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COUP-TFII AND ITS INTERACTING PROTEINS IN BREAST CANCER AND
ENDOCRINE RESISTANCE

By
Lacey Morgan Litchfield
B.S., University of Louisville, 2008
M.S., University of Louisville, 2011

A Dissertation
Submitted to the Faculty of the
University of Louisville School of Medicine
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Department of Biochemistry and Molecular Biology
University of Louisville, School of Medicine
Louisville, KY

May 2013

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A Dissertation Approved on

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ABSTRACT

COUP-TFII AND ITS INTERACTING PROTEINS IN BREAST CANCER AND ENDOCRINE RESISTANCE

Lacey M. Litchfield

April 5, 2013

Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) is an orphan nuclear receptor that functions as either a transcriptional activator or repressor. COUP-TFII expression is reduced in endocrine-resistant breast cancer cells and overexpression of COUP-TFII restores the ability of antiestrogens to inhibit cell proliferation. In this study, I tested the hypothesis that the activity of COUP-TFII in maintenance of endocrine sensitivity and cell differentiation is dependent on its interacting proteins. A direct interaction was identified between COUP-TFII and the phosphoprotein nucleolin, which was found to function as a coregulator for COUP-TFII-mediated transcription. COUP-TFII increased the expression of the tumor suppressor retinoic acid receptor B2 (*RARB2*) in a nucleolin-dependent manner. COUP-TFII and nucleolin expression were correlated in estrogen receptor α (ER α)+ invasive ductal carcinomas. COUP-TFII expression was inversely correlated with patient tumor grade.

Aberrant activation of the NF κ B pathway has also been implicated in endocrine resistance. COUP-TFII transfection suppressed NF κ B activity in endocrine-resistant breast cancer cells. COUP-TFII overexpression also reduced the expression of NF κ B

target genes (*IL6*, *ICAM1*, *TNFAIP3*, and *CCL2*) and subunits (*NFKB1*, *REL*, *RELA*, and *RELB*) through a mechanism involving interaction of COUP-TFII with NFκB subunits RelB and NFκB1 to inhibit NFκB DNA-binding. COUP-TFII also reduced the ability of coactivators SRC-1, SRC-3, and CBP to enhance NFκB activity. An inverse correlation between COUP-TFII and *IL6*, *ICAM1*, *TNFAIP3*, *NFKB2*, *REL*, *RELA*, and *RELB* was observed in breast tumors from tamoxifen-treated breast cancer patients. The combination of COUP-TFII overexpression, NFκB inhibition, and tamoxifen treatment inhibited the growth of endocrine-resistant breast cancer cells. Endocrine-resistant breast cancer cells were more sensitive to treatment with an NFκB inhibitor than endocrine-sensitive cells, reflecting their reliance on the NFκB pathway for survival.

To further explore the role of COUP-TFII-interacting proteins in endocrine sensitivity, mass spectrometry was used to identify proteins that interacted with COUP-TFII specifically when breast cancer cells were treated with tamoxifen versus vehicle control. HSP27 was selected for follow-up from the proteins identified as interacting specifically with COUP-TFII in cells treated with tamoxifen. HSP27 expression was reduced in an endocrine-resistant breast cancer cell line that has undergone epithelial to mesenchymal transition, LY2, as well as in a trastuzumab-resistant breast cancer cell line, JIMT-1. A concomitant reduction in COUP-TFII expression was also observed in these cells. Future studies will explore the role of COUP-TFII-HSP27 interaction in resistance to tamoxifen and trastuzumab. COUP-TFII may be both a useful biomarker to predict tamoxifen and/or trastuzumab sensitivity as well as a target to restore sensitivity to resistant cells.

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CHAPTER I – BREAST CANCER AND ENDOCRINE RESISTANCE

BREAST STRUCTURE AND DEVELOPMENT

The complex branched structure of the adult mammary gland is composed of two basic cellular compartments: luminal and basal cells. Luminal epithelial cells line the lumen of the mammary duct, while the basal compartment is composed of progenitor cells and contractile myoepithelial cells that do not directly contact the ductal lumen. Coordinated paracrine signaling as well as structural connections between these two compartments are necessary for both the proper development and function of the mammary gland [1].

In the late 1950s, endocrine ablation experiments in rats and mice demonstrated that sequential timing of treatment with 17- β -estradiol (E_2), progesterone, and prolactin, in the presence of cortisol and growth hormone, were the minimal hormones necessary for mammary gland development [1]. Sequential hormone excretion and/or hormone receptor expression is required for the formation of proper glandular structure. Estrogens mediate the beginning of ductal branching and elongation as well as initial side branching, followed by progesterone regulation of continued side branching and alveoli formation. Alveologenesis is completed by prolactin, which also regulates milk production during the late stages of pregnancy [1]. Milk is produced in the lobules and transported through the ductal system. The ducts and ductal-lobular units are extended during puberty and further expand during pregnancy and lactation [2]. Aberrant

proliferation can occur in both the duct and lobule structures to form ductal carcinoma or lobular carcinoma, respectively, although ductal carcinomas are more common, *i.e.*, 75% of invasive breast cancers are ductal in origin, while 11% are lobular (the remaining 14% comprise a heterogeneous set of less common classifications) [3].

BREAST CANCER SUBTYPES

Breast cancer is the most commonly diagnosed cancer in women, and the leading cause of cancer death in females aged 20-59 years [4]. Breast cancers can be divided into 5 basic subtypes based on their distinct gene expression data: normal breast-like, luminal A, luminal B, ERBB2+, and basal [5-7]. The luminal A and luminal B groups express a gene signature similar to that of the luminal epithelial cells of normal mammary tissue, while the basal subtype is more reminiscent of basal epithelial cells [6,7]. Luminal B is distinguished from luminal A by higher expression of the cell proliferation marker Ki67, a biomarker associated with recurrence, relapse, and overall worse outcome in breast cancer patients [8-11]. The ERBB2+ group is defined by overexpression of the HER2/neu (ERBB2) receptor, a member of the epidermal growth factor receptor (EGFR) family that is ligand-independent and constitutively active, a known oncogenic transformation associated with poor outcome in breast cancer patients [12]. Normal breast-like cancers express a gene signature similar to that of adipose tissue, basal epithelial cells, and other nonepithelial components of the breast [5,7]. Breast cancer subtype is also indicative of clinical outcome, with basal and ERBB2+ subtypes associated with a statistically significant decrease in survival [5,6]. Luminal A tumors

show the highest estrogen receptor α (ER α) expression of the five subtypes, while ER α -cancers generally fall in the ERBB2+ or basal subtypes [6,7].

ESTROGEN RECEPTOR FUNCTION

Estrogen's action as the ovarian hormone responsible for maintenance of estrus was first characterized in 1923 by Allen and Doisy using partially purified hormone from the hog ovary [13]. Further investigation revealed three forms of estrogen: estrone, estrinol, and estradiol, with estradiol being the primary form in premenopausal women [14]. A receptor for estradiol (ER α) was first reported in 1962 [15], with discovery of a second subtype, ER β , following in 1996 [16]. Estradiol binding to ER α results in a conformational change that activates the receptor, leading to its dimerization. Upon formation of a dimer, ER α translocates to the nucleus wherein it binds to estrogen response elements (EREs) in target gene promoters (Figure 1) [17,18]. ER α can also bind to DNA through a tethering mechanism, wherein a transcription factor such as AP-1, *e.g.*, c-Jun/Fos, binds to specific target sequences and ER α then binds through protein-protein interaction with AP-1 [19,20]. Such a tethering mechanism can also occur between ER α and Sp1 [21,22]. Binding of ER α and associated coactivators, *e.g.*, members of the SRC family or CBP/p300, leads to recruitment of RNA polymerase II for transcription initiation [21,23]. Work by Carroll and Brown using chromatin immunoprecipitation (ChIP)-sequencing (ChIP-seq) technology has identified a large set of estrogen-responsive genes, and expanded understanding of ERE location to include sequences present in far upstream enhancer regions which exert their influence on target gene transcription through complex chromatin looping [24,25]. Many identified ER α -

responsive genes have roles involved in cell proliferation and survival, cell cycle progression, growth factor signaling, and hormone synthesis [26]. ER β functions in much the same way to bind to target gene DNA and promote gene transcription. ER α and ER β share many EREs in common, though ER α takes preference in binding if both receptors are activated by ligand [27,28]. ER α and ER β are both activated by estradiol binding; however, there have been receptor-selective endogenous ligands, *e.g.*, 5 α -androstane-3 β ,17 β -diol (3 β -Adiol), and synthetic ligands, *e.g.*, 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) for ER β identified [29,30]. ER α and ER β may also recruit different coactivators, or corepressors instead of coactivators, resulting in alterations in their effects on downstream gene expression [29,31]. Rather than the proliferative activity of ER α , ER β has been reported to play a growth inhibitory role in many cell types [29,32].

Figure 1. Model of estrogen action in the breast and inhibition by aromatase inhibitors and antiestrogens.

In breast cells, estradiol binds ER α leading to its dimerization and subsequent DNA binding in the cell nucleus to regulate target gene expression [17,21]. The antiestrogens tamoxifen and fulvestrant inhibit estradiol binding to ER α . Binding of the selective estrogen receptor modulator (SERM) tamoxifen to ER α leads to inhibition of coactivator recruitment and target gene expression [33]. Binding of the selective estrogen receptor downregulator (SERD) fulvestrant results in altered ligand binding domain structure, decreased ER α dimerization, and increased proteasomal degradation [34]. Aromatase inhibitors (AI) inhibit estrogen action in the breast by decreasing the conversion of the androgens testosterone and androstenedione to estrogens in peripheral tissues and breast stromal cells by inhibiting the enzyme activity of aromatase (CYP17A1) [35]. Estradiol has higher affinity for ER α than estrone [16] and these two estrogens are interconverted by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) [36]. Androstenedione and testosterone can also be interconverted by 3 β -HSD (not pictured) [36].

BREAST CANCER TREATMENT

Since the early 1930s when estrogen was first shown to promote breast cancer formation in mice [37], targeting estrogen signaling has been of interest in the treatment and prevention of breast cancer. In 1971, Jensen demonstrated that breast cancers with higher ER content exhibited a greater response to endocrine ablation than ER- cancers [38]. Studies such as these paved the way to the use of modern antiestrogen therapies to target the proliferative action of estrogen in breast cancer. Selective estrogen receptor modulators (SERMs) such as tamoxifen (TAM) and raloxifene (RAL) exert antiproliferative effects on breast cancer by competing with estrogens for binding to ER α (Figure 1). First developed as the contraceptive ICI 46,474 [39,40], tamoxifen acts as a cell type-specific agonist/antagonist of the estrogen receptor (agonist in uterus and antagonist in breast) [33]. Binding of 4-hydroxytamoxifen, the active metabolite of tamoxifen, to the ligand binding domain (LBD) of ER α results in a repositioning of ER α helix 12 to cover the hydrophobic coactivator binding groove, preventing binding with coactivators [41] and resulting in an antagonist function in the breast. In other tissues, the increased expression of coactivators such as SRC-1 [42] or the presence of other tissue-specific pathways, as well as promoter-specific functions, allows tamoxifen to function as an agonist [43,44]. Treatment with tamoxifen leads to inhibition of cell cycle progression and induction of apoptosis in breast cancer cells [45,46]. The agonist activity of tamoxifen in the uterus leads to an increased risk of endometrial cancer [47]. Tamoxifen has greatly increased the survival rate of breast cancer patients since its initial

FDA approval in 1977 for the treatment of metastatic breast cancer, resulting in a 32% reduction in annual death rate [48-50].

Another method of targeting the estrogen receptor is through Selective Estrogen Receptor Downregulators (SERD) such as the pure antiestrogen fulvestrant (ICI 182,780), which is a steroidal 7 α -alkylsulphinyl analogue of estradiol (Figure 1) [34]. Binding of fulvestrant to ER α results in increased ER α turnover by the 26S proteasome [51]. Reducing the level of ER α blocks estrogen action on target genes in breast cancer [34].

Other treatment options for breast cancer patients with ER α + tumors include the use of aromatase inhibitors (AIs) [35], first proposed in 1973 [52]. In postmenopausal women, the ovaries no longer produce estrogens and progestins. Instead, estrogens are produced via the conversion of the androgens androstenedione and testosterone present in circulation from the adrenals by aromatase located in peripheral tissues, including stromal cells in breast tissue, to estrogens: estrone and estradiol, respectively by the enzyme aromatase (CYP19A1) (Figure 1) [36,53,54]. Aromatase inhibitors such as exemestane, letrozole, and anastrozole work by inhibiting CYP19A1 to stop the production of estrogens. In a five year study of 8,010 women, treatment with letrozole resulted in improved disease-free survival, overall survival, and time to distant recurrence for postmenopausal women, as compared to treatment with tamoxifen or a sequence of tamoxifen and letrozole [55], highlighting the clinical efficacy of aromatase inhibitors as a treatment of choice in postmenopausal women.

MECHANISMS OF ENDOCRINE RESISTANCE

Despite the initial efficacy of antiestrogen treatment, approximately 40% of patients relapse and die from metastatic disease because the cancer cells become endocrine-resistant [56]. This sequela is called acquired endocrine resistance since patients first respond and then relapse. Five years of tamoxifen therapy results in a 33.2% probability of recurrence at 15 years [49]. Many potential causes of endocrine resistance have been identified. Altered expression of ER α itself was identified to account for only 15-20% of acquired endocrine resistance [57], demonstrating that mechanisms other than direct loss of the antiestrogen target contribute to endocrine-resistant phenotypes. A variant form of ER α , ER α 36, has also been implicated in tamoxifen resistance, as ER α + women receiving tamoxifen treatment who also had increased expression of ER α 36 had shorter disease-free survival [58].

Altered coregulator expression

A proper coactivator/corepressor ratio is necessary for basal estrogen-regulated gene expression. Increased coactivator or reduced corepressor expression or altered function has been implicated in endocrine resistance. Overexpression of coactivator SRC-3 has been identified in breast cancer and is correlated with reduced responsiveness to tamoxifen therapy [59,60]. In endocrine-resistant LY2 breast cancer cells, treatment with tamoxifen increased nuclear colocalization of both SRC-1 and SRC-3 coactivators with ER α , indicating tamoxifen instead acts as an ER α agonist rather than antagonist in these cells [61]. Further, tamoxifen treatment itself can increase the expression of SRC-1 and SRC-3, promoting increased endocrine resistance [62]. The corepressor NCoR is

downregulated in a mouse model of progression to tamoxifen resistance [63]. Reduced NCoR expression was also found to be associated with shorter relapse-free survival of patients treated with tamoxifen [64]. In contrast, no correlation between the NCoR2/SMRT corepressor and recurrence was found through immunohistochemical (IHC) staining of breast tumors from tamoxifen-treated patients [65]. SMRT expression was instead correlated with shortened time to recurrence in untreated patients, suggesting a different role for this corepressor, such as in inhibition of the activity of transcription factors other than ER α [65].

Increased kinase signaling

Nongenomic ER signaling can occur via plasma membrane (PM)-associated forms of ER which, when activated by ligand, stimulate rapid cellular responses through subsequent effects on downstream kinase activation [66-70]. ER α localization to the PM may be mediated through palmitoylation at cysteine 447 leading to interaction with caveolin-1 at lipid rafts (caveolae) in a ligand-independent manner [71,72]. Increased localization of ER α and a shorter splice variant ER α 46 in lipid rafts occurs with HER2 overexpression, and ER α colocalizes with HER2 in MCF-7 cells [73]. In addition to HER2, PM-associated ER α interacts with other growth factor receptors/receptor tyrosine kinases (RTKs) such as EGFR and IGFR [74,75]. Elevated EGFR and HER2 expression has been reported in tamoxifen-resistant breast cancer cell lines, leading to downstream activation of the ERK pathway [76]. Increased expression of the coactivator SRC-3 and HER2 enabled tamoxifen to switch to an agonistic effect in breast cancer cells, recruiting ER α coactivators and stimulating cell proliferation [77]. Activation of EGFR and HER2

signaling can also promote cell proliferation independent of the involvement of ER, creating a mechanism for cells to bypass the normal inhibitory effects of tamoxifen and other antiestrogens [78].

Kinases other than RTKs also have roles in progression to tamoxifen resistance. Protein kinase C (PKC) activation is known to increase cell proliferation [79]. Elevated PKC α expression was detected in breast cancer cell lines that have undergone progression to tamoxifen or fulvestrant resistance, and treatment with a PKC inhibitor reduced cell proliferation [80]. Knockdown of PKC α expression with shRNA was able to restore cell sensitivity to tamoxifen but not fulvestrant, suggesting the involvement of other pathways [80]. Ligand-activated ER can interact with PI3K to activate Akt and lead to subsequent signaling [81]. Reciprocally, Akt can phosphorylate ER α at serine 167 to lead to ER α activation independent of ligand binding [82]. Tamoxifen can also activate Akt through phosphorylation at Serine 473 in ER+ breast cancer, which may play a further role in the contribution of Akt to tamoxifen resistance [83]. Inhibition of Akt with a PI3K inhibitor increased apoptosis in response to tamoxifen treatment [83].

Altered transcription factor expression/signaling

The nuclear receptor superfamily includes ligand/steroid activated receptors, such as ER, that act as transcription factors to modulate target gene expression. Alteration of nuclear receptor signaling has been implicated in development of many types of cancer, including breast cancer [84]. Muscat *et al.* recently demonstrated expression of nuclear receptors COUP-TFII (gene name *NR2F2*), thyroid hormone receptor β (*TR β*),

peroxisome proliferator-activated receptor γ (*PPAR* γ), and mineralocorticoid receptor (*MR*) to be predictive of metastasis-free survival in tamoxifen-treated patients [85].

Activation of the inflammatory NF κ B pathway leads to increased expression of genes involved in cellular processes such as proliferation, apoptosis resistance, and other oncogenic functions [86]. Several groups have reported an association between increased NF κ B pathway activation and tamoxifen resistance [87-92]. Elevated expression of one or more of the five subunits that comprise the NF κ B transcription factor homodimer or heterodimer has also been reported, including upregulation of RelA/p65 in tamoxifen-resistant breast cancer cell lines LCC9 and MCF-7/RR [87,88] and increased RelA/p65 and NF κ B1/p50 in an estrogen-independent breast cancer cell line LCC1 [93].

Increased AP-1 DNA binding was observed in a fulvestrant-resistant breast cancer cell line [94] as well as in a set of ER α + tamoxifen-resistant human breast tumor samples [20], suggesting AP-1 may provide an alternative pathway for transcription in the presence of antiestrogens. Increased activity of the c-Jun NH₂-terminal kinase (JNK), which phosphorylates and activates AP-1, was also identified in tamoxifen-resistant human breast tumors [20]. In addition, overexpression of c-Jun, a member of the AP-1 heterodimer, rendered MCF-7 cells unresponsive to endocrine therapy [95].

Dysregulated microRNA expression

MicroRNAs (miRNAs) are short, noncoding RNAs of 19-25 nucleotides in length that downregulate gene expression post-transcriptionally via binding to the 3'UTR of target mRNAs in a sequence-dependent manner leading to degradation or inhibition of translation. Altered expression of miRNAs in cancer has been widely reported, with

downstream targets in oncogenic systems such as apoptosis, proliferation, cell differentiation, angiogenesis, invasion, metastasis, and DNA repair [96]. Several miRNAs have been identified as targeting the expression of ER α , including miR-206 [97] and miR-221/222 [98]. MiR-221/222 was significantly upregulated in tamoxifen-resistant breast cancer cell lines and in HER2/neu+ human breast tumor samples [99]. Overexpression of miR-221/222 decreased the sensitivity of cells to treatment with tamoxifen [98]. Besides targeting ER α , miR-221/222 also targets the cell cycle inhibitor p27^{Kip1} [99]. A reduction in p27^{Kip1} also resulted in decreased growth inhibition by tamoxifen treatment in breast cancer cells [99].

In addition to these studies, microarray analysis identified twelve miRNAs that were differentially expressed between MCF-7 tamoxifen-sensitive and LY2 tamoxifen-resistant breast cancer cells [100]. Identified targets of these miRNAs had known roles in apoptosis (*PDCD4* and *BCL2*), estradiol metabolism (*CYP1B1*), epithelial to mesenchymal transition (*ZEB1*), and growth factor signaling (*ERBB3*). ER α itself was also identified as a target in this screen [100].

Altered DNA methylation

Changes in gene promoter methylation can also occur upon progression to antiestrogen resistance, either through activation of the expression of genes which promote proliferation through promoter hypomethylation (more common) or via downregulation of tumor suppressive genes through hypermethylation (less common) [101]. In one study, tamoxifen-resistant cell proliferation remained ER α -driven, while fulvestrant-resistant cell proliferation was driven by mechanisms other than ER α [101].

Many more changes in promoter methylation were found in the fulvestrant resistant cells, where altered methylation was identified for 72 genes compared to only 10 genes in tamoxifen-resistant cells [101].

CONCLUSIONS

Most breast tumors are initially driven by estrogens through activation of ER α . Targeting ER α by SERMs, *e.g.*, tamoxifen, and SERDs, *i.e.*, fulvestrant, and AIs, *e.g.*, letrozole, have been successful in increasing the overall survival of breast cancer patients in the last 25-30 yrs [33,48,102]. Unfortunately, ~40% of patients relapse on endocrine therapies [56] and understanding the mechanism by which tumors escape inhibition by AIs, SERMs, and SERDs and re-establishing endocrine-sensitivity is key to enhancing overall survival. Although many mechanisms have been identified as contributing to the endocrine-resistant phenotype, the field is currently lacking clinically relevant biomarkers and functional drug targets. More work is necessary to fully elucidate the complexity of interactions between the current observations and translate these findings into a unified model of the endocrine-resistance.

CHAPTER II - MULTIPLE ROLES OF COUP-TFII IN CANCER INITIATION AND PROGRESSION

The text of this chapter was published in *Journal of Molecular Endocrinology* 49(3): R135-48, 2012. doi:10.1530/JME-12-0144

INTRODUCTION

Steroid hormones and nuclear receptor ligands play critical roles in cancer initiation and progression and their antagonists have proven efficacy in the treatment and prevention of cancers. This is most notable in breast and prostate cancers and the use of all-*trans* retinoic acid for acute promyelocytic leukemia [103,104]. Steroid/nuclear receptors act as ligand-activated transcription factors to either positively or negatively regulate gene expression [84,105]. Activation of nuclear receptors occurs through binding a variety of ligands including hormones and vitamins/retinoids. Nuclear receptors (NR) have physiological roles to modulate gene expression during development and growth. As alteration of basal gene expression leads to many pathogenic outcomes - including cancer, maintenance of normal gene expression by nuclear receptors is vital. One such critical nuclear receptor is chicken ovalbumin upstream promoter transcription factor II (COUP-TFII). From the time of the identification of the COUP-TF family in 1986 [106], the many functions of COUP-TFs have continued to be explored. The role of

COUP-TFII in cancer is widely debated with evidence linking COUP-TFII to both tumor suppressive and oncogenic functions. This review will explore both the regulation and function of COUP-TFII and its connections to cancer.

COUP-TFI AND COUP-TFII

The COUP-TF family consists of two highly homologous subtypes, COUP-TFI and COUP-TFII, located on human chromosomes 5 and 15, respectively (Figure 2). COUP-TFs have been previously reviewed [107,108], but not in the specific context of separating COUP-TFI and COUP-TFII in cancer. COUP-TFs are ancient NRs and are located close to retinoid X receptors (RXRs) in the evolutionary tree [109,110]. As evolutionarily conserved transcription factors, COUP-TFs have major roles in development. The importance of COUP-TFII expression is evidenced by studies in knockout mice [111]. Homozygous mutation of COUP-TFII leads to embryonic lethality due to impaired angiogenesis and heart defects, resulting in hemorrhage and edema. These effects may in part be explained by the reduction in angiopoietin-1 expression in COUP-TFII-null mice [111]. Other important embryonic roles for COUP-TFII include regulation of limb growth and muscle development [112]. COUP-TFII-null mice display a reduction in expression of *Lbx1*, a protein required for proper muscle precursor cell migration, and in myogenin, which is necessary for muscle cell differentiation [112,113].

Based on the high sequence identity in their DNA binding domains DBDs (Figure 2), we anticipate that COUP-TFI and COUP-TFII regulate the same genes. However, this has not been empirically tested and it is worth noting that the N-terminus is divergent (Figure 2) and immunoprecipitation studies indicate differences in proteins interacting with COUP-TFI [114] and COUP-TFII [115], although, again, this has not been

Figure 2. Comparison of COUP-TFI and COUP-TFII protein homology. COUP-TFI (NP_005645.1) and COUP-TFII (NP_066285.1) amino acid sequences were obtained from the National Center for Biotechnology Information. The COUP-TF DNA binding domains (DBD) and ligand binding domains (LBD) share 99% and 97% amino acid homology, respectively. Positions noted as important in coactivator recognition are shown in orange.

systematically studied in cells in which both are expressed. COUP-TFI and COUP-TFII may have divergent functions in certain contexts as well. Differences in COUP-TFI and COUP-TFII function in breast cancer endocrine sensitivity, for example, have also been identified [116]. This review will focus specifically on COUP-TFII.

