Receptor Selective Coactivators: Characterization of a Novel Protein-Protein Interaction Module in Steroid Hormone Receptor Signaling

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RECEPTOR SELECTIVE COACTIVATORS:
CHARACTERIZATION OF A NOVEL PROTEIN-PROTEIN INTERACTION
MODULE IN STEROID HORMONE RECEPTOR SIGNALING

By

Sarath C. Dhananjayan

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

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Receptor Selective Coactivators: Characterization of a Novel Protein-Protein Interaction Module in Steroid Hormone Receptor Signaling

Abstract of a dissertation at the University of Miami.

Dissertation supervised by Zafar Nawaz PhD.
No. of pages in text. (121)

WW-domain binding protein-2 (WBP-2) was cloned as an E6-associated protein (E6-AP) interacting protein and its role in steroid hormone receptor (SHR) function was investigated. We show that WBP-2 differs from other SHR coactivators, as it specifically enhanced the transactivation functions of progesterone receptor (PR) and estrogen receptor (ERα), whereas it had no significant effect on the androgen receptor, glucocorticoid receptor or the activation functions of p53 or VP-16. We also demonstrated that, like other well characterized coactivators, WBP-2 contains an intrinsic activation domain. Depletion of endogenous WBP-2 with small interfering RNAs indicated that normal physiological protein level of WBP-2 was required for the proper functioning of ERα and PR. Moreover, chromatin immunoprecipitation (ChIP) assays demonstrate the hormone-dependent recruitment of WBP-2 onto an estrogen-responsive promoter. As we initially identified WBP-2 as an E6-AP interacting protein, we investigated whether WBP-2 and E6-AP function in concert. Our data shows that WBP-2 and E6-AP each enhance PR function and when co-expressed they additively enhance the transactivation functions of PR.
However, WBP-2 was also able to enhance the transactivation functions of ERα and PR in mouse embryonic fibroblast cells generated from E6-AP knockout mice lines, suggesting that the coactivation functions of WBP-2 was not dependent on E6-AP.

The further elucidate the molecular mechanism of action of WBP-2; we dissected the functional importance of the polyproline (PY) motifs contained within the WBP-2 protein. Mutational analysis suggests that one of three PY motifs, PY3 of WBP-2 was essential for its coactivation and intrinsic activation functions. In this study, we also demonstrate that the WBP-2 binding protein, Yes-kinase associated protein 1 (YAP1) acts as a secondary coactivator of ERα and PR. However, the coactivation function of YAP1 is revealed only in the presence of wild-type WBP-2 and not with the PY motif 3 mutant WBP-2. This is consistent with our observations that, unlike the wild-type WBP-2, the PY motif 3 mutant WBP-2 does not interact with YAP1.

Our quantitative reChIP assays demonstrates an estrogen-dependent recruitment and association of ERα with both WBP-2 and YAP1. The hormone-dependent recruitment of YAP1 to ERα responsive promoter is dependent on the physiological expression levels of WBP-2. This is consistent with, our observation that the coactivation functions of YAP1 is dependent on WBP-2, and is also in agreement with other known secondary coactivators that get recruited to SHR responsive promoter via their interaction with primary coactivators. Surprisingly, the association of WBP-2 with ERα and its recruitment to the ERα target promoter was abrogated by YAP1 knock-down, suggesting that WBP-2 and
YAP1 may stabilize each other at the promoter, and consequently, are functionally interdependent. Taken together our data establish the role of WBP-2 and YAP1 as selective coactivators for ERα and PR transactivation pathways.
ACKNOWLEDGEMENTS

This work would not have been possible without the support and guidance of my mentor, Dr. Zafar Nawaz. I am forever indebted to Dr. Nawaz for helping me learn how to be a better scientist and for always being optimistic and supportive throughout this research project. I want to thank my committee members, Dr. Kerry Burnstein, Dr. Amjad Farooq and Dr. Joyce Slingerland for their invaluable guidance and fruitful discussions during this thesis work. I would like to take this opportunity to thank all the past Nawaz lab members, especially Dr. Obaid Y. Khan for his patience and help during my lab rotation days and the initial part of my research project. I also thank my colleagues Sathish Srinivasan and Heath Catoe for making my hours in the lab enjoyable through their socio-economic and sometimes scientific chit-chats. I would like to acknowledge the Department of Defense for awarding me a pre-doctoral traineeship and the NIH and the Braman Family Breast Cancer Institute for funding Dr. Nawaz, without which this project would not have been possible.

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There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.

J.R.R. Tolkien
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ACRONYMS

AF-1  Activation Function 1
AF-2  Activation Function 2
AR    Androgen receptor
DBD   DNA Binding Domain
E6-AP E6-Associated Protein
ER    Estrogen Receptor
ERE   Estrogen Response Element
GR    Glucocorticoid Receptor
HRE   Hormone Response Element
LBD   Ligand Binding Domain
MR    Mineralocorticoid Receptor
PR    Progesterone Receptor
PRE   Progesterone Response Element
PY motif Proline-Tyrosine conserved polyproline motif
SHR   Steroid Hormone Receptors
TAD   Transcription Activation Domain
WBP-2 WW domain Binding Protein 2
WW domain 2 conserved tryptophan containing domains
YAP1  yes Associated Protein
CHAPTER 1
INTRODUCTION

1.1 STEROID HORMONE RECEPTORS

Steroid hormone receptors (SHR) belong to a large family of functionally related transcription factors known as the steroid and thyroid hormone receptor super-family of proteins. This super-family of receptors comprises of proteins that act as intracellular receptors for lipophilic ligands like steroid hormones, vitamins and thyroid hormones. Since these proteins are intracellular receptor cum transcription factors, ligand binding induces their second messenger functions that ultimately result in transcriptional activation of specific genomic target genes (Tsai and O'Malley, 1994). This section will focus on the key aspects of steroid hormone receptor biology and signaling.

1.1.1 HORMONES

Mammalian steroid hormones can be divided into five groups, estrogens, progestins, androgens, glucocorticoids, and mineralocorticoids. Vitamin D is a closely related sixth. Natural steroid hormones are derivatives of cholesterol and almost all of them contain the same signature cyclopentanophenanthrene ring structure (Figure 1.1). Steroid hormones are primarily lipids, which allow them to cross the plasma membrane and enter the nucleus. These lipophilic hormones are synthesized from cholesterol in primary tissues such as the gonads and the adrenal glands and are then transported via the circulatory system to their
target sites of action via the blood circulation (Jean D. Wilson, 1998). Once they reach the target organs, steroid hormones initiate and regulate a multitude of physiological and cellular processes, some example of which are listed in table 1.1.

**Table 1.1 Physiological roles of steroid hormone receptors**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Group</th>
<th>Principal target tissue(s)</th>
<th>Hormonal function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>Sex Hormones</td>
<td>Uterus, Breast, Vagina, Hypothalamus</td>
<td>Development of female sex characteristics</td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td>Seminal vesicles, Prostate, Testis</td>
<td>Development of male sex characteristics</td>
</tr>
<tr>
<td>Progesterone</td>
<td></td>
<td>Uterus, Oviduct</td>
<td>Maintenance of pregnancy</td>
</tr>
<tr>
<td>Hydrocortisone (cortisol)</td>
<td>Glucocorticoids</td>
<td>All cells</td>
<td>Regulation of energy utilization</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>Mineralocorticoids</td>
<td>Kidney</td>
<td>Regulation of electrolyte balance</td>
</tr>
</tbody>
</table>

Prepared using data collected from Jean D. Wilson, 1998.
Since steroid hormones are lipids they can readily cross the plasma membrane and bind to their cognate intracellular receptors known as steroid hormone receptors. Hormone binding to these receptors induces a conformational change that triggers a series of modifications including receptor phosphorylation and dimerization. Active receptor-dimers then translocate to the nucleus where they bind specific DNA enhancer regions known as hormone response elements (HRE) associated with SHR target genes. Though some target genes are regulated by more than one type of hormone, each hormone has its own set of target genes (Ming-Jer Tsai, 1998; Tsai and O'Malley, 1994).

1.1.2 CLASSIFICATION

SHRs are classified based on their cognate ligand. SHRs are primarily divided into 3 groups; type I - receptors that binds DNA as a homodimer, type II -
receptors that bind as heterodimers and orphan receptors – SHRs for which a natural ligand has not been identified yet. Type I SHRs are further divided into three groups: 1) sex hormone receptors, including estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR); 2) glucocorticoid receptor (GR); and 3) mineralocorticoid receptor (MR). Type II SHRs are also further classified into three groups: 1) vitamin D receptor (VDR); 2) vitamin A/retinoic acid receptor (RAR) and 3) thyroid hormone receptor (TR). The SHR subgroup includes three orphan receptors, namely, estrogen related receptor α (ERRα), estrogen related receptor β (ERRβ) and estrogen related receptor γ (ERRγ) (Mangelsdorf et al., 1995; Novac and Heinzel, 2004).

1.1.3 STRUCTURAL ORGANIZATION

Our basic knowledge and understanding of the structural organization of SHRs stems from the extensive research conducted on these receptors over the past few decades since their initial cloning.

Steroid hormone receptors are organized into three domains; an amino-terminal A/B domain, a central DNA binding domain and the carboxy terminal ligand binding domain (Figure 1.2). Although this overall structural organization is highly conserved among the various types of SHRs, amino acid sequence comparisons reveal that individual domains can vary to a great extent. As shown in figure 1.3, the DNA binding domain is the most conserved followed by the
ligand binding domain while the A/B domain is the least conserved across different types of steroid hormone receptors. The A/B domain is also known as the hypervariable domain that contains the activation function 1 (AF-1), and is essential for the transcriptional activities of SHRs. This will be discussed in detail in the following section. The ligand binding domain as the name suggests, encompasses the hormone binding pocket. Since the binding pockets are unique to specific ligands, the overall sequence identity of this domain among different SHRs is only moderately conserved. The cysteine-rich DNA binding domain comprises, two zinc-finger motifs that are involved in the recognition and binding of hormone responsive elements. Even though the two zinc-finger motifs are highly conserved, there are a few amino acid sequence variations among the DNA binding domain of various SHRs, as each hormone responsive element is unique to the specific receptor (de Boer et al., 1987; Giguere et al., 1986; Rusconi and Yamamoto, 1987; Wrange and Gustafsson, 1978).

**Figure 1.3: Conserved domains of SHRs.**
1.1.4 **FUNCTIONAL DOMAINS**

SHRs are organized in a modular fashion. Their structure can be dissected into a number of functional domains with specific roles in the biological functions of SHR (Figure 1.4) (Kumar et al., 1987).

![Figure 1.4: Functional domains of SHRs.](image)

**A/B Domain**

The amino-terminal (N-terminal) region of receptors contains the AF-1 (Bocquel et al., 1989; Danielian et al., 1992; Durand et al., 1994; Tasset et al., 1990; Tora et al., 1989; Webster et al., 1988). The size and sequence of the AF-1 varies considerably among different types of steroid receptors. Moreover, many SHR isoforms differ exclusively in their N-terminal regions as a result of alternative splicing and/or alternative promoter usage (Chambon, 1994; Kastner et al., 1990; Leid et al., 1992; Sartorius et al., 1994; Sartorius et al., 1993; Tora et al., 1988). AF-1 region of many receptors including GR, ER\(\alpha\) and \(\beta\), and PR has
been shown to be intrinsically unstructured and disordered. However, when expressed in tandem with the DNA binding domain this region appears to be structured suggesting the involvement of stabilizing intramolecular interactions (Bain et al., 2000; Bain et al., 2001; Dahlman-Wright et al., 1995; Kumar et al., 1987; Warnmark et al., 2001). Though AF-1 by itself has a poor propensity for secondary structure formation, in the presence of α-helix stabilizing compounds like trifluoroethanol, this region shows considerable α-helical properties in structural studies. Proline substitution mutations in this region disrupt, α-helix formation in the presence of trifluoroethanol and its transcriptional activity, suggesting that the formation of an α-helix is essential for AF-1 function (Dahlman-Wright and McEwan, 1996; Warnmark et al., 2000).

The N-terminal region of steroid receptors has an important role in coregulator interactions. Coregulators are accessory proteins that are recruited by SHRs to their target gene promoters to facilitate efficient transcription. Many coregulators have been shown to interact directly with AF-1 (Almlof et al., 1998; Ford et al., 1997; Henriksson et al., 1997; Hittelman et al., 1999).

**DNA Binding Domain (DBD)**

The central DNA binding domain (DBD) of SHRs is the most conserved domain and is responsible for targeting the receptor to their DNA response element. The DBD contains eight cysteine residues that form two coordinate complexes, known as zinc-finger motifs, each comprising of four cysteine residues and a zinc ion. These two zinc fingers form a structure that facilitates the binding of the receptor to the major groove of DNA (Figure 1.5) (Evans, 1988;
Gelmann, 2002). Amino acid residues at the base of the first zinc finger constitute the P-box, the intervening sequence between of the P-box and the second zinc finger constitutes the D-box (Figure 1.5). Both the P- and D-boxes are part of the reading helix, so called because it mediates sequence specific interactions with the hormone response element. The receptor binds DNA as a homodimer or heterodimer with each reading helix of a monomer making specific contacts with the major groove of the DNA sequence that forms one half-site of the hormone response element (Figure 1.6).

Figure 1.5: Twin Zinc-finger motifs in the DBD of SHRs.
Hormone Response Element (HRE)

HREs are cis-acting enhancer DNA sequences associated with receptor target genes that function in a position-independent manner. Generally, HREs comprise of two palindromic hexanucleotide half sites with a variable number of nucleotides in-between (spacer). The dyad symmetry of HREs permit the receptors to bind to these sites as dimers. Even a single base change in HREs can alter or completely abolish hormone responsiveness of the receptor (Chalepakis et al., 1988; Strahle et al., 1987). Consensus HRE sequences have been identified for most of the steroid hormone receptors (Table 1.2).
Table 1.2  Hormone responsive elements

<table>
<thead>
<tr>
<th>Receptor</th>
<th>P-box</th>
<th>D-box</th>
<th>HRE</th>
</tr>
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<tr>
<td>GR, PR, MR and AR</td>
<td>GSCKV</td>
<td>AGRND</td>
<td>AGAACAnnnTGTTCTCTTTGTnnnACAAGA</td>
</tr>
<tr>
<td></td>
<td>GSCKV</td>
<td>ASRND</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>EGCKA</td>
<td>PATNQ</td>
<td>AGGTCAAnnnTGACCTTCCAGTnnnACTGGA</td>
</tr>
<tr>
<td>TRα</td>
<td>EGCKG</td>
<td>KYDSC</td>
<td>AGGTCAATGACCTTCCAGTACTGGA</td>
</tr>
<tr>
<td>TRβ</td>
<td>EGCKG</td>
<td>KYEGK</td>
<td></td>
</tr>
<tr>
<td>RAR α, β &amp; γ</td>
<td>EGCKG</td>
<td>NRDKN</td>
<td>AGGTCAAnnnTGACCTTCCAGTnnnACTGGA</td>
</tr>
</tbody>
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Adapted from (Nawaz, 1992). GR – glucocorticoid receptor; PR – progesterone receptor; MR – mineralocorticoid receptor; AR – androgen receptor; ER – estrogen receptor; TR – thyroid hormone receptor; RAR – retinoic acid receptor.

As is evident from the table, HREs vary significantly in their sequence, number of spacer nucleotides and/or orientation. For instance, the consensus glucocorticoid response element (GRE) is recognized by other receptors including, PR (PRE), AR (ARE) and MR (MRE) (Cato et al., 1986; Ham et al., 1988; Otten et al., 1988; von der Ahe et al., 1985). The consensus estrogen response element (ERE) is similar to, but distinct from the canonical GRE (Brent et al., 1989; Klein-Hitpass et al., 1989; Peale et al., 1988). The thyroid hormone response element (TRE) and retinoic acid receptor response element (RARE), are identical to ERE except for the length of the spacer region (Brent et al., 1989).
**Hinge Domain**

The hinge domain serves as a flexible linker between the DBD and the ligand binding domain (LBD). This region also harbors the nuclear localization signal (NLS) and regulates the subcellular localization of the receptors. Due to its flexibility, this region imparts rotational freedom between the DBD and LBD, which is essential for proper orientation of the DBD toward the DNA major groove (Kumar et al., 1987).

