The Long Unwinding Road of RNA Helicases. Molecular Cell. 2007;27(3):339-52.
84. Linder P. Dead-box proteins: a family affair—active and passive players in RNP-remodeling. Nucleic Acids Research. 2006;34(15):4168-80.
85. Lee C-G. RH70, a Bidirectional RNA Helicase, Co-purifies with U1snRNP. Journal of Biological Chemistry. 2002;277(42):39679-83.
86. Rössler OG, Straka A, Stahl H. Rearrangement of structured RNA via branch migration structures catalysed by the highly related DEAD-box proteins p68 and p72. Nucleic Acids Research. 2001;29(10):2088-96.
87. Hönig A, Auboeuf D, Parker MM, O'Malley BW, Berget SM. Regulation of Alternative Splicing by the ATP-Dependent DEAD-Box RNA Helicase p72. Molecular and Cellular Biology. 2002;22(16):5698-707.
88. Dardenne E, Polay Espinoza M, Fattet L, Germann S, Lambert M-P, Neil H, et al. RNA Helicases DDX5 and DDX17 Dynamically Orchestrate Transcription, miRNA, and Splicing Programs in Cell Differentiation. Cell Reports. 2014;7(6):1900-13.
89. Wortham NC, Ahamed E, Nicol SM, Thomas RS, Periyasamy M, Jiang J, et al. The DEAD-box protein p72 regulates ER[alpha]-/oestrogen-dependent transcription and cell growth, and is associated with improved survival in ER[alpha]-positive breast cancer. Oncogene. 2009;28(46):4053-64.
90. Yager JD, Davidson NE. Estrogen Carcinogenesis in Breast Cancer. New England Journal of Medicine. 2006;354(3):270-82.
91. Bates GJ, Nicol SM, Wilson BJ, Jacobs AMF, Bourdon JC, Wardrop J, et al. The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumour suppressor2005 2005-02-09 00:00:00. 543-53 p.
92. Shin S, Rossow KL, Grande JP, Janknecht R. Involvement of RNA Helicases p68 and p72 in Colon Cancer. Cancer Research. 2007;67(16):7572-8.
93. Fang X, Yoon JG, Li L, Tsai YS, Zheng S, Hood L, et al. Landscape of the SOX2 protein-protein interactome. Proteomics. 2011;11(5):921-34.