COUP-TFII REGULATION OF GENE EXPRESSION

Mechanisms of regulation

COUP-TFII can activate or repress gene expression in both a tissue-specific and gene-specific manner through mechanisms involving direct binding to DNA response elements or binding to other transcription factors. Through binding to 5'-AGGTCA-3' direct repeats (DR) with variable spacing [117], COUP-TFII modulates the expression of target genes. Specific genes upon which COUP-TFII activates transcription include retinoic acid receptor $\beta 2$ ($RAR\beta 2$, *RARB2*) [115,118], phosphoenolpyruvate carboxykinase (PEPCK, *PCK1*) [119], *NGFI-A* [120,121], and cholesterol 7 α -hydroxylase (*CYP7A1*) [122]. COUP-TFII action may be potentiated by interaction with coactivators such as steroid receptor coactivator family members SRC-1/NCOA1, SRC-2/NCOA2, and SRC-3/NCOA3 [120,121], as well as PGC1 α [120], p300/CBP [121], orphan receptor coactivator (ORCA) [123], and nucleolin [115]. DNA binding of COUP-TFII can promote the binding of a second transcription factor, further activating gene transcription. This occurs for both the PEPCK and CYP7A1 genes, where COUP-TFII binding to the promoter recruits binding of glucocorticoid receptor (GR) to enhance gene expression [119,124]. COUP-TFII can also bind to Sp1 sites to cooperatively activate

gene expression, as was reported for regulation of *Otx2* expression during morphogenesis in the mouse eye [125].

Alternatively, binding of COUP-TFII to DRs may result in repression of gene expression. In the mechanism of “active repression,” COUP-TFII binding results in recruitment of corepressors, *i.e.*, nuclear corepressor (NCoR) [126] and silencing mediator of retinoid and thyroid receptors (SMRT) [127,128], resulting in repressed chromatin structure and a corresponding blockade of target gene transcriptional activation. COUP-TFII interaction with SMRT represses PPAR γ 1 and PPAR γ 2 expression to suppress adipogenesis [128]. Repression of the human oxytocin promoter by COUP-TFII binding has also been reported [129]. COUP-TFII represses *Pax2* expression in the retina via binding to a DR1 site (TGTTACAGTCCA) [125].

Through an alternative mechanism of transrepression, COUP-TFII can interact with other nuclear receptors and transcription factors to inhibit their normal transcriptional activity. Examples of this include inhibition of ER- and GR-induced gene expression in a gene-specific manner [119,130]. COUP-TFII can also repress AP-1 signaling through interaction with c-Jun [131]. Interaction of COUP-TFII with Runx2 inhibits osteoblast differentiation via blocking Runx2 binding to the osteocalcin promoter [132]. Other mechanisms of repression involve the modulation of ER, RXR, PPAR, and VDR activity by competing for DNA response element-binding or heterodimerization with the class II heterodimeric partner RXR [133].

Ingenuity Pathway Analysis

As summarized here, COUP-TFII regulates the expression of diverse gene targets. Table 1 contains a list of known COUP-TFII targets as identified using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com). These targets are also displayed in Figure 3. COUP-TFII has varying effects on expression of other nuclear receptors and transcription factors. COUP-TFII increased the expression of HNF-1 α [134], HNF-1 β [135], HNF-4 α [136], and RAR β [115,118,137], while it decreased the expression of Oct4 [138,139], Dax1 [140], and PPAR α [141]. As previously described, COUP-TFII has well known functions in repressing the transcriptional activity of other nuclear receptors and transcription factors. Although COUP-TFII increases HNF-4 expression, other reports highlight the repression of HNF-4 function by COUP-TFII. Specifically, COUP-TFII decreases transcriptional activation of *ALDH2* [142] and retinol binding protein 2 (RBP2) [143] by HNF-4. The HNF-4 activation of hepatic lipase is suppressed by COUP-TFII [144], while lipoprotein lipase expression is induced by COUP-TFII synergistically with PPAR γ [145]; part of the many of reported functions of COUP-TFII in the cholesterol processing pathway. A similar response occurs for apolipoproteins A-I, A-IV, and C-III, where COUP-TFII represses the RXR α -mediated expression of APOA-I [135,146,147] and HNF4-mediated expression of APOA-IV [148,149] and APOC-III [134,135,150,151]. HNF-4 and COUP-TFII binding to the sex hormone binding globulin (*Shbg*) promoter was reported in murine Sertoli cells [152]. SHBG expression is increased by HNF-4 and suppressed by COUP-TF in HepG2 hepatoblastoma cells [153]. Decreased SHBG expression is indicative of metabolic syndrome and may result in increased plasma androgen and estrogen levels, though the precise connection of COUP-TFII to these phenotypes has not been investigated [154].

Although COUP-TFII is classically known for its role in transrepression, COUP-TFII may also enhance the effect of a second nuclear receptor. Induction of cytochrome P450 family members cholesterol 7 α -hydroxylase CYP7A1 [122] and aldosterone synthase CYP11B2 [155,156] by COUP-TFII was reported, with COUP-TFII and HNF-4 acting to synergistically activate CYP7A1 [122]. CYP7A1 catalyzes the first step in the conversion of cholesterol to bile acid [122], while CYP11B2 catalyzes the final steps of aldosterone synthesis [155], implying that COUP-TFII transcriptional activation would increase the production of bile acid and aldosterone.

As shown in Table 1 and Figure 3, COUP-TFII opposes PPAR γ /RXR activation of PEPCK transcription in preadipocytes/fibroblasts, a result that was proposed to suppress adipogenesis [157]. COUP-TF also inhibited 9-*cis* retinoic acid/RXR-induced activation of the lactotransferrin promoter in transiently transfected ZR-75-1 and Hs578T breast cancer cells apparently by competing for DNA binding to a composite RARE/ERE in the gene promoter [158]. Concurrent binding of COUP-TFII and NF-Y to the hemoglobin epsilon promoter leads to a repression of gene expression [159]. In addition to the targets identified by IPA, COUP-TF was reported to play a dual regulatory role in the transcriptional regulation of the mitochondrial HMG-CoA synthase gene: alone COUP-TFI stimulated reporter gene activity from the HMG-CoA synthase promoter in transiently transfected HepG2 human hepatoma and rat Leydig tumor R2C cells, but it inhibited PPAR α -stimulated transcriptional activity by competing for the same DNA binding site [160].

Some of the IPA-identified COUP-TFII target gene relationships and mechanisms remain to be fully elucidated. In a study of the transcriptional regulation of murine

Figure 3. COUP-TFII (NR2F2) target genes.

COUP-TFII has been reported to modulate the expression of a variety of target genes both positively and negatively. A list of COUP-TFII target genes and corresponding network pathway were generated using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com).

Table 1. List of COUP-TFII-regulated genes identified by Ingenuity Pathway Analysis

Gene (protein)	Name	Location	Family	Regulation by COUP-TFII	Reference
ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	Cytoplasm	Enzyme	Decrease	[142]
ANGPT1	Angiotensinogen 1	Extracellular Space	Growth factor	Increase	[111]
APOA1	Apolipoprotein A-I	Extracellular Space	Transporter	Decrease	[135,146,147]
APOA4	Apolipoprotein A-IV	Extracellular Space	Transporter	Decrease	[148,149]
APOC3	Apolipoprotein C-III	Extracellular Space	Transporter	Decrease	[134,135,150,151]
CYP11B2	Aldosterone synthase, cytochrome P450, family 11, subfamily B, polypeptide 2	Cytoplasm	Enzyme	Increase	[155,156]
CYP7A1	Cholesterol 7 alpha-hydroxylase, cytochrome P450, family 7, subfamily A, polypeptide 1	Cytoplasm	Enzyme	Increase	[122]
GATA6	GATA binding protein 6	Nucleus	Transcription regulator	Decrease suggested	[161]
HBE1	Hemoglobin, epsilon 1	Cytoplasm	Transporter	Decrease	[159,162]
HNF1A	HNF1 homeobox A	Nucleus	Transcription regulator	Increase	[134,161]
HNF1B	HNF1 homeobox B	Nucleus	Transcription regulator	Increase	[135]
HNF4A	Hepatocyte nuclear factor 4, alpha	Nucleus	Transcription regulator	Increase	[136]
KDR	VEGFR-2; kinase insert domain receptor (a type III receptor tyrosine kinase)	Plasma Membrane	Kinase	Decrease	[163]
LIPC	Lipase, hepatic	Extracellular Space	Enzyme	Decrease	[144]
LPL	Lipoprotein lipase	Cytoplasm	Enzyme	Increase	[145]
LTF	Lactotransferrin	Extracellular Space	Peptidase	Decrease	[158]
NPPA	Natriuretic peptide A	Extracellular Space	Other	Increase	[164]
NR0B1	Dax1, nuclear receptor subfamily 0, group B, member 1	Nucleus	Ligand-dependent nuclear receptor	Decrease	[140]
NR1H4	FXR, nuclear receptor subfamily 1, group H, member 4	Nucleus	Ligand-dependent nuclear receptor	Unknown	[161]
NR1I2	PXR, nuclear receptor subfamily 1, group I, member 2	Nucleus	Ligand-dependent nuclear receptor	Unknown	[161]
NR5A2	LRH-1, nuclear receptor subfamily 5, group A, member 2	Nucleus	Ligand-dependent nuclear receptor	Unknown	[161]
NRP1	Neuropilin 1	Plasma Membrane	Transmembrane receptor	Decrease	[163]
PCK1	Phosphoenolpyruvate carboxykinase 1 (soluble)	Cytoplasm	Kinase	Decrease	[157]
POU5F1	Oct 4; POU class 5 homeobox 1	Nucleus	Transcription regulator	Decrease	[138,139]
PPARA	Peroxisome proliferator-activated receptor alpha	Nucleus	Ligand-dependent nuclear receptor	Decrease	[141]
RARB	Retinoic acid receptor, beta	Nucleus	Ligand-dependent nuclear receptor	Increase	[115,118,137,143]
RBP2	Retinol binding protein 2, cellular	Cytoplasm	Transporter	Decrease	[143]
SHBG	Sex hormone-binding globulin	Extracellular Space	Other	Decrease	[152,153]
SLC9A1	Solute carrier family 9, subfamily A (NHE1, cation proton antiporter 1), member 1	Plasma Membrane	Ion channel	Increase	[165]

hepatic development, COUP-TFII occupancy of *GATA-6*, *FXR*, *PXR*, and *LRH-1* promoters, as determined by chromatin immunoprecipitation (ChIP) assay, was reported during the postnatal period [161]. While an inhibitory relationship was suggested for the effect of COUP-TFII on *GATA-6*, the effect on *FXR*, *PXR*, and *LRH-1* expression is not yet known [161]. Several other target genes have been identified that highlight the critical function of COUP-TFII in the vascular system. These include an increase in angiopoietin 1 [111] and natriuretic peptide A [164] by COUP-TFII, and a decrease in VEGFR-2 and neuropilin 1 [163]. COUP-TFII enhances expression of the NHE1 solute exchanger [165,166].

In summary, as indicated by the IPA (Figure 3) and consistent with previous reports, COUP-TFII plays a role in many downstream pathways and may either activate or suppress gene expression.

Role in the retinoic acid pathway

COUP-TFs are classified as orphan members of the NR superfamily, because their endogenous ligand(s) is not known. However, Kruse *et al* demonstrated *in silico* binding of all-*trans* (atRA) and 9-*cis* (9cRA) retinoic acid to the crystal structure of the COUP-TFII LBD [120]. RA released the COUP-TFII LBD from the autorepressed conformation. While the investigators did not directly test binding of all-*trans* or 9-*cis* RA to COUP-TFII, they demonstrated that treatment with atRA or 9cRA increased COUP-TFII interaction with the coactivator SRC-3, with an EC₅₀ of 10-30 μ M. In agreement with this data, addition of 20 μ M atRA or 9cRA led to COUP-TFII's activation of a NGFI-A-luciferase reporter [120]. Although these concentrations of atRA

and 9cRA are greater than the physiological concentration of these retinoids, this finding provides novel insight into the ligand binding ability of COUP-TFII. Indeed, the function of this activation can be seen in the regulation of RAR β 2 by COUP-TFII, as COUP-TFII activation of RAR β 2 expression is increased with the addition of all-*trans* retinoic acid [115,118]. Treatment of MCF-7 breast cancer cells with atRA also increased COUP-TFII-binding to the *RARB2* promoter in a ChIP assay [115]. Retinoic acid induces the expression of COUP-TFII in certain breast cancer cell lines (*e.g.* T47D and ZR-75) but not others (*e.g.* MCF-7 and MDA-MB-231) (Figure 4) [115,167]. This indicates a potential feed forward loop, as treatment with retinoic acid may increase both the expression and activation of COUP-TFII, with downstream effects on retinoic acid receptor.

REGULATION OF COUP-TFII EXPRESSION

Tissue-specific regulation in humans

COUP-TFII has a widespread tissue distribution, with detectable expression in every human tissue type examined [168]. The regulation of COUP-TFII expression is tissue and cell-type specific, and can be modulated both transcriptionally and post-transcriptionally (Figure 4). Hyperinsulinemia is a risk for breast cancer [169,170]. COUP-TFII expression was repressed by insulin and glucose in the liver and pancreas of C57BL6/J mice and in mouse primary hepatic and pancreatic cell culture [171]. In contrast, we found that insulin treatment had no effect on COUP-TFII expression in

Figure 4. Regulation of COUP-TFII expression.

COUP-TFII expression has been shown to be modulated both transcriptionally and post-transcriptionally by a variety of transcription factors, signaling pathways, and various molecules, as diagramed here.

Figure 5. Insulin treatment does not affect COUP-TFII expression in human breast cancer cells.

MCF-7 and T47D human breast cancer cells were grown as in described in [115]. As T47D growth medium contains 6 mg/ml insulin, T47D cells were either grown in normal medium (with insulin) or in insulin-free medium to determine if this affected outcome. Prior to treatment with insulin, all cells were “starved” in low glucose medium (5 mM glucose) for 24 h [136,171]. Cells were treated for 6 h with the indicated concentrations of insulin. QRT-PCR was performed to measure *NR2F2* expression relative to *GAPDH* as a reference gene, as described in [115]. Insulin treatment had no statistically significant effect on COUP-TFII expression in these cell lines.

MCF-7 and T47D breast cancer cells (Figure 5). The lack of alteration in COUP-TFII expression with insulin in breast cancer cells highlights the importance of cell-specific regulation of COUP-TFII expression. There are currently no reports on the effect of insulin on COUP-TFII expression in other cancers.

miRNA regulation

MicroRNA (miRNA) expression is altered in a variety of conditions and disease states, including cancer, and results in important post-transcriptional regulation of crucial proteins [96]. While 115 miRNAs are predicted to target *NR2F2* (<http://cometa.tigem.it/site/index.php>), only one miRNA has been verified. miRNA-302 directly represses COUP-TFII expression in human embryonic stem cells [138]. Regulation of COUP-TFII expression by miRNA has not yet been reported in cancer cells.

DNA methylation

Methylation at CpG islands can result in suppression of gene transcription, and is known to be a hallmark of cancer progression. DNA methylation may also occur at intragenic and intergenic sites, as well as at the promoter [172,173]. Specifically, COUP-TFII has been found to be methylated in many cancers, including mantle cell lymphoma, acute myeloid leukemia, salivary gland adenoid cystic carcinoma, pancreatic adenocarcinoma, colon cancer, breast cancer ductal carcinoma *in situ*, as well as a tamoxifen-resistant breast cancer cell line [101,174-179]. *NR2F2* gene hypermethylation was associated with a concordant reduction in mRNA expression in mantle cell

lymphoma, pancreatic cancer, and tamoxifen-resistant breast cancer cells [101,174,177]. Whether this indicates a general trend of reduced COUP-TFII expression due to epigenetic modification across cancer types remains to be seen. Contrary to these reports, high levels of COUP-TFII mRNA expression were found in all cell lines in the NCI60 panel of human cancer cell lines [180].

Regulation by other transcription factors

COUP-TFII and Ets-1 have overlapping expression patterns in mesenchymal cells of the mouse gut, spleen, lungs, and other tissues [181]. Members of the ETS family (Ets-1, Ets-2, ETV, PEA3, Spi-1, and ERM) increased murine COUP-TFII-promoter activity in HeLa cells. Steroid receptor coactivators SRC-1/NCOA1, TIF2/SRC-2/NCOA2, and RAC3/SRC-3/NCOA3 enhanced the activation of the COUP-TFII promoter [181]. In agreement with this data, SRC-3 and RAR α increased COUP-TFII-promoter activity in HepG2 human hepatocellular carcinoma cells with atRA treatment. Reciprocally, siRNA knockdown of SRC-3 repressed COUP-TFII expression [182]. We observed that the protein expression (by immunohistochemical staining) of AIB1/SRC-3/NCOA3, PEA3, and SRC-1/NCOA1 were correlated with COUP-TFII in breast cancer patient samples [115].

Regulation by altered kinase activity and other signaling pathways

Several factors were reported to alter COUP-TFII expression in pathogenic states. More *et al* reported that expression of COUP-TFII, but not COUP-TFI, is stimulated by activation of the MAP kinase (MAPK) pathway. Breast cancer cell lines with increased

MAPK activity, *i.e.*, SKBR3, had a concomitant increase in COUP-TFII expression [183]. In contrast to the idea that MAPK activation increases COUP-TFII expression, MAPK has also been shown to phosphorylate and inactivate PP2A (protein phosphatase 2A), leading to a suppression of COUP-TFII expression in human peripheral blood CD34+ cells [184]. Inactivation of PP2A also inhibits sonic hedgehog-induced COUP-TFII expression in P19 cells [185]. PP2A is inhibited by the FOXO transcription factors, including FOXO1 [186]. COUP-TFII expression is induced by FOXO1 in pancreatic beta cells and hepatocytes [171], highlighting the highly cell type-specific nature of these pathways. MAPK activity may lead to increased COUP-TFII expression in certain conditions, while it may alternatively repress COUP-TFII in others. Taken together, these data suggest a possible feedback loop in certain cell types (Figure 4).

In addition to MAPK activation, Notch signaling is also dysregulated in many types of cancer. Increased Notch signaling has been implicated in carcinogenesis and metastasis and is also involved in regulation of endothelial cell proliferation and angiogenesis [187,188]. In breast cancer, Notch and its ligand Jagged1 upregulate the expression of Slug, a transcriptional repressor of E-cadherin important in metastatic progression [189]. Notch signaling has also been implicated in the amplification of HER2 and survival of tumor initiating cells [190] and cancer stem cells [188,191,192]. Activation of the Notch pathway confers cancer-like properties and apoptosis-resistance to normal breast epithelial cells [193]. Regulation of COUP-TFII by Notch signaling has been reported in endothelial cells of both arterial and venous origin and in mouse studies [163,194,195]. Notch can suppress COUP-TFII and Prospero-related homeobox domain 1 (Prox1), leading to an arterial rather than lymphatic phenotype in endothelial cells

[163,194,196]. COUP-TFII, in turn, can also suppress Notch signaling to result in vein rather than artery formation [195]. Transforming growth factor- β 1 (TGF β 1) suppresses COUP-TFII expression in keratinocytes and fibroblasts leading to induction of collagen type VII (*COL7A1*) expression [197] and in vascular progenitor cells to negatively regulate lymphvasculogenesis [198]. Whether COUP-TFII is regulated via Notch and TGF β 1 signaling has not yet been explored in cancer.

Amplification of Wnt/ β -catenin signaling has been widely reported in cancer [199]. In normal tissues, β -catenin signaling is controlled through signals leading to its phosphorylation by a multiprotein destruction complex and subsequent degradation. In breast and other cancers, increased expression of Wnt ligands leads to maintenance of β -catenin activation by preventing its degradation [199]. β -catenin signaling has many outcomes, such as normal mammary morphogenesis and ductal maturation; however, sustained activation, through a variety of mechanisms, leads to carcinogenesis [199]. ChIP assays demonstrated that β -catenin/TCF7L2 (T-cell factor 7-like 2 or transcription factor 7-like 2) bind the promoter of COUP-TFII to activate expression, resulting in suppression of adipocyte differentiation [128]. COUP-TFII is expressed in mouse liver and pancreatic β -cells and plays roles in the maintenance of glucose homeostasis and insulin sensitivity [171,200]. Boutant *et al* also reported that β -catenin/TCF7L2 induces COUP-TFII expression in the pancreas, and that COUP-TFII expression was necessary for normal β -cell function and glucose tolerance in mice [201]. The influence of β -catenin signaling on COUP-TFII expression in cancer has yet to be examined.

ROLE OF COUP-TFII IN CANCER

Angiogenesis

Many studies of COUP-TFII involve its regulation of the angiogenesis pathway. Under normal conditions, angiogenesis is not active after the time of vasculature development during embryogenesis. However, upon progression of a tumor's growth, activation of angiogenesis leads to the formation of new blood vessels to support the tumor [202]. COUP-TFII is necessary during normal development for angiogenesis and lymphangiogenesis, as evidenced by the impaired vessel formation and embryonic lethality in COUP-TFII knockout mice [111,203]. The expression of many pro-angiogenic factors is modulated by COUP-TFII, including members of the vascular endothelial growth factor (VEGF) family and their receptors. VEGF induces angiogenesis and lymphangiogenesis by activating tyrosine kinase receptors and upregulates endothelial cell proliferation and migration [204]. In a model of pancreatic islet tumorigenesis, ablation of COUP-TFII increased VEGFR-1 expression, impairing VEGFR-2 signaling and reducing angiogenesis [205]. Metastasis to regional lymph nodes was reduced as a result, implying that COUP-TFII may have a pro-angiogenic, pro-metastatic role in pancreatic cancer [205]. Similarly, ablation of COUP-TFII decreased tumorigenesis in B16-F10 melanoma and Lewis lung carcinoma mouse xenografts, and reduced tumorigenesis and metastasis in a spontaneous mouse mammary tumor model. These effects were attributed to a decrease in blood vessel density in COUP-TFII-deficient mice [206].

In addition to regulating VEGFR expression, COUP-TFII can also affect angiogenesis via regulation of angiopoietin-1 (Ang-1), through binding to an Sp1 site in

the promoter region. The induction of Ang-1 is partially responsible for the effects of COUP-TFII, as overexpression of Ang-1 allowed for recovery of angiogenesis in COUP-TFII-deficient mice [206].

Lymphangiogenesis can also contribute to metastasis by allowing the spread of tumor cells to lymph nodes [207,208]. COUP-TFII regulates tumor lymphangiogenesis via inducing expression of VEGF-C and neuropilin-2, a coreceptor for VEGF-C [203,209]. In a murine model of pancreatic islet tumorigenesis, COUP-TFII deletion resulted in impaired lymphangiogenesis and reduced metastasis [205]. Concordant with a role for COUP-TFII in lymphangiogenesis, Kang *et al* reported that Notch suppresses COUP-TFII expression, along with Prox1, in human primary dermal lymphatic endothelial cells to signal for arterial rather than lymphatic differentiation [163]. Suppression of COUP-TFII resulted in an increase in VEGF signaling by activating expression of VEGFR-2, a VEGF receptor whose signaling can feedback to increase activation of Notch signaling [163].

COUP-TFII induction by 9cRA was also shown to promote network formation but not cell fusion in SKBR3 breast cancer cells, suggesting a role in the endothelial transdifferentiation pathway as a necessary part of vascular formation [210]. Taken together, these data indicate that COUP-TFII may regulate angiogenesis and lymphangiogenesis, primarily through modulation of VEGF and its receptor in a cell context-dependent manner.

Invasion and metastasis

In addition to stimulation of angiogenesis, COUP-TFII may have other distinct roles in regulation of tumor growth and metastasis. Transfection with COUP-TFII in A549, H520, and H441 lung cancer cells and MDA-MB-231 breast cancer cells was reported to increase migration and invasion [211]. Navab *et al* found that COUP-TFII upregulated the expression of extracellular matrix-degrading proteinases matrix metalloproteinase 2 (MMP2) and urokinase-type plasminogen activator (uPA) [211]. MMP2 and uPA are known to play critical roles in cancer, particularly in angiogenesis and metastasis [212]. High levels of uPA are predictive of recurrence but also of a favorable response to adjuvant chemotherapy in breast cancer patients [213]. Interestingly, it has also been reported that uPA expression is dependent on Notch signaling in MDA-MB-231, MDA-MB-468, and HCC1143 breast cancer cells [214]. COUP-TFII and MMP2 expression were also positively correlated in a breast tumor microarray [115], further indicating a potential relationship between COUP-TFII and extracellular matrix degradation. In contrast, COUP-TFII decreased cell motility when transfected into LY2 tamoxifen-resistant breast cancer cells, while having no significant effect on invasion [116].

