Androgen and estrogen receptors in breast cancer co-regulate human UDP-glucuronosyltransferases 2B15 and 2B17

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Running title: Regulation of UGT2B15 and UGT2B17 in breast cancer

Key words: steroid metabolism, breast cancer, estrogen receptor, androgen receptor, UGT2B15, UGT2B17

Financial support: This work was supported by funding from the National Health and Medical Research Council of Australia (ID1020931 to P. I. Mackenzie; ID1085410 to R. Meech and P. I. Mackenzie; ID 1008349 to W. D. Tilley; ID 1084416 to W. D. Tilley and T. E. Hickey), Flinders Medical Centre Foundation (D. G. Hu and P. I. Mackenzie), Cancer Australia (ID 1043497 to W. D. Tilley and T. E. Hickey), and a Prostate Cancer Research Programs Transformative Impact Award from the US Department of Defense (W81XWH-13-2-0093 to W. D. Tilley and L. A. Selth). P. I. Mackenzie is an NHMRC Senior Principal Research Fellow. L. A. Selth was supported by a Young Investigator Award from the Prostate Cancer Foundation (Foundation 14 award). T. E. Hickey held a Postdoctoral Fellowship Award from the US Department of Defense Breast Cancer Research Program (BCRP; W81XWH-11-1-0592) and is currently an Early Career Development Fellow of the Royal Adelaide Hospital Research Foundation. R. Meech is an Australian Research Council Future Fellow.

Conflict of interest: The authors have no conflicts of interest to disclose.

Word count: 5986

Figures: 7

ABSTRACT

Glucuronidation is an enzymatic process that terminally inactivates steroid hormones, including estrogens and androgens, thereby influencing carcinogenesis in hormone-dependent cancers. While estrogens drive breast carcinogenesis via the estrogen receptor alpha (ERα), androgens play a critical role as prohormones for estrogen biosynthesis and ligands for the androgen receptor (AR). In this study, the expression and regulation of two androgen-inactivating enzymes, the UDP-glucuronosyltransferases UGT2B15 and UGT2B17, was assessed in breast cancer. In large clinical cohorts, high *UGT2B15* and *UGT2B17* levels positively influenced disease-specific survival in distinct molecular subgroups. Expression of these genes was highest in cases positive for ERα. In cell line models, ERα, AR and the transcription factor FOXA1 co-operated to increase transcription via tandem binding events at their proximal promoters. ERα activity was dependent on FOXA1, facilitated by AR activation, and potently stimulated by estradiol as well as estrogenic metabolites of 5α-dihydrotestosterone. AR activity was mediated via binding to an estrogen receptor half-site 3' to the FOXA1 and ERα binding sites. Although AR and FOXA1 bound the UGT promoters in AR-positive/ERα-negative breast cancer cell lines, androgen treatment did not influence basal transcription levels. *Ex vivo* culture of human breast tissue and ERα+ tumors provided evidence for up-regulation of *UGT2B15* and *UGT2B17* by estrogen or androgen treatment. ERα binding was evident at the promoters of these genes in a small cohort of primary tumors and distant metastases. Collectively, this data provides insight into sex steroid receptor-mediated regulation of androgen inactivating enzymes in ERα+ breast cancer, which may have subtype-specific consequences for disease progression and outcomes.

INTRODUCTION

Breast and prostate cancer are the most common forms of hormone-dependent cancer and account for a major proportion of cancer-related deaths in women and men, respectively. Breast cancer is predominantly driven by aberrant estrogen receptor alpha (ERα) signaling and prostate cancer by aberrant androgen receptor (AR) signaling. These diseases display multiple similarities of etiology and pathology (1). There has been mounting interest in the role of AR in breast cancer following identification of a subgroup (~12%) that lack expression of ERα but are AR+ and display features reminiscent of prostate cancers (2, 3). However, the large majority (>70%) of breast cancers express both sex hormone receptors, and the role of AR in ERα+ breast cancer may be anti-estrogenic and thereby tumor suppressive (4).

Inactivation and disposal of steroids regulates target cell exposure to steroid receptor agonists, and derangement of these pathways is evident in the pathology of hormone-dependent cancers (5, 6). Several enzymatic pathways inactivate steroids, but glucuronidation is the only irreversible event. Uridine 5'-diphospho (UDP) glucuronosyltransferases (UGTs) create steroid conjugates incapable of binding to cognate receptors. The resulting steroid glucuronides are more hydrophilic and more readily excreted from tissues than unconjugated steroids. Therefore, glucuronidation is a vital mechanism that regulates steroid receptor activity within hormone responsive tissues. Metabolism of estrogenic hormones has been widely investigated in breast cancer (6), but metabolism of androgenic hormones has yet to be extensively investigated in relation to this disease.

Among 19 human UGTs, only a few glucuronidate sex steroids to any significant extent (7, 8). The most potent natural androgens, testosterone (T) and 5α-dihydrotestosterone (DHT) are glucuronidated by UGT2B15 and UGT2B17. Testosterone is a major precursor for synthesis

of 17β-estradiol (E2), the most potent natural ERα ligand, while T and DHT are AR ligands. Hence, the activity of UGT2B15 and UGT2B17 functionally impacts ERα and AR activity, but the significance of *UGT2B15* and *UGT2B17* expression has not been examined in relation to the different molecular subtypes and clinical outcomes of breast cancer.

Primary breast tissues express *UGT2B15* and *UGT2B17* (9), with evidence of transcriptional up-regulation by ERα signaling in breast cancer cell lines (10, 11). One study also reported that DHT weakly stimulated expression of these genes in an ERα-dependent manner (10). The latter finding is feasibly due to metabolism of DHT into 3α-androstanediol (3α-diol) or 3β-androstanediol (3β-diol), compounds that have some estrogenic activity (12), but this has not been tested. Finally, regulation of *UGT2B15* and *UGT2B17* by AR signaling has not been thoroughly investigated in an ERα-positive context and never in an AR+, ERα-negative breast cancer context, in which the consequences of androgen metabolizing capacity may markedly differ. The present study was undertaken to fill these gaps in knowledge by examining expression in large clinical cohorts and elucidating mechanisms of transcriptional regulation by sex steroid receptor signaling in breast cancer, taking into account the molecular complexity of this disease.

MATERIALS AND METHODS

Chemicals and Cell Culture

Steroids and steroid receptor antagonists were purchased from Sigma-Aldrich (Australia), including 17β-estradiol (E2), 5α-dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (3α-diol), 5α-androstane-3β,17β-diol (3β-diol), methyltrienolone (R1881), 4-hydroxy-flutamide (Flut), fulvestrant (ICI 182,780) and bicalutamide (Bic). Enobosarm was provided by the

manufacturer (GTx, Inc, USA). The MCF7, ZR75-1 and MDA-MB-453 breast cancer cell lines were obtained from the American Type Culture Collection (USA) and the MFM223 line from the German Collection of Microorganisms and Cell Cultures (DMSZ); these were regularly verified for identity via short tandem repeat (STR) analyses. MCF7 and ZR75-1 cells were maintained in RPMI 1640 medium (Invitrogen, USA), MDA-MB-453 cells in DMEM (Gibco, USA), and MFM223 cells in EMEM (Gibco) containing either 5% (v/v; MCF7, ZR75-1, MDA-MB-453) or 10% (v/v; MFM223) fetal bovine serum (FBS; Gibco) at 37°C and 5% CO_2.