**Ligand Binding Domain (LBD)**

*Figure 1.7: Three-dimensional model of the SHR ligand binding domain. Unliganded LBD (Left panel); Agonist bound LBD (Right panel).*
Forming a major part of the carboxy terminus (C-terminus) of SHRs, the LBD is the hormone binding region of the receptor that is also responsible for dimerization, repression and transactivation functions. Although this region is only moderately conserved, alteration of the amino acid sequence in this region by insertion, deletion or point mutations results in total loss of hormone binding in almost all the characterized SHRs. Supporting this observation, the crystal structures of the receptors for progesterone, vitamin D, retinoid X, retinoic acid, estrogen (α and β) and androgen have shown that despite substantial variations in the amino acid sequence of these receptors (as low as 20% similarity), the three-dimensional structures of the different LBDs are remarkably similar (Brzozowski et al., 1997; Nolte et al., 1998; Shiau et al., 1998; Tanenbaum et al., 1998).

The LBD is comprised of 12 α-helices that form the steroid hormone binding pocket (Figure 1.7). Helix 12 folds over the ligand binding pocket forming a cap-like structure that is critical for stable hormone binding and also for subsequent transactivation functions. This transactivation function is enabled by the presence of the activation function-2 (AF-2) region in the LBD. This region is critical for the hormone-dependent transcriptional activity of the other SHRs (Barettino et al., 1994; Danielian et al., 1992; Durand et al., 1994; Lees et al., 1990; Saatcioglu et al., 1993). Structural models suggest that the positioning of helix 12 of the LBD is critical for the proper functioning of AF-2 (Bledsoe et al., 2002; Bourguet et al., 2000; Kallen et al., 2002; Renaud and Moras, 2000; Shiau et al., 2002; Watkins et al., 2003; Wurtz et al., 1996).
Figure 1.8: Structural basis for the binding of agonist and antagonist to the LBD of SHR. Agonist bound LBD of ERα (Top panel); Antagonist bound LBD of ERα (Bottom panel).
When ERα is bound to an agonist (DES – diethylstilbestrol), the helix-12 folds over the ligand binding pocket to form a cap like structure. In this ‘active’ conformation, the AF-2 of ERα is conducive for coactivator binding (Figure 1.8, top panel). However, when the LBD of ERα is occupied by an antagonist, such as 4-hydroxytamoxifen (4HT), the side-chain extension of 4HT causes a steric displacement of helix 12, resulting in an ‘inactive’ AF-2 conformation. This conformation blocks both coactivator interaction with AF-2 and agonist binding to the pocket (Figure 1.8, bottom panel). The conserved helix 12 alone is not sufficient to function as a transactivation domain; instead helix 12 together with additional regions of the LBD forms the AF-2 domain that acts as a coactivator ‘docking site’, when the LBD is occupied by an agonist (Brzozowski et al., 1997).

1.1.5 STEROID HORMONE RECEPTOR SIGNALING

Unliganded SHRs are thought to exist as heteromeric chaperone bound inactive complexes. Although it is not the case with all SHRs, the generalized view is that chaperone bound inactive receptors are localized to the cytoplasm. Steroid receptor chaperones include heat shock proteins 90 and 70, and immunophilin among others. Binding of the ligand to the LBD induces major conformational change in the receptor, which causes the dissociation of chaperones from the receptor and triggers a cascade of subsequent receptor modifications that result in the transcriptional activation or active-repression of target genes. The receptor modifications include phosphorylation of specific amino acid residues by various kinases and receptor dimerization (Denner et al., 1990a; Denner et al., 1990b; Moudgil, 1990). Dimerization of SHRs is aided by
two dimerization domains: 1) the D-box of the DBD (weak) and 2) the C-terminal helix 10 of the LBD (strong). Hydrophobic interactions between each helix 10 of the two receptor monomers are essential for stable dimerization that in turn facilitates the binding of SHRs to their HREs (Ming-Jer Tsai, 1998).

Active receptor dimers then translocate to the nucleus (some receptors like TR and RAR are already in the nucleus), where they bind to their cognate response elements and recruit accessory proteins known as coactivators. Coactivator proteins bring to the DNA a number of enzymatic activities, such as histone acetyltransferase (HAT), methylase and ATPase that stimulate target gene transcription. Coactivators also bridge and stabilize the interaction between
the receptor and the general transcription machinery. Following target gene transcription, the receptor, some coactivators and a few components of the general transcription machinery are thought to undergo degradation via the ubiquitin-proteasome pathway. This clears the promoter for another round of SHR-mediated target gene transcription (Figure 1.9) (Lonard and O'Malley, 2005; Lonard and O'Malley, 2006; McKenna et al., 1999; Nawaz and O'Malley, 2004).

1.2 Steroid Hormone Receptor Coregulators

Transcriptional coregulators are proteins that interact with transcription factors to either activate or repress transcription of target genes. Based on their function coregulators can be classified into: a) corepressors - proteins that repress transcription and b) coactivators - proteins that enhance transcription. A concise list of representative coregulators have been tabulated (Table 1.3).

Table 1.3 List of coregulators

<table>
<thead>
<tr>
<th>Coactivators</th>
<th>Corepressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRC1, SRC2 and SRC3</td>
<td>NCOR1</td>
</tr>
<tr>
<td>CBP/p300</td>
<td>SMRT</td>
</tr>
<tr>
<td>E6-AP</td>
<td>HDAC1, HDAC2 and HDAC3</td>
</tr>
<tr>
<td>CARM1</td>
<td>TBLR1</td>
</tr>
<tr>
<td>Swi/Snf/BRG</td>
<td>NRIP1</td>
</tr>
</tbody>
</table>

SRC – Steroid receptor coactivator; CBP – CREB binding protein; E6-AP – E6-associated protein; CARM1 – coactivator-associated arginine methyltransferase 1; Swi/Snf - SWItch/Sucrose non fermentable, BRG – Bramha regated protein; NCOR1 – Nuclear receptor corepressor 1; SMRT – Silencing mediator of retinoid and thyroid receptors; HDAC – Histone deactylase; TBLR1 - Transducin beta-like 1X-related protein 1; NRIP1 – Nuclear receptor interacting protein 1.
1.2.1 COREPRESSORS

Steroid receptors in general, translocate to the nucleus after binding to their ligand. However, a sub-group of SHRs including TR and RAR remain tethered to DNA in the nucleus in the absence of hormone. Various research groups have shown that the LBD of steroid receptors like TR, RAR encompasses a potent transferable repression domain. The ability of these receptors to repress target genes in absence of ligand suggested that repression of other NHRs could be mediated by other endogenous proteins called corepressors. These corepressor proteins are characterized by a “CoRNR box” sequence of amino acid (LXX I/H IXXX I/L), which interacts with the base of the AF-2 domain of unliganded and anti-hormone bound SHRs (Hu and Lazar, 1999; Nagy et al., 1997; Perissi et al., 1999; Webb et al., 2000).

Mammalian gene expression is driven by changes in its chromatin structure and the status of covalent histone modifications around the promoters. Acetylated histones are generally associated with transcriptionally active regions of the genome. Transcriptional corepressors have evolved with enzymatic activities, such as histone deacetylase and demethylase, so recruitment of these factors to active promoters will lead to transcriptional repression. The first identified corepressors were named N-CoR [nuclear receptor corepressor, (Horlein et al., 1995)] and SMRT [silencing mediator of retinoid and thyroid receptors, (Chen and Evans, 1995)]. Corepressor mediated repression of SHRs is facilitated by a large N-CoR/SMRT-containing repression complex, which comprises of a number of proteins including transducin α946-like protein (TBL1),
G-protein suppressor 2 (GPS2) and HDAC3 (histone deacetylase 3), among others. The HDAC3 component of the N-CoR/SMRT-containing repression complex provides the histone deacetylase activity that is necessary for transcriptional repression. However, HDAC3 is functionally dependent on the presence of N-CoR and SMRT in the complex. Taken together, corepressors are specialized proteins that interact with SHRs and repress transcription by functioning as huge multi-protein complexes with potent HDAC activity. As more and more data becomes available the complicated role played by corepressors in the regulation of SHRs and other transcription factors in being revealed (Goodson et al., 2005; Hassig and Schreiber, 1997).

### 1.2.2 Coactivators

Coactivators were initially envisioned as ‘transcriptional adaptors’ (Ptashne and Gann, 1990) or ‘bridging’ proteins that brought the basal transcription machinery and the enhancer DNA-element bound receptors together. This simplified view of the role of coactivators has since proven inadequate and had been replaced by a more complex and sophisticated mechanism involving multi-protein coactivator complexes that assemble a wide variety of enzymatic activities contributing to efficient target gene activation. When the first SHR coactivator, SRC-1 (steroid receptor coactivator 1), was identified by Onate et al., it was predicted that this new class of proteins may consist of 8-10 proteins, but in the last decade alone over 300 different coactivator proteins have been reported by various research groups. There are two major types of coactivators: 1) primary coactivators, which interact directly
with receptors and 2) secondary coactivators, which are proteins that do not interact with the receptors directly, but are constituents of a larger multi-protein coactivator complex that interact with the receptor (Lonard and O'Malley B, 2007).

**Primary coactivators**

As mentioned above, primary coactivators are proteins that can interact directly with SHRs. Amino acid sequence comparison of the different coactivators has revealed that most of the coactivators specifically interact with the steroid receptors via a consensus LXXLL motif (Heery et al., 1997; Li et al., 2003; Torchia et al., 1997). Generally, coactivators interact with agonist-bound SHRs. As discussed previously, binding of the agonist to the LBD of SHRs facilitates the formation of the ‘active’ conformation of the AF-2 domain, which provides a hydrophobic binding pocket for coactivator interactions. This hydrophobic binding pocket holds and stabilizes the leucine residues of the LXXLL helix in the coactivators. Many coactivators contain multiple LXXLL motifs, which may be useful in receptor-specific interactions and allosteric modulation of coactivator functions (Chang et al., 1999; Darimont et al., 1998; Heery et al., 1997; Nolte et al., 1998; Shiau et al., 1998).

**Secondary coactivators**

Secondary coactivators are proteins that do not directly interact with the receptor, but augment SHR transcriptional activity by their interaction with primary or other secondary coactivators. One of the best-studied examples of this type of coactivator is, CARM1 (coactivator-associated arginine
methyltransferase 1), which interacts with the C-terminus of p160 coactivators. This interaction between the C-terminus of p160 coactivators is responsible for the recruitment of CARM1 to the hormone responsive promoter. After recruitment, the methyltransferase activity has been shown to methylate histone H3 and other coregulators, which is essential for efficient SHR-mediated transcriptional activation (Chen et al., 1999; Lee et al., 2005a; Lee et al., 2005b; Rosenfeld et al., 2006).

Transcriptional activation by post-translational modifications

Eukaryotic transcription is one of the most complex and highly regulated multi-step process involving histone modifications (acetylation and methylation), ATPase dependent chromatin remodeling, initiation, elongation, alternative splicing, mRNA processing, and transcription termination (Lonard and O'Malley, 2006). SHR coactivators primarily concentrate their activities toward post-translational modifications (PTMs) of histones and chromatin. Some of the coactivators and their primary functions are listed in table 1.4.

Table 1.4 Coactivators and their functions

<table>
<thead>
<tr>
<th>Function</th>
<th>Coactivator(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyltransferase</td>
<td>p160 family (SRC 1 &amp; 3)</td>
</tr>
<tr>
<td>Methylase</td>
<td>CARM1</td>
</tr>
<tr>
<td>ATPase</td>
<td>BRG-1</td>
</tr>
<tr>
<td>Ubiquitin Ligase</td>
<td>E6-AP, RSP5</td>
</tr>
<tr>
<td>Ubiquitin Conjugation</td>
<td>UbcH7</td>
</tr>
<tr>
<td>RNA Helicase</td>
<td>p72</td>
</tr>
<tr>
<td>RNA transcript</td>
<td>SRA1</td>
</tr>
</tbody>
</table>
It is becoming increasingly evident that SHRs and their coactivators are also subject to post-translational modifications. For example, the p160/SRC family of coactivators exists as a multi-protein complex that plays vital roles assisting various transcriptional processes like formation of the pre-initiation and elongation complexes by promoting PTM of histones, SHRs, transcription machinery and even other coregulators. Coactivator-mediated histone modifications influence the transcriptional outcome of a particular gene locus, whereas PTM of SHRs and other coregulators (coactivators and corepressors) has a broader range of biological effects by influencing target gene transcription at a global level (Hassig and Schreiber, 1997; Lee et al., 2005b; Lonard and O'Malley, 2005; McKenna et al., 1999; Shao et al., 2004; Wu et al., 2005).

It is also conceivable that these multi-protein coactivator complexes may help script and coordinate the final chapter in SHR signaling, activation of SHR responsive genes, by temporally regulating the post-translational modification of the various components involved in SHR-mediated target gene activation. For instance, consider ubiquitination, which is one of the earliest identified and best understood PTM. Recently, the role of the ubiquitin-proteasome degradation system in various cellular processes including regulation transcription has gained prominence. The ubiquitin-proteasome system has been shown to have a positive role in transcription initiation as well as termination.
Post-translational modification: Ubiquitination

Ubiquitin is a 76 amino acid peptide that is attached to proteins to target them for degradation via the 26S proteasome, a multisubunit protease complex. Target (substrate) proteins, can be either monoubiquitinated with a single ubiquitin moiety or polyubiquitinated with multiple ubiquitin molecules linked to each other. The process of attaching ubiquitin to target proteins involves three sequential enzymatic reactions: first, the ubiquitin peptide is activated by the ubiquitin activating enzyme (E1); second, a ubiquitin conjugating enzyme (E2) catalyzes the attachment of the activated ubiquitin to an active site cysteine residue on itself and third, a ubiquitin protein ligase (E3) then accepts the ubiquitin molecule to its own active site cysteine residue, before transferring it to a lysine residue on the substrate (target) protein (Figure 1.10). There are numerous E3 ligases in the cell, each of which is specific for one or more proteins (Dhananjayan et al., 2005).

The primary evidence for the involvement of the ubiquitin proteasome system (UPS) in SHR signaling came from the observation that the proteasome-dependent degradation of

Figure 1.10: Ubiquitin proteasome system (UPS). The UPS consists of three enzymes, E1, E2 and E3 that activate and catalyze the attachment of ubiquitin to the substrate. The ubiquitinated substrate is then degraded by the 26S proteasome. Courtesy: www.myoops.org
ERα was essential for its transcriptional activity (Lonard et al., 2000; Nawaz et al., 1999a). This link between protein turn-over and transactivation suggested an intriguing scenario for the continuous assessment of hormone levels in the cell during SHR signaling (El Khissiin and Leclercq, 1999; Nawaz et al., 1999a; Reid et al., 2003; Welshons et al., 1993). Similar observations have been reported for other SHRs like TR, RAR, PR, VDR and AR (Rosenfeld et al., 2006). Unlike ERα, blocking the 26S proteasome mediated degradation of GR increased its transcriptional activities (Lin et al., 2002a; Lin et al., 2002b). This proteasome-dependent protein turnover is not limited to the receptors; coactivators including SRC-3 have been shown to be influenced by the UPS as well as other ubiquitin and ATP independent degradation systems (Li et al., 2006; Wu et al., 2007).

Apart from its use as a signal for protein degradation, ubiquitin also plays a critical role in the temporal regulation of transcription. A closer look at the transactivation functions of ERα revealed proteasome-dependent cyclic patterns of recruitment and dissociation of the receptor, coactivators, RNA polymerase II and elongation complex from the DNA (Dhananjayan et al., 2005). After transcription termination, dissociation of the RNA polymerase-associated elongation complex and the various transcription factors from the DNA also requires ubiquitin and the UPS. This global influence of the UPS on the various stages of SHR signaling probably accounts for the large number of UPS components that act as coactivator of SHRs (Dhananjayan et al., 2005; Lonard et al., 2000; Lonard and O'Malley B, 2007; Lonard and O'Malley, 2005; Lonard and O'Malley, 2006).
The primary focus of the Nawaz laboratory is to understand the ubiquitin-mediated degradation of SHRs and other coactivators. We were the first group to characterize ubiquitin-proteasome system enzymes as coactivators of SHRs. E6-AP is an E3 ligase that was identified and characterized in our laboratory as a coactivator of SHRs. My thesis project is a product of our continuing study of the molecular mechanism of E6-AP. In this project we have indentified and characterized an E6-AP-interacting protein, WW domain binding protein 2 (WBP-2) as a coactivators of ERα and PR. During the course of this study, we identified yes-associated protein 1 (YAP1) as a signaling partner of WBP-2. Rest of this introduction section will focus on these proteins and their known biological functions.