Estrogen receptor and clinical outcome

Nagasaki *et al* demonstrated that COUP-TFII expression was correlated with ER α status and indices of poor clinical outcome (clinical stage, lymph node status, histological grade) in human breast tumor samples, indicating COUP-TFII may play a role in cancer progression [209]. We also found that COUP-TFII and ER α expression were correlated in a human breast tissue/tumor microarray, but instead noted an inverse relationship

between COUP-TFII expression and TNM (tumor, node, metastasis) classification [115]. Similar findings were observed at the mRNA level by examining breast tumor mRNA transcriptomes in Oncomine [115]. COUP-TFII expression was significantly higher in ER α + breast cancer samples and significantly lower in metastatic samples [115]. These findings indicate a function for COUP-TFII in inhibiting tumor progression. A positive correlation with ER α is consistent with a previous report that siRNA knockdown of ER α in MCF-7 breast cancer cells decreased COUP-TFII expression and treatment with estradiol increased the expression of COUP-TFII [116]. ER α is a positive prognostic factor in breast tumors and is the target of endocrine-targeted cancer therapeutics such as the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene [48]. COUP-TFII, but not COUP-TFI, is reduced in tamoxifen-resistant human breast cancer cells, and re-expression of COUP-TFII can restore tamoxifen-sensitivity [116]. As ER α expression is important in keeping breast cancer cells responsive to treatment, the correlation of COUP-TFII and ER α further demonstrates a beneficial role for COUP-TFII, highlighting its potential importance in maintaining differentiation and endocrine sensitivity.

In contrast to a role for COUP-TFII in maintaining antiestrogen sensitivity, Holbeck *et al* reported that cancer cells in the NCI60 panel expressing low levels of COUP-TFII showed higher sensitivity to microtubule-targeting drugs vinblastine, colchicines, and taxol [180]. These data demonstrate that both cell type-specific as well as drug-specific mechanisms may determine the role of COUP-TFII in influencing treatment response.

Steroidogenesis

COUP-TFII expression was reported to be high in aldosteroma, with an inverse correlation to adrenal steroidogenesis [215]. These data also indicated an inverse correlation between COUP-TFII expression and CYP17A1 expression, with COUP-TFII inhibiting CYP17A1 in aldosteroma [215]. COUP-TFII competed with SF-1 for binding to overlapping sites within the promoters of the CYP17A1 [216,217], CYP11A1, and STARD1 genes in rat Leydig cells and to suppress testosterone production [216]. COUP-TFI and COUP-TFII both repressed angiotensin II-stimulated STARD1 (StAR) in bovine adrenal glomerulosa cells in primary culture [218]. COUP-TFII also competed with SF-1 for the human aromatase P450 promoter II in primary endometriotic stromal cells and suppressed aromatase expression.[219]. Overexpression of SF-1 in primary endometriotic stromal cells outcompeted the normal protective effect of COUP-TF (whether COUP-TFI or COUP-TFII was involved was unclear since both were equally expressed at the mRNA level) resulting in high local aromatase expression in endometriosis [219]. COUP-TFII was reported to bind the S1 silencer region of the human aromatase gene and suppress transcription in MCF-7 cells [220]. Indeed, the decreases in COUP-TFI, EAR γ , EAR-2, Snail and Slug in breast cancer were suggested to increase aromatase expression [221]. Thus, the downregulation of COUP-TFII expression that we observed in endocrine-resistant breast cancer cells [116] would be expected to increase aromatase and thus increase local estrogen production. However, whether increased COUP-TFII suppresses local androgen or estrogen biosynthesis in breast tissue is unknown. Local conversion of adrenal androgens to estrogens by aromatase is the target of AI therapy for post-menopausal women. However, there are

androgen metabolites, *e.g.*, 3 β -adiol, that bypass aromatase which activate ER α and ER β and may play a role in AI resistance [222,223]. Overall, the literature supports a negative role for COUP-TFII in regulating steroid hormone synthesis and further studies addressing COUP-TFII regulation of aromatase gene expression in local estrogen production in breast [224] and lung [225] adenocarcinomas would be of merit.

CONCLUSIONS

The studies reviewed here indicate that COUP-TFII is regulated and is functionally active to regulate target gene transcription in a cell type-dependent manner. There is evidence that COUP-TFII may perform both pro- and anti-tumorigenic roles. COUP-TFII has been reported to increase angiogenesis and lymphangiogenesis, both increase and decrease tumor metastasis, lead to favorable and unfavorable therapeutic outcome in cancer therapy, and suppress steroidogenesis. Qin *et al* reported that COUP-TFII was not expressed in tumor cells, but rather was instead found in high concentration in the surrounding blood vessels that support tumor growth and spread [206]. This indicates a crucial point of consideration about the nature of COUP-TFII in cancer formation and progression: the function of COUP-TFII within cancer cells *versus* in the surrounding tumor microenvironment and other cell types. Tissue type is clearly an important determinant in deciphering the oncogenic or tumor-suppressive nature of COUP-TFII. Many studies published to date involve the regulation and role of COUP-TFII during development and in non-cancerous disease states. The full applicability of these studies to our knowledge of the role of COUP-TFII in carcinogenesis and cancer

progression remains to be seen. Future studies are necessary to elucidate the complex nature of this vital nuclear receptor.

DISSERTATION SPECIFIC AIMS AND HYPOTHESES

Despite the initial efficacy of tamoxifen and other antiestrogens, approximately 40% of patients relapse and die from metastatic disease, in part because the cancer cells become endocrine-resistant [56]. Identification of proteins and pathways key in the progression to endocrine resistance is essential for the development of improved treatments for patients. Our lab has previously reported that the expression of COUP-TFII, but not COUP-TFI, is reduced upon progression to endocrine resistance, and that overexpression of COUP-TFII can restore endocrine sensitivity [116]. As reviewed in chapter 2, many reports on the cellular roles of COUP-TFII are contradictory and fail to provide insight necessary to understand the mechanism of COUP-TFII action in endocrine-resistant breast cancer [226]. The overall goal of this study was to identify and evaluate the function of proteins that interact with COUP-TFII to define the role of COUP-TFII in breast cancer and endocrine sensitivity. COUP-TFII may be both a useful biomarker to predict tamoxifen-sensitivity as well as a target to restore endocrine sensitivity to resistant cells. The overall **hypothesis** was that the activity of COUP-TFII in maintenance of endocrine sensitivity and cell differentiation is dependent on its interacting proteins.

SPECIFIC AIM 1: Characterize the interaction of COUP-TFII and nucleolin.

I tested the hypothesis that COUP-TFII and nucleolin interact directly in MCF-7 and T47D breast cancer cells. COUP-TFII and nucleolin interaction was evaluated in MCF-7 and T47D cells through co-immunoprecipitation. *In vitro* binding was assessed through a maltose binding protein (MBP) pulldown assay. COUP-TFII and nucleolin expression were analyzed in human breast cancer patient samples through immunohistochemistry.

SPECIFIC AIM 2: Determine how the interaction of COUP-TFII and nucleolin affects target gene expression in breast cancer cells.

I tested the hypothesis that nucleolin functions as a coregulator for COUP-TFII in the expression of the COUP-TFII target gene *RARB2*. The ability of nucleolin to function as a coactivator for COUP-TFII was analyzed in MCF-7 and T47D cells. Transient transfection, siRNA knockdown, luciferase assays, and other assays were used to determine the contribution of nucleolin to *RARB2* expression.

SPECIFIC AIM 3: Determine if COUP-TFII expression modulates NFκB activity in endocrine-resistant breast cancer cells.

I tested the hypothesis that COUP-TFII suppresses NFκB activity in breast cancer cells through a mechanism involving suppression of NFκB transcription. The effect of COUP-TFII on NFκB activity was assayed in endocrine-sensitive MCF-7 *versus* endocrine-resistant LCC9 breast cancer cells using luciferase assays and electrophoretic mobility shift assays (EMSA). NFκB subunit and target gene expression was examined using QRT-PCR and western blots.

SPECIFIC AIM 4: Determine the identity of proteins that interact with COUP-TFII in tamoxifen-treated MCF-7 human breast cancer cells.

I tested the hypothesis that 4-OHT treatment changes the interaction of COUP-TFII with other nuclear proteins in MCF-7 endocrine-sensitive cells and that these proteins are important in mediating the ability of antiestrogens to inhibit cell proliferation. MCF-7 breast cancer cells were transiently transfected with FLAG-tagged COUP-TFII and treated with ethanol (EtOH, vehicle control) or 4-OHT before immunoprecipitation was performed using a FLAG antibody to capture COUP-TFII and associated proteins. Mass spectrometry identified proteins interacting with COUP-TFII specifically when treated with EtOH *versus* 4-OHT. Further experiments including co-immunoprecipitation, transient transfection, western blots, and QRT-PCR were performed to analyze the role of the identified COUP-TFII-interacting proteins.

CHAPTER III - IDENTIFICATION AND CHARACTERIZATION OF NUCLEOLIN AS A COUP-TFII COACTIVATOR OF RETINOIC ACID RECEPTOR β TRANSCRIPTION IN BREAST CANCER CELLS

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INTRODUCTION

COUP-TFI (*NR2F1*) and COUP-TFII (*NR2F2*) are ‘orphan’ members of the steroid/nuclear receptor (NR) superfamily [107]. COUP-TFs regulate gene transcription in a cell- and gene- specific manner. COUP-TFII differs from COUP-TFI at the N-terminus, but is conserved within the DNA binding and ligand binding domains (DBD and LBD) [107]. Gene knockout mice demonstrated that COUP-TFI (*Nr2f1*) and COUP-TFII (*Nr2f2*) have distinct roles during embryogenesis, notably in the nervous and cardiovascular systems, respectively [111,227]. Although COUP-TFs are classified as orphan receptors, as they have no currently established physiological ligands, the crystal structure of the COUP-TFII LBD showed that its “auto-repressed conformation” was relieved by 9-*cis* and all-*trans* retinoic acids (9cRA and atRA) that bind the LBD with ~ 17-26 μ M affinity [120].

While the precise gene changes and epigenetic events that lead to breast tumorigenesis are still under investigation [228-230], estrogens are well-established risk factors in breast cancer [231]. Adjuvant endocrine therapies including the use of antiestrogens, *e.g.*, tamoxifen (TAM), and aromatase inhibitors (AI), *e.g.*, letrozole, are effective in reducing disease recurrence in many patients [102]. Antiestrogens, including TAM and fulvestrant, work by targeting estrogen receptor α (ER α) because of its proliferative activity in breast tumors [232,233]. AI work by blocking the synthesis of estrogens from androgenic precursors including androstenedione and testosterone [234].

Altered gene expression can dictate both the formation of tumors and patient response to treatment. In breast cancer, conflicting evidence has been reported on the nature of COUP-TFII in either promoting or inhibiting cancer formation, as well as influencing patient survival with adjuvant therapy. COUP-TFII is not expressed in basal-like, triple negative, dedifferentiated MDA-MB-231 and is lower in tamoxifen (TAM)/endocrine-resistant LCC9 and LY2 breast cancer cells than in parental endocrine-sensitive MCF-7 cells, whereas COUP-TFI expression is similar [116], suggesting a role in maintenance of differentiation and endocrine sensitivity. In agreement with this data, COUP-TFII was reduced in some ER α -null breast cancer cell lines [167]. These results suggest that, like ER α , loss of COUP-TFII may be considered an indicator of poor prognosis. Other reports suggested that COUP-TFII may play a role in mammary tumor formation in mice and that COUP-TFII expression in human breast tumors is associated with reduced survival [206,209]. These conflicting findings may be resolved through further investigation of the activities of COUP-TFII in breast cancer.

The function of COUP-TFs as transcription factors that can either suppress or stimulate gene transcription is dependent on interactions with other proteins. COUP-TFI [127] and COUP-TFII [119] interact with corepressors NCoR and SMRT. Proteins interacting with COUP-TFI include Sp1 [235]; the viral transactivator Tat [236]; CTIP1 and CTIP2, HDACs 1 and 2, and a nucleosome remodeling and deacetylation (NuRD) complex [237]; ER α [130,238]; AhR [239]; and many coregulators (reviewed in [156]). Twenty-four proteins interacted with HA-FLAG-COUP-TFI in stably-transfected HeLaS3 cells [114]. Interaction of ORCA with the COUP-TFII LBD stimulated transcriptional activation of the rat hydratase-dehydrogenase gene promoter in transiently transfected Bsc40 monkey kidney cells [123]. COUP-TFII interacted with the hinge domain of the glucocorticoid receptor α (GR α) and repressed phosphoenolpyruvate carboxykinase gene transcription [124]. No one has, to our knowledge, reported proteomic identification of COUP-TFII-interacting proteins.

The focus of the present study was to identify proteins that interact with COUP-TFII in MCF-7 cells to gain new insights into COUP-TFII's role in breast cancer. Nucleolin was identified among the nuclear proteins interacting with COUP-TFII. COUP-TFII-nucleolin interaction was confirmed by co-immunoprecipitation. This study reports a significant inverse association of COUP-TFII with breast tumor grade. Expression of the tumor suppressor retinoic acid receptor β 2 (RAR β 2), reduced in breast cancer [240,241], and dependent on COUP-TFII [118] was increased by nucleolin overexpression. Our data indicate that nucleolin plays a coregulatory role in COUP-TFII transcriptional regulation of *RARB2*.

MATERIALS AND METHODS

Chemicals

4-hydroxytamoxifen (4-OHT) and 9-cis and all-trans retinoic acid (9cRA and atRA) were from Sigma (St. Louis, MO). ICI 182,780 (Fulvestrant) was from Tocris (Ellisville, MO). Sequences of AS1411 (AGRO100, an antiproliferative, 26-mer G-rich oligonucleotide) and an inactive negative control C-rich control oligonucleotide (CRO) were reported [242] and purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Antibodies and reagents

The following antibodies were purchased: polyclonal COUP-TFII (Abcam, Cambridge, MA); monoclonal (mAB) anti-human COUP-TFII (R&D systems, Minneapolis, MN; PP-H7147-00. 2ZH7147H); mAB anti-FLAG M2 and β -actin (Sigma); polyclonal nucleolin (NB600-241, Novus Biologicals, Littleton, CO), monoclonal nucleolin/C23 (MS-3; Santa Cruz Biotechnology, Santa Cruz, CA); MBP-probe (R3.2; Santa Cruz Biotechnology); and HDAC-1 (Santa Cruz Biotechnology). HRP-conjugated secondary antibodies were from GE Healthcare (Piscataway, NJ).

Goat anti-rabbit and anti-mouse magnetic beads were from Thermo Scientific (Waltham, MA). *In vitro* transcription/translation used PROTEINScript II kit (Ambion, Austin, TX) or TNT Quick Coupled Transcription/Translation (Promega, Madison, WI).

Plasmid construction

Human COUP-TFII cDNA was amplified from DNA from MCF-7 cells using Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The forward primer contained an *EcoRI* restriction site (5'-CCGAATTCGATATGGCAATGGTAGTTAGCACG-3') and the reverse primer was designed to remove the stop codon from COUP-TFII and add a *XhoI* restriction site (5'-GTCCTCGAGTCGTTGAATTGCCATATACGGCCA-3'). The resulting fragment was cloned into pIRES-GFP-1a (Stratagene, Santa Clara, CA) to construct a C-terminal FLAG-tagged COUP-TFII expression plasmid (pIRES-COUP-TFII-FLAG). The inclusion of COUP-TFII-FLAG in the resulting pIRES-COUP-TFII-FLAG plasmid was verified by DNA sequencing and western blot analysis (Appendix I, Figure S1).

Cell culture

MCF-7 and T47D breast cancer cells were purchased from ATCC and used at passage < 10. T47D were grown in RPMI 1640 (Invitrogen) supplemented with 5% FBS and 6 µg/ml insulin (Sigma). MCF-7 cells were maintained as described [116].

Affinity purification and identification of COUP-TFII-FLAG interacting proteins

One mg of whole cell extract (WCE), prepared as described in [116], from MCF-7 cells transfected (24 h) with pIRES-COUP-TFII-FLAG as described in Methods S1 (Appendix I) was incubated with EZ view™ Red ANTI-FLAG® M2 Agarose Affinity gel (Sigma) overnight (~16 h) at 4°C with constant rotation. COUP-TFII-FLAG interacting proteins were eluted using two methods: 1) 0.1 M glycine, pH 3.5, 15 min at

room temperature with constant rotation; 2) an additional incubation with 0.1 M glycine, pH 3.5, 5 min at 95°C (Appendix I, Fig. S2A).

Protein identification by multidimensional protein identification technology (MudPIT)

Proteins eluted from the FLAG-affinity gel were trypsin digested and processed for mass spectrometry as detailed in Methods S1 (Appendix I). MS/MS spectra of the peptides were acquired by Q-TOF mass spectrometer (Waters, Milford, MA) in data dependent mode. Proteins were identified by comparing MS/MS spectra with sequences in Swiss-Prot database by ProteinLynx from Waters.

Co-immunoprecipitation (co-IP) and immunoblotting

Nuclear and cytosolic proteins were harvested in lysis buffer (10mM HEPES pH 7.9, 1.5mM MgCl₂, and 10mM KCl) containing 0.1 M DTT, protease and phosphate inhibitors (Roche, Indianapolis, IN). Following centrifugation, the supernatant containing cytosolic extract (CE) was collected. The pellet was resuspended in nuclear extraction buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.4 M NaCl, 0.2 M EDTA, and 25% (v/v) glycerol), 0.1 M DTT, protease and phosphatase inhibitors. Nuclei were disrupted by sonication and the nuclear extracts (NE) were collected after centrifugation.

For IP, 4 µg COUP-TFII polyclonal antibody, nucleolin monoclonal antibody, rabbit or mouse IgG (Abcam, Santa Cruz) were added to 250 µl prewashed MagnaBind goat anti-rabbit or anti-mouse IgG beads in RIPA buffer (Sigma) containing DTT, protease and phosphatase inhibitors for 30 min at 4°C. 200-400 µg NE was added and incubated for 4 h at 4°C. Antibody-bound beads were incubated with buffer without NE

as an additional negative control. Beads were washed 2x with RIPA buffer, resuspended in 1x Laemmli loading buffer (BioRad, Hercules, CA), separated by SDS-PAGE and analyzed by western blot [116].

In vitro transcribed-translated COUP-TFII interaction with purified recombinant maltose binding protein (MBP)–nucleolin fusion proteins

Extracts from *E. coli* expressing MBP-tagged nucleolin constructs, a gift from Dr. Nancy Maizels [243], were prepared in column buffer (CB, 20 mM Tris-HCl (pH 7.4), 0.2 mM NaCl, 1 mM EDTA) with 0.1 mM PMSF. 200 µg crude extract was incubated with 100 µL amylose resin (New England Biolabs, Ipswich, MA) for 2 h at 4°C. After washing with CB, 20 µL of *in vitro* transcribed/translated COUP-TFII (pIRES-COUP-TFII-FLAG) was added to the amylose resin for 2 h at 4°C. After washing 3x with CB, bound proteins were eluted with 50 µL of 1x Laemmli loading buffer.

Immunofluorescence staining of COUP-TFII and nucleolin

MCF-7 cells were grown on culture slides (BD Biosciences, Bedford, MA) and fixed with cold methanol. Cells were permeabilized with 0.2% Triton X-100. After blocking with 10% BSA in PBS for 1 h, primary monoclonal COUP-TFII (R&D) and polyclonal nucleolin (Novus Biologicals) antibodies were added (1:100) for 2 h. The cells were stained with secondary anti-mouse antibody labeled with DyLight™ 488 or anti-rabbit antibody labeled with rhodamine (TRITC) (Jackson ImmunoResearch, West Grove, PA) (1:500). Cells were incubated with Hoechst (2,5'-Bi-1H-benzimidazole,

Invitrogen) for 10 min. Images were captured using an Olympus FV1000 confocal microscope with a 40x objective lens using FluoViewTM software.

Immunohistochemistry of breast tissue microarrays (TMA)

COUP-TFII and nucleolin immunohistochemistry (IHC) was performed using commercial breast tissue microarrays BR961 and BR963 (U.S. Biomax) or an in-house TMA constructed following ethical approval from St. Vincent's University Hospital Ethics Board with tissue from 332 primary breast patients, following written informed consent, as previously described [61,244]. Data on the patients included pathological characteristics (tumor size, grade, lymph node status, estrogen receptor status) as well as treatment with radiotherapy, chemotherapy or tamoxifen. Follow-up data, median 7.72 years, was collected on the patients to determine disease free and overall survival. Staining was called by two independent observers using the Allred scoring system [61]. Xenografted MCF7 and HCT116 tumors were used as positive and negative controls, respectively (data not shown). Anti-Nucleolin antibody (Clone 4E2, Abcam) was diluted at 1:500 with overnight incubation at 4°C for BR961 and BR963. A metastatic melanoma was used as a positive control for nucleolin (data not shown). COUP-TFII and nucleolin staining were expressed as H-score: product of intensity (0 to 3 scale, 0 = no expression, 3 strongest expression) and frequency (fraction positive, 0-100%).

Statistical Evaluation of IHC

The univariate associations between COUP-TFII and nucleolin H-scores and categorical predictors used the Kruskal-Wallis test [61]. A multiple linear regression

model was used to fit with COUP-TFII and nucleolin H-scores against pathology, tumor grade, and TNM, classification. The TNM staging system classifies tumors according to disease progression based on the tumor size (T), regional lymph node involvement (N), and distant metastasis (M). Upon assignment of TNM, tumors can further be designated into a condensed grade/stage (I-IV) based on disease severity [245]. Comparisons in the ER α -positive invasive ductal carcinoma subset and among TNM classification within tumor grades were examined (t-test). Fisher's exact test was used for categorical variables to compare two proportions. Kaplan Meier estimates of survival functions were computed and the Wilcoxon test was used to compare survival curves. Two-sided P values of <0.05 were considered to be statistically significant.

Transient transfection

MCF-7 and T47D cells were transfected with a constant amount of total plasmid DNA, pcDNA 3.1 (Promega), pCMV-Tag2 (Stratagene), pcDNA 3.1-mCOUP-TFII (kindly provided by Drs. Sophia and Ming-Jer Tsai [246]), pCMV2-nucleolin [247] using Eugene 6 or HD (Roche) for 24 h prior to treatment with 10 μ M CRO (negative control), AS1411, or random oligomer (RO, 5'-GTTCAGCAGTCACGATTTCAGTCCAGT-3') for 6 or 24 h, as indicated. Where indicated, cells were co-treated with 1 μ M atRA or 9cRA for 6 h. Transient transfection of MCF-7 cells with the RARB promoter tk-luciferase reporter (kindly provided by Dr. Richard M. Niles [248]) and pTK-Renilla (Promega), for dual luciferase reporter assays, as described [130].

RNA Isolation, RT-PCR and Quantitative Real-Time-PCR (QRT-PCR)

RNA was extracted from cells using Trizol (Invitrogen) or RNeasy (Qiagen, Valencia, CA). The High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to reverse transcribe total RNA. QRT-PCR for *RARB2*, *NCL*, *ESR1*, *ESR2*, *GAPDH*, and 18S, using Taqman primers and probes as Assays-on-Demand, was performed in the ABI PRISM 7900 SDS 2.1 (Applied Biosystems, Carlsbad, CA). COUP-TFII (*NR2F2*) and *RRIG1* [249] mRNA expression was measured by QRT-PCR using the SYBR green method and normalized by GAPDH [116]. Analysis and fold differences were determined using the comparative CT method. Fold change was calculated from the $\Delta\Delta CT$ values with the formula $2^{-\Delta\Delta CT}$ and data are relative to EtOH-treated and control vector transfected cells.

siRNA transfection

For nucleolin, cells were transfected for 48 h with 25 nM (MCF-7) or 10 nM (T47D) nucleolin Stealth Select RNAi or Stealth RNAi Negative Control (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen). For COUP-TFII, MCF-7 cells were transfected for 48 h with 100 pmol NR2F2 Silencer Select siRNA (Ambion). Following transfection, cells were treated for 6 h with 1 μ M atRA.

Chromatin immunoprecipitation (ChIP)

MCF-7 cells were transfected with pIRES-COUP-TFII-FLAG or empty vector for 24 h and serum starved for 48 h in medium containing 5% dextran-coated charcoal stripped FBS (DCC-FBS) (Atlanta Biologicals, Lawrenceville, GA). Cells were treated with 1 μ M atRA or EtOH for 1 h before crosslinking with 1% formaldehyde for 5 min.