Clinical Tissues and Ex Vivo Culture

Normal breast tissues from reduction mammoplasties were obtained from Flinders Medical Centre (Adelaide, Australia). Primary ERα-positive breast tumor samples were obtained from the Burnside Private Hospital (Adelaide, Australia). All tissues were collected after provision of informed, written consent by the donors. This study was approved by the University of Adelaide Human Research Ethics Committee (approval numbers: H-065-2005; H-169-2011). Tissue pieces were cultured on hydrated gelatine sponges (3-4 per sponge) as previously described (13, 14). After a 36-hr pre-incubation period, hormones were added to the media as indicated and tissues harvested 24-hr later for RNA extraction. Representative pieces of uncultured tissue were fixed in 4% formalin in phosphate-buffered saline (PBS) at 4°C overnight then processed into paraffin blocks. Sections (2 μm) were: a) stained with haematoxylin and eosin and examined by a pathologist to quantify the relative proportion of tumor cells and b) immunostained for ERα and AR, as previously described (14). Treatment conditions included: vehicle (0.01% ethanol), E2 (10 nM), DHT (10 nM), Enobosarm (100 nM).

RNA Extraction and Transcriptional Analyses

Cell lines were cultured for 3 days in phenol red-free medium supplemented with 5% dextran-coated charcoal (DCC)-stripped FBS then seeded under the same conditions into six-well plates at 5 x 10^5 cells/well. Three days later, cells were treated for 24 h with treatments as indicated. RNA extraction, reverse transcription and quantitative real-time PCR (RT-qPCR) was performed as previously reported (11, 15, 16). For tissue samples, replicate pieces representing one condition were snap frozen in one tube containing RNAlater (Qiagen) and RNA isolated using RNeasy kits (Qiagen) according to manufacturer's protocol. Reverse transcription was performed using total RNA (500ng-1µg) and an iScript cDNA Synthesis Kit (Bio-Rad). The resulting cDNA was diluted 1:10 and used for transcriptional analysis as above.

Testosterone Glucuronidation Assay

MCF7 cells were pre-cultured for 72 hours in phenol red–free RPMI 1640 supplemented with 5% DCC-FBS in T175 flasks (Gibco) then treated with vehicle control (0.01% ethanol), E2 (10 nM), DHT (10 nM), 3α-diol (100 nM), or 3β-diol (100 nM) for 72 hours. Cells were scraped from flasks and whole cell lysates prepared in TE buffer (10 nM Tris-HCl and 1 mM EDTA, pH 7.6). Protein concentration was determined by Bradford assay (Bio-Rad), according to the manufacturer's protocol. The testosterone glucuronidation assay was conducted as previously reported (17).

Luciferase Reporter Assays

MCF7 cells were plated into 96-well dishes in 100µl of phenol red-free RPMI 1640 supplemented with 5% DCC-FBS and cultured for 24-48 h prior to transfection. Cells were transfected with wild type or mutated *UGT2B15* or *UGT2B17* promoter constructs, treated with hormones as indicated and assessed for luciferase activity as previously described (11, 15).

Promoter construct details are provided in Suppl Methods. Transfections were performed in triplicate, and experiments repeated at least twice.

Electrophoretic Mobility Shift and Supershift Assays (EMSAs)

MCF7 cells were cultured in T75 flasks for 6 days in phenol red-free RPMI 1640 supplemented with 5% DCC-FBS then treated overnight (~16 h) with 0.1% ethanol (vehicle), E2 (10 nM), DHT (10 nM), 3α-diol (100 nM), or 3β-diol (100 nM). Nuclear extracts were prepared and EMSAs performed as previously reported (11, 18). Probe and antibody information is provided in Supplemental Methods.

Small Interfering RNA Knockdown Experiments

ON-TARGET*plus*SMARTpool small interfering RNAs (siRNAs) were purchased from Dharmacon RNA Technologies (USA): 1) FOXA1 (NM_004496); 2) ERα (NM_000125); 3) AR (NM_001011645); and 4) ON-TARGET*plus* non-targeting pool siRNA (Scramble siRNA). Cells were cultured for 3 days in phenol red-free RPMI 1640 supplemented with 5% DCC-FBS then seeded into six-well plates at 5x 10^5 cells/well in 2 ml of fresh medium containing 8µl of LipofectAMINE 2000 (Invitrogen) and 100 nM siRNA. After 24 h, transfection media was replaced by fresh media supplemented with 5% DCC-FBS. After 48 h, cells were treated overnight as indicated. Cells were harvested for RNA isolation and transcriptional analyses as above.

Chromatin Immunoprecipitation (ChIP) Experiments

Standard methodologies were employed as described previously for ChIP followed by RT-qPCR (11) and for ChIP followed by exonuclease digestion and deep sequencing (ChIP-exo) (19). Immunoprecipitations were performed with Santa Cruz Biotechnology, Inc (USA) antibodies to ERα (HC-20), FOXA1 (H-120), AR (N-20), or rabbit pre-immune IgG control (sc-2027).

Statistical Analyses:

Log transformed data met the assumption of equal variance at a significance level of less than 0.01 (Levene's test). Analyses of transformed data were conducted by one- or two-way analysis of variance with the Tukey's post hoc test or independent t test using SPSS software (IBM, version 22). $P < 0.05$ was considered statistically significant. Recursive partitioning was used to dichotomize the METABRIC transcriptional data to assess the influence of relatively high versus low expression of each gene on patient survival using an R statistical language program as previously implemented (2). This was performed for all probes representing a gene.

RESULTS

Expression of UGT2B15 and UGT2B17 in clinical breast cancers

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) investigated 2,000 cases of breast cancer and redefined this heterogeneous disease into 10 molecular subgroups with distinct survival outcomes (2). The subgroups, called iClusters, were created by an integration of gene copy number variations and cis-acting transcriptomes (20, 21). Analysis of the microarray probes representing *UGT2B15* and *UGT2B17* in the METABRIC cohort revealed cases that displayed very high expression of these genes (Fig. 1A). Their expression was positively correlated in this (Suppl. Fig 1A) as well as an independent breast cancer cohort (n = 825 cases) from The Cancer Genome Atlas (TCGA) (22, 23) (Suppl. Fig 1B). In the METABRIC cohort, ERα+ cases had significantly higher expression of these genes compared to ERα-negative cases (Fig. 1B), and highest expression was observed in integrative cluster 1 (iCluster 1), predominantly comprised of ERα+, luminal B breast cancers (Fig. 1C). Integrative cluster 10, largely comprised of aggressive, triple negative breast cancers, had the lowest expression (Fig. 1C). Transcript levels of both enzymes significantly decreased with increasing

tumor grade (Fig. 1D). Higher levels of *UGT2B17* were significantly associated with increased survival outcomes in cases classified into iCluster 5, largely comprised of high-grade breast cancers with HER2 amplification and very poor survival outcomes, as well as all cases in the cohort classified as being HER2 amplified by SNP6 array analysis (Fig. 1E). Note that these groups contain both ERα+ and ERα-negative breast cancers. Higher levels of both *UGT2B15* and *UGT2B17* were associated with increased survival in iCluster 6 (Fig. 1F), dominated by ERα+, HER2-negative breast cancers with amplification of the 8p12 gene locus. Conversely, higher levels of *UGT2B15* were associated with poor survival in iCluster 9, comprised of ERα+ cancers with a luminal B phenotype and a high rate of *TP53* mutations (Fig. 1G). We also analyzed the relationship between expression of these genes and patient outcome in another publically available resource (KMplotter) containing normalized microarray data from multiple studies that collectively represent 3,455 cases of breast cancer (24). At present, this cohort can only be interrogated via the older PAM50 gene classifier. A high level of *UGT2B15* expression was associated with improved relapse-free survival in the entire cohort and in all PAM50 subgroups (Suppl. Fig. 2, upper row). While high *UGT2B17* expression was also associated with improved relapse-free survival in the entire cohort, this association was only sustained in the luminal B and HER2+ subtypes (Suppl. Fig. 2, lower row), the latter supporting results in Fig 1E. Collectively, these data suggest that expression of *UGT2B15* or *UGT2B17* impacts breast cancer survival in a gene- and disease subtype-specific manner.