1.3 E6-ASSOCIATED PROTEIN

E6-AP was initially identified as a protein that interacts with the viral E6 protein of human papilloma virus (HPV) type 16 and 18. This study also demonstrated that the E6-AP/E6 complex binds to p53 and facilitate its ubiquitination and degradation via the 26S proteasome (Huibregtse et al., 1991; Huibregtse et al., 1993a). Later, it was shown that the E6-AP/E6 complex actively participated in the degradation process in which E6-AP acts as the ubiquitin ligase and the E6 protein mediates the interaction between p53 and E6-AP (Huibregtse et al., 1993b). Subsequently, many such proteins with E3 ubiquitin ligase activity were identified. Since these proteins contained regions that are homologous to the E6-AP carboxy terminal domain, they are also known as hect domain containing E3 ligases (Huibregtse et al., 1995; Huibregtse et al., 1994).
Nawaz et al demonstrated that E6-AP was a bonafide coactivator of SHRs including PR, ER$_\alpha$, GR, AR, RAR$_\alpha$, and TR. Using an E3 ligase defective mutant (C833S) of E6-AP, they showed that the coactivation function of E6-AP was independent and separable from its E3 ligase activity. The coactivation functions of E6-AP were shown to be independent of E6 protein (Nawaz et al., 1999b). Role of the 26S proteasome mediated protein turnover in SHR signaling is a controversial and much debated topic in the field. Recent evidence suggests a correlation between SHR (ER$_\alpha$ and AR) protein levels and E6-AP expression. Studies conducted in E6-AP knockout (KO) mice, showed that even though the protein levels of AR were elevated, there was a reduction in the expression of the AR target gene, PSA (prostate specific antigen). Similarly, in human breast cancers with elevated ER$_\alpha$ protein expression, E6-AP expression was greatly reduced and vice versa, suggesting an inverse correlation between the expression levels of ER$_\alpha$ and E6-AP (Gao et al., 2005; Khan et al., 2006). Additionally, E6-AP has also been shown to be involved in ubiquitin-dependent degradation of AIB1 (amplified in breast 1 or SRC-3) in breast cancer cell lines (Mani et al., 2006).

Although E6-AP is a well-known SHR coactivator containing three consensus LXXLL motifs, very little is known about its molecular mechanism of action. In an attempt to identify proteins that interact with E6-AP, a yeast two-hybrid screen was performed using the E3 ligase defective (C833S) mutant of E6-AP as bait against the prey human brain cDNA library. UbcH7, an E2 conjugating enzyme, was identified as an E6-AP-interacting protein and was later
characterized as a coactivator of SHRs. WW domain binding protein-2 (WBP-2) was also identified in this yeast two-hybrid screen. This dissertation thesis documents the characterization of WBP-2 and YAP1 as coactivators of a subset of SHRs (ERα and PR) and provides some insights into the molecular mechanism of WBP-2 and YAP1.

1.4 WW Domain Binding Protein 2

WBP-2 is a 30Kda protein with two identifiable structural/functional domains: 1) a GRAM (glucosyltransferases, Rab-like GTPase activators and myotubularins) domain, found in glucosyltransferases, myotubularins and other putative membrane-associated proteins, and 2) three consensus polyproline (PY) motifs (PPXY) that specifically interact with WW-domains (Figure 1.11). WBP-2 was identified and cloned as a ligand for WW-domain containing protein YAP1. Further analysis revealed that proline rich PY motifs within the C-terminus of WBP-2 were essential for binding to the WW-domain of YAP1 (Chen and Sudol, 1995).

Figure 1.11: Schematic representation of the structural domains of WBP-2.

1.4.1 GRAM Domain

The GRAM domain belongs to a putative novel domain found in membrane-associated proteins including glucosyltransferases and myotubulin...
family of phosphatases. GRAM domains have been reported in ~180 different proteins according to the SMART (simple modular architecture research tool) database. Remarkably the crystal structure of one such protein, MTMR2 (myotubularin-related protein-2) showed that the GRAM domain forms five β strands that are part of a larger domain that is remarkably similar to pleckstrin homology (PH) domain. Multiple sequence alignments of various GRAM domains show high conservation of residues that form the hydrophobic core of the MTMR2 GRAM domain suggesting that all predicted GRAM domains may share the PH-like domain fold. Many protein modules share structural similarities with the PH domain but most of them have distinct functional roles and phospholipid binding preferences (Begley et al., 2003). The significance of the GRAM domain to the physiological roles of WBP-2 is still unknown.

1.4.2 PY MOTIF

Polyproline motifs are found in a wide variety of cellular proteins. Based on their consensus amino acid sequences, they are classified into 4 groups as follows: 1) PY – containing the PPXY consensus sequence (Chen and Sudol, 1995), 2) PPLP – contains the sensensus PPLP sequence (Bedford et al., 1997; Ermekova et al., 1997), 3) PR - proline/arginine-containing sequences (Bedford et al., 2000) and 4) p(S/T)P - phosphorylated serine/threonine-proline sites (Lu et al., 1999; Yaffe et al., 1997). These proline-rich sequence containing motifs can bind to different WW-domain subtypes (refer section 1.5.1). The PPXY motif has been suggested to play an important role in transcriptional activation, since this motif has been shown to be present in the transcriptional activation domains of a
wide range of transcription factors including c-Jun, AP-2 (Activating enhancer binding protein 2), NF-E2 (Nuclear factor, erythroid-derived 2), C/EBPα (CCAAT/enhancer-binding proteinα) and PEBP2/CBF (Polyomavirus enhancer-binding protein 2/ Core-binding factor) (Baichwal and Tjian, 1990; Kiekhaefer et al., 2004; Nerlov and Ziff, 1994; Williams and Tjian, 1991; Zhao et al., 2001).

1.4.3 KNOWN AND PREDICTED FUNCTIONS OF WBP-2

Nitsch et al had shown that WBP-2 interacted with Pax8, a Pax gene family of transcription factors. Pax8 has been shown to be an essential transcription factor involved in the morphogenesis of the thyroid gland. Though WBP-2 interacted with Pax8 it was not found to enhance the transcriptional activity of Pax8, suggesting that WBP-2 acts as an adaptor rather than a transcriptional coactivator (Nitsch et al., 2004). We identified WBP-2 as an E6-AP interacting protein in a yeast two-hybrid screen. Apart from the sequence and domain structure of WBP-2, very little was known about its physiological role. During the course of this study, we have characterized WBP-2 as a coactivator of ERα and PR (Dhananjayan et al., 2006). Furthermore, we have shown that the mechanism of action of WBP-2 involves the WW domain containing protein YAP1.
1.5 YES ASSOCIATED PROTEIN 1

YAP1 is a 65 kDa proline-rich phosphoprotein that binds to the SH3 domains of various signaling proteins including Nck, Crk and src. YAP1 was the first protein identified to contain the 'WW domain' protein module, which has since been found in numerous signaling molecules (Sudol et al., 1995). YAP1 is the prototypic example of a group I WW domain (Figure 1.12).

1.5.1 WW DOMAIN

WW domains are typically 35-40 amino acids in length and fold into a three-strand anti-parallel β sheet with two conserved tryptophan residues. WW domains can be divided into four major groups, I through IV, based on the type of proline-rich motif they recognize and bind (Table 1.5) (Huang et al., 2000; Kanelis et al., 2001; Macias et al., 1996; Verdecia et al., 2000).

<table>
<thead>
<tr>
<th>Type of WW domain</th>
<th>WW domain protein</th>
<th>Preferred proline-rich sequence</th>
<th>Protein ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>NEDD4, YAP1, WWOX1, AIP4</td>
<td>PPXY (PY)</td>
<td>WBP-2, ErbB4, p73</td>
</tr>
<tr>
<td>Group II</td>
<td>FE65, FBP11</td>
<td>PPLP</td>
<td>Mena</td>
</tr>
<tr>
<td>Group III</td>
<td>FBP30</td>
<td>PR</td>
<td>WBP11</td>
</tr>
<tr>
<td>Group IV</td>
<td>PinI</td>
<td>p(S/T)P</td>
<td>AP2A1</td>
</tr>
</tbody>
</table>

*NEPD4 - neural precursor cell expressed, developmentally down-regulated 4; YAP1 – yes associated protein 1; WWOX1 – WW domain containing oxidoreductase 1; AIP4 – Atropin interacting protein 4; FBP11 – Formin binding protein 11; FBP30 – Formin binding protein 30; WBP-2 – WW domain binding protein 2; ErbB4 – epidermal growth factor receptor 4; p73 – a 73 kDa protein member of the p53-family of transcription factors WBP11 – WW domain binding protein 11; AP2A1 – Adapter-related protein complex 2, alpha 1.
Group I is the largest group with many members. Group II and III are rather versatile in their binding preference since they can bind to PPLP, PR and even polyproline sequences containing glycine, methionine and arginine. Related WW domains from different proteins or even 2 WW domains from the same protein can have entirely different specificities for protein ligands. On the other hand, the same protein ligand can bind multiple classes of WW domains through separate proline-rich motifs (Ingham et al., 2005; Sudol et al., 2005).

1.5.2 PHYSIOLOGICAL FUNCTIONS

Recently, YAP2, a splice variant of human YAP1 that encodes a protein with two WW-domains was identified (Figure 1.12). Both YAP1 and 2 contain an activation domain, which is similar to the VP-16 activation domain (Komuro et al., 2003). However, the roles of YAP1 and 2 in transcription are not well defined. YAP1 and YAP2 have been shown to act as transcriptional coactivators for several genes but their transcriptional coactivation functions are dependent on
PY motif containing proteins as in the case of ErbB4 (Erythoblastic leukemia viral oncogene homolog 4) signaling pathway (Komuro et al., 2003). ErbB4 (HER4/ERBB4) is a type I receptor tyrosine kinase that regulates cell proliferation and differentiation. After binding its ligand heregulin or following activation by protein kinase C, the ectodomain of ErbB4 is cleaved by a metalloprotease (tumor necrosis factor-α-converting enzyme) and γ-secretase. This cleavage by γ-secretase facilitates the translocation of the carboxy-terminal fragment of ErbB4 (CTF) to the nucleus. The CTF interacts with YAP1 and YAP2 and enhances target gene expression by recruiting them to the promoter (Komuro et al., 2003).

In this regard, it has been suggested that YAP2 is a stronger coactivator of transcription than YAP1 with respect to the ErbB4 signaling pathway. Another WW domain containing protein, WWOX1 (WW domain containing oxidoreductase 1) has been shown to interact with the CTF of ErbB4. This interaction leads to transcriptional suppression of YAP2 in a dose dependent manner as WWOX1 competes with YAP2 to bind the CTF of ErbB4. WWOX1 is a potent tumor suppressor, which is predominantly localized to the cytoplasm. Upon interaction with the CTF of ErbB4, the WWOX1:CTF-ErbB4 complex is retained in the cytoplasm. This mislocalization of the CTF prevents it from entering the nucleus to carry out its genomic functions in association with YAP2 (Aqeilan et al., 2005).

Phosphorylation of YAP1 and YAP2 by specific kinases regulates their cellular distribution and transcriptional activation functions. Akt-dependent phosphorylation of YAP1 at Serine 127 leads to the nuclear export of YAP1 via
its interaction with 14-3-3 and results in the sequestration of YAP1 and YAP2 in the cytoplasm, thereby reducing their ability to function as coactivators of transcription (Basu et al., 2003; Zhao et al., 2007).

YAP1 has been shown to be a key determinant in the p73-mediated apoptotic signaling pathway, in which YAP1 interacts with and enhances the transcriptional activation of p73 target genes in response to DNA damage. YAP1 imparts selectivity to p73 by promoting the activation of a subset of p73 and/or p53 target genes. The PPXY (PY) motif of p73 has been shown to be essential for its interaction with YAP1, which in turn is critical for p73 protein stability. YAP1 competes with Itch (AIP4, WW domain containing protein) for binding to p73, and prevents Itch-mediated ubiquitin-dependent degradation of p73 (Dobbelstein et al., 2005; Howell et al., 2004; Levy et al., 2007; Strano et al., 2005; Strano et al., 2001).

YAP1 has been shown to interact with Runx2 (runt-related transcription factor 2 or PEBP2 – polyomavirus enhancer binding protein 2) in osseous cells and the PY motif of Runx2 was essential for this interaction. Runx2 recruits YAP1 to the osteocalcin gene promoter, where YAP1 represses Runx2-dependent activation of the osteocalcin gene. This YAP1-mediated repression of the osteocalcin gene appears to be cell-type independent it has been observed in more than one cell-type. YAP1-mediated repression of Runx2, however, seems promoter specific as it blocked the activation of TGFβR1, had no effect on the transcription of p21 or p60OSE2, and enhanced the transcription of Runx2 gene (Westendorf, 2006; Yagi et al., 1999; Zaidi et al., 2006; Zaidi et al., 2004).
Recently, YAP1 was characterized as an oncogene that is frequently amplified in breast cancers. Furthermore, overexpression of YAP1 in human nontransformed mammary epithelial cells results in phenotypic alterations that are hallmarks of tumorigenic transformation. Additionally, the PPXY motif containing proteins significantly enhance the oncogenic functions of YAP1 (Finn et al., 2007; Hicks et al., 2006). Taken together YAP1 seems to be a potent transcriptional activator, whose functions are dependent on PY motif containing proteins (p73, ErbB4 and Runx2) and a cohort of other DNA binding proteins and co-factors brought together by protein-protein interactions mediated by modular domains.

1.6 EMERGING ROLES OF THE PY-WW INTERACTION MODULE IN SHR SIGNALING

Protein-protein interaction modules are important for proper signal transduction process in any cell. There are numerous such modules that mediate various processes from cell cycle progression and arrest to apoptosis. One such protein interaction module, the PPXY motif and WW-domain (PPXY-WW module) has gained prominence in the last decade. WW-domains mediate their interactions with proteins that contain a short PPXY motif. PPXY motif containing proteins essentially act as ligands for WW-domain containing proteins. Phosphorylation of the terminal tyrosine in the PPXY motif or phosphorylation of WW-domains containing proteins by specific kinases abolishes their interaction, suggesting that this modification represents a negative regulatory mechanism for PPXY-WW module stabilization. Although the precise physiological roles of the
PPXY motif and WW-domain remain undetermined, their presence in diverse proteins involved in signaling, regulatory, transcription and cytoskeletal functions, as well as their rapidly emerging role in signaling mechanisms that underlie several human diseases including cancer, clearly underscores their importance.

Many new roles of PPXY motif and WW-domain proteins in other cellular processes are just emerging. It is interesting to note that the PPXY motif is found in the transcriptional activation domains of many transcription factors and mutations in the PPXY motif either reduce or abolish their transcriptional activities. This observation suggests that the PPXY motif proteins play a role in transcriptional activation by interacting with WW-domain containing proteins that serve as transcriptional coactivators. Since, the interaction between WW-domain and PPXY motif is highly specific, it suggests that PPXY-WW complex is a more specialized coactivator complex that specifically activates a selective subset of transcription factors. In general it has been shown that transcriptional coactivator complexes such as CBP, SRC-1 and p/CAF (p300/CBP associated factor) coactivate the transcriptional activities of a variety of hormone receptors without exerting any specificity.

In this study, we observed that the complex of PPXY motif containing protein, WBP-2 and WW-domain containing oncoprotein, YAP1 specifically coactivates ERα and PR-dependent gene transcription and this protein complex has no significant effect on the transactivation functions of other steroid hormone receptors. The importance of the PY-WW interaction module is revealed by the observation that neither WBP-2 nor YAP1 is recruited to the ERα target gene
promoter in the absence of the other, indicating that these two proteins are functionally linked. Elucidating the mechanism of action of these novel ERα and PR selective coactivators will help us better understand their specific roles in the global temporal regulation of ERα and PR.
CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 COMMON MATERIALS

The common materials used in this study are listed in Table 2.1.