ChIP was performed using MAGnify ChIP (Invitrogen). Lysates were incubated with anti-FLAG M2 antibody (Sigma) or mouse IgG (Invitrogen). The following primers were used for PCR to amplify the region of the *RARB2* promoter containing a COUP-TFII binding site [118]: F 5'-CAGGGCTGCTGGGAGTTTTTA-3' and R 5'-GGCATCCCAGTCCTCAAACAGC-3'. Quantitation was performed as described in [250].

Statistical analysis

Values are reported as $\pm$ SEM. Student's *t* test was used for comparisons between control and treatment. One way ANOVA was used for multiple comparisons followed by Student-Newman-Keuls or Dunnett's post-hoc tests using GraphPad Prism. *P* values considered statistically significant are indicated.

RESULTS

Identification of COUP-TFII-associated proteins

C-terminal FLAG-tagged COUP-TFII was overexpressed in MCF-7 cells (~2-fold higher expression compared to COUP-TFII endogenous expression; Appendix I, Fig. S1 and S2) and interacting proteins were captured by immunoprecipitation (IP) with anti-FLAG-affinity gel (Appendix I, Fig. S2A). The negative control was MCF-7 cells transfected with the pIRES-GFP-1a parental vector and parallel purification of nonspecific interacting proteins (Appendix I, Table S1). The capture of COUP-TFII-FLAG by the anti-FLAG affinity gel was demonstrated (Appendix I, Fig. S2C). Serial glycine steps eluted COUP-TFII-FLAG-associated proteins (Appendix I, Fig. S3). In the

first elution, 18 proteins having a ‘moderate association’ with COUP-TFII including hsp70, an established NR chaperone that interacts with COUP-TFI [114], were identified (Appendix I, Table S2). The second elution identified 36 more ‘strongly associated’ nuclear proteins, *i.e.*, ribnucleoproteins, histones, DNA repair proteins, and RNA binding proteins, and nucleolin (Table 2), reflecting COUP-TFII nuclear localization.

Nucleolin is a multifunctional protein with roles in processes including transcription, ribosome biogenesis, DNA replication, histone chaperone/chromatin remodeling, apoptosis, and macropinocytosis [251-254]. There are several examples of nucleolin functioning as a transcription factor or as a coregulator through its interactions with other proteins [255-257]. Because nucleolin plays multiple nuclear roles and is a target of anticancer therapy [252], we selected nucleolin for follow-up studies.

Table 2. Identification of COUP-TFII interacting proteins in MCF-7 cells.

WCE from pCOUP-TFII-FLAG-transfected MCF-7 cells (Fig. 6) was incubated with anti-FLAG affinity gel, eluted with 0.1 M glycine, pH 3.5 for 5 min. at 95°C, and subjected to MudPIT peptide identification. Matched (No) indicates the number of sequenced peptides that match the full length protein. Coverage indicates the % of the total protein matched. (Only matches of > 1 peptide match and/or > 3% coverage are included.)

Protein name	Accession (GI)	Mass (Mr)	pI	Matched (No)	Coverage (%)
Histones					
Histone H1.0	P07305	20719	11.2	1	6.7
Histone H2A type 2-C	Q16777	5919	11.8	1	16.1
Histone H1.4	P10412	21721	11.4	7	4.1
Proteins involved in cell cycle & proliferation					
Antigen KI-67	P46013	358525	9.8	6	0.5
Regulator of chromosome condensation	P18754	44941	7.7	4	3.1
Poly [ADP-ribose] polymerase 1 (PARP-1)	P09874	112881	9.2	7	5.7
Proteins involved in transcription					
Activated RNA polymerase II transcriptional coactivator p15	P53999	14255	9.9	1	8.7
DNA Repair Proteins					
DNA topoisomerase 1	P11387	90669	9.6	5	1.6
DNA topoisomerase 2-beta (TopoII β)	Q02880	183152	8.4	4	2.0
ATP-dependent DNA helicase 2 subunit 1	P12956	69668	6.6	3	2.1
ATP-dependent DNA helicase 2 subunit 2	P13010	82521	5.7	3	3.0
Peptidyl-prolyl cis-trans isomerase B	P23284	22728	9.6	3	10.6
RNA binding proteins					
RNA-binding protein FUS	P35637	53394	9.5	3	2.7
Splicing factor, arginine/serine-rich 1	Q07955	27597	10.5	4	12.6
Splicing factor, arginine/serine-rich 9	Q13242	25526	8.9	2	5.4
Splicing factor, arginine/serine-rich 7	Q16629	27350	11.8	2	3.8
Other nuclear proteins					
Nonhistone chromosomal protein HMG 14	P05114	10522	10.0	2	24.2
Nucleolin Protein C23	P19338	76167	4.6	3	1.7
Heat shock cognate 71 kDa protein	P11142	70854	5.4	2	4.3
GTP-binding nuclear protein Ran	P62826	24408	7.6	2	9.7

Heat shock 70 kDa protein 1
(hsp70, *HSPA1*)

P08107

70009

5.6

2

4.4

Endogenous COUP-TFII and nucleolin interact in MCF-7 and T47D cells

We next examined endogenous nucleolin-COUP-TFII interaction in untreated MCF-7 cells. IP with a COUP-TFII antibody detected nucleolin interaction in the NE (Fig. 6A), although nucleolin is in CE as well (Appendix I, Fig. S4A). We did not detect COUP-TFII-nucleolin interaction in CE because COUP-TFII is not in the CE (Appendix I, Fig S4A). Reciprocal co-IP of COUP-TFII with nuclear nucleolin was detected (Appendix I, Fig. S4A). Another example of a COUP-TFII- nucleolin co-IP in MCF-7 cells is also provided (Appendix I, Fig. S4C and D). To examine whether COUP-TFII interacts with nucleolin in another luminal breast cancer cell line, we performed IP with a COUP-TFII antibody in T47D cells and confirmed nucleolin-COUP-TFII interaction in the NE (Fig 6B). Immunofluorescence microscopy revealed a pattern of co-localization of endogenous nucleolin and COUP-TFII in the nucleus, but not within the nucleolus nor in the cytoplasm, of MCF-7 cells (Fig. 6C). These data confirm endogenous COUP-TFII-nucleolin nuclear interaction. Because the focus of this study is COUP-TFII-interacting proteins, we did not evaluate COUP-TFI-nucleolin interaction.

Figure 6. Endogenous nuclear nucleolin-COUP-TFII interaction in MCF-7 and T47D cells.

NE (200 μ g protein) from MCF-7 cells (A) and (400 μ g protein) from T47D (B) cells were immunoprecipitated with COUP-TFII antibody or with rabbit IgG (negative control), followed by western blot analysis for nucleolin and COUP-TFII. 5% input NE serves as loading control. C, Immunofluorescent staining of endogenous COUP-TFII (green) and nucleolin (red) in the nuclei (Hoechst, blue) of MCF-7 cells. Merged images are shown at the right. Bar is 10 μ m. D, schematic representation of the N-terminal maltose binding protein (MBP)-tagged recombinant nucleolin proteins used for MBP pull-down assays. MBP was fused to the N-termini of the RNA binding domains (RBD) and/or the arginine/glycine-rich domain (RGG) of nucleolin. E, *In vitro* transcribed/translated COUP-TFII was incubated with the MBP-nucleolin fragments or MBP. Interacting proteins were captured with amylose resin. Eluted proteins were probed for COUP-TFII (top) and MBP (bottom, control).

Direct interaction of COUP-TFII with the RGG domain of nucleolin

To determine if COUP-TFII interacts directly with nucleolin and which domain(s) are involved, *in vitro* transcribed/translated COUP-TFII and MBP-tagged recombinant nucleolin polypeptides were incubated with an amylose affinity resin (Fig. 6D and E). The MBP fusion proteins contain the RNA binding domains (RBD1,2,3,4) and/or the arginine-glycine repeat (RGG). Nine RGG repeats are present in the C-terminus of nucleolin. Only these domains were investigated because the N-terminal domain of nucleolin cannot be expressed in *E. coli* [247]. COUP-TFII was bound to all MBP-tagged polypeptides containing the RGG domain but not with MBP-RBD1,2, MBP-RBD3,4, or MBP alone. RGG9 appears to interact with COUP-TFII with weaker affinity compared to RGG4, perhaps because of the lower abundance of the MBP-RGG9 protein relative to MBP-RGG4. Overall, these results indicate that the C-terminal RGG domain is the minimal domain required for COUP-TFII-nucleolin interaction.

Immunohistochemical COUP-TFII and nucleolin staining in human breast tissue and tumor tissue microarrays

Nuclear COUP-TFII and nucleolin immunoreactivity were examined in two independent human breast tissue microarrays (TMAs, Fig. 7). In the TMAs from U.S. Biomax, significant differences in COUP-TFII staining were observed between TNM classes of tumor grades II ($d = -51.7$, $p = 0.078$) and II~III ($d = -58.5$, $p = 0.046$) (Fig. 7C). COUP-TFII and nucleolin staining were correlated in invasive ductal carcinomas

($\phi_p = 0.31$, $p=0.0281$; $\phi_s = 0.30$, $p=0.0334$). Normal breast tissue was also positive for COUP-TFII expression (Average H-score 127.5, SEM 5; data not shown). In a separate breast TMA [61], staining of a total of 332 patient tumors showed ~47% were positive for nuclear COUP-TFII (Table 3, Fig. 7F). There was a significant association between tumor grade and COUP-TFII with high grade tumors tending to be COUP-TFII negative (Table 3). COUP-TFII was also significantly positively associated with ER α , SRC-1, PEA3, MMP2, and phospho-Src and negatively associated with HER2 (Table 3). High SRC-1 (*NCOA1*) was associated with a favorable response to TAM [258], a finding that corresponds to COUP-TFII's role in 4-OHT-mediated inhibition of breast cancer cell proliferation [116]. However, breast tumors from aromatase inhibitor-resistant patients show high expression of SRC-1 and a reduction in disease-free survival [259]; thus, the relationship between COUP-TFII and SRC-1 expression will require further investigation. SRC-1 and PEA3 synergistically activated COUP-TFII-promoter luciferase activity in transiently transfected HeLa cells [181]. PEA3 directly activates MMP2 transcription [244]; hence COUP-TFII may be correlated with MMP2 through the PEA3-COUP-TFII connection, although this hypothesis will require further analysis beyond the scope of the present study. The relationship between COUP-TFII and phospho-Src may be because activation of Src is part of the MAPK pathway that increases COUP-TFII expression [183]. According to Kaplan Meier, COUP-TFII did not associate with disease free survival in tamoxifen-treated patients ($p = 0.4471$, Fig. 7H and I).

Figure 7. COUP-TFII and nucleolin in breast cancer tissue microarrays.

A and B, COUP-TFII immunostaining at 200x: A, benign breast tissue (H-score 30) and B, invasive ductal carcinomas, grade 2 (H-score 153). Bar is 200 μ m. C, Average $\pm$ SEM of H-score for nuclear COUP-TFII staining in ER α -positive invasive ductal carcinomas by tumor grade. * significantly different from T2N0M0 ($p < 0.05$). D and E, Nucleolin immunostaining at 400x: D, benign breast tissue (H-score 13) and E, invasive ductal carcinomas, grade 3 (H-score 151). Bar is 100 μ m. F and G, immunohistochemical localization of COUP-TFII (100 $\times$, inset: 200 $\times$) on a tissue microarray constructed from archival tissue from 332 breast cancer patients [61] showing positively (F) and negatively (G) stained cores COUP-TFII at 200x, bar is 100 μ m. H and I, Kaplan-Meier estimates of disease-free survival functions were computed, and the Wilcoxon test was used to compare survival curves. In addition, the Wilcoxon rank sum test was used to compare two medians. The data are not statistically significant.

Table 3. COUP-TFII staining in breast tumor microarray.

Associated expression of COUP-TFII with ER α , PR, HER2, SRC-1, AIB1, Pea3, AIB1, MMP2, and phospho-Src (psrc) staining in 560 human breast tumors [61,244]. Statistical analysis was performed using the Fisher's exact test, and a P value of <0.05 is considered to be significant (**bold** values).

	Total Population n=321	% COUPTFII positive 47%	p value
ER			
neg	99	34.3	
pos	222	52.3	0.004
PR			
neg	133	50.4	
pos	146	44.5	0.339
Her2			
neg	267	50.2	
pos	62	32.3	0.011
Grade			
1	24	54.2	
2	122	54.1	
3	112	33.0	0.003
Node			
neg	156	47.4	
pos	167	46.1	0.824
Recurrence			
neg	200	46.0	
pos	132	47.7	0.822
SRC-1			
neg	214	41.6	
pos	113	56.6	0.011
AIB1			
neg	88	25.0	
pos	221	55.7	<.001
Pea3			
neg	120	35.8	
pos	120	58.3	0.001
MMP2			
neg	29	20.7	
pos	284	48.2	0.005
psrc			
neg	226	42.0	
pos	87	56.3	0.031

atRA enhances COUP-TFII binding to the RARB2 promoter

COUP-TFII is required for atRA- or 9cRA- induced *RARB2* expression in breast cancer cells [118,131] and binds the *RARB2* promoter in electrophoretic mobility shift assays [118]. To examine COUP-TFII interaction with the *RARB2* promoter, we first examined recruitment of endogenous COUP-TFII to the *RARB2* promoter using the R&D systems COUP-TFII antibody, but were unable to detect product in the PCR reactions (data not shown) likely due to lower COUP-TFII protein in MCF-7 cells compared to C3H10T1/2 CH3 mouse embryo mesenchymal cells that express high levels of COUP-TFII [260]. To obviate this difficulty, MCF-7 cells were transfected with an empty vector or COUP-TFII-FLAG and ChIP was performed after FLAG IP. ChIP revealed for the first time that COUP-TFII binds the *RARB2* promoter and atRA increased COUP-TFII occupancy at the *RARB2* promoter by 72% in MCF-7 cells (Fig. 8A; Appendix I, Fig. S5).

AS1411 inhibits COUP-TFII-stimulated RARB2 gene expression in MCF-7 and T47D breast cancer cells

Once establishing the presence of COUP-TFII at the promoter of its target gene *RARB2*, we sought to determine if nucleolin functions as a coactivator for COUP-TFII-mediated *RARB2* expression. *RARB2* was increased in MCF-7 cells transfected with COUP-TFII and nucleolin overexpression potentiated the *RARB2* induction in a concentration-dependent manner (Fig. 8B). Further, because the G-rich, G-quartet forming DNA aptamer AS1411 binds and reduces nucleolin activity [252,261], we hypothesized that AS1411 would inhibit COUP-TFII-stimulated *RARB2* expression.

AS1411 inhibited the COUP-TFII-induced increase in *RARB2*, while CRO (negative control) had no effect (Fig. 8C). Although nucleolin did not affect basal *RARB2* expression, nucleolin abrogated the inhibition of *RARB2* transcription by AS1411 (Fig. 8C). COUP-TFII also increased luciferase activity from a *RARB* gene promoter-reporter and AS1411 abrogated luciferase induction (Fig. 8D).

Figure 8. COUP-TFII increases *RARβ2* transcription in MCF-7 cells.

A, ChIP of COUP-TFII-FLAG to the *RARB2* promoter in MCF-7 cells transfected with empty vector (EV) or COUP-TFII (CII) and treated with EtOH or 1 μ M atRA for 6 h. * $P < 0.05$ versus E control, $^{\#} P < 0.05$ versus CII-EtOH. B-C, Cells were transfected with parental or expression plasmids for COUP-TFII or nucleolin for 24 h and were treated with EtOH, 10 μ M CRO or AS1411 for 24 h post-transfection. For C, cells were transfected with 0.5 μ g and 1 μ g of nucleolin and COUP-TFII expression vector, respectively. QRT-PCR was performed to determine *RARB2* expression. Values are the average of 6 separate experiments $\pm$ SEM. D, Cells were transfected with pcDNA or pcCOUP-TFII and treated with 10 μ M RO or AS1411 for 24 h. Dual luciferase activity was expressed relative to the pcDNA-transfected, no-treatment control. Values are mean $\pm$ S.E.M. of two separate experiments. For B-D, * $P < 0.05$ versus vector control, ** COUP-TFII alone, or $\blacktriangle$ between the indicated values.

To be sure that any effect of AS1411 on *RARB2* is not cell-line specific, MCF-7 and T47D cells with similar nucleolin expression (Appendix I, Fig. S6) were tested. T47D has ~40% lower COUP-TFII than MCF-7 (Appendix I, Fig. S6). Both atRA and 9cRA increased *RARB2* expression in MCF-7 and T47D cells, with greater induction in T47D, and increased *NR2F2* (COUP-TFII) expression in T47D (Fig. 9A-B).

Pretreatment of MCF-7 cells with AS1411, but not negative control CRO, reduced atRA-induced *RARB2* expression (Fig. 9C). Transfection of MCF-7 cells with a nucleolin expression vector increased basal *RARB2* except in AS1411-treated cells. Nucleolin and atRA additively increased *RARB2* expression in MCF-7 cells and AS1411, but not CRO, reduced *RARB2* induction. Nucleolin significantly abrogated the inhibition of *RARB2* expression by AS1411. COUP-TFII mRNA levels were not significantly reduced by AS1411 (Appendix I, Fig. S7). Neither ER antagonists ICI 182,780 nor 4-OHT blocked atRA-induced *RARB2* expression, indicating that ER is not involved in COUP-TFII-activated *RARB2* expression (Fig. 9C). ICI and 4-OHT increased basal *RARB2* in MCF-7, in agreement with *RARB*-luciferase reporter activation in transfected MCF-7 cells [262].

Pretreatment of T47D cells with AS1411, but not CRO, reduced atRA-induced *RARB2* expression and co-transfection with an expression vector for nucleolin significantly abrogated the inhibition of *RARB2* expression by AS1411 (Fig. 9D). AS1411 had no effect on MCF-7 cell viability for the treatment times used in these experiments (Appendix I, Fig. S8), commensurate with previous findings that MCF-7 viability is inhibited only after 6 d of AS1411 treatment [261].

Figure 9. Regulation of RAR β 2 transcription.

A and B, QRT-PCR for *RARB2* (B RAR β 2) and *NR2F2* (C COUP-TFII) in MCF-7 or T47D cells treated with EtOH, 1 μ M atRA, or 1 μ M 9-cis-RA for 24 h. Values are the average of 3-5 separate experiments. * $P < 0.05$ versus EtOH. C, MCF-7 and D, T47D cells were transfected with 2 μ g pCMV-tag2 (-) or pCMV-tag2-nucleolin (+) for 24 h prior to 24 h treatment with EtOH, 10 μ M CRO, or 10 μ M AS1411. Where indicated, cells were treated with 1 μ M atRA, 100 nM 4-OHT, or 100 nM ICI 182,780 for 6 h. QRT-PCR for *RARB2* expression. Values are the average of 6 (MCF-7) and 4-10 (T47D) separate experiments $\pm$ SEM. * $P < 0.05$ versus EtOH or ** between the indicated values.

RARB2 expression is inhibited by nucleolin or COUP-TFII knockdown

siNucleolin reduced nucleolin (*NCL*) mRNA by 37-58% and protein by 22-35% (Fig. 10A) in MCF-7 cells. In parallel, basal *RARB2* was reduced 32-56% (Fig. 10A). To determine if nucleolin knockdown inhibited atRA-induced *RARB2*, T47D cells were transfected with siNucleolin and untreated or treated with atRA (Fig. 10B). atRA had no effect on nucleolin knockdown (Fig. 10B). Analogous to the MCF-7 cells, siNucleolin reduced *RARB2* mRNA (Fig. 10B). siNucleolin inhibited the atRA induction of *RARB2* ~36%. Taken together, results from AS1411 and siNucleolin studies indicate a functional role for nucleolin as a coactivator of COUP-TFII-regulated atRA-induced *RARB2* expression in T47D and MCF-7 cells.

siCOUP-TFII reduced COUP-TFII protein and, consequently, *RARB2* transcription and protein (Fig. 10C). *RARβ2* stimulates retinoic acid receptor-induced gene 1 (*RRIG1*) transcription [263]. siCOUP-TFII reduced basal and atRA-induced *RRIG1* expression. Transfection with COUP-TFII increased *RRIG1*. These data substantiate COUP-TFII's regulation of functional *RARβ2*.

Effects of AS1411 on nucleolin-COUP-TFII nuclear interaction

AS1411 reduced the nuclear/cytoplasmic ratio of the nucleolin-interacting protein PRMT5 (Protein Arginine Methyltransferase 5) in DU145 prostate cancer cells [247]. AS1411 or CRO did not change nuclear COUP-TFII or nucleolin or cytosolic nucleolin in MCF-7 cells (Fig. 11A-B). There was no change in relative COUP-TFII-nucleolin interaction in MCF-7 cells treated with AS1411 (Fig. 11C- D).

Figure 10. Reduction of COUP-TFII or nucleolin decreases RAR β 2 transcription in MCF-7 cells.

MCF-7 (A) and T47D (B) cells were transfected with control siRNA or an siRNA targeting nucleolin for 48 h. T47D cells were treated with EtOH or 1 μ M atRA for 24 h. QRT-PCR for nucleolin (*NCL*) and *RARB2*. Values are the average of triplicates. C, Western blot showing COUP-TFII and RAR β 2 expression after transfection with siCOUP-TFII. Values are relative to β -actin. MCF-7 were transfected with siControl or siCOUP-TFII for 48 h and treated with 1 μ M atRA for 6 h. QRT-PCR was also performed for *RRIG1*. $P < 0.001$ * versus control or ** versus atRA.

Figure 11. Effects of AS1411 on nuclear nucleolin-COUP-TFII interaction.

A, Representative western blots of CE and NE (30 μ g) from MCF-7 cells: untreated (untx) or treated with 10 μ M CRO or AS1411 for 24 h were probed for nucleolin or COUP-TFII, then stripped and reprobed for HDAC1 and α -tubulin. B, Relative nucleolin and COUP-TFII expression (normalized to respective loading controls and untreated sample protein ratios were set to 1 for NE and CE). Bars are the mean $\pm$ SEM of 4-8 separate experiments. C, NE or CE (200 μ g) from MCF-7 cells treated as above were IPed with COUP-TFII antibody or rabbit IgG. The Ab-no NE lane was a negative control: COUP-TFII antibody incubated with beads and buffer without NE. D, The ratio of nuclear nucleolin/COUP-TFII is the mean $\pm$ SEM of 3 experiments.

DISCUSSION

COUP-TFII plays an undefined role in breast cancer [116]. In this report, we identify nucleolin as a new functional partner for COUP-TFII. We have demonstrated for the first time that COUP-TFII binds nucleolin *in vitro* and *in vivo*. In breast tumors, nuclear nucleolin correlates with ER α and cell proliferation [264,265]. Here IHC staining revealed a correlation between nuclear COUP-TFII and nucleolin staining in invasive ductal carcinoma, a finding that reflects a previous report showing overexpression of COUP-TFII in A549 lung adenocarcinoma cells increased *in vitro* tumorigenicity and migration [211]. We demonstrate that nucleolin acts as a coactivator of endogenous COUP-TFII transcriptional activity for *RARB2* in breast cancer cells. Similarly, nucleolin acted as a coregulator by interacting with c-Jun and Sp1 and increasing cytosolic phospholipase A2 (*cPLA2*) gene transcription [256].

We report a positive correlation between COUP-TFII and ER α , SRC-1, Pea3, MMP2, and phospho-Src expression, an inverse correlation of COUP-TFII with tumor grade and reduced COUP-TFII in ER α -positive, invasive ductal carcinomas with increased TNM stage within tumor grades II and III and HER2 positivity. These data are in agreement with Oncomine analysis demonstrating *NR2F2* is higher in ER α + tumors and lower in metastatic breast tumors in microarray data [266] (Appendix I, Fig. S9). These observations indicate that COUP TFII may play a role in “phenotype maintenance” and that its function may be restricted to the luminal breast cancer subtypes. We speculate that the precise role of COUP-TFII in breast cancer depends on cellular context, which is consistent with the role of other nuclear receptors in breast cancer [267], and remains to be fully elucidated.

Other COUP-TFII-interacting proteins identified here include PARP-1, which also interacts with nucleolin [268], TOPOII β , involved in transcriptional activation by NRs [269,270], and DNA topoisomerase I (TopoI), which localizes to active transcription sites [271]. Reflecting its NR chaperone role and interaction with COUP-TFI [114], Hsp70 interacted with COUP-TFII. Other COUP-TFII-interacting proteins including hnRNP A2/B1, RPS20, RPL15, and RPL21 were also identified as binding with nucleolin to a c-myc G-quadruplex affinity column [272].