ERα and AR transcriptionally regulate UGT2B15 & UGT2B17 in an estrogen sensitive context

As expected, E2 significantly increased transcripts of *UGT2B15* and *UGT2B17* (Fig. 2A-B) and an ERα-regulated positive control gene (*GREB1*; Suppl. Fig. 3A), effects that were abolished by co-treatment with fulvestrant (ICI), an ERα inhibitor. Treatment with DHT or its metabolites, 3α-diol or 3β-diol, dose-dependently increased *UGT2B15* and *UGT2B17* transcripts (Fig. 2A-B) and the control gene (Suppl. Fig 3A) to varying degrees. These stimulatory effects were all significantly attenuated by co-incubation with fulvestrant, supporting the hypothesis that DHT indirectly stimulates ERα activity via conversion to diols. Individual hormone treatments also significantly increased levels of glucuronidated testosterone (Fig 2C), indicative of enhanced enzyme activity. Surprisingly, fulvestrant also inhibited the activity of R1881 (Fig 2A-B), a synthetic androgen that is not metabolized to estrogenic compounds, although direct binding of R1881 to ERα at high doses has been reported (25). While this data collectively suggested lack of direct regulation by AR, subsequent analyses revealed that the stimulatory effects of DHT were also significantly attenuated by treatment with flutamide, an AR antagonist (Suppl Fig 3B) and treatment with the ERα and AR inhibitors combined resulted in greater inhibition of steroid-mediated up-regulation of *UGT2B15* and *UGT2B17* than either inhibitor alone (Fig. 2D). These complementary effects were not evident for regulation of *GREB1* by DHT, which was completely dependent on ERα (Suppl Fig 3C). Consistent with a direct role for AR in up-regulating the UGT genes, siRNA-mediated ablation of AR in MCF7 cells significantly reduced both E2- and DHT-mediated transcriptional effects (Fig 2E). The same patterns of hormone regulation and the influence of AR loss were evident for the *UGT2B15* gene in ZR75-1 cells, another model of ERα+AR+ breast cancer (Suppl Fig 3D). The *UGT2B17* gene was not detected under basal or hormone stimulation in ZR75-1 cells. Collectively, these findings indicate that AR

directly regulates *UGT2B15* and *UGT2B17* in ERα+AR+ breast cancer cells, acting co-operatively with ERα.

Steroid-induced transactivation of the proximal promoters of UGT2B15 and UGT2B17

Highly conserved estrogen response units (ERUs) reside in the proximal promoters of the *UGT2B15* and *UGT2B17* genes, comprised of three estrogen response element (ERE) half sites and two AP-1 binding sites, all required for E2-mediated transcriptional regulation (11). A FOXA1 binding site has recently been identified in the region between two of the ERE half sites (11, 16) but its importance in breast cancer cells is unknown. The prototypical ERU, including the FOXA1 binding site, is depicted in Fig. 3A. As expected, E2 significantly enhanced basal promoter activity in an ERα-dependent manner (Fig 3B). All other steroids including DHT, 3α-diol, 3β-diol, and R1881 stimulated *UGT2B15* promoter activity at levels similar to or greater than that induced by E2, and these stimulatory effects were all significantly repressed to some degree by the ERα inhibitor. However, the AR inhibitor also significantly reduced DHT-induced promoter activity (Fig. 3C), supporting direct regulation by both sex steroid receptors.

An ERU mutant *UGT2B15* promoter construct dramatically reduced transactivation by E2 and obliterated transactivation by other tested steroids (Fig 3D; left panel) and mutation of the FOXA1 site abolished all steroid-induced promoter activity (Fig 3D; right panel). Transactivation of the wild type (Fig. 3E; left panel) and FOXA1-mutated (Fig. 3E; right panel) *UGT2B17* promoter constructs by steroids mirrored that of the *UGT2B15* constructs, but with more attenuated stimulatory effects.

To confirm the direct involvement of FOXA1, EMSA assays were performed. A major protein/DNA complex formed on a DNA probe containing the *UGT2B15* promoter FOXA1 site following incubation with nuclear extracts from vehicle and steroid-treated MCF7 cells (Fig 4A).

Addition of a FOXA1 antibody reduced the intensity of the complex and also induced formation of super-shifted complexes. Furthermore, mutation of the FOXA1 site abolished the formation of FOXA1 protein/DNA complexes. Although the *UGT2B15* and *UGT2B17* gene promoters are highly conserved, a unique but prevalent -155A/G SNP occurs within the FOXA1 binding site in the *UGT2B17* promoter (15). Therefore, a *UGT2B17* probe with an A-containing FOXA1 site was tested (Fig 4B). A major complex was formed on the A-containing *UGT2B17* FOXA1 probe, which was super-shifted by addition of FOXA1 antibody and abolished by mutating the FOXA1 site or adding a 100-fold molar excess of un-labelled probe. Therefore, endogenous FOXA1 in MCF7 cells can bind the "A-containing" FOXA1 binding site SNP in the *UGT2B17* promoter. These results confirm a critical role for FOXA1 in steroid-mediated transactivation of the *UGT2B15* and *UGT2B17* promoters, even in the context of a common SNP variant in *UGT2B17*.

Recruitment of ERα, FOXA1 and AR to the UGT2B15 and UGT2B17 promoters

ERα occupancy at the *UGT2B15* and *UGT2B17* proximal promoters and a positive control locus (*pS2/TIF1*) was significantly stimulated by E2 in MCF7 cells (Fig. 4C-E). Of the other steroids tested, 3β-diol stimulated the highest level of ERα enrichment, a compound with significantly less affinity for ERα than E2 (26). FOXA1 bound to the *UGT2B15* and *UGT2B17* loci before ligand stimulation, consistent with its role as a pioneer factor (27). Interestingly, FOXA1 binding was significantly increased by all hormone treatments (Fig. 4C-E), contrary to observations at other genomic loci (28). This steroid-induced increase in FOXA1 occupancy is consistent with a recent study showing that FOXA1 binding can be altered by activation of steroid receptors, including ERα, in breast cancer cells (29). The AR did not display a strong enrichment over IgG controls under any treatment condition; maximal AR enrichment (2.6-fold) occurred at the positive control locus for ERα binding (*pS2/TIFF1*) when cells were stimulated with R1881.

Low enrichment of AR may be a technical issue due to low AR protein levels in MCF7 cells. However, the requirement for ERα, FOXA1, and AR in the regulation of *UGT2B15* and *UGT2B17* by steroids was confirmed by siRNA-mediated ablation (Suppl Fig 4). Analysis of genome-wide ERα, FOXA1 and AR chromatin binding in MCF7 and ZR75-1 cells generated by ChIP-exo (19) revealed the presence of all three factors in the proximal promoters of the UGT genes (Figure 5A-B; magnified views in Suppl Fig 5). ChIP-exo generates sharper peaks than ChIP-seq due to the inclusion of an exonuclease step following immunoprecipitation of protein-DNA complexes, allowing better discernment of relative binding positions. This data suggests that AR binds to or overlaps the ERE half site located 3' to the FOXA1 and ERα peaks in the promoters of *UGT2B15* and *UGT2B17*. The tandem pattern of chromatin binding strongly supports the concept that ERα, AR and FOXA1 interact to regulate transcription of these two androgen inactivating enzymes in an estrogen sensitive breast cancer context.

Regulation of UGT2B15 and UGT2B17 by Enobosarm, a new selective AR modulator

Enobosarm (GTx024) is a selective AR modulator (SARM) (30) currently being tested for clinical efficacy in women with ERα+AR+ breast cancer (NCT02463032). This compound dose-dependently activated an AR reporter construct transfected into MCF7 cells (Suppl Fig 6A), up-regulated *UGT2B15* and *UGT2B17* in an AR-dependent manner (Suppl Fig6B) and dose-dependently increased activation of the wild type UGT promoter constructs (Fig 5C). Using selective ERE mutant constructs of the *UGT2B15* promoter, we show that the previously defined "3' ERE half site" is critical for AR-mediated up-regulation of promoter activity (Fig 5D).