Table 2.1 Common materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Manipulating Enzymes</td>
<td>Restriction endonucleases, Ligases, Polymerases, etc,</td>
<td>Invitrogen (San Diego, CA), New England Biolabs (Bethesda, MD)</td>
</tr>
<tr>
<td>Radioisotope</td>
<td>$^{35}$S Methionine</td>
<td>GE Healthcare (formerly Amersham Biosciences)</td>
</tr>
<tr>
<td>Hormones</td>
<td>Estradiol, Tamoxifen, Progesterone, RU486, Dexamethazone, etc,</td>
<td>Sigma (St. Louis, MO)</td>
</tr>
<tr>
<td>Growth Media</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>Luria broth and agar</td>
<td>Difco-BRL (Bethesda, MD)</td>
</tr>
<tr>
<td>Mammalian cells</td>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM), Roswell Park Memorial Institute – 1640 (RPMI-1640)</td>
<td>Invitrogen (San Diego, CA)</td>
</tr>
<tr>
<td></td>
<td>Fetal bovine serum (FBS)</td>
<td>Invitrogen (San Diego, CA) and Atlanta Biologicals (Lawrenceville, GA)</td>
</tr>
<tr>
<td>Yeast</td>
<td>Yeast extract peptone dextrose medium</td>
<td>Difco-BRL (Bethesda, MD)</td>
</tr>
<tr>
<td>Western Blot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transfer membrane</td>
<td>Nitrocellulose membrane</td>
<td>Whatmann GmbH (Dassel, Germany)</td>
</tr>
<tr>
<td>Western blotting reagent</td>
<td>ECL (enhanced chemiluminescence)</td>
<td>GE Healthcare (formerly Amersham Biosciences)</td>
</tr>
</tbody>
</table>
reagent

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
<td>ERα (HC-20 and D-12)</td>
</tr>
<tr>
<td></td>
<td>E6-AP</td>
</tr>
<tr>
<td></td>
<td>YAP1</td>
</tr>
<tr>
<td></td>
<td>WBP-2</td>
</tr>
<tr>
<td></td>
<td>FLAG (M2 monoclonal antibody) and FLAG M2 peroxidase conjugated</td>
</tr>
<tr>
<td><strong>Secondary antibodies</strong></td>
<td>Goat-anti-rabbit-HRP</td>
</tr>
<tr>
<td></td>
<td>Goat-anti-mouse-HRP</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assay Kits</th>
<th>RNeasy mini kit</th>
<th>Qiagen (Valencia, CA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total RNA isolation and cDNA synthesis</strong></td>
<td>QuantiTect reverse transcription kit</td>
<td></td>
</tr>
<tr>
<td><strong>Real-time PCR analysis</strong></td>
<td>iQ SYBR green supermix</td>
<td>Bio-Rad Laboratories (Hercules, CA)</td>
</tr>
<tr>
<td><strong>Transfection Reagents</strong></td>
<td>Fugene 6 and Fugene HD</td>
<td>Roche Diagnostics (Indianapolis, IN)</td>
</tr>
<tr>
<td></td>
<td>JetSi</td>
<td>Polyplus-transfection (Newyork, NY)</td>
</tr>
<tr>
<td></td>
<td>Lipofectamine RNAiMAX</td>
<td>Invitrogen (San Diego, CA)</td>
</tr>
<tr>
<td></td>
<td>GenePORTER 2</td>
<td>Genlantis (Sandiego, CA)</td>
</tr>
</tbody>
</table>

2.1.2 BACTERIAL STRAINS

E. coli strain DH5α was used in all cloning and site-directed mutagenesis experiments. Chemically competent E. coli DH5α cells were procured from Invitrogen (San Diego, CA). This bacterial strain was also used for the expression and purification of glutathione S-transferase (GST) fusion proteins.
2.1.3 Yeast Strains

The yeast-two-hybrid screening assays for the identification of E6-AP interacting proteins were carried out in Y190. The yeast strains grown in yeast extract peptone dextrose (YEPD) medium. The agar version of YEPD typically consisted of 1% (mass/volume) yeast extract, 2% peptone, 2% glucose/dextrose and 2% agar. The broth version of YEPD typically consisted of 1% yeast extract, 2% peptone and 1% glucose/dextrose in distilled water.

2.1.4 Mammalian Cell-lines

All the cell lines utilized in the study were maintained in an atmosphere of 5% CO₂ at 37°C. The appropriate media requirements and the origin of the various cell lines used in this study are below in Table 2.2.

<table>
<thead>
<tr>
<th>Cell line a</th>
<th>Culture Conditions b</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>DMEM, 10% FBS &amp; 1% penicillin-streptomycin</td>
<td>Human cervical cancer</td>
</tr>
<tr>
<td>MCF-7</td>
<td>DMEM, 10% FBS &amp; 1% penicillin-streptomycin</td>
<td>Human breast adenocarcinoma</td>
</tr>
<tr>
<td>T47D</td>
<td>RPMI-1640, 10% FBS &amp; 1% penicillin-streptomycin</td>
<td>Human breast ductal carcinoma</td>
</tr>
<tr>
<td>LnCaP</td>
<td>RPMI-1640, 10% FBS &amp; 1% penicillin-streptomycin</td>
<td>Metastatic prostate carcinoma</td>
</tr>
<tr>
<td>E6-AP KO MEFs</td>
<td>DMEM, 10% FBS, 1% penicillin-streptomycin &amp; 7 µl/liter β-mercaptoethanol (10⁻⁴ M)</td>
<td>E6-AP KO mouse</td>
</tr>
</tbody>
</table>

aKO – Knockout; MEF – Mouse embryonic fibroblasts. bDMEM – Dulbecco’s modified Eagle’s medium; RPMI – Roswell park memorial institute; FBS – Fetal bovine serum.
2.1.5 siRNAs Utilized in This Study

During the initial phases of this study when ready to use small interfering RNAs (siRNAs) were not commercially available, we utilized the siRNA target finder program from Ambion Inc (Austin, TX) to design siRNA that specifically knocks down WBP-2. The sequences used were as follows from the amino terminus: Seq#1(AS) 5’-AACGTGCCAGAAGCCTTCAAACCTGTCTC-3’, Seq#1(S) 5’-AATTGAAGGCTTCTTCTGGGCACGCTGTCTC-3’. Ambion siRNA construction kit was used to construct and purify siRNA molecules for transfection assay. Table 2.3 lists all the siRNAs with their sequences that were procured from Dhharmacon Inc (Lafayette, CO).

Table 2.3 List of siRNAs

<table>
<thead>
<tr>
<th>Gene and Product Namea</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ON-TARGETplus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scrambled</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>WBP-2</td>
<td>NA*</td>
<td>NA*</td>
</tr>
<tr>
<td><strong>YAP1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCACCUAUCACUCUCGCAAGUU</td>
<td>UCUCGAGAGUGAUAGGUGCUU</td>
<td></td>
</tr>
<tr>
<td>UGAGAACAAUGACGACCAGUU</td>
<td>UUGGUCGUCAUUGUUCUCAUU</td>
<td></td>
</tr>
<tr>
<td>GGUCAGAGAUACUUCUUAUUAU</td>
<td>UUAAGAAGUAUCUCUGACCUU</td>
<td></td>
</tr>
<tr>
<td>CCACCAAGCUAGAUAAAGAUU</td>
<td>UCUUUAUCUAGCUUGGUGUU</td>
<td></td>
</tr>
</tbody>
</table>

aAll the products described above were procured from Dhharmacon (Lafayette, CO); NA – proprietary, Dhharmacon does not provide sequence info on their control siRNAs; NA* - sequence information was not provided on orders placed before Feb 2007
2.2 METHODS

2.2.1 DNA CONSTRUCTION

Bacterial expression vectors

Bacterial expression vectors for the expression and purification of GST-WBP-2 was constructed by serial digestion of the cDNA cloning plasmid pBlueScript-WBP-2 (a gift from Sudol M, Geisinger Medical Center, PA) with BamHI-EcoRI and subcloned in-frame with GST into pGEX4T (GE Healthcare).

Yeast expression vectors

The ubiquitin ligase defective mutant E6-AP was cloned into the yeast two-hybrid plasmid by HindIII-digesting (and filling) pGEM E6-AP (C833S) and re-digesting with BamHI. The resulting BamHI-HindIII (filled) fragment was inserted into the BamHI-EcoRI (filled) sites of pGAD10 (clontech, Cambridge, UK)

Mammalian expression and reporter vectors

The mammalian expression plasmids for progesterone receptor-B (pCR3.1.PR-B), glucocorticoid receptor (pCR3.1.GR), androgen receptor (pCR3.1.AR), estrogen receptor α(pCR3.1.ER), p53, SRC-1 (pBIND.SRC-1), E6-AP (pCR3.1.E6-AP) and GAL4-VP16 have been described previously (Allan et al., 1992; Nawaz et al., 1999b; Onate et al., 1995; Tilley et al., 1989). The mammalian expression vectors for WBP-2 were constructed by digesting pBlueScript-WBP-2 with BamHI-EcoRI, the resulting fragment was then subsequently cloned into the corresponding sites in pBK-RSV (Stratagene, La
vector and in-frame with GAL4 DNA binding domain in pBIND (Invitrogen, San Diego, CA) vector. Expression vectors encoding both wild-type and the WW-domain mutant of YAP1 (pcDNA3.1) that have been previously described (Chen and Sudol, 1995) were a generous gift from Dr. Sudol, M. (Geisinger Medical Center, PA).

The progesterone/glucocorticoid/androgen-responsive reporter (PRE-TATA.LUC.), estrogen-responsive reporter (ERE-TATA.LUC), p53-responsive reporter (p21 promoter-LUC) and GAL4-VP-16 responsive reporter (17mer-LUC) plasmids also have been described previously (el-Deiry et al., 1993; Liu et al., 1999; Onate et al., 1998; Xu et al., 1996). The reporter plasmid pGS5 with multiple copies of Gal4 response element was procured from Invitrogen (San Diego, CA).

Site-directed mutagenesis of the PY motifs of WBP-2

The GeneEditor™ in vitro Site-Directed Mutagenesis System from Promega Corporation was used to generate WBP-2 PY motif mutants. Mutations within the three poly-proline motifs (PY motifs) were generated in pBlueScript vector. The oligonucleotide primers used in the process were as follows: 5’-GGA ATG TAC CCC TGC GCTGCT GGC GCC CCC TAT CCA CCG CCC-3’ coding for AAGA (first PY motif); 5’-TAC GTG CAG CCC CCA GCAGCG CCC GCC CCT GGG CCC ATG GAA-3’ coding for AAPA (second PY motif); 5’-AGC CAG CCG CCA GCTGCC TAC GCC CCA CCG GAA GAT AAG coding for AAYA (third PY motif). Mutants were screened and confirmed by sequencing. The cDNAs of PY mutants of WBP-2 were then digested with BamHI-HindIII and
inserted into the corresponding sites of pBK-RSV vector. Subsequently, the cDNA of WBP-2 that carried a mutation in only the third (most carboxy-terminal) PY motif was cloned into pBIND (in-frame with Gal4 DNA binding domain) and pGEX-4T (in-frame with GST) vectors using the BamH1-HindIII digestion sites.

2.2.2 BACTERIAL TRANSFORMATION

2 µl (1-100 ng) of plasmid DNA or ligation reaction was mixed with 50 µl of chemical competent cells on ice. The sample was incubated on ice for 10 min, heated to 42°C in a water bath for 45 sec, and immediately placed on ice for 2 min. To allow the cells to recover, 900 µl of SOC (super optimal medium with catabolite repression effect) was added. Then, the cells were grown for 1 hr to allow plasmid antibiotic resistance markers to be expressed. To select transformants, dilutions were plated on the appropriate antibiotic-containing agar plates.

2.2.3 ELECTROPHORESIS

Agarose gel electrophoresis

DNA was electrophoresed through 1-2% agarose gels in TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA) or TAE (40 mM Tris acetate, 2 mM EDTA) buffer. Prior to electrophoresis, 6X DNA loading dye (10 mM Tris-HCl - pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA.) was added to DNA samples.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
The Laemmli method of SDS-PAGE was used to separate proteins by molecular weight. Polyacrylamide gels consisted of a 4% stacking gel [4% v/v acrylamide (29:1; Sigma), 125 mM Tris-HCl, pH 6.8, 0.1% w/v SDS] and a 10% or 12% separating gel [10% or 12% v/v acrylamide (29:1; Sigma), 375 mM Tris-HCl, 0.1% w/v SDS]. Gels were cast and run using the Bio-Rad Mini-PROTEAN 3 apparatus. Before loading, protein samples were mixed with an equal volume of 2X SDS sample buffer (80 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% w/v SDS, 15% v/v glycerol, 0.01% w/v bromophenol blue) and heated to 100°C for 5 min. Samples were electrophoresed in running buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS) at 100 V until the bromophenol dye migrated off the gel. Gels were either stained with agitation in 0.025% Coomassie Brilliant Blue R250, 40% methanol, 7% acetic acid and destained in 10% acetic acid or were further analyzed by Western blotting.

2.2.4 Western Blot Analysis

Following SDS-PAGE, the separated proteins were transferred onto nitrocellulose membrane (Whatman, Germany) in Western blot transfer buffer (25 mM Tris, 0.2 M glycine and 20% methanol) using the Bio-Rad Mini Trans-Blot transfer cell at either 400 mA (2 hr express transfer at 4°C) or 25 V (overnight at 4°C). Membranes were immersed in blocking buffer [5% non-fat milk powder in 1X phosphate buffered saline with 0.05% Tween 20 (PBST)] overnight at 4°C. Dilutions of primary antibodies (ERα – 1:1000, E6-AP – 1:1000, YAP1 – 1:1000, β-actin – 1:4000 and WBP-2 – 1:500) were prepared in dilution buffer (1% non-fat milk powder in PBST) and incubated with the membranes for appropriate
durations according to the specific antibody. Membranes were washed thrice in PBST and incubated with HRP-conjugated (Horse radish peroxidase) secondary anti-mouse or anti-rabbit (as specified by the primary antibody). After washing 3 times with PBST the membranes were developed with ECL chemiluminiscent detection reagent (GE Healthcare) and exposed with X-ray films. Bands were visualized after the films were further processed using a Kodak developer.

2.2.5 Yeast Two-Hybrid Screening

The yeast two-hybrid screening assay was performed as described previously (Nawaz et al., 1999b). A catalytically inactive form of E6-AP (C833S), in which the active site cysteine residue is substituted with serine, was used as bait. The prey cDNA library, fused to the Gal4 activation domain, was derived from human brain cells (Clontech). After transformation the yeast cells were grown in culture medium containing X-gal (Substrate for β-galactosidase). Protein-protein interaction positive colonies appear blue colored due to the metabolism of X-gal (5-bromo-4-chloro-3-indoly-β-D-galactoside) by β-galactosidase. The positive colonies were then assayed for β-galactosidase activity by β-gal assay.

β-galactosidase assay

Yeast cells were grown in 5ml minimal medium (-leucine /-tryptophan) broth to mid-log phase. After centrifugation cells were resuspend in 5ml filter-sterilized Z-buffer [60mM Na$_2$HPO$_4$, 40mM NaH$_2$PO$_4$, 10mM KCl 1mM MgSO$_4$, 50mM β-mercaptoethanol (pH 7.0)] and placed on ice. Next the optical density was measured (OD$_{600}$) and the cell were diluted 10x or 20x (40 or 80µl brought to
0.8mL with Z-buffer). 1 drop of 0.1% SDS and 2 drops of chloroform were added to each tube followed by vortexing for 15 sec and equilibration at 30°C for 15 min. 160µl of 4mg/ml ortho-Nitrophenyl-β-galactoside (ONPG) was added and vortexed for another 10 sec. 15-20 min after incubation at 30°C the reaction was quenched by the addition of 400µl of 1M sodium carbonate. Cell debris was cleared by centrifugation and the samples were measured at OD\textsubscript{420} and OD\textsubscript{550}. β-gal activity (in Miller units) was calculated using the formula:

\[
U = 1000 \times \frac{[(OD_{420}) - (1.75 \times OD_{550})]}{[\text{Time} \times (Vol) \times OD_{600}]}
\]

Where, Vol is volume of culture used in assay in mls, and Time is minutes at 30°C.

2.2.6 WBP-2 ANTIBODY GENERATION

Since WBP-2 specific antibodies were not available through any commercial vendor, we approached Alpha Diagnostic International Inc., to generate a peptide antibody against the WBP-2 protein. A unique 17 amino acid (N'-NDMKNVPEAFKGTKKGT-C') peptide sequence was selected within WBP-2 proteins spanning amino acids 35-49, based on hydrophilicity, antigenicity and accessibility scale using various bioinformatic protein-profiling programs. Possible cross homology of the peptide sequence was checked by BLAST (Basic Local Alignment Search Tool) analysis and was found to be conserved in WBP-2 proteins across species. This peptide was synthesized \textit{in vitro} and covalently attached to a carrier protein; KLH (Keyhole Limpet Hemocyanin), via a cysteine residue added to the amino-terminus and was injected into rabbits for polyclonal
antibody generation. Antibodies generated were concentrated by affinity purification. Anti-WBP-2 antibody was used at 1:500 dilutions in immunoblotting, and at 1:50 for chromatin immunoprecipitation (ChIP) and immunoprecipitation (IP) assay.