Nucleolin is a key target of the anticancer aptamer AS1411, although AS1411 interacts with other proteins, *e.g.*, NEMO to inhibit NF κ B activation and PARP-1 [252]. AS1411 has pleiotropic effects on nucleolin, *e.g.*, inhibiting nucleolin binding to the AU-rich element in the 3' UTR of *BCL2* in MCF-7 cells causing apoptosis [273] and stimulating macropinocytosis [251]. AS1411 is used to functionally inhibit nucleolin [274]. Here, AS1411 and siNucleolin reduced COUP-TFII-induced *RARB2* expression in MCF-7 and T47D cells and cotransfection with nucleolin reduced AS1411-inhibition. AS1411 did not alter nuclear COUP-TFII-nucleolin interaction, indicating that the mechanism for AS1411 inhibition of *RARB2* expression is independent of reducing nuclear COUP-TFII protein, a result different from AS1411 reducing nuclear PRMT5 in DU145 cells [247]. The inhibition of atRA- and COUP-TFII- regulated *RARB2* expression by AS1411 may also be independent of its effect on nucleolin and may indicate a potential adverse 'side effect' of AS1411 that may be a concern if this drug is used for breast cancer therapy.

In conclusion, COUP-TFII interacting proteins were identified in MCF-7 breast cancer cells. Endogenous COUP-TFII and nucleolin interact in both MCF-7 and T47D

luminal breast cancer cells. A coregulatory role for nucleolin in COUP-TFII-mediated *RARB2* transcription was described (Fig. 12). Retinoids, *e.g.* 9cRA and atRA, and RAR β have long been associated with tumor suppressive properties such as reduced cell proliferation, inflammation, and solid tumor formation, as well as enhanced apoptosis (reviewed in [275]). RAR β 2 expression is reduced in breast tumors and restoration of RAR β 2 expression increases sensitivity to tumor growth inhibition by retinoids [276]. Ours is the first demonstration that atRA increased COUP-TFII-*RARB2* promoter interaction by ChIP. Since serum carotenoids levels are inversely associated with breast cancer risk in women with high mammographic density [277], the increase in *RARB2* in response to COUP-TFII-nucleolin interaction is consistent with a role for COUP-TFII in phenotype maintenance. In agreement with a previous report [118], we observed a much higher induction of *RARB2* in T47D in response to atRA compared to MCF-7 despite, as newly reported here, lower COUP-TFII expression. These data indicate that other mechanisms are also involved. COUP-TFII protein staining in TMAs correlates with ER α expression and an inverse correlation of COUP-TFII with tumor grade in ER α -positive, invasive ductal carcinomas was detected, a finding that correlates with reduced COUP-TFII expression in endocrine-resistant breast cancer cells [116]. Together, these data suggest that COUP-TFII may be important in differentiated ER α -expressing, retinoid-responsive, epithelial breast cancer cells and that reduced COUP-TFII leads to tumor advancement, including endocrine resistance.

Figure 12. Model of nucleolin-COUP-TFII interaction and upregulation of *RARB2* expression.

COUP-TFII binds its response elements as either a homodimer or as a heterodimer with RXR [107]. Previous reports demonstrated that 1) 9cisRA and atRA bind COUP-TFII and increase COUP-TFII transcriptional activity [120]; 2) Nucleolin acts as a transcriptional coregulator by interacting with cJun and Sp1 [256]; 3) COUP-TFI and COUP-TFII increase *RARB2* expression in cooperation with RAR α and CBP [118]. Here we demonstrated that 1) nucleolin interacts directly with nuclear COUP-TFII; 2) atRA and 9-cisRA increased *RARB2* mRNA; 3) AS1411, used as a functional inhibitor of nucleolin [252,274], inhibited COUP-TFII-upregulation of *RARB2* gene transcription; 4) siRNA knockdown of nucleolin reduces induction of *RARB2* and reduced RAR β 2 protein.

CHAPTER IV - COUP-TFII INHIBITS NF κ B ACTIVATION IN BREAST CANCER CELLS AND RESTORES ENDOCRINE SENSITIVITY

INTRODUCTION

Selective estrogen receptor modulators (SERMs) such as tamoxifen (TAM) exert antiproliferative effects on breast cancer by competing with estrogens for binding to estrogen receptor α (ER α), leading to an inhibition of downstream genes, including those involved in breast cancer proliferation. Fulvestrant is a selective ER α downregulator (SERD) because it causes proteasomal degradation of ER α in addition to its SERM-like ability to antagonize estrogen-activated ER α transactivation [278]. TAM inhibits cell cycle progression and induces apoptosis [45,46]. TAM has greatly increased the survival rate of breast cancer patients since its initial FDA approval in 1977, resulting in a 31% reduction in annual death rate [48,279]. Despite the initial efficacy of SERM and aromatase inhibitor treatment, approximately 40% of patients relapse and die from metastatic disease because the cancer cells become refractory to endocrine therapies [56].

The mechanisms for acquired endocrine resistance are complex and a better understanding of maintenance of endocrine sensitivity is clearly needed. Reduced expression of the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) has a demonstrated role in acquired endocrine

resistance in a breast cancer cell line model of human disease progression [116]. Restoration of COUP-TFII expression, which is reduced in endocrine-resistant breast cancer cell lines, inhibited cell proliferation and motility and increased apoptosis in TAM and fulvestrant-treated endocrine-resistant LCC9 and LY2 breast cancer cells [116]. Conversely, inhibition of COUP-TFII expression caused MCF-7 cells to become resistant to SERM and fulvestrant inhibition of proliferation [116]. A mechanism for the involvement of COUP-TFII in maintenance of endocrine sensitivity has not yet been determined. Since COUP-TFII acts as either a transcriptional activator or repressor in a gene- and cell-specific manner [226], the mechanism of action may involve modulation of specific target genes including tumor suppressors and oncogenes.

The NF κ B family of dimeric transcription factors has important functions related to immune and inflammatory responses, and recent studies have demonstrated NF κ B activation plays a role in acquired endocrine-resistant breast cancer [87,88,280]. Inactive NF κ B is located in the cytoplasm bound to I κ B, the inhibitor of NF κ B. Also present in the cytoplasm is the IKK complex containing the regulatory subunit NEMO (nuclear factor- κ B essential modulator or IKK γ) and the catalytic subunits IKK α and IKK β . Upon activation by a variety of stimuli, the IKK complex phosphorylates the inhibitor I κ B leading to its degradation, freeing the now activated NF κ B to translocate to the nucleus to regulate target gene transcription. Because NF κ B activation results in expression of genes that lead to anti-apoptotic and pro-proliferative activities, increased NF κ B signaling promotes cancer cell survival [281].

Dysregulation of the NF κ B subunits p65 (*RELA*), RelB (*RELB*), p50 (*NFKB1*), p52 (*NFKB2*), and c-Rel (*REL*) results in altered activation of the NF κ B pathway in

breast cancer. Increased expression of p50/NFκB1, p52/NFκB2, and c-Rel was detected in breast tumors compared to adjacent normal tissue [282]. Elevated p50/NFκB1-DNA binding correlated with metastatic relapse and reduced disease-free survival in patients with ERα-positive tumors [283]. An increase in NFκB activity has been reported in endocrine-resistant breast cancer cells [87,280,284]. The amount of RelA/p65 protein was also elevated [87]. The expression and DNA binding of p50 and p65 are also enhanced in LCC1 estrogen-independent, TAM-sensitive breast cancer cells compared to MCF-7 estrogen-dependent cells, further demonstrating a role for NFκB in the pathway to endocrine resistance [93].

Since COUP-TFII expression is decreased in endocrine-resistant breast cancer cells, we hypothesized COUP-TFII may play a part in the normal suppression of NFκB activity in endocrine-sensitive breast cancer cells by regulating the transcription of components of the NFκB pathway. In this study, we observed that COUP-TFII overexpression inhibited NFκB activity in LCC9 endocrine-resistant breast cancer cells. This suppression of the NFκB pathway resulted in reduced expression of downstream NFκB target genes as well as NFκB subunits and increased sensitivity to 4-hydroxytamoxifen (4-OHT) treatment. Our results provide a mechanism by which COUP-TFII maintains endocrine sensitivity by suppressing NFκB expression and activity in breast cancer cells.

MATERIALS AND METHODS

Chemicals

4-hydroxytamoxifen (4-OHT) and BMS-345541 were purchased from Sigma-Aldrich (St. Louis, MO). Tumor necrosis factor α (TNF α) was purchased from PeproTech (Rocky Hill, NJ).

Antibodies

The following antibodies were purchased: monoclonal (mAb) anti-human COUP-TFII (R&D systems, Minneapolis, MN); polyclonal RelA/p65, polyclonal and mAb RelB, and mAb NF κ B1 p105/p50 (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal c-Rel, polyclonal NF κ B1 p105/p50, and polyclonal NF κ B2 p100/p52 (Cell Signaling, Danvers, MA), β -actin (Sigma-Aldrich). HRP-conjugated secondary antibodies were from GE Healthcare (Piscataway, NJ).

Cell culture and transient transfection

MCF-7 cells were purchased from ATCC and used at passage < 10. LCC9 cells were kindly provided by Dr. Robert Clarke [285]. MCF-7 and LCC9 cells were maintained as in [116]. Cells were transiently transfected as indicated in figure legends using FuGENE HD (Roche, Indianapolis, IN) as per the manufacturer's protocol.

Luciferase assay

To analyze NF κ B activity, MCF-7 and LCC9 cells were transiently transfected with pGL4.32[luc2P/NF- κ B-RE/Hygro] (Promega, Madison, WI) containing five copies of a NF κ B response element, pGL4-hRluc-TK (*Renilla*, Promega), and pcDNA3.1 or pcDNA3.1-mCOUP-TFII (kindly provided by Drs. Sophia and Ming-Jer Tsai [246]) for

48 h before performing dual luciferase assay (Promega). Where indicated, cotransfection was performed with plasmids for CBP (kindly provided by Dr. Margarita Hadzopoulou-Cladaras [286]), SRC-1 (kindly provided by Dr. Bert W. O'Malley [287]), GRIP1/SRC-2, or ACTR/SRC-3 (kindly provided by Dr. Michael Stallcup [288]).

NF κ B pathway PCR array

LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-mCOUP-TFII for 24 h before treating with 10 ng/ml TNF α for 6 h. RNA was isolated using RNeasy (Qiagen, Valencia, CA) and cDNA was prepared using RT² First Strand Kit (SABiosciences/Qiagen, Valencia, CA). Human NF κ B Signaling Pathway PCR Array (SABiosciences/Qiagen) was run according to manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-mCOUP-TFII for 48 h followed by treatment with 10 ng/ml TNF α for 6 h. Following centrifugation, cells were resuspended in 10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol (DTT, Sigma-Aldrich), 1X protease and phosphatase inhibitors (Roche). The cells were lysed with a 27 gauge syringe and centrifuged to obtain cytosolic extract. The resulting nuclear pellet was resuspended in EMSA buffer (10 mM Tris HCl, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton-X 100, 12.5% glycerol, pH 7.9 (Sigma-Aldrich), 1 mM DTT, 1X protease and phosphatase inhibitor (Roche)) and sonicated to prepare nuclear extract (NE).

NF κ B sense (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and antisense (5'-GCC TGG GAA AGT CCC CTC AAC T-3') oligonucleotides (Integrated DNA Technologies, Coralville, IA) were annealed and labeled with [32 P]- γ ATP (PerkinElmer, Waltham, MA) using T4 polynucleotide kinase (Promega) to prepare labeled DNA probe. The [32 P]NF κ B response element (RE) probe was purified using the QIAquick Nucleotide Removal Kit (Qiagen).

25 μ g NE from the indicated cells was incubated with poly-dIdC (Sigma-Aldrich), [32 P]NF κ B-RE (labeled probe), and EMSA buffer for 1 h before separating on a 4% non-denaturing polyacrylamide gel (Bio-Rad, Hercules, CA). For *in vitro* binding assay, *in vitro* transcribed/translated COUP-TFII was prepared as described in [115] and incubated in increasing concentrations with NE from LCC9 cells treated with 10 ng/ml TNF α for 6 h.

NF κ B family transcriptional assay

MCF-7 or LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNF α for 6 h before preparing NE. 15 μ g NE was used for TransAM NF κ B assays (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Briefly, NE was added to 8 well strips containing immobilized NF κ B RE oligonucleotide. Primary antibodies specific for NF κ B subunits RelB, RelA, p50, p52, and c-Rel were used to identify subunit-specific NF κ B activation. Secondary HRP-conjugated antibody and provided buffers provided colorimetric quantitation via spectrophotometric analysis at 450 nm.

Coimmunoprecipitation

Nuclear extracts were prepared from MCF-7 cells as in [115]. Dynabeads protein A (Life Technologies, Carlsbad, CA) were incubated with COUP-TFII antibody (Abcam, Cambridge, MA) or negative control IgG (Santa Cruz) for 30 minutes prior to the addition of 400 µg MCF-7 NE for 4 h at 4°C with rotation. Following washes, protein was eluted with Elution Buffer (Life Technologies, Carlsbad, CA) and pH was adjusted with 1M TrisHCl.

Cell proliferation assay

MCF-7 and LCC9 cells were grown for 48 h in medium containing 5% charcoal-stripped fetal bovine serum (CSS) and transfected with pcDNA or pcDNA-mCOUP-TFII for 24 h before treatment with 1 µM 4-OHT, 10 ng/ml TNFα, and 0.01-5 µM BMS-345541 in DMSO for 5 days. Cell proliferation was assayed using CellTiter Aqueous One Solution Cell Proliferation Assay (MTS; Promega).

RNA Isolation, RT-PCR and Quantitative Real-Time-PCR (QRT-PCR)

RNA isolation, RT-PCR, and QRT-PCR were performed as in [115]. The following SYBR green primers were used for QRT-PCR analysis, relative to *GAPDH* control: *RELB* [289], *REL*, *NFKB1* and *NFKB2* [290], *IL6* [291], *A20/TNFAIP3* [292], *ICAM1* [293], *CCL2* [294], *NR2F2* and *GAPDH* [183], *Nr2f2* (Forward 5'-CCCCCATAGATATGGCAATGGTAGTCAGCACG-3', Reverse 5'-TTGATTTATTTATTGAATTGCCATATATGGCC-3'). *p65/RELA* was measured using TaqMan Gene Expression Assay (Applied Biosystems) relative to 18S control.

Microarray analysis

Published microarray data was obtained from Gene Expression Omnibus (GEO) (dataset GSE17705) consisting of 298 ER α + patients treated with tamoxifen for 5 years and analyzed to assess the correlation between COUP-TFII (*NR2F2*) expression and NF κ B subunit (*NFKB1*, *NFKB2*, *REL*, *RELA*, *RELB*) and target gene (*ICAM1*, *IL6*, *CCL2*, *TNFAIP3*) expression. Gene expression data was log₂ transformed and quantile normalized prior to analysis. Correlation analysis was performed using the Spearman Rho (ρ S) statistic to assess whether *NR2F2* expression was inversely correlated with NF κ B subunit/target genes.

Statistical analysis

Statistical analyses were performed as in Chapter III [115].

RESULTS

COUP-TFII suppresses NF κ B activity

To determine if NF κ B activity is differentially regulated by COUP-TFII in endocrine-sensitive versus -resistant cells, MCF-7 (sensitive) and LCC9 (resistant) cells were transfected with a luciferase reporter containing five tandem repeats of a NF κ B responsive element as well as pcDNA3.1 parental plasmid or pcDNA3.1-COUP-TFII (Fig. 13A). Basal NF κ B activity in LCC9 is ~5 fold higher than MCF-7 cells. When treated with TNF α , a dramatic induction of NF κ B activity is observed in LCC9 but not

MCF-7 cells. A statistically significant, dose-dependent decrease in the $\text{TNF}\alpha$ -induced $\text{NF}\kappa\text{B}$ activation occurs in LCC9 cells upon transfection with increasing amounts of COUP-TFII. These data demonstrate that COUP-TFII suppresses $\text{NF}\kappa\text{B}$ activity in endocrine-resistant breast cancer cells. The expression of $\text{NF}\kappa\text{B}$ subunits *NFKB2*, *REL*, and *RELB* was higher in LCC9 compared to MCF-7 cells (Fig. 13B), accounting for the higher $\text{NF}\kappa\text{B}$ activity in LCC9 cells.

Figure 13. COUP-TFII suppresses NF κ B activity in LCC9 cells.

(A) MCF-7 and LCC9 cells were transfected with a NF κ B luciferase reporter and increasing concentrations of pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNF α for 6 h before performing dual luciferase assay (Promega). Firefly luciferase values are shown relative to protein concentration for a representative experiment of quadruplicate values $\pm$ SEM. * Significantly different $P < 0.05$ from pcDNA+TNF α in LCC9. (B) Comparison of NF κ B subunit mRNA expression in MCF-7 versus LCC9 cells. Values are the mean of 4-8 separate experiments $\pm$ SEM. *: $P < 0.05$ versus MCF-7.

Identification of COUP-TFII effect on NFκB pathway

An NFκB pathway PCR array was used to determine the effect of COUP-TFII on the NFκB pathway in LCC9 endocrine-resistant cells treated with TNFα using a 3-fold cutoff (Fig. 14A). As displayed in the resulting heatmap, a general decrease in the expression of NFκB pathway genes was detected upon COUP-TFII overexpression (Fig. 14B). Many genes suppressed by COUP-TFII greater than 3-fold were NFκB - responsive genes, *e.g.*, *CCL2*, *IL6*, *LTA*, *ICAM1*, and *TNFAIP3*, as well as NFκB subunits (*RELB*), reflecting a role for COUP-TFII in downregulation of the NFκB pathway. These genes were selected for further analysis.

COUP-TFII suppresses NFκB target gene expression

To extend and confirm the results from the NFκB pathway PCR array, MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII and treated with TNFα to determine the effect of COUP-TFII on endogenous NFκB target gene expression. COUP-TFII expression following transient transfection was verified via western blot (Appendix I, Fig. S10). Four NFκB target genes with known roles in breast cancer progression were examined through QRT-PCR to validate the results from the NFκB pathway PCR array: interleukin 6 (IL6), intercellular adhesion molecule 1 (ICAM1), TNFα-induced protein 3 (A20, TNFAIP3), and chemokine (C-C motif) ligand 2 (CCL2) (Fig. 15). TNFα increased the expression of *IL6*, *ICAM1*, *TNFAIP3*, and *CCL2* in both MCF-7 and LCC9 cells (Fig 15A-D). COUP-TFII overexpression reduced the basal expression of *IL6* and *CCL2* in LCC9 cells (Fig. 15A and D) and *ICAM1* and

TNFAIP3 in both MCF-7 and LCC9 cells (Fig. 15B and C). The TNF α -induction of *IL6* and *ICAM1* was reduced by COUP-TFII in LCC9 cells (Fig. 15A and B), while the TNF α -induction of *TNFAIP3* and *CCL2* was reduced in MCF-7 cells (Fig. 15C and D). The suppression of *IL6*, *ICAM1*, *TNFAIP3*, and *CCL2* expression by COUP-TFII is in agreement with the data from the NF κ B PCR array.

Figure 14. COUP-TFII inhibits expression of multiple genes in the NF κ B pathway. LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 24 h and treated with 10 ng/ml TNF α for 6 h before preparing RNA and cDNA. (A) SABiosciences NF κ B Pathway Array was used to identify genes with expression altered greater than 3-fold by addition of COUP-TFII. (B) Heat map displaying fold change of individual genes in the array.

Figure 15. COUP-TFII inhibits expression of NF κ B target genes.

MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNF α for 6 h before preparing RNA and cDNA. mRNA expression of NF κ B target genes identified in the NF κ B pathway array (A. *IL6*, B. *ICAM1*, C. *TNFAIP3*, D. *CCL2*) was analyzed by QRT-PCR. Note that relative expression of each gene was normalized to pcDNA-transfected, control treated cells within each cell line. Values are the mean of 2-7 separate experiments $\pm$ SEM. Bars indicate $p < 0.05$ between the indicated samples to indicate effect of COUP-TFII on basal or TNF α -induced gene expression in each cell line.

COUP-TFII suppresses NFκB subunit gene expression

RELB expression was decreased by COUP-TFII overexpression in the NFκB pathway PCR array. Increased NFκB subunit expression has been reported in several lineages of endocrine-resistant breast cancer cells [87,88,91], but correlation with COUP-TFII expression has not yet been examined. Consistent with these reports, we observed higher *NFKB2*, *REL*, and *RELB* expression in LCC9 versus MCF-7 cells (Fig. 13B). TNFα increased the expression of *NFKB1*, *NFKB2*, *REL*, and *RELB*, but not *RELA* in both MCF-7 and LCC9 cells (Fig. 16A-E). COUP-TFII overexpression inhibited the basal expression of *REL* and *RELA* in LCC9 cells (Fig. 16C and E). The TNFα-induction of *NFKB1*, *REL*, and *RELB* was significantly reduced by COUP-TFII in LCC9 cells (Fig. 16A, C, and D). At the protein level, COUP-TFII overexpression decreased basal NFκB1 p105, NFκB2 p100, and RelB in MCF-7 cells and NFκB2 p100 in LCC9 cells (Fig. 17). COUP-TFII inhibited the TNFα-induced expression of NFκB2 p100, RelA, RelB, and c-Rel in MCF-7 cells and NFκB1 p105, RelA, and RelB in LCC9 cells (Fig. 17). Thus, COUP-TFII inhibits TNFα-induced increases in NFκB1 and RelB at both the mRNA and protein levels in LCC9. Differences between protein and mRNA results for NFκB subunit expression may have been due to the treatment conditions used for the experiment, as NFκB mRNA and protein levels may be altered at different timepoints.

Figure 16. COUP-TFII inhibits mRNA expression of NF κ B subunits in LCC9 cells. MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml $TNF\alpha$ for 6 h before preparing RNA and cDNA. mRNA expression of NF κ B subunits (A. *NFKB1*, B. *NFKB2*, C. *REL*, D. *RELB*, E. *RELA*) was analyzed by QRT-PCR. Bars indicate $p < 0.05$ between the indicated samples to indicate effect of COUP-TFII on basal or $TNF\alpha$ -induced gene expression in each cell line.

Figure 17. COUP-TFII inhibits protein expression of NFκB subunits.

MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNFα for 6 h before preparing whole cell extracts.

Expression of NFκB subunits (NFκB1 p105/p50, NFκB2 p100, RelA p65, RelB, c-Rel) was analyzed by western blot with specific antibodies and compared to β-actin control.

Note: NFκB2 p52 was not detectable.

COUP-TFII and NFκB expression are inversely correlated in breast cancer patient samples

To examine the relationship between COUP-TFII and NFκB in breast cancer patients, publically available microarray gene expression data from 298 ERα+ patients treated with tamoxifen for five years was analyzed using the Spearman Rho (ρ_S) statistic to determine if there was a correlation between COUP-TFII expression and the expression of NFκB subunits and target genes. COUP-TFII (*NR2F2*) expression was inversely correlated with NFκB subunit genes *NFKB2*, *REL*, *RELA*, and *RELB* and NFκB target genes *ICAM1*, *IL6*, and *TNFAIP3* (Table 4). In contrast, COUP-TFII (*NR2F2*) was positively correlated with NFκB target gene *CCL2* (Table 4).

Table 4. Correlation of COUP-TFII expression to NFκB target and subunit gene expression in ERα+ breast cancer patients treated with tamoxifen.

Spearman correlations of gene expression between COUP-TFII (NR2F2) and select NFκB-related genes obtained by analysis of microarray data (GSE17705) from 298 ERα+ breast cancer patients treated with tamoxifen for five years.

Classification	Gene	Spearman Rho	p-value	Correlation to COUP-TFII
NFκB target	<i>ICAM1</i>	-0.35	0.0000	Inverse
	<i>IL6</i>	-0.16	0.0063	Inverse
	<i>TNFAIP3</i>	-0.23	0.0000	Inverse
	<i>CCL2</i>	0.14	0.0152	Positive
NFκB subunit	<i>NFKB1</i>	0.08	0.1638	
	<i>NFKB2</i>	-0.20	0.0004	Inverse
	<i>REL</i>	-0.28	0.0000	Inverse
	<i>RELA</i>	-0.34	0.0000	Inverse
	<i>RELB</i>	-0.12	0.0428	Inverse

COUP-TFII decreases NFκB-DNA binding

COUP-TFII can repress gene transcription by direct DNA binding and by direct protein:protein interaction with other nuclear receptors and transcription factors [108,226]. To determine if COUP-TFII inhibits NFκB-DNA binding, NE from LCC9 endocrine-resistant cells treated with TNFα to activate NFκB was incubated with increasing concentrations of *in vitro* transcribed/translated COUP-TFII protein and a [³²P]NFκB RE for an EMSA. In the absence of NE (lane 1), the [³²P]NFκB RE did not form a retarded band; however, addition of NE from TNFα-treated LCC9 cells formed a retarded band (Fig. 18A). The intensity of the NFκB-DNA complex was decreased after competition with 100-fold excess of unlabeled NFκB oligomer (Appendix I, Fig. S11). COUP-TFII alone did not bind to the [³²P]NFκB RE oligomer (Fig. 18A); however, the amount of NFκB retarded complex was first slightly increased and then decreased with the addition of COUP-TFII in increasing concentrations (Fig. 18A). This suggests that COUP-TFII directly inhibits NFκB-DNA binding *in vitro*. A similar effect was seen using NE from MCF-7 and LCC9 cells transfected with COUP-TFII. In both MCF-7 endocrine-sensitive cells and LCC9 endocrine-resistant cells, transfection with COUP-TFII decreased the amount of [³²P]NFκB RE bound complex (Fig. 18B). No increase was seen in [³²P]NFκB RE-binding with TNFα treatment in either MCF-7 or LCC9 cells (Fig. 18B), although induction of NFκB activation with TNFα treatment was detected in LCC9 cells under identical treatment conditions (Fig 13A). These data suggest that COUP-TFII interacts with and decreases NFκB-DNA complex formation.