AR does not regulate UGT2B15 or UGT2B17 in an estrogen insensitive context

AR activity sustains a luminal phenotype in MDA-MB-453 cells, a model of AR+ERα-negative breast cancer, by mimicking the action of ERα in MCF7 cells (31). This type of breast cancer

also has similarities to prostate cancer (3). Interrogation of our previously published ChIP-seq data (31) shows that AR and FOXA1 occupy the proximal promoter regions of *UGT2B15* and *UGT2B17* in MDA-MB-453 cells, similar to ERα and FOXA1 occupancy in MCF7 cells and AR and FOXA1 occupancy in LNCaP prostate cancer cells (Fig. 6A-B). However, treatment with DHT did not up-regulate expression of these genes in the MDA-MB-453 or MFM223 breast cancer cell lines (Fig 6C), which both have a "luminal AR" phenotype (32). *UGT2B17* expression was undetectable under any treatment conditions in these AR+ ERα-negative cell lines. In contrast, DHT potently downregulates both genes in LNCaP cells (33). Thus, while ERα and AR positively co-regulate transcription of the two major androgen metabolizing enzymes in an estrogen-sensitive breast cancer context, AR alone does not appear to have this capacity in an estrogen-insensitive context.

Estrogen and androgen regulation of UGT2B15 and UGT2B17 in clinical tissues

Normal breast tissue from reduction mammoplasties (n = 8) were cultured *ex vivo* on gelatin sponges to sustain tissue architecture, cellular heterogeneity and hormone receptor expression as previously described (14). In 3/8 cases, all three treatments (E2, DHT, Enobosarm) up-regulated transcript levels of *UGT2B15* or *UGT2B17* compared to the vehicle control (Suppl Fig 6C). In other cases, one or two agents demonstrated stimulatory action and in a few cases there were no effects, demonstrating the expected heterogeneity of primary tissue responses. This methodology also provides a unique pre-clinical model for testing drug responses (13, 34). In primary ERα+ breast tumors (n = 13) cultured *ex vivo* under estrogenic conditions, treatment with Enobosarm up-regulated *UGT2B15* in a total of 7/13 cases and *UGT2B17* in 8/13 cases (Fig 7A-B). Two cases demonstrated no stimulatory effect on either gene above baseline, 4 demonstrated stimulatory effects on both genes, 3 demonstrated upregulation of *UGT2B15* alone and 4

demonstrated upregulation of *UGT2B17* alone. Collectively, these data indicate that therapeutic activation of AR can significantly upregulate expression of these UGT genes in primary ERα+ breast cancers.

A previous study investigated genome-wide ERα chromatin binding in primary breast tumors (35). The cohort consisted of ERα+, progesterone receptor (PR)-positive and HER2-negative tumors associated with good disease outcomes (n = 8); ERα+, PR-negative and/or HER2+ tumors associated with poor outcomes (n = 7) and ERα+ distant metastases (n = 3). Interrogation of that data revealed no evidence for ERα occupancy at the promoters of *UGT2B15* or *UGT2B17* in normal breast tissues (0/2 cases) or ERα+ tumors associated with a good outcome (0/8 cases). However, ERα occupancy was evident at the *UGT2B15* (2/7 cases) and *UGT2B17* (1/7 cases) promoters in tumors associated with poor outcomes and in 2/3 metastases (Fig. 7C-D). Although the study represented a small number of cases, it was the first study to investigate genome-wide ERα chromatin interactions in clinical breast tissues.

DISCUSSION

Herein, the potential clinical significance and hormone-mediated regulation of two steroid glucuronidation enzymes, UGT2B15 and UGT2B17, was investigated in relation to breast cancer. These enzymes can functionally impact both ERα and AR activity by directly or indirectly reducing levels of their activating ligands in the tissue microenvironment. While positive regulation of these genes via ERα activity at their proximal promoters has been previously described in breast cancer cell lines (10, 11), we provide the first evidence that this activity is exquisitely dependent on the pioneer factor FOXA1, is facilitated by AR binding to an adjacent ERE half site, and is potently stimulated by metabolites of DHT normally considered ‘weak’

estrogens. These data are consistent with the fact that *UGT2B15* and *UGT2B17* levels were highest in clinical ERα+ breast cancers and in low grade tumors, which are known to be characterized by high levels of both ERα and AR. Importantly, higher expression of one or both of the UGT enzymes was associated with better survival outcomes in a number of molecular subgroups of ERα+ disease, which may have clinical implications. For example, therapeutic activation of AR with Enobosarm significantly increased expression of one or both genes in *ex vivo* cultured normal human breast tissues and ERα+ breast tumors. Whether this plays a mechanistic role in attenuating the estrogenic signal or determining clinical responses of women treated with this compound for estrogen sensitive breast cancer (NCT02463032) remains to be determined.

In breast malignancies, the levels of estrogenic or androgenic hormones can greatly exceed levels measured in plasma or in adjacent non-malignant breast tissues (36, 37). The altered intracrinology involves deregulation of steroid metabolizing genes such as 17β-HSDs, sulfotransferases (SULTs), 5α-reductases and estrogen metabolizing UGTs (38-40). Our data indicate that androgen metabolizing UGTs are also involved, particularly in the context of ERα-positive and/or HER2-positive clinical breast cancers in which relative expression of *UGT2B15* and/or *UGT2B17* could discern cases with distinct survival outcomes. While it is tempting to speculate on how relative levels of either enzyme influences steroid intracrinology and hence disease outcome in various breast cancer contexts, it is important to note that UGT2B15 and UGT2B17 have different potencies in the inactivation of androgen hormones and that one or both have been shown to inactivate xenobiotics and drugs relevant to breast cancer, including bisphenol AF (41), exemestane (42), the histone deacetylase inhibitor vorinostat (43) and the major active tamoxifen metabolite 4-OH-tamoxifen (42, 44). Differential activity of the two

enzymes in the glucuronidation of androgens or other compounds may explain gene-specific influences on breast cancer outcomes. We recently found that tamoxifen and its metabolites upregulate *UGT2B15* via a mechanism involving ERα (45), which may have implications for resistance to this drug. Collectively, these studies indicate that the influence of *UGT2B15* and *UGT2B17* expression in breast cancer includes, but also has implications that extend beyond, their ability to metabolize androgen hormones.

Both metabolites of DHT (3α- and 3β-diol) could recruit ERα to the proximal promoters of *UGT2B15* and *UGT2B17* to stimulate transcription, which in part explains the ERα-dependent stimulatory effects of this androgen. Indeed, rapid conversion of DHT into 3α- and 3β-diol has been previously reported in MCF7 cells (46, 47) and all 4 human 3α-hydroxysteroid dehydrogenases (HSDs) capable of this conversion are expressed in breast tissues (48). However, the action of DHT also stimulated AR binding to the promoters at a site 3' to the ERα binding site and ablation or chemical inhibition of AR reduced estrogen- and androgen-mediated transcriptional regulation of these genes. The co-operative activity of AR and ERα at these loci is suggestive of an 'assisted loading' mechanism described for the glucocorticoid receptor, a steroid receptor closely related to AR (49). Co-operative regulation of *UGT2B15* by ERα and AR was also evident in ZR75-1 cells, another luminal breast cancer model, indicating that the findings are not limited to one cell line.

There has been increasing interest in the capacity of AR signaling to drive breast cancer, particularly in ERα-negative tumors. The MDA-MB-453 cell line has emerged as the prototypical and most studied model of this type of breast cancer, in which AR is expressed at high level and treatment with DHT can increase proliferation. Indeed, AR signaling in this cell line shares many similarities to AR signaling in LNCaP cells (2, 31). However, we show that AR

and FOXA1 occupy the promoter regions of *UGT2B15 a*nd *UGT2B17* in MDA-MB-453 cells in a similar manner to their occupancy in LNCaP cells, but androgen treatment did not influence their transcription in two models of AR+, ERα-negative breast cancer. Since AR potently downregulates these genes in LNCaP cells (14) and we show that AR up-regulates them in ERα+ breast cancer cells, regulation of these genes by AR is highly context dependent.