2.2.7 PREPARATION OF MOUSE EMBRYONIC FIBROBLASTS FROM E6-AP KNOCKOUT MOUSE

After setting up matting between E6-AP heterozygous mice, the female genital-plug was checked every morning (appearance of the plug was considered as day 1 of pregnancy). 14-17 days later the embryos were removed under sterile conditions followed by removal of the liver and heart, rest of the parts were washed in phosphate buffered saline (PBS) to remove as much blood as possible. Next the embryos were finely teased and incubated overnight in 3-5 ml of cold 0.25% trypsin-EDTA solution to allow trypsin to diffuse into tissues (trypsin has negligible activity at 4°C). Without disturbing the tissue pellet, excess trypsin was aspirated out leaving about 2 volumes of the tissue and incubated at 37°C for 30 min. After incubation the tissues were broken-up by vigorous pipetting in culture medium [DMEM with 10% FBS and 7µl/liter β-mercaptoethanol (10⁻⁴ M)] followed by centrifugation at 1x g for 1 min at room temperature to sediment any remaining clumps. The suspension was plated in 100mm culture dishes or 75cm² flasks (3 dishes or flasks per embryo). When confluent parts of the cells were frozen in cell freeing medium [9 parts FBS + 1 part dimethyl sulfoxide (DMSO)], remaining cells were passaged a few times (always maintain the cell at relatively high density).
During the first few passages the genotype of the cell (E6-AP knockout) were confirmed by PCR with E6-AP gene specific primers as follows: forward 5'-ACTTCTCAAGGTAAGCTGAGCTTG-3' and reverse 5'-TGCATCGCATTGTCCTGAGGTGTC-3'. The PCR conditions include, initial denaturation at 95°C for 5 min; followed by 35 cycles: 95°C for 1 min, 56°C for 1 min, 72°C for 1 min; and final extension at 72°C for 5 min. This primary culture grows rapidly for about 7-12 passages, all the transfection assays were performed within these passages. Primary cells are especially difficult to transfect, after extensive search GenePORTER 2 (Gentalis) was found to be the most reliable and efficient transfection reagent for these primary cells.

2.2.8 **In Vitro Interaction Assay**

*In vitro* expression of radiolabeled E6-AP, PR, ERα and YAP1 were performed by *in vitro* transcription and translation (TNT) from rabbit reticulocyte extract in the presence of [35S]-methionine according to the manufacturer’s recommended conditions (Promega, Madison, WI). GST-WBP-2 was expressed in *E.coli* DH-5α cells and purified on glutathione-sepharose beads. The purified and glutathione-bound WBP-2 was incubated with *in vitro* translated E6-AP, PR, ERα and YAP1 in NETN buffer [50mM NaCl; 1mM EDTA; 20mM Tris pH (8.0); 0.1% Nonidet P-40] overnight at 4°C. After washing four times with NETN buffer, WBP-2 bound E6-AP, PR, ERα and YAP1 were eluted and separated on 7.5-10% SDS-polyacrylamide gels. Gels were then dried in a gel drier (Bio-Rad, Hercules, CA) and analyzed by autoradiography.
2.2.9 WW DOMAIN ARRAY SCREENING

TranSignal WW domain arrays (Panomics, Redwood City, CA) was used to identify possible WBP-2 interacting proteins. Panomics’ TranSignal WW Domain Arrays consisted of 67 different human WW domains from various proteins. The arrays were constructed by expressing recombinant conserved binding sites of individual WW domains fused to GST. These recombinant GST fusion proteins were expressed, affinity purified and spotted onto a membrane in duplicates. FLAG-tagged wild-type WBP-2 and PY3 mutant WBP-2 were expressed and purified from bacteria using anti-FLAG affinity gels. Affinity purified full-length WBP-2 proteins were used in this screening assay. After blocking the membrane with 1X blocking buffer (proprietary, supplied with product) for 2 hr at RT, the membranes were incubated with either 10 µg/ml of purified FLAG-tagged WBP-2 or 1X of the positive control extract (proprietary, supplied with product) for 1 hr at 4°C. The membranes were washed 3 times with the wash buffer (supplied with product), followed by incubation with 1:5000 diluted anti-FLAG HRP conjugated antibody (diluted in 1X wash buffer) for 1 hr at RT. After thoroughly washing the membranes 3 times, they were developed with ECL chemiluminiscent detection reagent (GE Healthcare) and exposed with X-ray films. Protein spots were visualized after the films were processed using a Kodak developer.

2.2.10 IMMUNOPRECIPITATION ASSAY

Cells were washed in TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl) and lysed in ice-cold RIPA buffer containing salt [400 mM NaCl,
1XPBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF (10µl/ml), Aprotinin (30 µl/ml) and 100 nM Sodium orthovanadate (10 µl/ml)] by pipetting up and down. Thereafter, cell lysates were placed on ice for 30 min. In order to bring the salt concentration of cell lysates to 150 mM NaCl, 150 µl of NaCl free RIPA buffer [1XPBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF (10µl/ml), Aprotinin (30 µl/ml) and 100 nM Sodium orthovanadate (10 µl/ml)] was added to the lysates. After centrifugation at 4°C (21,000g), lysates were incubated with 20µl of Protein A sepharose and rocked at 4°C for 30 minutes. After centrifugation, supernatants were transferred to fresh tubes and lysates were mixed either with serum, anti-ERα antibody (HC-20, Santa Cruz) or anti-YAP1 (Cell Signaling Tech.) at 4°C for 2 hours on rocker. Afterward 20µl of Protein A sepharose beads were added and lysates were incubated for an additional hour at 4°C on a rocker. Finally, after extensive washing with NaCl-free RIPA buffer, immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting using an anti-ERα, anti-E6-AP, anti-WBP-2 or anti-YAP1 antibody.

2.2.11 TEMPORARY TRANSFECTION

*Luciferase reporter gene assays*

HeLa and MCF-7 cells were maintained in DMEM containing 10% FBS. T47D and LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% FBS. E6-AP knockout MEFs were maintained in DMEM containing 10% FBS and 10^-4 M β-mercaptoethanol. 24 hours prior to transfection, 3X10^5 cells were
plated in one well of a 6-well plates in appropriate culture media (as mentioned above) supplemented with 5% charcoal stripped FBS (CFBS). Cells were transfected with the indicated amount of DNA using FuGene 6 transfection reagent (Roche Diagnostics) or GenePORTER 2 (Gentalis) for MEFs. After 4 hours, cells were treated with the indicated hormones and harvested 24 hours later. Luciferase assays were performed using Promega’s luciferase assay system as per manufacturer instructions.

*Endogenous target gene coactivation assay in MCF-7*

MCF-7 cells were maintained in DMEM containing 10% FBS, 24 hours prior to transfection, cells were plated in 6-well plates at a concentration of 3X10^5 cells/well. This concentration is critical to achieve 80-90% confluency (Fugene HD is optimally efficient at this cell density), on the day of transfection, replace growth media with phenol red-free DMEM supplemented with 5% CFBS. Fugene HD was used to transfec indicated amount of DNA as per protocol provided with the product. Cells were cultured for an additional 48 hrs, followed by real-time quantitative PCR (QPCR) analysis as described below.

*siRNA transfection in MCF-7*

Lipofectamine RNAiMAX (Invitogen) was the most efficient and consistently reliable reagent available for siRNAs transfection in MCF-7 cells. Reverse transfection was recommended by the supplier for MCF-7. Cells were maintained in DMEM containing 10% FBS. 25nM siRNAs (Dharmacon Inc) diluted in OPTI-MEM I (Invitrogen Inc) was first coated on the surface of the 6-well plates followed by the addition of appropriate amounts Lipofectamine
RNAiMAX and incubated at room temperature (RT) with gently shaking for 10 mins. 3X10^5 cells counted and re-suspended in phenol red-free DMEM with 5% CFBS without antibiotics medium was gently layered on top of the transfection complex and swirled to mix well. Cell were incubated for another 48 hrs and analyzed by QPCR (described below) or Western blotting.

2.2.12 RNA EXTRACTION AND REAL-TIME PCR ANALYSIS

RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) as per the supplier’s protocol. After determining the quantity and purity of the RNA samples, 1µg of total RNA per sample was used for cDNA synthesis. cDNA synthesis was performed using QuantiTect reverse transcription kit (Qiagen, Valencia, CA) following the instructions provided with the product. This cDNA synthesis kit includes a DNase step that efficiently removes any possible plasmid or genomic DNA contamination. After dilution, 10ng of cDNA samples were used to perform real-time PCR analysis using an icyclerQ PCR detection system (Bio-Rad, Hercules, CA) in iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). PCR conditions were 95°C for 30 sec, followed by 60°C for 1 min for a total of 40 cycles. The cDNAs of various genes were analyzed using specific primers listed in Table 2.4. To avoid variations from different samples, the relative mRNA levels of the gene of interest were normalized against the GAPDH mRNA content of the same sample.
Table 2.4  List of cDNA PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reverse Primer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GAAGGTGAAGGTCGGAGTC</td>
<td>GAAGATGGTGATGGGATTTTC</td>
</tr>
<tr>
<td>pS2</td>
<td>GCGCCCTGGTCCTGTGTCCA&lt;sup&gt;T&lt;/sup&gt;</td>
<td>GAAACCACAATTCTGTCTTTTCA</td>
</tr>
<tr>
<td>GREB1</td>
<td>ATCAGCTGCTCGGACTTGCTG&lt;sup&gt;G&lt;/sup&gt;</td>
<td>TGAGCTCCGGTCCTGACAGATG</td>
</tr>
<tr>
<td>WBP-2</td>
<td>CTGGTCTGTGCTGGTCTC</td>
<td>AGGGAAGGGAAGGAAGGG</td>
</tr>
<tr>
<td>YAP1</td>
<td>GCAGATGGAGAAGGAGAGG</td>
<td>GTGTTGGTAACTGGCTACG</td>
</tr>
</tbody>
</table>

<sup>a</sup> All primers were purchased from Sigma-Genosys (The Woodlands, TX) and the sequences read 5'-3'.

2.2.13  CHROMATIN IMMUNOPRECIPITATION ASSAY (CHIP)

Classical ChIP

MCF-7 cells were grown in phenol red-free DMEM with 5% CFBS for 72 hr before treatment with either vehicle (ethanol) or 10 nM E₂ (estradiol) for 1 hr. Cells were then fixed with 1% formaldehyde for 10 min at room temperature (RT) in an orbital shaker. After washing with ice-cold PBS the fixing reaction was stopped with glycine stop-fix solution (0.125M glycine in 1X PBS). Cells were then washed, scraped off the culture dish in ice-cold cell scraping buffer [1X PBS with 0.5mM phenylmethylsulphonyl fluoride (PMSF)] and pelleted. The pellets were re-suspended in ice-cold lysis buffer [50mM HEPES-KOH pH-8.0, 1mM EDTA, 140 mM NaCl, 10% Glycerol, 0.5% NP-40, 0.25% Triton X-100 with 1X protease inhibitor cocktail (Sigma) and 1mM PMSF] and incubated at 4°C in an end-over-end shaker for 30 min. The nuclei released from the previous lysis
reaction was then pelleted and re-suspended in ice-cold shearing buffer [10mM Tris-Cl pH-8.0, 1mM EDTA, 140mM NaCl, 0.1% SDS, 0.1% Na Deoxycholate, 1% Triton X-100 with 1X protease inhibitor cocktail (Sigma) and 1mM PMSF] followed by sonication to shear DNA. A Branson Sonifier® S-250D digital ultrasonic processor with a micro probe specially adapted for ChIP assays was used under optimized conditions to produce DNA fragment between 100-1000 basepairs in length. The sonication conditions were as follows, 14% amplitude (power), 15 sec pulse-on, 5 sec pulse-off for a total of 8 cycles (2 min pulse-on). The resulting DNA fragments were clarified by centrifugation and diluted with buffer Y [0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 167mM NaCl, 16.7mM Tris-Cl pH-8.1 with 1X protease inhibitor cocktail (Sigma) and 1mM PMSF]. These diluted DNA fragments were then pre-cleared with purified IgG (Rabbit or Mouse depending on the specific antibody) and incubated over-night with specific antibody (ERα, WBP-2 or YAP1) against the protein of interest at 4°C. The enriched protein-DNA complexes were precipitated with Protein A agarose/Salmon Sperm DNA beads (Millipore/Upstate). After extensive washing, once with TSE-I (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl pH-8.1, 150mM NaCl), TSE-II (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl pH-8.1, 500mM NaCl), TSE-III (0.25M LiCl, 1% NP-40, 1% Na deoxycholate, 1mM EDTA, 10mM Tris-Cl pH-8.1, 150mM NaCl) and twice with TE (10mM Tris-Cl pH-8.1, 1mM EDTA) the protein-DNA complexes were eluted in the elution buffer (1% SDS, 0.1M NaHCO₃). Both the eluates and the previously saved input DNA-protein complexes were then reverse cross-linked at 65°C over-night.
followed by proteinase K and RNase I digestion. The immunoprecipitated DNA and total input DNA samples were purified using QIAquick PCR purification Kit (Qiagen, Valencia) and eluted in 50 µl and 100 µl of H₂O respectively.

The input DNA samples were further diluted 1:10 before PCR analysis. Each PCR reaction contains 6 µl of immunoprecipitated or input, 0.5 µM of each primer, 0.4 mM dNTP mixture, 1X Titanium Taq PCR buffer (Clontech, Palo Alto), and 1X Titanium TaqDNA polymerase (Clontech) in a total volume of 25 µl. The primers for the pS2 promoter were: forward, 5’-GGCCATCTCTCACTATGAATCACTTCTGC-3’and reverse, 5’-GGCAGGCTCTGTTTGCTTAAAGAGCG-3’. PCR was performed for 29 cycles with 1 minute of denaturing at 94 °C, annealing at 62 °C and extension at 68 °C in a MJ Research DNA Engine.

Quantitative ChIP and Re-ChIP

ChIP assays were performed as described above, the resulting DNA samples were then quantified using real-time PCR analysis. Re-ChIP assays were used to identify other proteins bound to the primary ChIP enriched protein-DNA complexes and also to study co-occupation of a single gene locus by two different proteins. Re-ChIP assays were performed by eluting the cross-linked immunocomplexes from the primary ChIP in 10 mM dithiothreitol at 37°C for 30 min, subsequently the samples were diluted 1:50 fold in 1% Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris-HCl (pH 8.1). These diluted samples were subjected to another round of ChIP assay (hence re-ChIP) with a different antibody as described above. Input DNA samples were further diluted 1:10 with
DEPC (Diethyl pyrocarbonate) treated water. 5 μl of immunoprecipitated and diluted input DNA samples were mixed with iQ SYBRGreen supermix (Bio-Rad) and run in an icyclerQ PCR detection system (Bio-Rad). PCR conditions were 95°C for 30 sec, followed by 60°C for 1 min for a total of 40 cycles. The primers utilized for the real-time PCR analysis of pS2 and GREB1 gene loci are listed in Table 2.5.

**Table 2.5  List of genomic PCR primers**

<table>
<thead>
<tr>
<th>Gene Locus</th>
<th>Forward Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reverse Primer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pS2</td>
<td>CACCCCCGTGAGCCACTGT</td>
<td>CTGCAGAAGTGATCATAGTGGAGA</td>
</tr>
<tr>
<td>GREB1</td>
<td>GTGGCAACTGGGTCATTCTGA</td>
<td>CGACCCACAGAAATGAAAAGG</td>
</tr>
</tbody>
</table>

<sup>a</sup>All primers were purchased from Sigma-Genosys (The Woodlands, TX) and the sequences read 5’-3’.
CHAPTER 3
WBP-2: SELECTIVE COACTIVATOR OF ER AND PR SIGNALING

3.1 ISOLATION AND CHARACTERIZATION OF WBP-2 AS AN E6-AP-INTERACTING PROTEIN

Yeast two-hybrid screening system was used to isolate cDNA clones that encode E6-AP-interacting proteins. Since E6-AP could activate the degradation of its binding proteins a catalytically inactive form of E6-AP-C833S was used as bait to avoid potential degradation of interacting proteins. E6-AP (C833S) was fused in frame with the Gal4 DNA-binding domain and introduced into S. cerevisiae. The prey cDNA library, fused to the Gal4 activation domain, was derived from human brain cells. We isolated 12 colonies with cDNAs encoding proteins that strongly interacted with E6-AP. Surprisingly, all twelve colonies contained identical cDNAs. A sequence similarity search in the GenBank database revealed that the cDNAs encoded the carboxy-terminus of WBP-2.