C

Figure 18. COUP-TFII decreases NFκB-DNA binding.

(A) NE (25 μ g) from TNF α -treated (6 h) LCC9 cells was incubated alone or with increasing amounts of *in vitro* transcribed and translated COUP-TFII. [32 P]NF κ B RE DNA probe was added for 1 h prior to EMSA. Values are relative bound [32 P]NF κ B RE/total [32 P]NF κ B RE. (B) 25 μ g NE prepared from MCF-7 and LCC9 cells transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with TNF α for 6 h was incubated with [32 P]NF κ B RE DNA probe for 1 h to perform EMSA. (A-B) Bottom panels contain lighter exposure of unbound [32 P]NF κ B RE DNA probe. (C) TransAM NF κ B family assays were performed to measure NF κ B subunit activation using 15 μ g NE from MCF-7 and LCC9 cells transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with TNF α for 6 h. Values are the average of two determinations $\pm$ SEM. * Significantly different $p < 0.05$ from pcDNA-transfected cells.

COUP-TFII inhibits RelB, RelA, and p50 NFκB activation

To examine the effect of COUP-TFII on specific NFκB subunit interaction with the NFκB RE, TransAM NFκB family assays were performed (Fig. 18C). RelB activity was higher in MCF-7 cells, while NFκB1 p50 activity was greater in LCC9. RelA and c-Rel activation were not statistically different between the two cell lines. NFκB2 p52 activation was low in MCF-7 or LCC9 (data not shown), consistent with the lack of p52 protein expression seen in western blots (Fig. 17). RelB activation was significantly decreased by COUP-TFII-transfection in TNFα-treated MCF-7 cells. Activation of RelA and p50 was inhibited by COUP-TFII in both MCF-7 and LCC9 TNFα-treated cells as well as untreated LCC9 cells. COUP-TFII inhibited NFκB1/p50 more than RelA. These data suggest that COUP-TFII interferes with the binding of NFκB subunits RelB, RelA, and p50 to the NFκB RE.

COUP-TFII interacts with NFκB subunits

As both COUP-TFII overexpression and *in vitro* incubation with COUP-TFII protein led to decrease in NFκB-DNA binding (Fig. 18A and B), reduced p50 and RelB activation (Fig. 18C), and inhibited target gene expression (Fig. 15A-D), co-immunoprecipitation was used to determine if COUP-TFII interacts with NFκB subunits to suppress their activity. Immunoprecipitation of COUP-TFII in MCF-7 cells revealed interaction between COUP-TFII and the NFκB subunits RelB and NFκB1 p105/p50 (Fig. 19A).

Figure 19. COUP-TFII interacts with NFκB subunits and inhibits coactivator function in NFκB activation.

(A) 500 μg NE from MCF-7 cells treated with TNFα for 6 h was immunoprecipitated (IP) using a COUP-TFII antibody and separated on a 10% SDS PAGE gel prior to transfer to a PVDF membrane. Interaction with NFκB subunits NFκB1 p105/p50 and RelB was identified using specific antibodies on western blot. (B) LCC9 cells were transfected with a NFκB luciferase reporter and pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h +/- plasmids for the coactivators SRC-1, SRC-2, SRC-3, and CBP. Cells were treated with 10 ng/ml TNFα for 6 h before performing dual luciferase assay. Firefly luciferase values are shown relative to protein concentration for a representative experiment of quadruplicate values ± SEM. * Significantly different P<0.05 from pcDNA-transfected cells. Bars connect values which are significantly affected by COUP-TFII-transfection vs. coactivator alone.

Inhibition of coactivator function by COUP-TFII reduces NFκB transcriptional activity

COUP-TFII has also been reported to inhibit the activity of androgen receptor (AR) by competing with coactivators for AR interaction [295]. SRC-1, SRC-2, SRC-3, and CBP function as coactivators of NFκB-mediated transcription [296]. Each of these coactivators also have known interactions with COUP-TFII [226]. To determine if COUP-TFII represses NFκB signaling by interfering with coactivator function, LCC9 cells were cotransfected with a NFκB luciferase reporter with either pcDNA3.1 or pcDNA3.1-COUP-TFII plasmids (as in Fig. 13A), and plasmids for SRC-1, SRC-2, SRC-3, or CBP. The cells were treated with 10 ng/ml TNFα for 6 hours to activate NFκB. SRC-1, SRC-3, and CBP increased NFκB activity in LCC9 cells compared to parental vector pcDNA (Fig. 19B). SRC-2 transfection had no effect on NFκB activity. Cotransfection with COUP-TFII decreased the activation of NFκB activity by the coactivators SRC-1, SRC-3, and CBP (Fig. 19B), implicating COUP-TFII inhibition of coactivator function, *e.g.*, competing with NFκB for coactivator interaction, as part of the mechanism of NFκB suppression.

Endocrine-resistant cells are sensitive to NFκB-inhibition

Cell proliferation assays were performed to determine phenotypic effects of the both the activation of NFκB pathway in endocrine-resistant cells and the suppression of NFκB activity by COUP-TFII. MCF-7 and LCC9 cells overexpressing COUP-TFII were treated with combinations of TNFα to activate NFκB, 4-OHT to assess endocrine-sensitivity, and BMS-345541, an NFκB inhibitor that functions by inhibiting IκB kinase

and subsequent downstream NF κ B activation [297]. BMS-345541 treatment was performed with a range of concentrations (0.01-5 μ M) determined to be effective in inhibiting NF κ B activity in serum starved MCF-7 and LCC9 cells (Appendix I, Fig. S12). The proliferation of MCF-7 cells was largely unaffected by treatment with BMS-345541 (Fig. 20A). MCF-7 cells displayed reduced viability with 4-OHT or combined transfection with COUP-TFII and 4-OHT treatment (Fig. 20A), as previously reported [116] and in agreement with MCF-7's phenotype as endocrine-sensitive breast cancer cells. Treatment with TNF α did not significantly affect the response of MCF-7 to the compounds tested (Fig. 20B).

When LCC9 cells were treated with increasing concentrations of the NF κ B inhibitor BMS-345541, a marked reduction in cell proliferation was observed (Fig. 20C), reflecting LCC9's increased reliance on a functional NF κ B pathway for cell growth as endocrine-resistant cells [87,88]. Reduced LCC9 cell viability was observed upon combined transfection with COUP-TFII and treatment with 4-OHT, as has been previously reported [116]. LCC9 cells transfected/treated with a combination of COUP-TFII, 4-OHT, and BMS-345541 exhibited a reduction in the higher range of BMS-345541 concentrations (Fig. 20C). Interestingly, when LCC9 cells were treated with TNF α , 4-OHT increased cell proliferation (Fig. 20D). This finding could have important translational implications if tamoxifen therapy may cause similar effects in patients with high levels of inflammation and NF κ B activation. This observed induction of LCC9 cell proliferation with TNF α and 4-OHT treatment was blocked by transfection with COUP-TFII and/or treatment with higher concentrations of BMS-345541 (Fig. 20D).

Figure 20.
Overexpression of COUP-TFII increases endocrine sensitivity in breast cancer cells.
 MCF-7 (A-B) and LCC9 (C-D) cells were serum-starved for 24 h before being transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 24 h. Cells were treated with concentrations of BMS-345541 indicated, as well as 1 μM 4-OHT and 10 ng/ml TNFα for 5 days before performing MTT assay to assess changes in cell proliferation. $p < 0.05$ ** vs. untreated, ^ vs. 4-OHT, # vs. COUP-TFII, " vs. COUP-TFII + 4-OHT, * vs. BMS-only at each concentration.

DISCUSSION

The NF κ B pathway is constitutively activated in many types of cancer, including breast, gastrointestinal, genitourinary, hematological, and gynecological cancers, through a variety of mechanisms [86]. NF κ B target genes have roles in oncogenic transformation, including apoptosis resistance [292,298], invasion [299], and epithelial to mesenchymal transition [300]. Here we demonstrate that COUP-TFII inhibits TNF α -induced NF κ B activity in LCC9 endocrine-resistant breast cancer cells. Consistent with previous reports that the NF κ B pathway is activated during progression to breast cancer endocrine-resistance [87,88,91-93,301], LCC9 cells exhibited greater basal and TNF α -induced NF κ B activity than MCF-7 parental, endocrine-sensitive breast cancer cells. This inhibition led to downregulation of TNF α -induced NF κ B target gene expression of *IL6*, *ICAM1*, *TNFAIP3*, and *CCL2*, which had reduced basal and/or TNF α -induced expression upon COUP-TFII overexpression. The inflammatory cytokine IL6 is a negative prognostic indicator for breast cancer patients [302], and may have a role in trastuzumab resistance [303]. Downregulation of the glycoprotein adhesion molecule ICAM1 at the mRNA and protein levels led to a strong suppression of human breast cell invasion [304]. The ubiquitin-editing enzyme A20/TNFAIP3 protects MCF-7 cells from TNF α -induced apoptosis, and cells overexpressing TNFAIP3 are resistant to tamoxifen [292,298,305]. The chemokine ligand CCL2 may play a role in breast cancer development and progression, invasion and angiogenesis [299].

NF κ B subunit expression is regulated by the NF κ B pathway in a positive feedback loop. Specifically, RelA activates expression of RelB [306] and NF κ B2 [307], while NF κ B1/p50 expression is regulated by a combination of p50 and RelA [308].

Similarly, c-Rel is also capable of autoregulation [309]. Because COUP-TFII suppresses genes that are targets of the NFκB pathway, this would also lead to reduced expression of the NFκB subunits as observed. Consistent with our data showing higher NFκB subunit and target gene expression in LCC9 endocrine-resistant cells, NFκB subunits *NFKB1*, *NFKB2*, *RELA*, and *REL* as well as the NFκB gene targets *ICAM1*, *TNFAIP3*, and *CCL2* are also upregulated in inflammatory breast cancer patient samples [310]. A previous study showed that overexpression of COUP-TFII inhibited 15Δ-PGJ2-induced IL-8 mRNA expression and promoter activity in U937 human myeloid cells, a result which the authors attributed to COUP-TFII competing with PPARγ to form a heterodimer with RXR or by forming a COUP-TFII/RXR heterodimer and competing with PPARγ/RXR for DNA binding [311].

COUP-TFII was recently identified as one of four NRs that are significant predictors of metastasis-free survival in tamoxifen-treated breast cancers, independent of ERα expression [85]. We previously observed that COUP-TFII expression was reduced with histological grade [115] and this was confirmed in [85]. Here we report for the first time that COUP-TFII, *i.e.*, *NR2F2* gene expression was inversely correlated with NFκB subunit genes *NFKB2*, *REL*, *RELA*, and *RELB* and NFκB target genes *ICAM1*, *IL6*, and *TNFAIP3* in microarray data from 298 ERα+ breast tumors from patients treated with TAM for five years (Table 4). Although COUP-TFII inhibited NFκB target gene *CCL2* in LCC9 cells (Fig. 15), COUP-TFII (*NR2F2*) was positively correlated with *CCL2* in the breast tumors (Table 4), indicating potential differences between the patient population and cell line responses. This might be due to the effect of the breast tumor

microenvironment in the patients, and expression of CCL2 by tumor-associated macrophages and other cells [299].

Mechanistically, I have demonstrated that COUP-TFII interacts with NFκB subunits RelB and NFκB1 p105/p50 and inhibits NFκB DNA-binding. COUP-TFII also inhibited the ability of coactivators SRC-1, SRC-3, and CBP to enhance NFκB activation. The effects of COUP-TFII on NFκB lead to inhibition of cell proliferation, with endocrine-resistant LCC9 cell proliferation showing the most reduction when treated with an NFκB inhibitor. Overall, these data indicate a general downregulation of NFκB target gene expression by COUP-TFII, which could have important phenotypic consequences on endocrine sensitivity. As NFκB is highly activated in endocrine-resistant cells, blockage of downstream target gene expression by COUP-TFII may be a mechanism by which COUP-TFII enables breast cancer cells to maintain endocrine sensitivity. Natural product inhibitors of NFκB activity such as parthenolide [88], curcumin [312,313], and ellagic acid [314], as well as synthetic inhibitors [89,297] provide compounds for both experimental and future translational purposes. These data point to potential for the combination of NFκB inhibitors with antiestrogen therapies, *e.g.* tamoxifen, to promote endocrine sensitivity, as well as the availability of COUP-TFII as a marker for response.

CHAPTER V - IDENTIFICATION OF COUP-TFII-INTERACTING PROTEINS IN TAMOXIFEN-TREATED BREAST CANCER CELLS

INTRODUCTION

COUP-TFII is an orphan nuclear receptor that may play multiple roles in endocrine resistance. COUP-TFII expression is reduced in endocrine-resistant breast cancer cell lines, and overexpression of COUP-TFII restores endocrine sensitivity [116]. Part of the mechanism of maintenance of endocrine sensitivity by COUP-TFII involves its ability to suppress activation of NF κ B pathway, as described in Chapter IV. As detailed in Chapter II, many pathways have been reported to be amplified in endocrine resistance and play important roles as both prognostic indicators and areas for potential therapeutic intervention, such as COUP-TFII. As outlined in previous chapters, the role of COUP-TFII in multiple pathways involved in cell differentiation, apoptotic resistance, migration, invasion, and others seems to be dependent on cellular context and interacting partners [226].

Mass spectrometry-based analyses to identify protein interactions can yield important information about disease states [315]. Protein interaction networks provide detailed pathways to enhance understanding of a protein in a particular cellular context [316]. Analyzing the roles of proteins within a particular protein complex or network produces valuable information about the specific activities of the proteins within the cell

[317]. Proteomics is especially useful when studying drug resistance, resulting in additional angles from which to target a particular pathway [317]. As described in Chapter III, a previous study to identify COUP-TFII-interacting proteins in MCF-7 cells identified nucleolin as a coactivator for COUP-TFII-mediated transcription of retinoic acid receptor B2 (*RARB2*) [115].

The focus of the present study was to identify proteins that interact with COUP-TFII in ethanol- versus tamoxifen-treated MCF-7 cells to define interacting proteins changed by treatment with tamoxifen. The goal of this study was to better understand the function of COUP-TFII in maintenance of breast cancer cellular response to tamoxifen treatment and how loss of COUP-TFII expression confers endocrine resistance to breast cancer cells. Heat shock protein 27 (HSP27) was identified amongst the proteins interacting with COUP-TFII more strongly in tamoxifen-treated cells. Given conflicting reports linking HSP27 to both increased and decreased drug resistance [318-325], HSP27 was selected for additional studies.

HSP27 interaction with COUP-TFII was confirmed in MCF-7 breast cancer cells by co-immunoprecipitation experiments. Regulation of HSP27 expression by estradiol and tamoxifen was investigated, as well as the expression and phosphorylation of HSP27 in both endocrine-resistant and trastuzumab-resistant breast cancer cells. Further studies are necessary to fully determine the relationship between HSP27 and COUP-TFII.

MATERIALS AND METHODS

Chemicals and antibodies

4-hydroxytamoxifen (4-OHT) and 17 β -estradiol (E₂) were purchased from Sigma-Aldrich (St. Louis, MO). The following antibodies were purchased: polyclonal COUP-TFII (Abcam, Cambridge, MA); monoclonal (mAB) anti-human COUP-TFII (R&D systems, Minneapolis, MN; PP-H7147-00); mAB anti-FLAG M2 and β -actin (Sigma-Aldrich); mAB HSP27 (Santa Cruz, Santa Cruz, CA), polyclonal phospho-HSP27 (Ser82) and mAB HER2 (c-ErbB2/c-Neu, Millipore, Billerica, MA), HRP-conjugated secondary antibodies were from GE Healthcare (Piscataway, NJ).

Cell culture

MCF-7 breast cancer cells were purchased from ATCC and used at passage < 10. Endocrine-resistant LCC2 [326], LCC9 [285], and LY2 [327] breast cancer cells were obtained from Robert Clarke (Georgetown University). MCF-7, LCC2, LCC9, and LY2 cells were maintained in improved MEM (IMEM) (Cellgro, Manassas, VA) supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (P/S, Cellgro). JIMT-1 trastuzumab-resistant breast cancer cells [328] were obtained from Gail Sonenshein (Tufts University) and maintained in DMEM (Cellgro) supplemented with 10% FBS and 1% P/S. All cells were maintained in a 37°C incubator containing 5% CO₂.

Immunoprecipitation of COUP-TFII-FLAG-interacting proteins

MCF-7 cells were transfected with pIRES-COUP-TFII-FLAG for 24 h using FuGENE HD (Roche, Indianapolis, IN) according to manufacturer's instructions. Cells were 'serum-starved' using phenol red-free IMEM supplemented with 5% dextran-coated

charcoal-stripped serum (Atlanta Biologicals) for 24 h before being treated with 100 nM 4-OHT or vehicle control EtOH for 2 h. One mg of whole cell extract (WCE) prepared in lysis buffer (10% glycerol, 50 mM HEPES pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 2 mM DTT, protease inhibitor cocktail (Roche), 10 mM NaF, and 0.25 mM NaVO₄) [329] was incubated with EZview™ Red ANTI-FLAG® M2 Affinity gel (Sigma-Aldrich) for 4 h at 4°C with constant rotation. Washes and elution was performed in spin chromatography columns (BioRad, Hercules, CA). COUP-TFII-FLAG-interacting proteins were eluted by incubation with 100 µl 6M urea + 100mM (NH₄)HCO₃ for 30 min with constant rotation. Three elution volumes were subsequently pooled for further analysis.

Identification of COUP-TFII-interacting proteins by 2D-LC-MS/MS

COUP-TFII-FLAG protein complexes were analyzed with a Thermo LTQ linear ion trap using a previously described 2D-LC-MS/MS approach [329]. Database searching was performed with tandem mass spectra extracted by ReadW and converted to mzXML format. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using SequestSorcerer (Sage-N, Milpitas, CA). Sequest was set up to search a FASTA-formatted human protein database (Human RefSeq, 2007). Searches were performed with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.2 Da. Iodoacetamide derivative of cysteine was specified as a fixed modification. Oxidation of methionine was specified as a variable modification.

Scaffold (version Scaffold 3.0.00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein probabilities

were assigned by the Protein Prophet algorithm [330]. Protein identifications were accepted if they could be established at greater than 95.0% probability. Proteins that contained similar peptides but could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Co-immunoprecipitation (co-IP) and immunoblotting

Nuclear and cytosolic proteins were prepared as in Chapter III [115]. 4 µg COUP-TFII polyclonal antibody (Abcam) or rabbit IgG (Invitrogen, Carlsbad, CA) were added to 50 µl Dynabeads Protein A (Invitrogen) in PBS (Cellgro) for 20 min at 4°C. 400 µg NE was added and incubated for 4 h at 4°C with rotation. Beads were washed 3x with PBS and bound proteins were eluted by incubation with Elution Buffer (Invitrogen). Proteins were separated by SDS-PAGE and analyzed by western blot [115,116].

Cell treatment

Cells were 'serum-starved' for 48 h prior to treatment with ethanol (EtOH, vehicle control), 10 nM E₂, or 100 nM 4-OHT for 6 h, as indicated.

RNA Isolation, RT-PCR and Quantitative Real-Time-PCR (QRT-PCR)

RNA isolation, cDNA preparation, and QRT-PCR were performed as in [115]. QRT-PCR for HSP27 (HSPB1) and 18S, used Taqman Gene Expression Assays (Invitrogen). COUP-TFII (NR2F2) and GAPDH were measured using the SYBR green method as in [116]. All QRT-PCR was performed in the ViiA 7 Real-Time PCR system

(Applied Biosystems, LifeTechnologies). Fold change was calculated from the $\Delta\Delta CT$ values with the formula $2^{-\Delta\Delta CT}$ and data are relative to EtOH-treated cells.

Statistical analysis

Values are reported as $\pm$ SEM. Student's *t* test was used for comparisons between control and treatment. *P* values considered statistically significant are indicated.

RESULTS

Identification of COUP-TFII-associated proteins in ethanol- and tamoxifen-treated cells

To identify COUP-TFII-interacting proteins which are increased in their association with COUP-TFII either in EtOH (vehicle control) or 4-OHT treated MCF-7 cells, 2D-LC-MS/MS was performed following immunoprecipitation of COUP-TFII-FLAG and interacting proteins from EtOH and 4-OHT-treated MCF-7 cells. Efficiency of FLAG affinity purification is demonstrated in Figure 21. The majority of COUP-TFII-FLAG was bound by the FLAG affinity gel and not lost in flow through or washes (Figure 21A). COUP-TFII-FLAG was detected by western blot in both EtOH and 4-OHT-treated cells and not in vector control-transfected cells (Figure 21B). From the mass spectrometry analysis, proteins were identified as interacting with COUP-TFII specifically in EtOH or 4-OHT-treated cells, as well as a group of proteins which were found in both EtOH and 4-OHT treated samples to varying degrees (Table 5). While three independent IP-mass spectrometry experiments were performed, COUP-TFII was not identified in the second experiment, and data from this experiment were not included in the summary of proteins identified as interacting with COUP-TFII. COUP-TFII-

interacting proteins (from experiments 1 and 3) were subsequently categorically assigned as enriched in the ethanol or tamoxifen-treated groups, or equal/indistinguishable between the two (Table 5) based on their overall presence (number of spectra) in the replicate experiments.

Figure 21. Efficiency of COUP-TFII-FLAG purification by FLAG affinity gel.

A, 20 μ g WCE from MCF-7 cells transfected with COUP-TFII-FLAG (input), 25 μ l flow through from incubation of WCE with FLAG affinity gel, 25 μ l IP washes, and 10 μ l of elutions 1 and 2 (10% of elution volume) were analyzed by western blot to detect the presence of COUP-TFII-FLAG. B, 20 μ g WCE from empty vector pIRES or COUP-TFII-FLAG-transfected cells treated with EtOH or 4-OHT (input) or 10 μ l of pooled elutions 1-3 were analyzed by western blot to detect the presence of COUP-TFII-FLAG before and after immunoprecipitation.

Table 5. COUP-TFII-interacting proteins in ethanol- and tamoxifen-treated MCF-7 cells.

COUP-TFII-FLAG-transfected MCF-7 cells were treated with EtOH (Et) or 100 nM 4-OHT (T) for 2 h. 1 mg WCE was incubated with anti-FLAG affinity gel. COUP-TFII-FLAG and interacting proteins were eluted with 6 M urea and 100 mM (NH₄)HCO₃. 2D-LC-MS/MS was used to identify proteins. The number of assigned spectra for identified proteins (Values) was normalized to number of spectra for COUP-TFII (bottom row in this Table) in each experiment. Qualitative assignment to ethanol, tamoxifen, and equal-interacting groups was made based on normalized values.