In summary, the *UGT2B15* and *UGT2B17* enzymes are transcriptionally regulated by sex hormone signaling in ERα-positive breast cancer cells and are highly expressed in a subset of primary breast cancers, which has significance for survival outcomes in distinct genetic subgroups of this disease. Apart from their influence on the level of active steroids in the microenvironment, these enzymes may also determine the level of active xenobiotics and drugs relevant to breast cancer, which could have implications for response to a range of adjuvant hormone or other therapies. Collectively, these observations add new knowledge about the complexity of breast cancer intracrinology that has implications for other hormone dependent cancers. The findings provide a basis for further exploration of the role of UGTs in moderating the steroid milieu and modulating drug responses in breast and other cancers.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

ACKNOWLEDGEMENTS

We acknowledge Geraldine Laven-Law, Dhilushi Wijayakumara and Apichaya Chanawong for technical assistance and the clinical personnel (particularly Dr. Stephen Birrell and Dr. Clive Hoffman) and patients from Adelaide clinics who supported the study.

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FIGURE LEGENDS

Figure 1. *UGT2B15* and *UGT2B17* expression in clinical breast cancers. (A) Heatmap showing relative expression of *UGT2B15* (2 probes) and *UGT2B17* (3 probes) in the METABRIC cohort of 2,000 breast cancers. The degree of red or blue color indicates high and low expression, respectively. The ten integrative clusters (iClusters) defined by the METABRIC study are indicated by colors that correspond to the boxes in Fig. 1C. (B) *UGT2B15* and *UGT2B17* gene expression is significantly higher in ERα-positive compared to ERα-negative breast cancers. (C) Levels of *UGT2B15* and *UGT2B17* in the iClusters. (D) *UGT2B15* and *UGT2B17* expression significantly decreases with increasing tumor grade. (E) High expression of *UGT2B17* is associated with increased disease-specific survival in iCluster 5, representing cases in which HER2 amplification is the key genomic feature (left panel) and in all cases with HER2 amplification as determined by SNP6 array (right panel). (F) High expression of *UGT2B15* (left panel) and *UGT2B17* (right panel) is associated with better disease-specific survival in iCluster 6. (G) High expression of *UGT2B15* is associated with decreased disease-specific survival in iCluster 9. Box and whisker plots in Fig 1B-D represent the median and interquartile range (25-75 percentiles; ***$p<0.0001$).

Figure 2. Regulation of *UGT2B15* and *UGT2B17* by estrogenic and androgenic steroids. Relative mRNA levels of (A) *UGT2B15* and (B) *UGT2B17* in MCF7 cells treated for 24h with estrogen (E2), androgen (DHT), metabolites of DHT (3α-diol, 3β-diol) or a synthetic androgen (R1881) alone or in combination with the ERα inhibitor fulvestrant (ICI) at concentrations as indicated. (C) Levels of glucuronidated testosterone, indicative of UGT2B15/17 enzyme activity, in MCF7 cells treated with hormones as indicated. (D) Influence of ERα inhibition (ICI) and/or

AR inhibition with flutamide (Flut) on up-regulation of *UGT2B15* (upper panel) and *UGT2B17* (lower panel) by DHT in MCF7 cells. (E) Influence of siRNA-mediated silencing of AR (siAR) versus a non-target siRNA (siCtrl) on E2- or DHT-mediated induction of *UGT2B15* (upper panel) or *UGT2B17* (lower panel) in MCF7 cells. All data shown are means ± SEM of three independent experiments. Asterisks above columns represent comparisons with the vehicle control, set at 1. Asterisks above a line represent comparisons between selected columns (One-way analysis of variance followed by Tukey's post hoc multiple comparison test; *$p<0.05$; **$p<0.01$;***$p<0.001$).

Figure 3. Estrogen and androgen regulation of the *UGT2B15* and *UGT2B17* promoters. (A) Schematic depiction of highly conserved estrogen response units (ERU) in the *UGT2B15* and *UGT2B17* proximal promoters, positioned between -454 and -172 nucleotides in the *UGT2B15* promoter and between nucleotides -396 and -112 in the *UGT2B17* promoter relative to their translation start sites (ATG). From 5' to 3', the six key binding motifs are 5'-AP-1 and 3'-AP-1 sites, an imperfect estrogen response element (ERE), a 5'-ERE half-site, a FOXA1 site, and a 3'-ERE half-site. MCF7 cells were transfected with wild type (WT) promoter constructs or mutated constructs as indicated by a backslash over the relevant motif. (B) Stimulation of the WT *UGT2B15* promoter by estrogen (E2), androgen (DHT), metabolites of DHT (3α-diol, 3β-diol) or a synthetic androgen (R1881), alone or together with the ERα inhibitor fulvestrant (ICI). (C) Inhibition of DHT-stimulated WT *UGT2B15* promoter activity by ICI and an AR inhibitor, flutamide (Flut). (D) Influence of *UGT2B15* promoter mutations on steroid-mediated transactivation. (E) Activity of WT and FOXA1-mutant *UGT2B17* promoters in response to steroids alone or in combination with ICI. Relative luciferase activities of promoter constructs

are expressed as fold induction over that of the empty vector (set to1) after normalizing transfection efficiency with *R. reniformis* luciferase. Data shown are means ± SD of three independent experiments performed in triplicate. (One-way analysis of variance followed by Tukey's post hoc multiple comparison test, *p<0.05; **p<0.01;***p<0.001).

Figure 4. FOXA1, ERα and AR enrichment at *UGT2B15* and *UGT2B17* promoters. (A) EMSA blot to demonstrate FOXA1 binding to DNA fragments representing the *UGT2B15* and (B) *UGT2B17* promoters. MCF7 cells were treated with vehicle (lanes 1-8), estrogen (E2; lanes 9-12), androgen (DHT; lanes 13-14), DHT metabolites 3α-diol (lanes 15-16), or 3β-diol (lanes 17-18). EMSAs were performed with 50,000 cpm (~1 ng) of indicated ^{32}P-labeled probes. FOXA1-bound DNA complexes are labelled **A** for the prostate specific antigen (PSA) control probe, **B** for *UGT2B15* or *UGT2B17* probes, **C** for non-specific complexes and **SS** for antibody-induced super-shifted complexes. For super-shift assays, FOXA1 antibody was added (lanes 4, 7, 12, 14, 16, and 18). For competition assays, unlabeled probes were added at a 10-fold (lane 11) or 100-fold (lane 10) molar excess. In lane 8, a mutated FOXA1 probe was added. (C-E) ChIP-qPCR data showing enrichment of ERα, FOXA1 and AR chromatin binding at the (C) *UGT2B15*, (D) *UGT2B17* or (E) *pS1/TFF* promoters in MCF7 cells following stimulation with indicated steroids. Data are presented as relative fold enrichment over the normal rabbit immunoglobulin (IgG) controls (set to 1). Bars represent means ± SEM of three independent experiments. (One-way analysis of variance followed by Tukey's post hoc multiple comparison test, *p<0.05; **p<0.01;***p<0.001 compared to the relevant IgG control).

Figure 5. AR regulation of *UGT2B15* and *UGT2B17* promoters in ERα+ breast cancer cells. (A-B) Representative genome browser images showing enrichment of DNA sequences (peaks) pulled down by immunoprecipitation of FOXA1, ERα or AR by the process of ChIP-exo, indicative of chromatin binding at the proximal promoters of (A) *UGT2B15* and (B) *UGT2B17* in two models of ERα+, AR+ breast cancer (MCF7, ZR75-1). (C) Wild type (WT) *UGT2B15* and *UGT2B17* promoter constructs were transfected into MCF7 cells followed by treatment with the selective AR modulator, Enobosarm (Eno), as indicated. (D) Influence of site-specific mutations (MT) in the *UGT2B15* promoter constructs transfected into MCF7 cells followed by treatment with Enobosarm (100 nM) alone or in combination with the AR inhibitor, bicalutamide (Bic; 1μM). Data in (C) and (D) are means ± SD of two independent experiments performed in triplicate. Asterisks above columns represent comparisons with the appropriate vehicle control, set to 1. Asterisks above lines represent comparisons between selected columns. (One-way analysis of variance followed by Tukey's post hoc multiple comparison test, *$p<0.05$; **$p<0.01$).