We subsequently verified that WBP-2 interacts with E6-AP both in vivo and in vitro. As shown in Figure 3.1A, WBP-2 strongly interacts with E6-AP in a yeast two-hybrid assay. To further confirm that the WBP-2 and E6-AP interaction observed in the yeast two-hybrid assay is due to direct physical association of WBP-2 with E6-AP, we performed GST-pull down assays. In this assay, E6-AP preferentially interacted with WBP-2 (Figure 3.1B).
3.2 WBP-2 INTERACTS WITH ERα AND PR

WBP-2 interacts with E6-AP, and E6-AP has been shown to act as coactivator for certain nuclear and steroid hormone receptors. To determine whether WBP-2 interacts with steroid hormone receptors, we assayed WBP-2 binding to ERα and PR in GST-pulldown assays. Figure 3.2A shows strong WBP-2 interaction with full-length ERα in the presence of estradiol and reduced binding in the absence of hormone and presence of anti-estrogen (tamoxifen). Under similar conditions, WBP-2 was unable to interact with control GST protein. Similarly, WBP-2 and PR interaction was observed in the presence of progesterone, while only minimal binding was observed in the absence of
hormone (Figure 3.2B). These findings indicate that WBP-2 can interact with ERα and PR in the presence of hormone in vitro.

![Figure 3.2: WBP-2 interacts with ERα and PR. Interaction of WBP-2 with ERα and PR was determined in a GST pull down assay. ERα and PR were radio labeled with [35S] by in vitro transcription and translation kit. The labeled ERα and PR proteins were then incubated overnight at 4°C with E. coli expressed GST alone (control) or GST-WBP-2 bound to beads either in the absence of hormone (-H), presence of estradiol (E2) or tamoxifen (T) for ERα or in the presence of progesterone for PR. Bound proteins were analyzed by SDS-PAGE and autoradiography with 10% and 20% of in vitro translated ERα and PR respectively, as input.]

3.3 WBP-2 CO-PRECIPITATE WITH ERα IN CELLULAR LYSATES

Next we wanted to determine the physiological interaction of WBP-2 with E6-AP and ERα in cells. Immunoprecipitation assays using MCF-7 cell lysates treated with vehicle (ethanol), estradiol or tamoxifen showed that both WBP-2 and E6-AP strongly interact with ERα in the presence of estradiol (Fig. 3.3). However, both WBP-2 and E6-AP exhibited a weak interaction with ERα in the absence of hormone and tamoxifen, which is consistent with our in vitro assays.
3.4 **WBP-2 selectively enhances the transcriptional activities of ERα and PR**

Since E6-AP acts as a coactivator for nuclear hormone receptors and WBP-2 physically associates with E6-AP and steroid hormone receptors, we next tested whether WBP-2 regulates receptor-dependent activation of target gene expression using transient transfection assays in HeLa cells. HeLa cells were co-transfected with mammalian expression plasmids containing one of the following receptors, PR, ERα, GR and AR receptors along with the luciferase reporter plasmids driven by their cognate hormone response element. These assays were carried out with or without the coexpression of WBP-2. In the absence of hormone, WBP-2 had little effect on the transactivation functions of PR and ERα. However, WBP-2 significantly enhanced (~11-17 fold) the hormone-dependent transcriptional activities of PR and ERα (Figure 3.4). WBP-2 had only minimal effect on the transcriptional activities of GR and AR both with and without their

<table>
<thead>
<tr>
<th>WB</th>
<th>5% Input</th>
<th>IP: IgG</th>
<th>IP: ERα</th>
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<tbody>
<tr>
<td></td>
<td>-H</td>
<td>E₂</td>
<td>T</td>
</tr>
<tr>
<td>ERα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E6-AP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBP-2</td>
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</table>

**Figure 3.3: WBP-2 interacts with E6-AP and ERα in cells.** MCF-7 cells were treated with either no hormone (-H), estradiol (E₂) or tamoxifen (T) for twenty four hours and cells were lysed with RIPA lysis buffer. Lysates were clarified with Protein A sepharose beads and incubated with either anti-ER antibody or rabbit pre-immune serum (Alpha Diagnostics). The lysates were precipitated with Protein A sepharose beads that bind to rabbit IgGs followed by electrophoresis. 5% of the lysates were used as input and analyzed by Western blot with anti-ER, anti-E6-AP or anti-WBP-2 antibodies.
cognate ligands (Figure 3.5). Similarly WBP-2 had only negligible effect on other nuclear receptors like retinoic acid receptor-alpha and thyroid receptor (data not shown). These data suggest that WBP-2 selectively modulates the ligand-dependent transcriptional activities of ERα and PR.

![Figure 3.4: WBP-2 specifically modulates the hormone-dependent transcriptional activity of ERα and PR.](image)

HeLa cells were transiently transfected with receptor expression plasmid for PR, ER and their cognate hormone response elements in the presence or absence of WBP-2. Later, cells were treated with respective hormones as follows: PR, progesterone (10⁻⁷ M); ER, estradiol (10⁻⁸ M). Cells were harvested after 24 hrs and assayed for luciferase activity and bars represent mean ± SD from three different determinations. The data is presented as fold activation. The activity of each receptor in the presence of hormone and in the absence of WBP-2 was defined as 1-fold, and the data for other bars were scaled accordingly.
Since HeLa cells express the viral E6 protein that could functionally interact with WBP-2 via E6-AP, we wanted to demonstrate the coactivation function of WBP-2 in cells that lack E6 expression. As shown in Figure 3.6, WBP-2 stimulated the hormone-dependent transcriptional activity of endogenous PR in the E6-negative T47D cells (Figure 3.6). Similarly, WBP-2 could also stimulate the hormone-dependent transcriptional activity of endogenous ERα in the E6 negative MCF-7 cells. Furthermore, WBP-2 has no significant effect on the transcriptional activity of ERα in the presence of anti-estrogen (tamoxifen).
suggesting that WBP-2 only enhances the hormone-dependent transcriptional activity of receptors (Figure 3.7). These data also demonstrates that WBP-2 can enhance the transactivation function of endogenous PR and ERα, and that the coactivation function of WBP-2 is not dependent on the viral E6 protein. This is consistent with our previously published data, indicating that the coactivation function of E6-AP is not dependent on the viral E6 protein (16).

**Figure 3.6: WBP-2 enhances PR transactivation in T47D cells.**

T47D cells were transiently transfected with progesterone response element containing reporter plasmid in the presence or absence of WBP-2. Later, cells were treated with progesterone (10^{-7}M). Cells were harvested after 24 hrs and assayed for luciferase activity and bars are mean ± SD from three different determinations. The activity of receptor in the presence of hormone and absence of WBP-2 was considered as 1-fold and scaled accordingly.
To further confirm that WBP-2 selectively modulates the transcriptional activities of ER\(\alpha\) and PR, we investigated the effect of WBP-2 on the transcriptional activities of non-receptor transcription factors like p53 and VP-16. As shown in Figure 3.8, the transcriptional activities of p53 and the VP-16 activation domain were not enhanced by exogenously expressed WBP-2 at non-saturating conditions (as shown by dose response). These observations suggest that WBP-2 preferentially modulates the hormone-dependent transcriptional activity of a subset of SHRs.

**Figure 3.7: WBP-2 enhances ER\(\alpha\) transactivation in MCF-7 cells.** MCF-7 cells were transiently transfected with estrogen response element containing reporter plasmid in the presence or absence of WBP-2. Later, cells were treated with either vehicle (-H), estradiol (10^-8 M) or antiestrogen, tamoxifen (10^-6 M). Cells were harvested after 24 hrs and assayed for luciferase activity and bars are mean ± SD from three different determinations. The activity of receptor in the presence of hormone and absence of WBP-2 was considered as 1-fold and scaled accordingly.
To confirm that WBP-2 is indeed required for PR activation, HeLa cells were transiently transfected with siRNAs directed against either WBP-2 or against GAPDH control, together with the PR and PR-responsive reporter plasmids. Expression of siRNA directed against WBP-2 reduced the endogenous protein levels of WBP-2, while the control siRNA had no effect on WBP-2 protein levels (Figure 3.9A). Depletion of endogenous WBP-2 reduced PR transcriptional activity of ERα and PR.

**Figure 3.8:** WBP-2 had no significant effect on the transcriptional activity of p53 and VP-16 activation domains. HeLa cells were transiently transfected with expression plasmid for either p53 or VP-16 activation domain fused to GAL4, along with their respective reporter plasmids, p21 promoter-LUC or 17mer-LUC in the presence or absence of WBP-2. Cells were harvested after 24 hrs and assayed for luciferase activity and bars are mean ± SD from three different determinations. The activity of the transcription factor in the absence of WBP-2 was defined as 1-fold, and the data for other bars were scaled accordingly.
activity by 75% (Figure 3.9B), indicating that WBP-2 is required for the normal transcriptional activities of the PR.

**Figure 3.9:** Physiological level of WBP-2 is essential for normal PR signaling. (A) Expression analysis of WBP-2 protein after siRNA treatment. HeLa cells were transiently transfected with PR expression plasmid and its response element either in presence of WBP-2 specific siRNA (in vitro synthesized) or control GAPDH siRNA. 4hrs post transfection cells were treated either with progesterone (10^{-7} M) or vehicle (ethanol). Twenty-four hours after transfection cells were harvested and lysed. Cell lysates were run on a 4-20% gradient gel, transferred onto nitrocellulose paper. Protein levels were assessed by Western Blot using WBP-2 specific antibodies. Equal loading of samples was confirmed using β-actin specific antibodies. (B) Depletion of endogenous WBP-2 levels reduces transcriptional activity of PR. Part of the cell lysates were used to measure luciferase activity. The activity of PR in the presence of progesterone and control siRNA (GAPDH) was taken as 100-fold activation and the data for other bars were scaled accordingly.
Figure 3.10: WBP-2 expression level is critical for ERα-mediated endogenous target gene activation. (A) WBP-2 mRNA expression levels after siRNA treatment. MCF-7 cells were transfected with control siRNA (siScrambled), siGAPDH or siWBP-2. Then cells were treated with vehicle (−H) or E2 (+H) for 12 hr before being collected for RNA preparation. Real-time PCR was performed with specific primers for WBP-2. (B) Depletion of endogenous WBP-2 levels reduces the expression levels of ER target gene pS2. The mRNA levels of pS2 gene were quantified using pS2 specific primers by real-time PCR analysis. All the samples were normalized against calnexin transcripts, and plotted as relative fold mRNA levels. The relative mRNA level of both WBP-2 and pS2 under GAPDH knockdown condition was taken as 1-fold and other bars are scaled accordingly.
We further investigated whether disruption of endogenous WBP-2 expression affects estrogen-dependent biological actions of ERα in MCF-7 cells that express high levels of ERα. MCF-7 cells were transfected with non-specific siRNA (siScrambled), control siRNAs (siGAPDH) or siRNA against WBP-2 (siWBP-2) and then treated with either vehicle or estradiol. The expression of estrogen-regulated gene, pS2 was measured by quantitative real-time PCR. In these assays, WBP-2 mRNA levels were quantified to measure the efficacy of the siWBP-2. We found that there was a 66% decrease (average between cells treated with and without estradiol) in WBP-2 mRNA levels in cell treated with siWBP-2 in comparison to the control (siGAPDH and siScrambled) siRNA treated cells (Figure 3.10A).

Estradiol treatment significantly enhanced the relative mRNA levels of pS2 in cells that were transfected with either of the control siRNAs (siScrambled or siGAPDH) (Figure 3.10B). Importantly, cells treated with siWBP-2 had a 51% reduction in the estradiol-induced pS2 mRNA level and a 49% reduction is estradiol-induced GREB1 mRNA level, suggesting that WBP-2 is one of the factors required for the biological functions of ERα in MCF-7 cells (Figure 3.10B). Though siRNA mediated gene knockdown is adequately specific to the intended target gene, off-target effects cannot be completely ruled out and so ‘Wobble complementation’ assays can be employed to study the re-introduction of WBP-2 in siRNA-mediated knockdown assays.
3.6 Recruitment of WBP-2 onto an Estrogen-Responsive Promoter in MCF-7 Cells

To further substantiate the coactivation function of WBP-2, we employed classical ChIP to study the recruitment of WBP-2 onto an ERα-responsive promoter in MCF-7 cells. Formaldehyde crosslinked protein-chromatin complexes were pre-cleared with pre-immune serum and immunoprecipitated with ERα and WBP-2 specific antibodies from MCF-7 that were treated with or without estradiol. The genomic DNA fragments associated with ERα or WBP-2

Figure 3.11: WBP-2 is recruited onto ERα-responsive promoter. Chromatin Immunoprecipitation (ChIP) was performed using MCF-7 cells in the absence (-H) or presence (+H) of estradiol. Primers specific for pS2 promoter were used to amplify the genomic DNA associated with either ERα (IP: ERα) or WBP-2 (IP: WBP-2) in MCF-7 cells. The PCR products were separated on agarose gel and the band intensities were quantified using the Image J (NIH) software. Input band intensity was considered as 100% and the other bands were scaled accordingly.
were amplified by PCR using primers specific for estrogen receptor binding site within the pS2 promoter locus. ChIP analyses demonstrated the recruitment of WBP-2 onto ER\(\alpha\)-responsive promoter in the presence of estrogen (Figure 3.11) indicating that WBP-2 is recruited to the pS2 promoter in a hormone-dependent manner, like other characterized coactivators.

3.7 WBP-2 REVERSES TRANSCRIPTIONAL INTERFERENCE BETWEEN ER\(\alpha\) AND PR

Previously, it has been demonstrated that the transcriptional activity of one receptor can be squelched by the overexpression of another receptor, indicating that both receptors compete for limited pools of common factors (Ming-Jer Tsai, 1998). We used a transient transfection assay to determine if WBP-2 can relieve ER\(\alpha\)-induced squelching of the transcriptional activity of PR. We observed that PR-mediated transcriptional activity was reduced by 75% upon co-expression of ER\(\alpha\) in the presence of estradiol (Figure 3.12; compare lanes 2 and 3). Addition of WBP-2 reversed this squelching in a dose-dependent manner (Figure 3.12; compare lanes 3 through 8). In control cells that are not treated with estradiol, WBP-2 transfection increased PR-mediated transcriptional activity three to four fold (Figure 3.12; compare lanes 2 and 9). Thus co-expression of WBP-2 can reverse the interference between ER\(\alpha\) and PR, suggesting that WBP-2 is one of the limiting factors that are necessary for efficient PR and ER\(\alpha\) transcriptional activity.
3.8 **Co-expression of WBP-2 and E6-AP Additively Enhance ERα and PR Activity**

Since E6-AP has been reported to interact with SHRs (Nawaz et al., 1999b) and WBP-2 (this study), we wished to further explore the functional interaction between E6-AP and WBP-2. HeLa cells were transiently transfected with wild-type E6-AP and WBP-2 expression plasmids either alone or together.
WBP-2 and E6-AP each transfected alone significantly enhanced the activity of PR (Figure 3.13). However, when coexpressed, WBP-2 and wild-type E6-AP additively enhanced the transactivation function of PR (Figure 3.13). To guard against saturation of PR activity when expressing E6-AP and WBP-2 exogenously in these assays, we performed dose response curves (data not shown). This data suggests that E6-AP and WBP-2 may functionally cooperate with each other.

**Figure 3.13: WBP-2 and E6-AP additively enhance PR transactivation.** Cells were transiently transfected with PR expression plasmid and progesterone responsive reporter plasmid in the absence or presence of WBP-2 or E6-AP or both. Cells were treated with or without progesterone (10^{-7} M). Data is expressed as mean ± SD of three different experiments and plotted as fold activation.
3.9 Coactivation functions of WBP-2 does not require E6-AP

To better understand the functional interaction between WBP-2 and E6-AP, we wanted to determine whether the coactivation functions of WBP-2 was dependent on E6-AP. To address this question, we performed transient co-transfection assays in primary MEF cells generated from the E6-AP knockout mouse line (as described in methods). E6-AP knockout MEFs were transiently co-transfected with expression vectors for PR, wild-type WBP-2 and the progesterone receptor specific reported plasmid. In these assays wild-type E6-AP was utilized as a positive control.