		Experiment 1				Experiment 2				
		Values		Normalized		Values		Normalized		
Identified Proteins	MW (kDa)	Et	T	Et	T	Et	T	Et	T	Qualitative enrichment
myosin-9 (MYH9)	227	102	24	3.00	0.67	10	4	3.33	4.00	Ethanol
spectrin alpha chain (SPTAN1)	285	4	2	0.12	0.06	11	2	3.67	2.00	Ethanol
Calmodulin (CALM)	17	10		0.29		7	2	2.33	2.00	Ethanol
spectrin beta chain (SPTBN1)	275	4	0	0.12		4		1.33		Ethanol
tubulin alpha-1B chain (TUBA1B)	50	41	50	1.21	1.39	6	10	2.00	10.00	Tamoxifen
RNA-binding protein FUS (FUS)	53	25	26	0.74	0.72	6		2.00		Tamoxifen
serum albumin preproprotein (ALB)	69	10	8	0.29	0.22	2	2	0.67	2.00	Tamoxifen
heterogeneous nuclear ribonucleoprotein U (HNRNPU)	89	9	10	0.26	0.28	2	2	0.67	2.00	Tamoxifen
keratin, type I cytoskeletal 10 (KRT10)	59	65	59	1.91	1.64	2	2	0.67	2.00	Tamoxifen
60S acidic ribosomal protein P1 (RPLP1)	12	4	5	0.12	0.14	2	2	0.67	2.00	Tamoxifen
probable ATP-dependent RNA helicase DDX5 (DDX5)	69	28	27	0.82	0.75	2	2	0.67	2.00	Tamoxifen
homeobox prox 1 (PROX1)	28	14	16	0.41	0.44	2	2	0.67	2.00	Tamoxifen
heat shock cognate 71 kDa protein (HSPA8)	71	48	53	1.41	1.47	2	4	0.67	4.00	Tamoxifen
78 kDa glucose-regulated protein precursor (HSPA5)	72	23	30	0.68	0.83	2	2	0.67	2.00	Tamoxifen
heat shock protein beta-1 (HSPB1)	23	10	14	0.29	0.39		2		2.00	Tamoxifen
F-actin-capping protein subunit alpha-1 (CAPZA1)	33	6	3	0.18	0.08		2		2.00	Tamoxifen
histone H4 (HIST1H4)	11	34	27	1.00	0.75	8	4	2.67	4.00	Equal
filamin-A (FLNA)	280	35	40	1.03	1.11	8	2	2.67	2.00	Equal
myosin-14 (MYH14)	228	7		0.21		4	2	1.33	2.00	Equal

keratin, type II cytoskeletal 8 (KRT8)	54	77	93	2.26	2.58	5	2	1.67	2.00	Equal
myosin-Ib (MYO1B)	125	8	1	0.24	0.03	4	2	1.33	2.00	Equal
keratin, type I cytoskeletal 9 (KRT9)	62	63	38	1.85	1.06	2	2	0.67	2.00	Equal
2,4-dienoyl-CoA reductase, mitochondrial precursor (DECR1)	36	6	6	0.18	0.17	3	1	1.00	1.00	Equal
keratin, type II cytoskeletal 1 (KRT1)	66	53	47	1.56	1.31	1		0.33		Equal
myosin-Ic (MYO1C)	122	4		0.12		3		1.00		Equal
X-ray repair cross- complementing protein 6 (XRCC6)	70	59	56	1.74	1.56	3		1.00		Equal
plasminogen activator inhibitor 1 RNA-binding protein (SERBP1)	45	21	21	0.62	0.58		1		1.00	Equal
COUP transcription factor 2 (NR2F2)	46	34	36	1.00	1.00	3	1	1.00	1.00	

Functional Annotation Clustering of COUP-TFII-interacting proteins

The Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.7) [331,332] was used to identify functionally related groups amongst the identified COUP-TFII-interacting proteins in the ethanol, tamoxifen, and equally enriched groups (Table 6). This bioinformatic analysis allows for detection of biological themes amongst groups of proteins to identify pathways that may be relevant in a particular state [331,332]. Gene lists compiled from Table 5 were entered into the DAVID database and functional annotation clustering was performed using high stringency for analysis. Repeated terms were removed from the resulting list (Table 6).

Common themes amongst the groups were calmodulin-binding (ethanol and equal) and RNA splicing/alternative splicing (ethanol and tamoxifen) (Table 6), suggesting these may have importance to COUP-TFII. As calmodulin was identified amongst the COUP-TFII-interacting proteins (Table 6), this may indicate that this interaction has significance. A role for COUP-TFII in RNA splicing has not yet been reported, and this may also be a new area for study given potential interactions with DEAD box protein 5 (DDX5), RNA-binding protein FUS (FUS), and heterogeneous nuclear ribonucleoprotein U (HNRNPU) with tamoxifen treatment (Table 5 and 6).

Table 6. Functional Annotation Clustering of COUP-TFII-interacting proteins.
Proteins identified as enriched in the EtOH or tamoxifen-treated samples or proteins identified as interacting with COUP-TFII relatively the same with either treatment were entered into the DAVID database to identify biological relationships between the proteins.

Term	Count	p-value	Fold Enrichment	Bonferroni
ETHANOL				
Cluster 1: MYH9, SPTAN1, SPTBN1				
Cortical cytoskeleton	3	4.3E-05	195.64	0.003
Calmodulin-binding	3	1.2E-04	120.22	0.004
Cell cortex	3	1.2E-04	115.50	0.008
Actin-binding	3	4.9E-04	58.41	0.018
Plasma membrane part	3	7.9E-02	4.35	0.996
Alternative splicing	3	3.4E-01	1.93	1.000
Cluster 2: MYH9, SPTAN1, CALM, SPTBN1				
Cytoskeletal protein binding	4	5.8E-05	25.76	0.002
Acetylation	4	2.6E-03	7.30	0.091
Non-membrane-bounded organelle	4	8.4E-03	4.92	0.435
Plasma membrane	4	2.6E-02	3.38	0.831
Phosphoprotein	4	5.4E-02	2.65	0.871
Polymorphism	4	2.2E-01	1.67	1.000
TAMOXIFEN				
Cluster 1: DDX5, FUS, HNRNPU				
RNA splicing	3	0.018	12.99	0.918
Cluster 2: DDX5, HSPA8, HNRNPU, TUBA1B, HSPA5				
Nucleotide-binding	5	0.012	4.75	0.568
Cluster 3: ALB, HSPB1, HSPA5				
Negative regulation of apoptosis	3	0.027	10.42	0.978
Cluster 4: ALB, HSPA8, HSPA5				
Cytoplasmic membrane-bounded vesicle	3	0.079	5.81	0.997
Cluster 5: DDX5, HSPA8, HNRNPU, HSPA5				
ATP-binding	4	0.036	4.84	0.923
Cluster 6: ALB, FUS, HSPA5				

Metal ion binding	3	0.910	0.78	1.000
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EQUAL				
Cluster 1: MYO1B, MYO1C, MYO14				
Myosin	3	3.1E-04	102.86	0.019
Calmodulin-binding region	3	9.5E-04	57.44	0.026
Cluster 2: MYO1B, MYO1C, MYO14, FLNA				
Actin-binding	4	2.3E-04	28.32	0.015
Cytoskeletal protein binding	4	5.7E-03	9.37	0.310
Cluster 3: KRT1, KRT8, KRT9				
Intermediate filament	3	7.2E-04	67.26	0.044
Cytoskeletal keratin	3	1.8E-03	40.42	0.013
Structural molecule activity	3	8.3E-02	5.58	0.996
Cluster 4: MYO1B, MYO1C, MYO14, XRCC6				
ATP-binding	4	2.7E-02	5.27	0.824
Nucleotide-binding	4	5.1E-02	4.15	0.962
Cluster 5: XRCC6, HIST1H4, MYO1C				
Nucleoplasm	3	1.5E-01	3.95	1.000
Intracellular organelle lumen	3	4.2E-01	1.96	1.000

Endogenous nuclear interaction of COUP-TFII with HSP27

Heat shock protein beta-1 (*HSPB1*), also known as heat shock protein 27 (HSP27), is a member of the small heat shock protein family of chaperones that are involved in maintenance of protein stability and prevention of abnormal protein folding or aggregation [333,334]. HSP27 is increased expression in breast carcinoma and other cancers and can inhibit caspase-dependent apoptosis [335-337]. HSP27 expression has been reported to be increased by chemotherapy in breast cancer patients and to be correlated with reduced disease free survival [318]. HSP27 is known to interact with ER β [338], ER α , progesterone receptor (PR), and androgen receptor (AR) [339]. Based on these reports, HSP27 was selected from the list of proteins identified to interact with COUP-TFII, and enriched in cells treated with 4-OHT, for further investigation.

Endogenous COUP-TFII-HSP27 interaction was examined in MCF-7 cells. HSP27 is abundantly present in both the NE and CE of MCF-7 cells, while COUP-TFII is only present in the NE (Fig. 22A). HSP27 was detected via western blot following IP of NE with a polyclonal COUP-TFII antibody (Fig. 22A). No HSP27 was present in the negative control IgG or antibody-only lanes (Fig. 22A). Interaction of COUP-TFII with calmodulin, another protein identified by mass spectrometry to interact with COUP-TFII, although enriched when cells were treated with EtOH (Table 5), was also verified through IP (Fig. 22A).

To examine whether COUP-TFII-HSP27 interaction was altered following treatment with 4-OHT, a similar IP was performed using NE prepared from MCF-7 cells treated with EtOH (vehicle control) or 4-OHT for 2 h (Fig. 22B). No significant change in COUP-TFII-HSP27 interaction was observed following treatment with 4-OHT as

compared to EtOH (Fig. 22B). COUP-TFII and HSP27 expression and subcellular localization were also not affected by treatment (Fig. 22B). COUP-TFII interaction with calmodulin was also verified via IP in MCF-7 cells (Fig. 22A), although follow-up experiments to elucidate the function of this interaction have not yet been performed.

Figure 22. Endogenous nuclear interaction of COUP-TFII with HSP27 and calmodulin in MCF-7 cells.

400 μ g NE from MCF-7 cells was immunoprecipitated with polyclonal COUP-TFII antibody or negative control rabbit IgG (negative control) and analyzed by western blot for COUP-TFII and interacting proteins identified via mass spectrometry. 40 μ g NE or CE was used as input control. A, COUP-TFII interacts with HSP27 and calmodulin in MCF-7 cells. B, COUP-TFII interacts with HSP27 in EtOH and 4-OHT-treated MCF-7 cells.

Regulation of HSP27 by estradiol and tamoxifen in endocrine-resistant breast cancer cells

The promoter region of HSP27 contains a Sp1 and half ERE site reported to be responsible for the upregulation of HSP27 expression by E₂ through a Sp1/ER complex [340]. To examine both the relative expression of HSP27 in breast cancer cell lines with increasing endocrine resistance, as well as the effect of E₂ and 4-OHT treatment on HSP27 expression, MCF-7, LCC1, LCC2, LCC9, and LY2 cells were treated with 10 nM E₂, 100 nM 4-OHT, or EtOH (vehicle control) for 6 h. Increased HSP27 expression was observed in LCC1 estrogen-independent but tamoxifen-sensitive cells [326] as compared to MCF-7 estrogen-dependent, tamoxifen-sensitive cells. LCC2 cells, which are both estrogen-independent and tamoxifen-resistant [326], had reduced HSP27 expression compared to MCF-7. LCC9, estrogen-independent, tamoxifen and fulvestrant-resistant cells [285], displayed a further reduction in HSP27 expression. LY2, a breast cancer cell line that is resistant to tamoxifen, fulvestrant, and LY117018 (a raloxifene analog) [327] and has undergone epithelial to mesenchymal transition, had undetectable expression of HSP27 by QRT-PCR.

Previous reports have indicated regulation of HSP27 by estrogen signaling [340]; however, this was not confirmed in these experiments. E₂ treatment had no statistically significant effect on HSP27 expression in any of the cell lines examined (Fig. 23), reflecting potential differences the breast cancer cell lines used by investigators, the methods of detection, altered endocrine responses of the tamoxifen-resistant cell lines, etc. 4-OHT treatment in LCC1 cells lead to a slight increase in HSP27 expression (Fig. 23), while no effect was observed in other cell lines. Increased HSP27 expression in

response to 4-OHT treatment would be consistent with reports that cellular stress and drug treatment induce HSP27 expression [336,341].

HSP27 and COUP-TFII expression is reduced in endocrine-resistant and trastuzumab-resistant breast cancer cells

To follow up on the observed differences in steady state mRNA expression of *HSPB1* (Fig. 23), HSP27 expression was also analyzed by western blot of WCE from endocrine-sensitive MCF-7 cells and endocrine-resistant LCC2, LCC9, and LY2 cells. As seen at the mRNA level (Fig. 23), a reduction in HSP27 expression was prominent in LY2 cells (Fig. 24A). LCC2 and LCC9 cells displayed HSP27 expression approximately equivalent to MCF-7 cells. Interestingly, the lower molecular weight band of HSP27 seen in MCF-7, LCC2, and LCC9 was absent in LY2 cells (Fig. 24A). HSP27 expression was also examined in a trastuzumab-resistant breast cancer cell line, JIMT-1 (Fig. 24B). Trastuzumab (Herceptin) is a monoclonal antibody used for the treatment of HER2+ breast cancer patients [12]. JIMT-1 cells display clinical resistance to trastuzumab, as they were derived from a pleural effusion of a patient whose disease had progressed to trastuzumab-resistance [328]. Similar to LY2 cells, HSP27 expression was reduced in JIMT-1 cells, and the lower band seen in MCF-7 cells was not present (Fig. 24B).

Phosphorylation of HSP27 by MAPK at serine 15, 78, and 82 has also been shown to be important to its cellular function and localization [319,325]. Cell stress, such as heat shock, chemotherapy, or cytokine exposure, can induce phosphorylation of HSP27 [337]. Phosphorylation of HSP27 at Ser82 was also examined in the same cell

lines, and the pattern of p-Ser82-HSP27 was found to mirror the expression of HSP27 in all 5 cell lines examined (Fig. 24A and B).

To investigate a possible correlation between HSP27 and COUP-TFII expression, COUP-TFII expression was also analyzed by western blot. As previously reported [116], COUP-TFII expression is reduced in the endocrine-resistant LCC2, LCC9, and LY2 cells as compared to MCF-7 (Fig. 24A). COUP-TFII expression was also much lower in JIMT-1 cells compared to MCF-7 endocrine-sensitive cells, suggesting a potential role for COUP-TFII in trastuzumab-resistance (Fig. 24B).

COUP-TFII/HSP27 and HER2 expression are inversely correlated

We have previously reported an inverse correlation between COUP-TFII and HER2 expression in tamoxifen-treated patient tumors via immunohistochemistry [115]. To determine if COUP-TFII and HSP27 expression are correlated to HER2 expression in the endocrine-resistant and trastuzumab-resistant cell lines, a western blot was performed using a HER2 antibody. HER2 expression was undetectable in MCF-7 and LCC2 cells (Fig. 24A). Very low expression of HER2 was detected in LCC9 cells, while LY2 cells had the greatest HER2 expression of the endocrine-resistant cell lines (Fig. 24A). JIMT-1 cells are trastuzumab-resistant and, as part of their identified phenotype, have been reported to have increased expression of HER2 [328]. JIMT-1 cells had the highest HER2 expression detected in any of the cell lines examined (Fig. 24B). These data suggest a trend towards an inverse pattern of expression between COUP-TFII/HSP27 and HER2 in the cell lines examined herein.

Figure 23. Regulation of HSP27 expression by E₂ and 4-OHT in TAMs and TAMR cells.

QRT-PCR for *HSPB1* (HSP27) in MCF-7, LCC1, LCC2, LCC9, and LY2 cells treated with EtOH, 10 nM E₂, or 100 nM 4-OHT for 6 h. Each bar is the average of triplicate determinations in two experiments $\pm$ SEM. * Significantly different $p < 0.05$ from MCF-7 EtOH-treated cells.

Figure 24. Expression of HSP27, HER2, and COUP-TFII in endocrine-resistant and trastuzumab-resistant cells.

Western blot of WCE (40 μg) from (A) TAMS MCF-7 and TAMR LCC2, LCC9, and LY2 breast cancer cells or (B) MCF-7 and trastuzumab-resistant JIMT-1 breast cancer cells to evaluate the expression of HER2, phospho-HSP27 (Ser82), HSP27, and COUP-TFII relative to β-actin.

DISCUSSION

The role of HSP27 in breast cancer response to antiestrogens and other treatments has been controversial. Examination of HSP27 expression by immunohistochemistry in ER α + tamoxifen-treated breast cancer patients with metastatic disease revealed no statistically significant association between HSP27 and response to tamoxifen therapy [320]. This is in contrast to an early study which reported that patients with HSP27+ tumors responded better to tamoxifen and had a lower failure rate than patients with HSP27- tumors, implicating loss of HSP27 in progression to tamoxifen-resistance [321]. Another group reported increased resistance to doxorubicin in MDA-MB-231 cells overexpressing HSP27 [342]. However, HSP27 expression was reduced in doxorubicin-resistant MCF-7/adr cells. This was also correlated to reduced heat shock factor 1 expression and increased NF κ B p65 and mutant p53 expression in the MCF-7/adr cells [324]. A trastuzumab-resistant cell line, SKBR3 HR, expresses increased levels of HSP27 compared to the parental SKBR3 cell line. Knockdown of HSP27 expression resulted in increased trastuzumab sensitivity, although no correlation between HSP27 and trastuzumab sensitivity could be established across a panel of cell lines [322]. Further evaluation of the reduced HSP27 expression in endocrine-resistant and trastuzumab-resistant breast cancer cells (Fig. 24A and B) and the interaction of HSP27 with COUP-TFII (Fig. 22A and B) identified herein may provide more clues. Evaluation of the specific identity of the lower molecular weight band of HSP27 present in MCF-7, LCC2, and LCC9 but not LY2 and JIMT-1 cells (Fig. 24A and B) may also be of interest.

The phosphorylation status of HSP27 may also contribute to its ability to modulate chemoresistance [322,323]. Decreased phosphorylation of HSP27 at Ser82

with increasing endocrine resistance or trastuzumab resistance corresponding to decreased HSP27 expression was identified in this study (Fig. 24A-B), but phosphorylation of other sites may also be important. Phosphorylation of HSP27 at Ser78 is regulated downstream of the HER2-p38 MAPK pathway [343], and may also play a role in trastuzumab sensitivity. Induction of apoptosis was associated with increased phosphorylation of HSP27 in response to TNF α or 4-OHT treatment, suggesting HSP27 phosphorylation may play a role in treatment response [325]. Phosphorylation of HSP27 also increases its nuclear translocation, while unphosphorylated HSP27 remains distributed throughout the cell [319]. It would be interesting to see if phosphorylation plays a role in HSP27 interaction with COUP-TFII. Much remains to be determined about the role of phosphorylation in HSP27 function and effect on drug sensitivity. Further investigation into whether phosphorylation of HSP27 is tumor-promoting or tumor-suppressive will yield insight into the complex cellular actions of HSP27.

In addition to HSP27, other cancer-associated proteins were found to interact with COUP-TFII through this proteomic study, which will require further investigation. The interaction between COUP-TFII and calmodulin was also verified herein (Fig. 22A). Calmodulin, a calcium binding protein, also interacts with EGFR [344], HER2 [345], ER β [346], and ER α [347,348]. Binding of calmodulin to ER α stabilizes ER α [349], and tamoxifen inhibits the interaction of ER α and calmodulin [350]. Calmodulin appears to play a role in HER2 stimulated SKBR3 cell proliferation since calmodulin inhibition decreased HER2-stimulated cell growth [345]. Further investigation of the function of the interaction between COUP-TFII and calmodulin would be of interest. Also found to

interact with COUP-TFII was DEAD box protein 5 (DDX5), which interacts with ER α where it functions as a transcriptional coactivator [351]. DDX5 also interacts with other ER α and COUP-TFII coactivators SRC-1, SRC-2, and SRC-3 [352]. Interaction between DDX5 and COUP-TFII has not yet been reported, but would be another potential area to pursue.

In conclusion, novel COUP-TFII-interacting proteins have been identified through a proteomic screen and their relative interaction with COUP-TFII following treatment with EtOH or 4-OHT has been qualitatively analyzed. COUP-TFII-HSP27 interaction in MCF-7 cells and reduced expression of HSP27 in endocrine-resistance and trastuzumab-resistance raises many questions about the function of these proteins in breast cancer cells. Of particular interest will be analyses of HSP27 localization and phosphorylation in endocrine-resistant and trastuzumab-resistant cells, and the effect of COUP-TFII on these properties.

CHAPTER VI – RESEARCH IMPLICATIONS

Breast cancer is the most common type of cancer diagnosed in women and the second leading cause of female cancer death [4]. The use of antiestrogen therapy such as selective estrogen receptor modulators (SERMs) greatly increases survival, with five years of tamoxifen therapy reducing mortality rate by 32% and recurrence rate by 45% [50]. Despite the initial efficacy of this treatment, approximately 40% of patients undergoing antiestrogen therapy progress to metastatic disease and die [56]. Identification of novel treatment combinations, biomarkers, and drug targets is of great importance in increasing the effectiveness of antiestrogen therapy for patients. In this study, I have described the function of the orphan nuclear receptor COUP-TFII in breast cancer and endocrine resistance.

COUP-TFII-interacting proteins in MCF-7 breast cancer cells were identified in studies described in Chapter III. The interaction between COUP-TFII and nucleolin was verified through co-immunoprecipitation and an *in vitro* binding assay [115]. Nucleolin is a multifunctional protein that has roles in mRNA stability, ribosome biogenesis, apoptosis, and the modulation of gene expression as a coregulator [253-257]. As COUP-TFII transcriptional action is largely modified by interaction with coregulators [226], a role for nucleolin as a coregulator for transcription of a COUP-TFII-regulated gene retinoic acid receptor B2 (*RARB2*) was explored. Nucleolin enhanced COUP-TFII

activated *RARB2* expression, and siRNA-mediated knockdown of COUP-TFII or nucleolin reduced *RARB2* expression [115]. This indicates that both COUP-TFII and nucleolin are necessary for the basal expression of *RARB2*. Binding of COUP-TFII to the promoter of *RARB2* was also verified, as well as COUP-TFII regulation of a *RARB2*-target gene, retinoid receptor-induced gene-1 (*RRIG1*) [115]. Supporting evidence for the importance of COUP-TFII in breast cancer was provided by immunohistochemical (IHC) staining of breast cancer tumor tissue microarrays, where an inverse correlation was found between COUP-TFII expression and tumor grade. COUP-TFII and nucleolin expression were also positively correlated in ER α + invasive ductal carcinomas [115]. These data enhance our understanding of the regulation of COUP-TFII's transcriptional activity, as well as the characteristics of COUP-TFII expression in breast cancer tissue.

Several reports have implicated increased NF κ B activity as a driving pathway in endocrine-resistance breast cancer [87,88,280-284]. As COUP-TFII expression is reduced in endocrine-resistant breast cancer, and COUP-TFII can repress the activity of other transcription factors, Chapter IV explores a role for COUP-TFII in suppression of NF κ B activity in breast cancer. Endocrine-resistant LCC9 cells displayed much greater basal and TNF α -induced NF κ B activity than endocrine-sensitive MCF-7 cells. Transfection of COUP-TFII in endocrine-resistant LCC9 cells reduced NF κ B activation. COUP-TFII suppressed both the basal and TNF α -induced expression of NF κ B target genes *IL6*, *CCL2*, *ICAM1*, and *TNFAIP3* in LCC9 cells, as well as the basal expression of *ICAM1* and *TNFAIP3* and the TNF α -induced *CCL2* and *TNFAIP3* in MCF-7 cells. COUP-TFII also suppressed the basal expression of

the *REL* and *RELA* NF κ B subunits in LCC9 cells and the TNF α -induced expression of *NFKB1*, *REL*, and *RELB* in LCC9. As the NF κ B subunits are also downstream targets of the NF κ B pathway themselves [306-309], this provides further evidence of the decreased NF κ B activation with COUP-TFII transfection. The correlation between COUP-TFII and NF κ B target gene and subunit expression was further examined through analysis of data from a microarray of gene expression in breast tumors from ER α + breast cancer patients treated with tamoxifen. Consistent with the data in Chapter IV, an inverse correlation was found between COUP-TFII and *ICAM1*, *IL6*, *TNFAIP3*, *NFKB2*, *REL*, *RELA*, and *RELB* in the patient samples. This suggests that the same trend seen in the breast cancer cells is also true for breast cancer patients.

To further investigate the mechanism of COUP-TFII regulation of NF κ B activation, the impact of COUP-TFII on NF κ B DNA-binding was analyzed through EMSA. COUP-TFII suppressed DNA-binding in both an *in vitro* assay where *in vitro* transcribed/translated COUP-TFII was incubated with nuclear extract from LCC9 cells treated with TNF α , as well as in an EMSA with nuclear extract from MCF-7 and LCC9 cells transfected with COUP-TFII. Further, co-immunoprecipitation revealed that COUP-TFII interacts with NF κ B subunits RelB and NF κ B1. This suggests COUP-TFII interaction with NF κ B subunits inhibits binding to the NF κ B RE and decreases NF κ B activation. COUP-TFII also inhibited induction of NF κ B activity by coactivators SRC-1, SRC-3, and CBP, implying that COUP-TFII and NF κ B compete for these coactivators, a mechanism that has been recently reported for COUP-TFII action on other transcription factors [295].

Identification of COUP-TFII as a negative regulator of NF κ B activity in breast cancer cells provides valuable information about the possible mechanism by which COUP-TFII helps prevent the progression to endocrine-resistance. It may partly explain the ability of COUP-TFII to increase endocrine sensitivity [116], as well as why endocrine-resistant cells (which have lower COUP-TFII expression) have induced NF κ B activity. These data also point to future use for COUP-TFII as both a biomarker as well as a target for the enhancement of endocrine sensitivity. LCC9 endocrine-resistant cells were found to be more sensitive to treatment with an NF κ B inhibitor, and showed markedly reduced cell proliferation as compared to MCF-7 cells. This may be due to their increased dependence on the NF κ B pathway for cell survival, and may provide a target for therapeutic intervention.