Figure 6. Androgen regulation of *UGT2B15* and *UGT2B17* promoters in ERα-negative breast cancer cells. (A-B) Representative genome browser images showing enrichment (peaks) of DNA sequences pulled down by immunoprecipitation of FOXA1 or AR by the process of ChIP-seq, indicative of chromatin binding at the proximal promoters of (A) *UGT2B15* and (B) *UGT2B17* in MDA-MB-453 breast cancer cells, an AR+, ERα-negative model of breast cancer. For comparison, ERα and FOXA1 peaks are shown for the same loci in MCF7 breast cancer cells and AR and FOXA1 peaks in prostate cancer cells (LNCaP). (C) RT-qPCR data corresponding to indicated cell lines treated with estrogen (E2) or androgen (DHT). Data shown

are means ± SEM of three independent experiments. One-way analysis of variance followed by Tukey's post hoc multiple comparison test (***p<0.0001; NS = not significant).

Figure 7. Sex hormone regulation of *UGT2B15* and *UGT2B17* in clinical samples. (A) Relative mRNA levels of *UGT2B15* and *UGT2B17* in *ex vivo* cultured pieces of ERα+AR+ primary breast tumors (n = 13 independent cases) following treatment with the selective AR modulator, Enobosarm (Eno; 100nM). (B) Representative genome browser images of ERα occupancy at the *UGT2B15* and (C) *UGT2B17* proximal promoters in clinical samples representing normal breast tissue (n = 2), good outcome ERα+, PR+, HER2- tumors (n = 3), poor outcome ERα+, PR+/-, HER2+/- tumors (n = 3) and ERα+ distant metastases (n = 3).

Figure 1

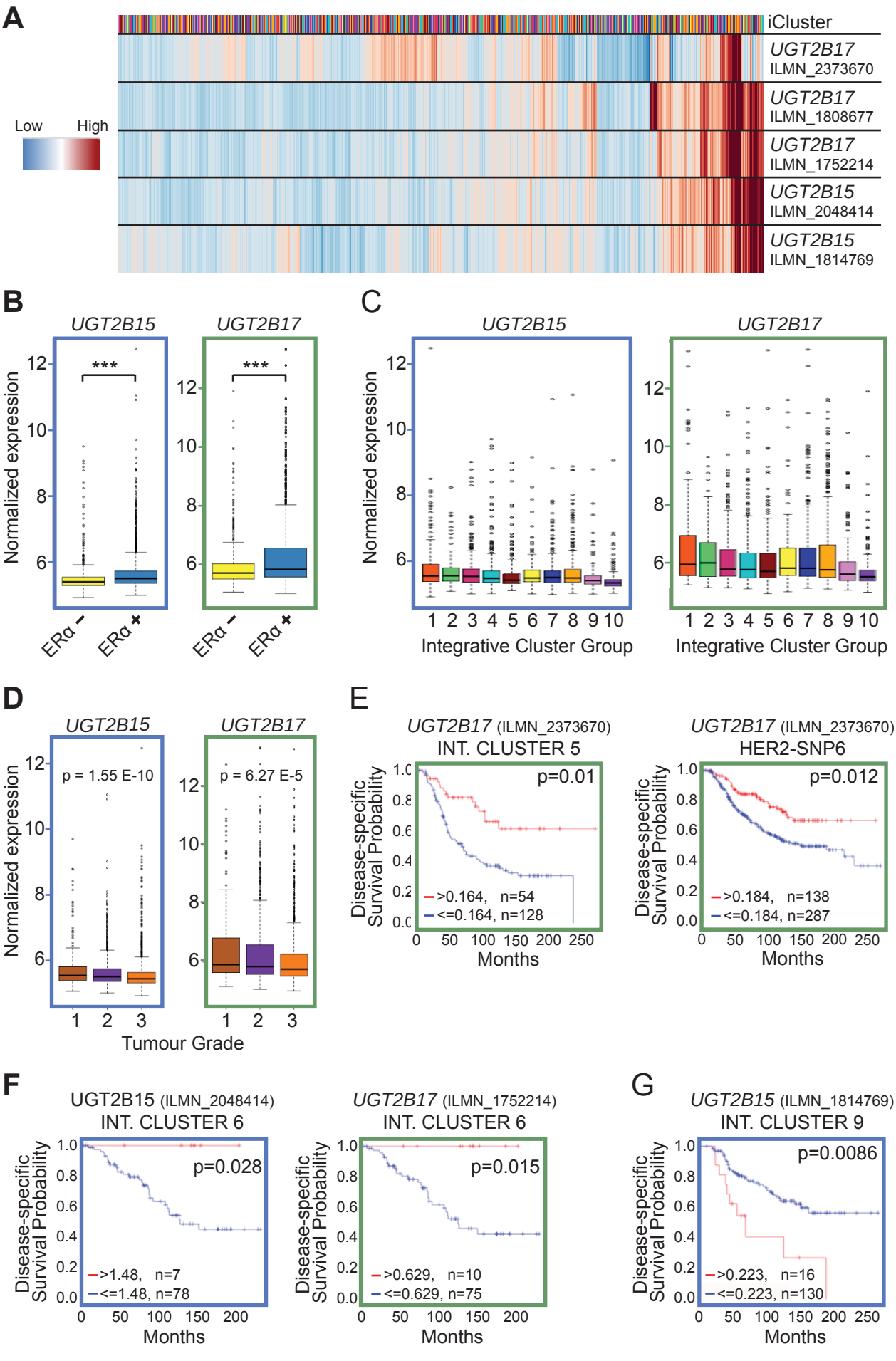

A
iCluster
UGT2B17
ILMN_2373670
UGT2B17
ILMN_1808677
UGT2B17
ILMN_1752214
UGT2B15
ILMN_2048414
UGT2B15
ILMN_1814769
Low
High
B
UGT2B15
UGT2B17
Normalized expression
***
***
ERα –
ERα +
C
UGT2B15
UGT2B17
Normalized expression
1 2 3 4 5 6 7 8 9 10
Integrative Cluster Group
D
UGT2B15
UGT2B17
p = 1.55 E-10
p = 6.27 E-5
Normalized expression
Tumour Grade
E
UGT2B17 (ILMN_2373670)
INT. CLUSTER 5
p=0.01
>0.164, n=54
<=0.164, n=128
Disease-specific Survival Probability
Months
UGT2B17 (ILMN_2373670)
HER2-SNP6
p=0.012
>0.184, n=138
<=0.184, n=287
F
UGT2B15 (ILMN_2048414)
INT. CLUSTER 6
p=0.028
>1.48, n=7
<=1.48, n=78
UGT2B17 (ILMN_1752214)
INT. CLUSTER 6
p=0.015
>0.629, n=10
<=0.629, n=75
G
UGT2B15 (ILMN_1814769)
INT. CLUSTER 9
p=0.0086
>0.223, n=16
<=0.223, n=130