Figure 3.14: Coactivation functions of WBP-2 does not require E6-AP. E6-AP knockout mouse embryonic fibroblast cells were transiently transfected with PR expression plasmid and progesterone responsive reporter plasmid in the absence or presence of WBP-2 or E6-AP. Cells were treated with or without progesterone (10⁻⁷ M). Data is expressed as mean ± SD of three different experiments and plotted as fold activation.
Our data clearly showed that WBP-2-mediated enhancement of PR transactivation in the absence of E6-AP was not significantly reduced, suggesting that the coactivation activity of WBP-2 in not dependent of E6-AP (Figure 3.14). These observations were corroborated by similar results obtained with ERα in an identical experimental set-up (Figure 3.14).

3.10 CARBOXY-TERMINAL PY MOTIF 3 (PY3) OF WBP-2 IS ESSENTIAL FOR ITS COACTIVATION FUNCTION

WBP-2 contains three proline-rich motifs containing the sequence PPXY (P: proline; X: any amino acid and Y: tyrosine) (Chen and Sudol, 1995). These PPXY sequences or PY motifs in WBP-2 are binding/recognition sites for a subclass of WW-domains present in certain other proteins. Since WBP-2 enhances the transcriptional activities of ERα and PR, we wanted to determine whether the PY motifs of WBP-2 are essential for its ability to modulate the transcriptional activity of these receptors. To this end, we mutated each of the three PPXY motifs of WBP-2 to AAXA (A: alanine) (Figure 3.15) and tested the
ability of the mutant WBP-2 proteins to modulate PR transcriptional activity by transient transfection assays.

![Figure 3.16: The PY motif 3 of WBP-2 is essential for its coactivation functions.](image)

**Figure 3.16:** The PY motif 3 of WBP-2 is essential for its coactivation functions. **(A)** PY motif 3 of WBP-2 is required for its coactivation function. HeLa cells were transfected with PR expression and reporter plasmids in the presence or absence of either wild-type or one of the seven PY motif mutants of WBP-2. PY1 represents the construct where the first PY motif of WBP-2 is mutated (similarly PY2 and PY3 signifies mutation of the second and third motifs respectively), PY1-2 carries double mutations at the first and second PY motifs (PY1-3 and PY2-3 contains mutations at corresponding motifs) and PY1-2-3 is the triple mutant where all three motifs are mutated. Cells were treated with progesterone (10^{-7}M) and luciferase activity was measured. Data is expressed as mean ± SD of three different transfections and plotted as fold activation. The activity of PR in the presence of hormone and in the absence of WBP-2 was defined as 1-fold, and the data for other bars were scaled accordingly. **(B)** **Wild-type and PY3 mutant WBP-2 expressed at equal level.** Protein levels were analyzed by Western blot using anti-WBP-2 antibody. Control lane represents cells that were transfected with empty vector. β-actin expression was used as loading control.

As shown in Figure 3.16A, wild-type WBP-2 enhanced the transcriptional activity of PR in a hormone-dependent manner in transient transfection assays.
Similarly, the WBP-2 proteins that contained mutations in either PY motif 1 or PY motif 2 were able to enhance PR activity, suggesting that PY motifs 1 and 2 of WBP-2 were nonessential for its coactivation functions (Figure 3.16A). In contrast, the WBP-2 construct that contained mutations in the carboxy-terminal PY motif (PY3) of WBP-2 (alone or in combination with other motifs mutants) was unable to enhance the transcriptional activity of PR suggesting that the PY3 motif is the minimum essential motif of WBP-2 required for its transcriptional function (Figure 3.16A).

To confirm that the loss of coactivation function of the PY3 motif mutant WBP-2 was not due to the loss of expression of the mutant WBP-2, we analyzed the expression of both the wild-type and the PY3 mutant WBP-2 by Western blot analysis. Figure 3.16B, demonstrated that both the wild-type and the PY3 mutant WBP-2 proteins were expressed at approximately equal levels.

3.11 PY3 MOTIF MUTANT WBP-2 CAN INTERACT WITH ERα AND PR

Next, we wanted ascertain whether the mutation of the PY motif 3 of WBP-2 affects the interaction between WBP-2 and the receptor. To accomplish this, we analyzed the interaction between the PY3 mutant WBP-2 with ERα and PR in GST-pulldown assays. In these assays wild-type WBP-2 was used as a positive control. As shown in Figure 3.17, both wild-type WBP-2 and PY3 motif mutant WBP-2 can still interact with ERα and PR in a hormone-dependent manner suggesting that loss of coactivation function of the PY3 mutant WBP-2 was not due to loss of its interaction with the receptors. Taken together these
results demonstrate that the carboxy-terminal PY motif 3 of WBP-2 is essential for its ability to enhance SHR-mediated transcription.

Figure 3.17: Wild-type and PY3 mutant WBP-2 interact with ERα and PR. Interaction of wild-type and PY3 mutant WBP-2 with ERα and PR were determined in a GST pull down assay. ERα and PR was labeled with [35S] by in vitro transcription and translation kit. The labeled ERα and PR proteins were then incubated overnight at 4°C with E. coli expressed GST alone (control), GST-WBP-2 (wild-type) or GST-PY3 mutant WBP-2 bound to beads either in the absence of hormone (-H), presence of hormone (+H: estradiol for ER and progesterone for PR). Bound proteins were analyzed by SDS-PAGE and autoradiography with 10% of in vitro translated ER and PR as input.
4.1 **Identification of WBP-2 Interacting Proteins**

As mentioned in the introduction, WBP-2 contains three polyproline (PY) motifs and we have shown that one of these motifs is essential for the coactivation activity of WBP-2. The next important step in understanding the mechanism behind the coactivation functions of WBP-2 is to identify the WW-domain containing proteins that interact with WBP-2. In pursuit of this aim we utilized Panomics' TranSignal WW-domain Array (Panomics, Redwood City, CA). This array is a dot-blot based membrane array that consists of WW-domains from 42 different proteins. The protein-protein interactions were analyzed by immunoblotting for FLAG-tagged WBP-2, which were bound to the WW-domains on the membrane. Our screening data suggested that WBP-2 interacts with a wide variety of WW-domain containing proteins, as listed in table 4.1. In this study, I focused on YAP1, one of the proteins that we identified in this assay (Figure 4.1).

**Table 4.1 List of WBP-2 interacting proteins**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>WW-domain(s)</th>
<th>Interaction</th>
<th>Protein name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SMURF1</strong></td>
<td>D1</td>
<td>-</td>
<td></td>
<td>E3 ubiquitin ligase</td>
</tr>
<tr>
<td></td>
<td>D2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SMURF2</strong></td>
<td>D1</td>
<td>-</td>
<td></td>
<td>E3 ubiquitin ligase</td>
</tr>
<tr>
<td></td>
<td>D2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WWP1</strong></td>
<td>D1</td>
<td>++</td>
<td>WW-domain</td>
<td>Nedd-4-like</td>
</tr>
<tr>
<td>D2</td>
<td>++</td>
<td>Containing protein 1</td>
<td>Ubiquitin protein ligase</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>++</td>
<td>NEDD4</td>
<td>Neuronal precursor cell-expressed developmentally downregulated 4</td>
<td></td>
</tr>
<tr>
<td>D4</td>
<td>++</td>
<td>Caveolin-3</td>
<td>Membrane protein</td>
<td></td>
</tr>
<tr>
<td>PABPN1</td>
<td>+</td>
<td>Poly(A)-binding protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAS7</td>
<td>+</td>
<td>Growth-arrest specific 7 isoform b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YAP1</td>
<td>++</td>
<td>Yes-associated protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAIAP1</td>
<td>D1</td>
<td>-</td>
<td>Membrane associated guanylyl kinase-1</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITCH</td>
<td>D1</td>
<td>+</td>
<td>Atropin-1 interacting protein 4</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3</td>
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<tr>
<td>D4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAZ</td>
<td>++</td>
<td>Transcriptional coactivator with PDZ domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WWOX1</td>
<td>D1</td>
<td>++</td>
<td>WW-domain containing oxidoreductase, isoform 1</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>+/-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APBB3</td>
<td>+</td>
<td>FE65-like protein</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Information provided by Panomics, Inc (Redwood City, CA).
Initially, Sudol et al, identified WBP-2 as a binding partner for YAP1 using a functional screen, which we confirmed with our WW domain array screening assay. Next we wanted to know if this interaction is mediated by the PY motif of WBP-2 and the WW-domain of YAP1. For this purpose we used both wild-type and coactivation-dead PY3 mutant WBP-2 in GST pull-down assays. Figure 4.2, demonstrates that while the wild-type WBP-2 was able to interact with YAP1, mutation of the PY motif 3 of WBP-2 completely abrogated this interaction. Together with our previous observations we concluded that the PY3 motif of WBP-2, though not essential for its interaction with the receptors was indispensable for its interaction with the WW-domain containing protein YAP1.
4.3 **WBP-2 and YAP1 co-precipitate with ERα in cellular lysates**

We have previously shown that WBP-2 interacts with ERα in MCF-7 cells; subsequently we have identified YAP1 as a WBP-2-interacting protein. Next we
wanted to know if WBP-2 and YAP1 form a complex with ERα. To this end, we performed IP assays in MCF-7 cells treated with and without estradiol. Our data shows that WBP-2 and YAP1 interact with ERα in a hormone-dependent manner (Figure 4.3).

4.4 **ESTROGEN INDUCES ASSOCIATION OF ERα WITH WBP-2 AND YAP1 AT ERα RESPONSIVE GENE PROMOTERS**

To further investigate the association of ERα with WBP-2 and YAP1 at ERα responsive pS2 and GREB1 promoters, we performed quantitative re-ChIP assays in MCF-7 cells. Cross-linked and sheared DNA-protein complexes from MCF-7 cells were immunoprecipitated with either non-specific purified IgG (Mock) or ER-α specific antibody. After immunoprecipitation the cross-linked immunocomplexes from each of the primary ChIPs were eluted and subjected to another round of immunoprecipitation with antibodies specific for WBP-2 or YAP1. The immunocomplexes after re-ChIP were eluted; reverse cross-linked and the associated genomic DNA fragments were analyzed by QPCR with pS2 and GREB1 promoter specific primers.

We observed that in ERα/WBP-2 ChIP assays where ERα antibody was used in the first ChIP and WBP-2 specific antibody was used in the re-ChIP, there was a significant enrichment in the estrogen-induced association of WBP-2 with ERα in comparison with the IgG/WBP-2 (mock) ChIP (Figure 4.4). Similar results were observed with ERα/YAP1 ChIP when compared to its IgG/YAP1 (mock) ChIP (Figure 4.4). These observations suggest that WBP-2 and YAP1 are
recruited to and are associated with ERα at the pS2 and GREB1 promoter loci in an estrogen-dependent manner.

Figure 4.4: Recruitment of WBP-2 and YAP1 to ERα responsive promoter. MCF-7 cells were treated with vehicle (-H) or E2 (+H, 10⁻⁷ M) for 1 hour. Sheared chromatin-protein complexes were immunoprecipitated with non-specific purified IgG (mock) or an antibody to ERα, the products were subject to re-ChIP analysis using antibodies against WBP-2 or YAP1. The bound DNA fragments were quantified by real-time PCR. The bound DNA fragments were quantified by real-time PCR. A mock ChIP reaction was included in the analysis as a control. The data are represented as fold enrichment over 1% total chromosome input DNA before immunoprecipitation and are mean ± SD from three independent experiments.

4.5 YAP1 AND WBP-2 SYNERGISTICALLY ENHANCE PR TRANSACTIVATION FUNCTIONS

WBP-2 was first identified as a protein-binding partner of the WW-domain containing protein, YAP1. YAP1 has been shown to be a transcriptional coactivator (Strano et al., 2005; Strano et al., 2001; Yagi et al., 1999). Thus, we wanted to know whether YAP1 modulates the steroid receptor-dependent target gene expression. To determine the role of YAP1 in steroid hormone receptor
transactivation, HeLa cells were co-transfected with mammalian expression plasmids for the PR along with reporter plasmids containing its cognate hormone response element, with or without an expression vector for YAP1. YAP1 (alone) had no effect on PR-mediated transactivation either in the absence or presence of hormone. In contrast, when YAP1 was coexpressed with WBP-2 the hormone-dependent transcriptional activity of PR was synergistically enhanced (~24-fold), which is three times more than the activity observed with wild-type WBP-2 alone (Figure 4.5). These data suggest that the hormone-dependent transcriptional coactivation activity of YAP1 is revealed only in the presence of WBP-2.

The PY motifs of WBP-2 have been shown to interact with the WW-domain of YAP1 (Chen and Sudol, 1995). In this study we have identified that the PY motif 3 of WBP-2 is essential for its coactivation functions, hence we asked whether the PY motif 3 of WBP-2 is required for YAP1 to function as a steroid receptor coactivator. As shown in figure 4.5, when coexpressed together, wild-type WBP-2 and wild-type YAP1 greatly enhanced the transactivation function of PR. However, coexpression of the PY3 mutant WBP-2 and wild-type YAP1 failed to enhance the transcriptional activity of the progesterone receptor (Figure 4.5) indicating that the PY motif 3 of WBP-2 is essential for YAP1 to function as a secondary coactivator.
4.6 ENDOGENOUS ERα TARGET GENEACTIVATION IS SYNERGISTICALLY ENHANCED BY YAP1 AND WBP-2

To further substantiate that YAP1 and WBP-2 synergistically enhance ERα and PR transactivation functions as observed by reporter gene assays, we performed QPCR analysis of ERα target gene pS2 in MCF-7 cells. MCF-7 cells were co-transfected with either control plasmid or expression plasmids of wild-type WBP-2, PY3 motif mutant WBP-2, and wild-type YAP1 alone or in
combination. Cells were treated with either vehicle or estradiol (E\(_2\)) and expression of estrogen-regulated gene, pS2 was measured by QPCR. As control, we also examined the mRNA levels of ER\(\alpha\), WBP-2 and YAP1. There was no change observed in ER\(\alpha\) mRNA levels (data not shown) whereas, the mRNA levels of exogenously expressed WBP-2 (wild-type and PY3 mutant) and wild-type YAP1 were significantly increased in cells that were transfected with their respective expression plasmids (Figure 4.6A).

As shown in figure 4.4B, wild-type WBP-2 enhanced the relative mRNA levels of ER\(\alpha\) target gene, pS2 in the presence of estradiol. Neither YAP1 nor the PY3 mutant WBP-2 had any significant effect on pS2 mRNA levels. Whereas, when YAP1 was coexpressed with wild-type WBP-2, the hormone-dependent transcriptional activity of ER\(\alpha\) was synergistically enhanced, more than that observed with WBP-2 alone (Figure 4.6B). Furthermore, this synergistic enhancement of pS2 relative mRNA levels was not observed when YAP1 was co-expressed with the PY3 motif mutant WBP-2. Taken together, both our reporter gene assays and endogenous target gene assays demonstrate that YAP1 can function as a secondary transcriptional coactivator of PR and ER\(\alpha\) in a WBP-2 dependent manner (Figure 4.6B).
4.7 ESTROGEN-INDUCED RECRUITMENT OF WBP-2 AND YAP1 ONTO ERα RESPONSIVE pS2 PROMOTER IS MUTUALLY INTERDEPENDENT

Our initial observations showed that YAP1 acts as a secondary coactivator of ERα only when co-expressed with WBP-2. We also show that estrogen enhances the recruitment of YAP1 to ERα responsive pS2 promoter. To test further, if the recruitment of YAP1 is dependent on WBP-2 and conversely, is WBP-2 dependent on YAP1 for its recruitment to ERα responsive promoter, we performed quantitative re-ChIP assays as described above in MCF-7 cells. In these assays MCF-7 cells were treated with siRNAs against WBP-2, YAP1 or a non-specific scrambled siRNA.

In cells treated with non-specific scrambled siRNA, estrogen-induced association of ERα with WBP-2 (ERα/WBP-2) and YAP1 (ERα/YAP1) were consistent with our earlier observations when compared to their respective mock ChIP assays. Whereas in cells treated with siRNA against WBP-2, the estrogen-induced enhancement of the association of YAP1 with ERα was completely lost, suggesting that the recruitment and association of YAP1 with ERα at the pS2
promoter locus is dependent on the normal endogenous expression levels of WBP-2. Interestingly, the converse relationship also holds true, where the association of WBP-2 with ERα and its recruitment to the pS2 promoter locus was abolished in cells that were treated with siRNA against YAP1 (Figure 4.7).