Chapters III and IV have highlighted the importance of interacting proteins on COUP-TFII function. The focus of the experiments described in Chapter V is to identify proteins which differentially interact with COUP-TFII upon treatment with tamoxifen. Many proteins were identified which could provide a starting point for future studies. DAVID analysis was used to identify common themes amongst the interacting proteins, including RNA splicing, calmodulin-binding, and ATP-binding. As calmodulin was also found in the interacting proteins with EtOH (vehicle control) treatment, and verified through co-immunoprecipitation in Chapter V, this could be an interesting pathway for further investigation.

Also in Chapter V, the interaction between COUP-TFII and HSP27 was verified through co-immunoprecipitation. Despite reports that HSP27 increase cell proliferation and plays an oncogenic role in cancer progression [335-337], reduced

HSP27 expression was found with increasing endocrine-resistance. Reduced HSP27 expression was also present in JIMT-1 trastuzumab-resistant breast cancer cells. Conflicting reports have identified HSP27 as both increasing [322,342] and decreasing drug resistance [321,324], and the interaction and positive correlation of COUP-TFII and HSP27 merits further study. Phosphorylation of HSP27 at Serine 15, 78, and 82 also plays a role in cellular localization and function [319,325], and has not been widely studied in resistance to antiestrogens or other drugs. A correlation of HSP27 phosphorylation at Ser82 and drug resistance was not found in the endocrine-resistant and trastuzumab-resistant cell lines studied in Chapter V, but further studies will investigate phosphorylation at other serines. Subcellular localization of HSP27 in these cell lines will also be examined.

Overall, this study has provided important new information about the many roles of COUP-TFII in breast cancer and confirmed the importance of COUP-TFII-interacting proteins to its cellular function. Further investigation of the mechanisms responsible for the ability of COUP-TFII to enhance endocrine sensitivity is crucial for its use as a biomarker or therapeutic target. Identification of biomarkers to enable detection of patients who would respond favorably to endocrine therapy would be of great use, as would discovery of a target to reduce endocrine resistance in patients.

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APPENDIX I - SUPPLEMENTARY MATERIAL

Methods S1

Transient transfection with pCOUP-TFII-FLAG. MCF-7 cells, purchased from ATCC, and used at passages <10, were transiently transfected with 2 µg pCOUP-TFII-FLAG using the P-20 program of the Amaxa Nucleofection System. Cells were 'serum-starved' in phenol red-free IMEM supplemented with 5% dextran coated charcoal stripped FBS (DCC-FBS) for 24 h before treatment with vehicle control ethanol (EtOH) for 2 h as indicated in the experimental design (Fig. 1).

Protein identification by multidimensional protein identification technology (MudPIT). Proteins eluted from the FLAG-affinity gel (Fig. 1) were digested by trypsin, desalted with C18 spin columns, dried by speedvac, and fractionated by strong cation exchange (SCX). The desalted samples were dissolved with loading buffer containing 20% acetonitrile (ACN), 100 mM acetic acid (HAc), and 2 mM NH₄Acetate and then loaded on a pre-cleaned SCX cartridge from Michrom Bioresources. After washing twice, peptides were eluted stepwise with 20 µl of HAc/NH₄Ac buffers with increasing ionic strength and pH (15 buffers and up to 10 mM HAc/615 mM NH₄Ac, pH 6.5). The SCX fractions were concentrated by speedvac and diluted to 7 µl with 5% ACN/0.1% formic acid. 5 µl of the samples were loaded to CapLC capillary LC system from Waters and peptides in the samples

were separated with a 3.5 μm Symmetry C18 column (75 μm $\times$ 150 mm, Waters). MS/MS spectra of the peptides were acquired by Q-TOF mass spectrometer (Waters) in data dependent mode. Proteins were identified by comparing MS/MS spectra with sequences in Swiss-Prot database by ProteinLynx from Waters.

Immunofluorescence staining of pCOUP-TFII-FLAG. Cells were grown on coverslips, washed with PBS, fixed with cold methanol/acetone 1:1 for 5 min, and washed twice with cold PBS. After blocking with 1% goat serum and 0.3 % Triton X-100 in PBS for 30 min, primary FLAG antibody (Sigma) was added (1: 300 dilution) for 1 h. After removing the primary antibody, the cells were stained with secondary anti-mouse antibody labeled with ZenonTM Alexa Fluor 488 (Molecular Probes). Cells were then incubated with ProLong[®] Gold antifade reagent with DAPI (Molecular Probes). Images (Fig. S1) were captured using a Zeiss Axiovert200 inverted microscope with a 63x objective lens using AxioVision Release 4.3 software.

Figure S1: Nuclear localization of COUP-TFII in transfected MCF-7 cells.

A, MCF-7 cells were either non-transfected (control) or transfected with pCOUP-TFII-FLAG for 48 h. Immunofluorescence staining was performed for FLAG as described in Supplementary Materials and Methods. Cells were counterstained with DAPI (blue) to image nuclei. The bar is 20 µm. Overlap images indicate localization of COUP-TFII-FLAG in the nucleus. B, Western blots of CE (30 µg) or NE (10 µg) from untransfected MCF-7 cells (control) or transfected with pIRES-GFP-1a parental vector or pCOUP-TFII-FLAG with FLAG or ERα (AER320, ThermoFisher) antibodies. The two bands are likely due to protein degradation. C, Ponceau S staining shows protein levels.

Figure S2: Overexpression of COUP-TFII-FLAG in MCF-7 cells and immunocapture of COUP-TF-FLAG by the anti-FLAG agarose affinity resin.

A, Briefly, MCF-7 cells were transiently transfected with C-terminal FLAG-tagged COUP-TFII or empty vector for 24 h as described in Materials and Methods. WCEs were prepared and incubated with EZ view Red ANTI-FLAG M2 Affinity gel (Sigma) for 16 h. After rinsing, proteins were eluted with serial glycine elutions: 1. 15 min at room temperature for proteins associating with immobilized COUP-TFII-FLAG with moderate affinity and 2. 5 min at 95°C to elute proteins bound to the immobilized COUP-TFII-FLAG with high affinity. Immunoprecipitating proteins were analyzed by MudPIT. Non-specific proteins were subtracted from the total interacting proteins to identify proteins specifically interacting with COUP-TFII-FLAG. **B**, 30 µg of WCE from LCC9, MCF-7 and MCF-7 cells transiently transfected with pCOUP-TFII-FLAG were separated by SDS-PAGE and western blots were performed for COUP-TFII, FLAG and β-actin. Quantitation of the COUP-TFII/β-actin in lanes 2 and 3 indicate a 2-fold increase COUP-TFII in the transfected cells. TAM-R LCC9 cells served as a negative control, as we reported lower COUP-TFII in LCC9 cells compared to parental MCF-7 cells (Riggs et al Cancer Res. 66: 10188-98, 2006). Note FLAG signal was only detected in the transfected cells (lane 2), indicating specificity. **C**, 1 mg of protein in WCE from COUP-TFII-FLAG over-expressing MCF-7 cells was immunocaptured on anti-FLAG agarose affinity beads. COUP-TFII and interacting proteins were eluted with 6 M urea. 30µg of WCE were separated by SDS-PAGE in parallel to 30 µg unbound IP supernatant and 30µg eluted protein. COUP-TFII-FLAG-affinity bead binding is confirmed by decreased FLAG in the Supernatant (flow-thru = unbound proteins) and enriched FLAG in the eluted samples.

Figure S3: Testing elution methods for retrieval of COUP-TFII-FLAG.

~ 1 mg of WCE from MCF-7 cells transfected with pCOUP-TFII-FLAG was immunocaptured on the anti-FLAG affinity resin and eluted with sequential glycine incubations: 1) 10 or 15 min room temperature (lanes 2 and 4) or 5 min. at 95°C (lanes 3 and 5) in two different experiments (Exp A or B), as indicated (lanes 2-5). Samples of the indicated eluates were separated by SDS-PAGE and immunoblotted using anti-FLAG antibody. 20 µg of the IP supernatant, containing unbound COUP-TFII-FLAG, was run in parallel as a control (lane 1). As seen in lane 5, incubation with glycine for 15 min. followed by a 5 min. incubation of fresh glycine at 95°C (Exp. B) eluted the most intact COUP-TFII-FLAG (~50 kDa). The increased temperature and incubation time of glycine in Exp. B resulted in a FLAG-tagged degradation product(s) of ~20 kDa (lanes 3-5).

Figure S4: Endogenous nuclear nucleolin-COUP-TFII interaction in MCF-7 cells.

A, Equal amounts (100 μ g) of protein of CE and NE from MCF-7 cells were immunoprecipitated with nucleolin mAb (lanes 3 and 4), mouse (*m*) IgG (negative control for mAb, lanes 5 and 6), COUP-TFII antisera (lanes 7 and 8), or rabbit (*r*) IgG (negative control for IPs using COUP-TFII polyclonal antiserum, lanes 9 and 10), followed by western blot for nucleolin and COUP-TFII. 20% (20 μ g) input NE and CE serve as loading controls (lanes 1 and 2). B, The relative amount of nucleolin and COUP-TFII in the nucleolin IP was plotted relative to expression of each protein in the input (set to 100). COUP-TFII in rabbit IgG IPs was not graphed because of the contamination of the heavy IgG chain (lanes 7 and 8, COUP-TFII blot). Western blots demonstrate that: 1) nucleolin interacts with COUP-TFII in the NE of MCF-7 cells (lane 7); 2) nucleolin is not IP'ed with rabbit IgG (lanes 9 and 10); 3) more COUP-TFII interacts with nucleolin in NE IP'ed with nucleolin antibody than with mouse IgG (lane 3 versus lane 5). C, MCF-7 cells were treated with EtOH, 10 nM E_2 , or 100 nM 4-OHT for 1 h prior to separation of NE and CE. 200 μ g of NE or CE were IP'ed with polyclonal COUP-TFII antibody and immunoblotted with a monoclonal antibody (mAb) against nucleolin. The blot was stripped and re-probed with mAb against COUP-TFII. D, 10% input for NE and CE used in IP in part C.

Figure S5: ChIP of COUP-TFII-FLAG on the *RARB2* promoter in MCF-7 cells. A, Chromatin immunoprecipitation was performed in MCF-7 cells transfected with pIRES-COUP-TFII-FLAG or empty vector, serum starved for 48 h, and treated with 1 μ M atRA for 1 h. Following Q-PCR using primers to the *RARB2* promoter as described in Materials and Methods, duplicate samples were run on a 2% agarose gel. B, Only EV – EtOH set to 1. atRA increased COUP-TFII-FLAG binding to the *RARB2* promoter 32%. Signific. different $p < 0.05$: * to EV – EtOH, ** to EV – atRA, ‡ to CII – EtOH.

Figure S6: Expression of COUP-TFII and nucleolin in T47D and MCF-7 cells. A, WCE (50 μ g) were Western blotted for COUP-TFII and nucleolin expression. The blot was stripped and re-probed for β -actin. B, The ratio of nucleolin/ β -actin and COUP-TFII/ β -actin for each cell lines was plotted. These data are the average of 3 separate experiments. The lower COUP-TFII expression in T47D agrees with the higher CT values for *NR2F2* in T47D.

Figure S7: Effect of cell treatments on *NR2F2* (COUP-TFII) expression in MCF-7 cells. A, Schematic diagram of transfection and treatment of MCF-7 cells. MCF-7 cells were transfected with equal amounts of pTAG2 control vector or pCMV-nucleolin for 24 h., treated with 10 μ M CRO or AS1411, as indicated for 24 h, and 1 μ M atRA was added for the last 6 h. RNA was harvested and Q-PCR performed. B, *NR2F2* values were normalized to GAPDH. Values are the average of 6 separate experiments $\pm$ SEM. * signific. different, $p < 0.05$ in one way ANOVA followed by Bonferroni multiple comparison test. Note that there was no statistical difference between the pTAG+AS1411 versus Nucl+AS1411 or AS1411 sample measurements of *NR2F2*.

Figure S8: Neither AS1411 nor CRO inhibit MCF-7 cell viability after 4 d. MCF-7 cells were treated with the indicated concentrations of AS1411 or CRO and cell viability was measured by an MTT assay (A490nm, Promega CellTitre assay). Values are the average of 4 determinations $\pm$ SEM.

Figure S9: Oncomine examination of *NR2F2* expression in breast tumors. Data mining in Oncomine microarray data sets for *NR2F2* in breast cancer identified that: A, *NR2F2* expression is significantly higher in ERα+ breast tumors ($p < 0.007$). B. *NR2F2* is significantly lower in metastatic breast tumors ($p < 0.05$). Data are from (van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002; 347: 1999-2009).

Table S1: Identification of proteins in MCF-7 WCE that non-specifically (NS) interact with the anti-FLAG-affinity resin.

1 mg protein in WCE from EMPTY-FLAG vector-transfected MCF-7 cells was incubated with anti-FLAG affinity gel, eluted with 0.1 M glycine, pH 3.5 for 15 min. at RT, and subjected to MudPIT peptide identification. Matched number (No) indicates the number of sequenced peptides that match the full length protein. Coverage indicates the % of the total protein matched.

Protein name	Accession (GI)	Mass (Mr)	pI	Matched (No)	Coverage (%)
Cytoskeletal proteins					
Actin cytoplasmic 1	P60709	41710	5.4	4	15.5
Tropomyosin alpha-4 chain	P67936	28505	4.7	4	17.3
Tropomyosin alpha-3 chain	P06753	29015	4.8	4	18.1
Myosin-9	P35579	226392	4.6	4	3.0
Tropomyosin beta chain	P07951	32970	4.7	5	23.6
Tropomyosin alpha-1 chain	P09493	32689	4.7	4	14.8
Myosin light polypeptide 6	P60660	16788	4.6	3	22.7
Histones					
Histone H4	P62805	11229	11.4	3	29.4

Table S2: Identification of proteins ‘moderately’ associated with COUP-TFII in MCF-7 cells.

1 mg protein in WCE from pCOUP-TFII-FLAG transfected, EtOH-treated MCF-7 cells was incubated with anti-FLAG affinity gel, eluted with 0.1 M glycine, pH 3.5 for 15 h at RT, and subjected to MudPIT peptide identification. Matched number (No) indicates the number of sequenced peptides that match the full length protein. Coverage indicates the % of the total protein matched. This table excludes proteins that were nonspecifically associated with the anti-FLAG affinity gel as summarized in Table S1.

Protein name (<i>gene</i>)	Accession (GI)	Mass (Mr)	pI	Matched (No)	Coverage (%)
Ribonucleoproteins					
Heterogeneous nuclear ribonucleoprotein D0	Q14103	22435	11.3	2	10.2
Histones					
Histone H2A.Z	P0C0S5	13414	10.9	2	7.9-16
Core histone macro-H2A.1	O75367	39445	10.1	2	5.4
Histone H1.5	P16401	22435	11.3	3	10.2
Histone H1.2	P16403	21221	11.3	3	10.8
Histone H3.1	P68431	15263	11.3	2	10.4
Other Proteins					
Heat Shock 70 kDa protein B (<i>HSPA7</i>)	P48741	26890	7.6	3	16.2
Heat Shock 70 kDa protein 1 HSP70 (<i>HSPA1A</i>)	P08107	70009	5.6	3	6.2
Chromobox Protein Homolog 3 (<i>CBX3</i>)	Q13185	19708	5.1	3	6.4

Figure S10. Expression of COUP-TFII in transfected MCF-7 and LCC9 cells. MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNF α for 6 h before preparing whole cell extracts. Expression of COUP-TFII was analyzed by western blot and compared to β -actin control.

Figure S11. Specificity of protein complex binding to NF κ B-RE DNA probe.

Full EMSA picture from Fig. 6B. 25 μ g NE prepared from MCF-7 and LCC9 cells transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with TNF α for 6 h was incubated with [32 P]NF κ B-RE DNA probe +/- 100X cold unlabeled DNA probe for 1 h to perform EMSA. Binding to [32 P]NF κ B-RE DNA probe is inhibited by the addition of excess cold DNA probe.

Figure S12. BMS-345541 inhibits NFκB activity in MCF-7 and LCC9 cells.

MCF-7 and LCC9 cells were serum-starved for 24 h before being transfected with a NFκB luciferase reporter for 48 h and treated with various concentrations of an NFκB inhibitor, BMS-345541 +/- 10 ng/ml TNFα for 6 h. Dual luciferase assay was performed to verify that the BMS-345541 inhibited NFκB activity at these concentrations. Firefly luciferase values are shown relative to pGL4-hRluc-TK *Renilla* (Promega). Note higher NFκB activity in serum-starved MCF-7 cells as compared to normal media (Figure 1).

*: $P < 0.05$ versus DMSO control for each cell line.

CURRICULUM VITAE

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EDUCATION

- 2004-08 B.S. Biology, concentration Genetics/Subcellular (*summa cum laude*), University of Louisville, Louisville, KY
GPA 3.875
- 2008-11 M.S. Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, KY
- 2008-13 Ph.D. Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, KY
GPA 3.948

RESEARCH EXPERIENCE

- 2007-08 Undergraduate Research
The role of nucleolin in the mechanism of novel anticancer agent, AS1411
University of Louisville School of Medicine
Mentor: Dr. Paula J. Bates
- 2008-13 Doctoral Research
COUP-TFII and its interacting proteins in breast cancer and endocrine resistance
Department of Biochemistry and Molecular Biology
University of Louisville School of Medicine
Mentor: Dr. Carolyn M. Klinge

HONORS

- 2005-Present Golden Key International Honour Society

2008	University honors scholar, University of Louisville, Louisville, KY
2008	<i>Summa cum laude</i> graduate, University of Louisville, Louisville, KY
2008-10	Integrated Programs in Biomedical Sciences Fellowship
2010-12	NIEHS T32-ES011564 Predoctoral Fellowship
2012	First place award in poster competition at Research!Louisville, University of Louisville School of Medicine, Louisville, KY, September 18, 2012. Litchfield, L.M. and Klinge, C.M. COUP-TFII suppresses NFκB activation in endocrine-resistant breast cancer cells. (Abstract #56)
2013	John M. Houchens Award for most meritorious dissertation
2013	Graduate Dean's Citation

PUBLICATIONS

1. **Litchfield, L.M.**, Riggs, K.A., Hockenberry, A.M., Oliver, L.D., Barnhart, K.G., Cai, J., Pierce, W.M., Ivanova, M.M., Bates, P.J., Appana, S.N., Datta, S., Kulesza, P., McBryan, J., Young, L.S., Klinge, C.M. Identification and Characterization of Nucleolin as a COUP-TFII Coactivator of Retinoic Acid Receptor β Transcription in Breast Cancer Cells. *PLoS ONE* 7(5): e38278, 2012. doi:10.1371/journal.pone.0038278
2. **Litchfield, L.M.** and Klinge, C.M. Multiple roles of COUP-TFII in cancer initiation and progression. *Journal of Molecular Endocrinology*, 49(3): R135-48, 2012. doi:10.1530/JME-12-0144
3. Manavalan, T.T.*, Teng, Y.*, **Litchfield, L.M.***, Muluhngwi, P., Al-Rayyan, N., Klinge, C.M. Reduced expression of miR-200 family members contributes to antiestrogen resistance in LY2 human breast cancer cells. *PLoS ONE*, In publication. *equal contribution
4. **Litchfield, L.M.** and Klinge, C.M. COUP-TFII inhibits NFκB activation in breast cancer cells and restores endocrine sensitivity. In submission.

PROFESSIONAL MEMBERSHIPS

2011-Present	The Endocrine Society
2012-Present	American Association for Cancer Research
2012-Present	Women in Cancer Research
2012-Present	Kentucky Academy of Science

ORAL PRESENTATIONS

1. **Litchfield, L.M.**, Emberts, C.G., and Klinge, C.M. COUP-TFII-Nucleolin Interaction Regulates RAR β 2 Expression in Human Breast Cancer Cells. Oral Presentation at the 2011 Colloquium of Biochemistry and Molecular Biology, University of Louisville School of Medicine, August 26, 2011.

POSTER PRESENTATIONS

Abstracts of poster presentations (national):

1. **Litchfield, L.M.**, Emberts, C.G., and Klinge, C.M. COUP-TFII-Nucleolin Interaction Regulates RAR[β 2] Expression in Human Breast Cancer Cells (Abstract P2-10) Abstracts of the 93rd annual meeting of the Endocrine Society, Boston, MA, June 5, 2011.
2. **Litchfield, L.M.**, Cai, J., Pierce, W.M., Appana, S.N., Bates, P.J., Datta, S., Kulesza, P., Young, L.S., and Klinge, C.M. Identification and functional characterization of COUP-TFII-nucleolin interaction in breast cancer cells and tumors. AACR Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications meeting, San Francisco, CA, October 14, 2011.
3. **Litchfield, L.M.** and Klinge, C.M. COUP-TFII suppresses NF κ B activation in endocrine-resistant breast cancer cells. (Abstract #2214) The 103rd annual meeting of the American Association for Cancer Research, Chicago, IL, April 2, 2012.
4. **Litchfield, L.M.** and Klinge, C.M. COUP-TFII suppresses NF κ B activation in endocrine-resistant breast cancer cells. (P6-04-30) CTRC-AACR San Antonio Breast Cancer Symposium, San Antonio, TX, December 8, 2012.

Abstracts of poster presentations (local):

1. Fox, J.M., **Litchfield, L.M.**, Emberts, C.G., Clark, B.J., and Klinge, C.M. Characterization of the impact of nucleolin on COUP-TFII-mediated regulation of ER α , ER β , RAR β and StAR transcription in MCF-7 Human Breast Cancer Cells. (Abstract MED-19) Research!Louisville, University of Louisville School of Medicine, Louisville, KY, October 14, 2009.
2. Oliver, L.D., **Litchfield, L.M.**, and Klinge, C.M. The effect of COUP-TFII-nucleolin interaction on RAR β 2 expression in human breast cancer cells. SROP poster presentation. University of Louisville School of Medicine, Louisville, KY, August 1, 2010.
3. **Litchfield, L.M.**, Emberts, C.G., and Klinge, C.M. The effect of COUP-TFII-nucleolin interaction on RAR β 2 expression in human breast cancer cells. (Abstract GRD-40) Research!Louisville, University of Louisville School of Medicine, Louisville, KY, October 13, 2010.
4. Oliver, L.D., **Litchfield, L.M.**, and Klinge, C.M. The effect of COUP-TFII-nucleolin interaction on RAR β 2 expression in human breast cancer cells. Posters at the Capitol. Frankfort, KY, February 10, 2011.
5. **Litchfield, L.M.**, Emberts, C.G., and Klinge, C.M. The effect of COUP-TFII-

- nucleolin interaction on RAR β 2 expression in human breast cancer cells. 13th annual Institute for Molecular Diversity and Drug Design (IMD3) Symposium, March 8, 2011.
6. Barnhart, K.G., **Litchfield, L.M.**, and Klinge, C.M. Effect of COUP-TFII on RAR β 2 and NF κ B Expression in Breast Cancer Cells. SROP poster presentation. University of Louisville School of Medicine, Louisville, KY, August 1, 2011.
 7. **Litchfield, L.M.**, Emberts, C.G., and Klinge, C.M. COUP-TFII-Nucleolin Interaction Regulates RAR β 2 Expression in Human Breast Cancer Cells. (Abstract GRD-52) Research!Louisville, University of Louisville School of Medicine, Louisville, KY, October 11, 2011.
 8. **Litchfield, L.M.**, Emberts, C.G., and Klinge, C.M. COUP-TFII-Nucleolin Interaction Regulates RAR β 2 Expression in Human Breast Cancer Cells. (Abstract #76) 10th annual Brown Cancer Center Retreat, Louisville, KY, October 28, 2011.
 9. **Litchfield, L.M.** and Klinge, C.M. COUP-TFII suppresses NF κ B activation in endocrine-resistant breast cancer cells. (Abstract #31) 14th annual Institute for Molecular Diversity and Drug Design (IMD3) Symposium, March 13, 2012.
 10. **Litchfield, L.M.** and Klinge, C.M. COUP-TFII suppresses NF κ B activation in endocrine-resistant breast cancer cells. (Abstract #56) Research!Louisville, University of Louisville School of Medicine, Louisville, KY, September 18, 2012.
 11. **Litchfield, L.M.** and Klinge, C.M. COUP-TFII suppresses NF κ B activation in endocrine-resistant breast cancer cells. 11th annual Brown Cancer Center Retreat, Louisville, KY, October 26, 2012.
 12. **Litchfield, L.M.** and Klinge, C.M. COUP-TFII suppresses NF κ B activation in endocrine-resistant breast cancer cells. 15th annual Institute for Molecular Diversity and Drug Design (IMD3) Symposium, March 12, 2012.