Figure 2

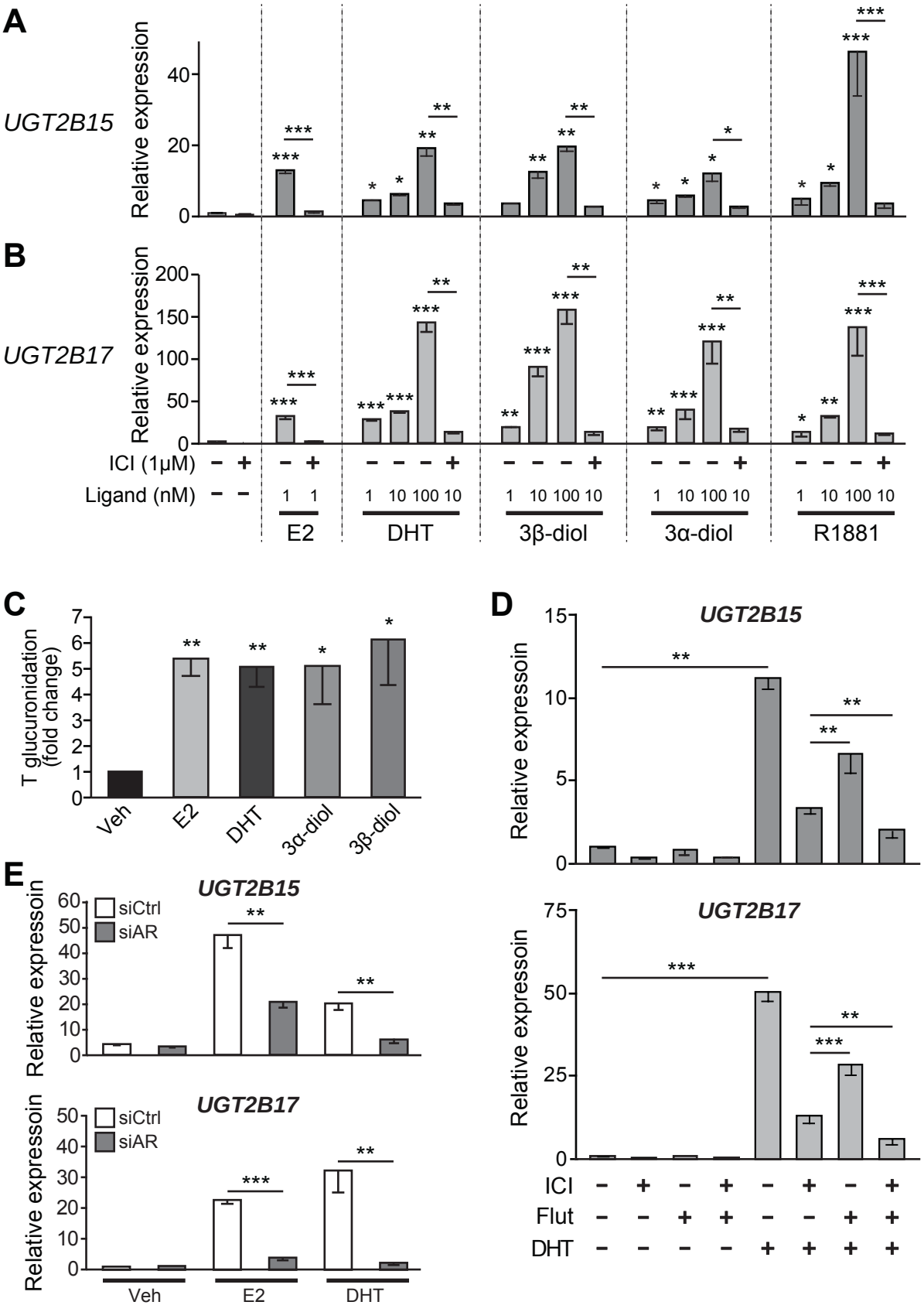
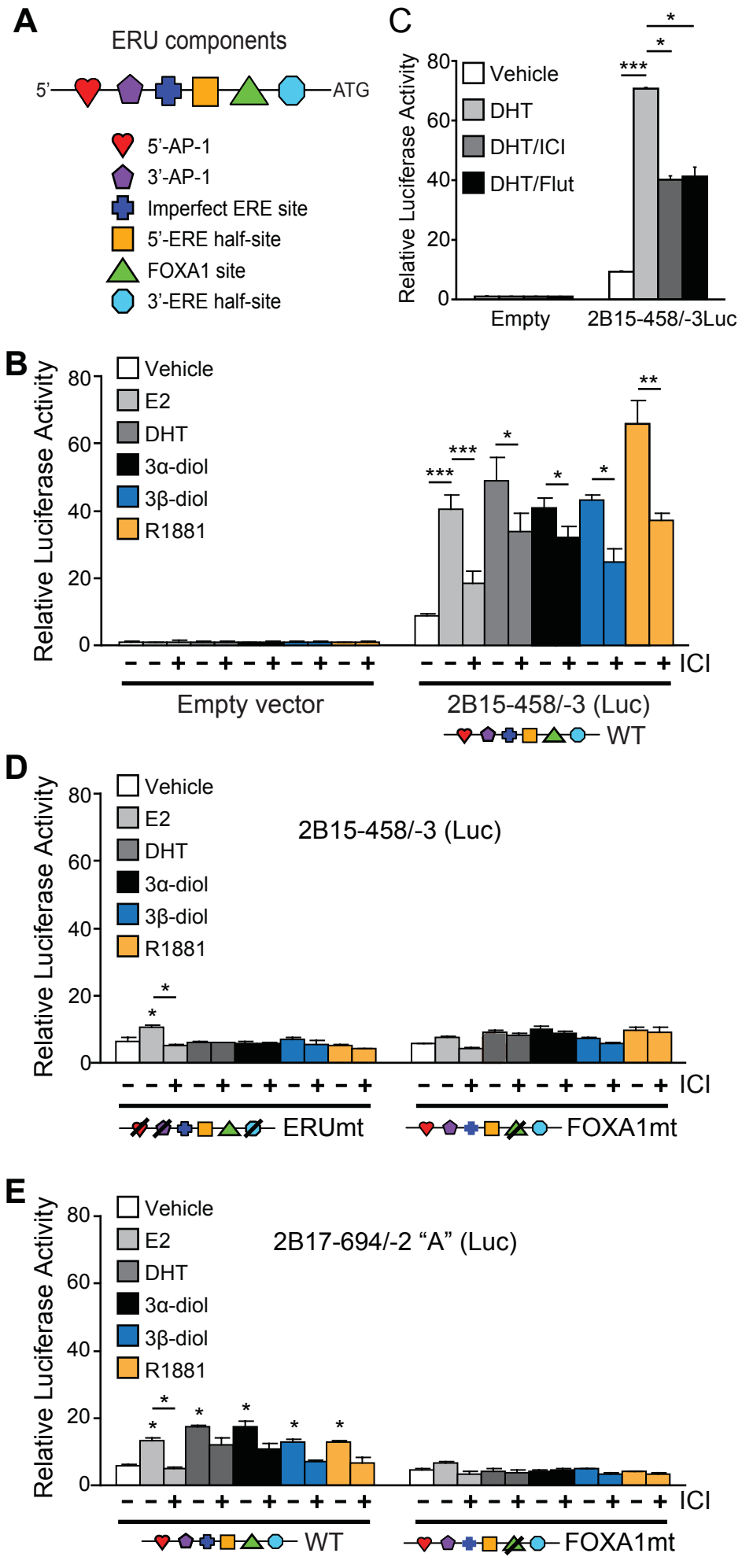

A
UGT2B15
Relative expression
B
UGT2B17
Relative expression
ICI (1μM)
Ligand (nM)
E2
DHT
3β-diol
3α-diol
R1881
C
T glucuronidation (fold change)
Veh
E2
DHT
3α-diol
3β-diol
D
UGT2B15
Relative expressoin
UGT2B17
Relative expressoin
ICI
Flut
DHT
E
UGT2B15
Relative expressoin
siCtrl
siAR
UGT2B17
Relative expressoin
siCtrl
siAR
Veh
E2
DHT