**Figure 4.7: Recruitment of WBP-2 and YAP1 to ERα responsive promoter is mutually interdependent.** MCF-7 cells were pre-treated with non-specific siRNA (siScramble), siRNA against WBP-2 (siWBP-2) or YAP1 (siYAP1) before treatment with vehicle (-H) or E2 (+H, 10⁻⁷ M) for 1 hour. Sheared chromatin-protein complexes were immunoprecipitated with non-specific purified IgG (mock) or an antibody to ERα, the products were subject to re-ChIP analysis using antibodies against WBP-2 or YAP1. The bound DNA fragments were quantified by real-time PCR. The bound DNA fragments were quantified by real-time PCR. A mock ChIP reaction was included in the analysis as a control. The data are represented as fold enrichment over 1% total chromosome input DNA before immunoprecipitation and are mean ± SD from three independent experiments.
These observations suggest the intriguing possibility that the recruitment of WBP-2 and YAP1 to the pS2 promoter locus and their association with ERα may be mutually interdependent. When either YAP1 or WBP-2 expression is reduced, the binding to pS2 ERE of the other is affected.
CHAPTER 5
DISCUSSION

ERα and PR are members of a superfamily of hormone-dependent transcription factors known as steroid hormone receptors (SHR) that stimulate target gene expression in response to estrogens and progestins, respectively. Upon ligand binding, these receptors undergo a series of modification steps that include conformational change, receptor dimerization, phosphorylation and finally, recruitment of other protein coregulators. Coregulators can be broadly classified into two groups, namely, coactivators and corepressors, that, as the names suggest either facilitate or attenuate receptor-mediated signaling. A vast majority of coactivators possess chromatin modifying activities (including histone acetyltransferase (HAT), methyl transferase, ATPase, ubiquitin-conjugation and ubiquitin-ligase) that help in opening up chromatin for receptor binding. To date, there are over 300 different known coactivators with varying functions that range from mediating protein-protein interactions to receptor degradation and promoter clearance. The identification and characterization of coregulators is critical for the better understanding of the molecular strategies underlying SHR-mediated gene transcription (Ming-Jer Tsai, 1998; Tsai and O'Malley, 1994).

In this study we describe the identification and characterization of a novel protein, WBP-2, with previously unknown biological function, as a coactivator that selectively modulates ERα and PR mediated target gene activation. We also
propose a plausible molecular mechanism of action for WBP-2 and present data in support of this mechanism.

5.1 Identification of WBP-2

Coactivators are known to form and function as multi-protein complexes. We used a yeast two-hybrid screening system to identify proteins that interact with E6-AP. WBP-2 was one of the proteins identified from this screen. Our GST pulldown assays confirmed the direct interaction between WBP-2 and E6-AP.

5.2 Characterization of WBP-2 as a Novel Coactivator of ERα and PR

Since WBP-2 was identified as an E6-AP-interacting protein, we wanted to study its possible role as a coactivator of SHRs. We took a systematic approach toward our working hypothesis that WBP-2 acts as a coactivator of SHRs, by constructing a set of questions that would sufficiently test our hypothesis.

1. Does WBP-2 interact with SHRs?

Our data demonstrates that WBP-2 preferentially interacts with the liganded forms of ERα and PR both in in vitro GST pull down assays and in coprecipitation experiments in cells that endogenously express high levels of ERα and PR respectively. Most characterized SHR coactivators have been shown to interact with the receptor only in the presence of hormone via their LXXLL motif(s) (Heery et al., 1997; Li et al., 2003; Torchia et al., 1997), but WBP-2 is distinct from other coactivators in this aspect, as it interacts with ERα and PR in a hormone-dependent manner despite the lack of a consensus LXXLL motif.
2. Does WBP-2 enhance the transactivation functions of SHRs?

A vast Majority of coactivators exhibit little receptor specificity. Instead they enhance the transactivation function of a variety of steroid/nuclear hormone receptors. Our data from transient transfection assays in HeLa cells showed that, unlike other coactivators, WBP-2 is unique, as it exhibits receptor selectivity and preferentially coactivates the hormone-dependent transcriptional activities of PR and ERα, having little effect on the transactivation functions of GR and AR. Furthermore, WBP-2 had no effect on other non-receptor transcription factors like p53 or VP-16. These observations were corroborated by our data demonstrating the selective coactivation functions of WBP-2 on endogenous ERα and PR in MCF-7 and T47D respectively. Additionally, WBP-2 had no effect on the AR in LnCaP cells, which express high levels of AR.

3. Does knockdown of WBP-2 affect ERα and PR transactivation function?

Targeted depletion of coactivators in mice and cell lines has demonstrated that coactivators are required for proper functioning of steroid hormone receptors (32, 33). siRNA-mediated depletion of endogenous WBP-2 resulted in a concomitant decrease in receptor-mediated transactivation functions both in reporter gene assays and endogenous target gene assays. These experiments suggest that, like other coactivators, WBP-2 may play an active role in the proper functioning of steroid hormone receptors.
4. Is WBP-2 recruited to ER\(\alpha\) responsive promoter?

Our classical ChIP analyses demonstrate the hormone-dependent recruitment of WBP-2 onto an endogenous ER\(\alpha\) responsive pS2 promoter. Like other characterized coactivator proteins such as E6-AP (Khan et al., 2006), UbcH7 (Verma et al., 2004), and SRC family members (Onate et al., 1998), WBP-2 is also recruited to the ER\(\alpha\) target gene promoters in a hormone-dependent manner.

5. Is WBP-2 a component of the limited pool of coactivators?

The existence of modulatory proteins in the steroid/nuclear hormone receptor transactivation pathway is supported by the findings that the transcriptional activity of one receptor can be squelched by the overexpression of another receptor, indicating that both receptors compete for a limited pool of common factors. Our results indicate that overexpression of WBP-2 reverses the squelching effect of ER\(\alpha\) on PR transactivation in a dose-dependent manner and are consistent with previously published studies indicating that authentic coactivators usually can reverse the squelching between two competent active receptors (19).

6. Is WBP-2 functionally dependent on E6-AP?

Since we identified WBP-2 as an E6-AP-interacting protein and since E6-AP is a characterized SHR coactivator, it is possible that WBP-2 may be functionally dependent of E6-AP. Our reporter gene assays from HeLa cells, indicated that WBP-2 and E6-AP additively enhanced the transactivation function of PR, but further investigations into this relationship using mouse embryonic...
fibroblasts from E6-AP knockout lines revealed that WBP-2 does not require E6-AP for its coactivation functions.

As mentioned in the introduction, most coactivators have some enzymatic activity that facilitates receptor transactivation whereas WBP-2 does not have any enzymatic activity. The WBP-2 protein contains three PPXY sequences known as the PY motifs. The PY motifs are present in the transcriptional activation domains of several transcription factors, including c-Jun (Baichwal and Tjian, 1990), AP-2 (Williams and Tjian, 1991), C/EBPα (Nerlov and Ziff, 1994), NF-E2 (Kiekhaefer et al., 2004), KROX-20 (Vesque and Charnay, 1992), MEF2B (Molkentin et al., 1996), and PEBP2 (Yagi et al., 1999), suggesting that the PY motifs may play vital role in gene transcription. PY motifs in these proteins have been previously shown to mediate protein-protein interactions and they represent potential transactivation domains that could function by recruiting additional strong transactivator proteins to the promoters of target genes (Sudol et al., 2005; Sudol et al., 2001). Thus we hypothesized that the PY motif(s) might play a significance role in WBP-2 function.

7. Is the PY motif(s) of WBP-2 essential for its coactivation functions?

Our data support the hypothesis that the PY motif(s) is involved in gene transcription, because mutation of the third PY motif (PY3) totally abolished both the intrinsic activation function of the molecule, as well as the coactivation function of WBP-2. Although neither ERα nor PR contain WW domains (specific binding domain of PY motifs), we wanted to confirm that the loss of the coactivation function of WBP-2 was not due to lack of interaction with the
receptors. Our GST pull down assays showed that both the wild-type and coactivation-dead mutant (PY3) WBP-2 preferentially interact with the liganded receptors (ERα and PR). These data suggested that the PY motif 3 of WBP-2 is essential for its transcriptional activities (intrinsic activation and coactivation functions) but not for its interaction with the receptors.

Taken together, the above listed observations confirmed that WBP-2 is a *bona fide* coactivator of a subset of SHRs namely ERα and PR. Furthermore, the coacivation function of WBP-2 is not dependent on E6-AP, but the PY motif 3 of WBP-2 is a critical mediator required for its transcriptional activities.

### 5.3 Molecular Mechanism of WBP-2 Functions

Specific protein-protein interactions and multiprotein complexes are important for a multitude of cellular processes including gene transcription. These interactions are facilitated by various protein-protein interactions modules. For example, the interaction between poly-proline (PY) motif containing proteins and WW domain (WW) containing proteins is facilitated by the PY-WW module. As a direct result of the first part of this study, we have observed that the PY motif 3 of WBP-2 is essential for its coactivation and intrinsic activation functions, but mutation of the PY motif 3 of WBP-2 does not affect its interaction with the receptors. These two key insights into the possible mechanism of action of WBP-2 led us to postulate that there may be additional WW domain containing protein(s) involved in WBP-2-mediated coactivation of ERα and PR.
8. **What are the WW domain containing protein(s) that interacts with WBP-2?**

As described in the introduction, PY motifs are modular protein domains that specifically recognize and bind to WW domains. The PY-WW protein-protein interaction module is well documented, so we performed a WW domain array screen for the identification of possible WBP-2-interacting proteins. There were a number of proteins that interacted with WBP-2 including YAP1. Since YAP1 was a known transcriptional coactivator of various signaling pathways like p73, Runx2 and ErbB4, we explored YAP1 as a candidate signaling partner for WBP-2.

9. **Does the PY-WW module mediate the interaction between WBP-2 and YAP1?**

Even though WBP-2 was initially identified as a binding partner for YAP1, we wanted to establish that the interaction between WBP-2 and YAP1 is facilitated by the PY-WW module. Our data clearly showed that unlike the wild-type WBP-2, the PY motif 3 mutant WBP-2 failed to interact with YAP1, indicating that one of the PY motifs (PY3) of WBP-2 was necessary and sufficient for its interaction with YAP1.

10. **Does YAP1 interact with the receptor?**

Having shown that WBP-2 interacts with the receptors in a hormone-dependent manner, we then asked if YAP1 exists is a complex with WBP-2 and ERα. Immunoprecipitation assays from MCF-7 and T47D cells have shown that YAP1 and WBP-2 co-precipitate with the ERα and PR respectively. This suggested that WBP-2 and YAP1 may exist in a complex with ERα. Next we studied the functions of this complex.
11. Are WBP-2 and YAP1 recruited to ERα responsive promoter?

YAP1 has been reported to be recruited to p73 and Runx2 target gene promoters (Strano et al., 2005; Strano et al., 2001; Zaidi et al., 2004), and we have shown that WBP-2 is recruited to ERα responsive pS2 promoter in a hormone-dependent manner. Since our data suggested that WBP-2 and YAP1 may form a complex with the receptor, we assayed the recruitment of WBP-2 and YAP1 in association with ERα at the pS2 promoter locus. Our quantitative re-ChIP assays demonstrated that estrogen enhanced both the recruitment and association of WBP-2 with ERα at the pS2 promoter locus. Identical results were obtained for the recruitment and association of YAP1 with ERα at the same locus, indicating that WBP-2 and YAP1 co-occupy the pS2 promoter locus with ERα in an estrogen-dependent manner.

12. IS WBP-2 and YAP1 functionally linked?

In this report, we have shown that YAP1 acts as a secondary coactivator of steroid hormone receptors. However, the coactivation function of YAP1 is revealed only in the presence of wild-type WBP-2 and not in the presence of the coactivation-dead (PY3) mutant WBP-2, suggesting that PY-WW module is critical in this functional relationship between WBP-2 and YAP1. Our data is consistent generally with previously published reports that YAP1 stimulates gene transcription by binding to the PY motif of ErbB4 protein (Aqeilan et al., 2005; Komuro et al., 2003).
13. Is the recruitment of YAP1 to pS2 promoter dependent on WBP-2?

Since the coactivation activity of YAP1 was revealed only in the presence of WBP-2, we quantified the recruitment of YAP1 to the pS2 promoter under WBP-2 knock down conditions. Our data strongly suggests that the recruitment and association of YAP1 with ERα at the pS2 promoter is dependent on the physiological expression levels of WBP-2. The above observation is consistent with other secondary coactivators, which are recruited to the promoters of SHR target via their interaction with primary coactivators that can directly interact with the receptor. Intriguingly, recruitment and association of WBP-2 is in turn dependent on YAP1 levels. Based on these observations, we postulate that the PY motif 3 of WBP-2 binds to the WW domain of YAP1 and recruits YAP1 to the target gene promoter by interacting with receptor, but the stability of this WBP-2-YAP1-ERα complex is dependent on the PY-WW module.

Taken together, several lines of evidence support our proposed molecular mechanism of WBP-2 that the PY motif 3 of WBP-2 recruits the WW domain containing protein YAP1 to the ERα responsive promoter and stimulates ERα target gene activation. First, WBP-2 interacts with YAP1 via the PY3 motif. Second, the secondary coactivation activity of YAP1 is revealed in the presence of WBP-2. Third, the PY motif 3 of WBP-2 is essential for its coactivation functions as well as the secondary coactivation functions of YAP1. Fourth, estrogen enhances the recruitment and association of WBP-2 and YAP1 with ERα at the pS2 promoter locus. Fifth, the recruitment and association of WBP-2 with ERα at the pS2 promoter is dependent on YAP1 and vice versa.
5.4 PROPOSED MODEL FOR THE COACTIVATION FUNCTIONS OF WBP-2 AND YAP1

Coactivators play a central role in the stimulation, regulation and termination of SHR signaling. Numerous coactivators have been shown to form and function as a multi-component complex that aggregates various enzymatic activities that serve to initiate and enhance SHR-mediated transcriptional activation of target genes. In my thesis project, I have identified and characterized two novel proteins, WBP-2 and YAP1 that specifically enhance the transactivation functions of a subset of SHR including ERα and PR. Based on our observation we propose a model for the coactivation functions of WBP-2 and YAP1.

WBP-2 and YAP1 form a complex, which is mediated by specific interactions between the PY motif 3 of WBP-2 and the WW domain of YAP1. Upon hormone induction the WBP-2-YAP1 complex binds to ERα and is recruited to the ERα responsive promoters. Once on the promoter, the WBP-2-YAP1 complex stimulates ERα-mediated transactivation by virtue of the potent transactivation domain contained within the C-terminus of YAP1. This results in the transcription and up-regulation of ERα target genes. Taken together we predict that, when the receptor-WBP-2-YAP1 complex is recruited to the hormone-responsive promoter, it acts at one of the many sub-steps required to modulate the transactivation of steroid hormone-responsive target gene. Additionally, we have observed that WBP-2 can interact with WW domain containing oxidoreductase 1 (WWOX1), a known WW domain containing tumor
suppressor protein. When WBP-2 interacts with WWOX1, the WBP-2-WWOX1 complex is retained in the cytoplasm and prevents YAP1 from binding to WBP-2. In summary, WWOX1 negatively regulates the coactivation functions of the WBP-2-YAP1 complex resulting in the attenuation of the mitogenic signaling via ER and PR (Figure 5.1). The data pertaining to the role of WWOX1 in the molecular mechanism of WBP-2 is preliminary and is beyond the scope of this thesis dissertation.

Figure 5.1: Proposed model for the molecular mechanism of WBP-2. The PY motif containing protein WBP-2, interacts with the WW domain of oncogenic YAP1. This WBP-2:YAP1 complex translocate to the nucleus and is recruited to ER-responsive gene promoters. Estrogen induced association of WBP-2 and YAP1 with ERα at these promoters result in enhanced ERα signaling. Another WW domain containing protein, WW domain containing oxidoreductase 1 (WWOX1), negatively regulates WBP-2 by sequestering it in the cytoplasm (Data not shown in the dissertation). Cytoplasmic retention of the WBP-2:WWOX1 complex impinges on the coactivation function of the WBP-2:YAP1 complex resulting in the attenuation of ERα signaling.
The coactivation functions of these two proteins also represent the identification of a novel protein-protein interaction module, the PY-WW module, which we have characterized to be specific for ER and PR. Since ERα signaling is a mitogenic pathway, WBP-2-YAP1 complex-mediated enhancement of ERα signaling may be biologically relevant in estrogen dependent breast cancers. Taking into account the recent observation that YAP1 is an oncogene, it will be very rewarding to further explore the roles of this novel module in the functions of ERα and PR. Elucidating the precise mechanism of action of these proteins will greatly advance our understanding of the importance of the PY-WW module in SHR signaling, as well as the global regulation of transcription.
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