Figure 3


A
ERU components
5'
ATG
5'-AP-1
3'-AP-1
Imperfect ERE site
5'-ERE half-site
FOXA1 site
3'-ERE half-site
C
Relative Luciferase Activity
Vehicle
DHT
DHT/ICI
DHT/Flut
Empty
2B15-458/-3Luc
B
Vehicle
E2
DHT
3α-diol
3β-diol
R1881
ICI
Empty vector
2B15-458/-3 (Luc)
WT
D
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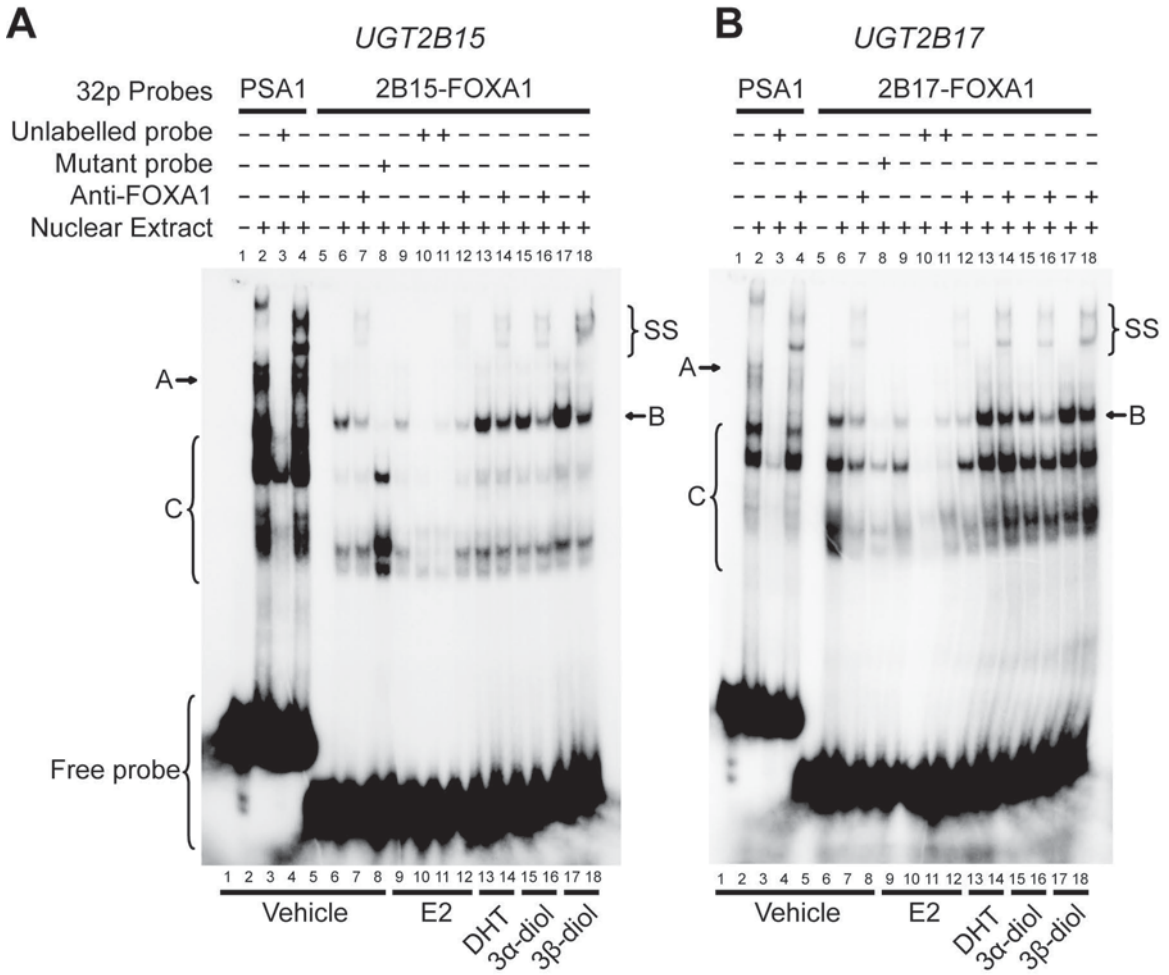
ERUmt
FOXA1mt
E
2B17-694/-2 "A" (Luc)
WT
FOXA1mt

Figure 4


A
UGT2B15
32p Probes
PSA1
2B15-FOXA1
Unlabelled probe
Mutant probe
Anti-FOXA1
Nuclear Extract
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
SS
A
B
C
Free probe
Vehicle
E2
DHT
3α-diol
3β-diol
B
UGT2B17
PSA1
2B17-FOXA1
SS
A
B
C
Vehicle
E2
DHT
3α-diol
3β-diol

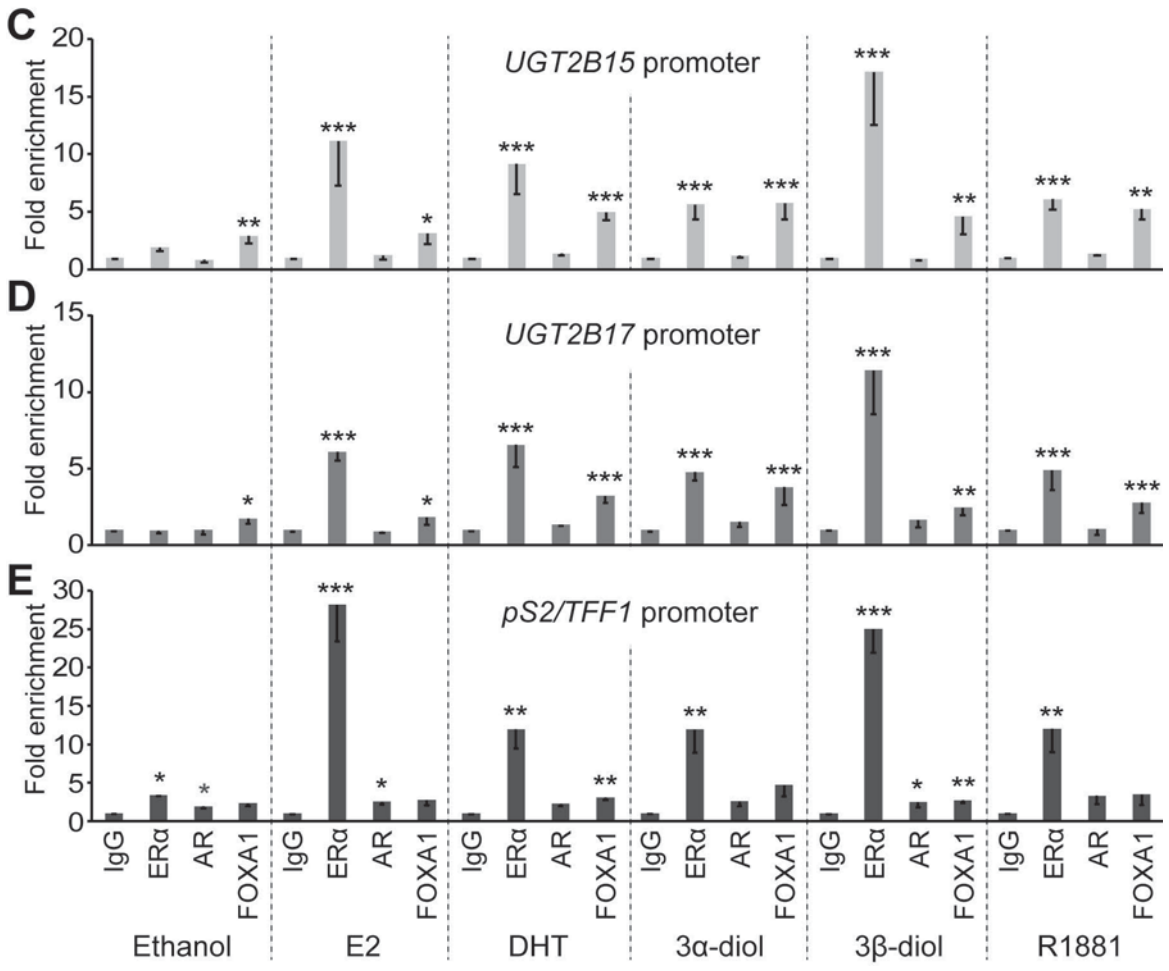

C
Fold enrichment
UGT2B15 promoter
D
Fold enrichment
UGT2B17 promoter
E
Fold enrichment
pS2/TFF1 promoter
IgG
ERα
AR
FOXA1
Ethanol
E2
DHT
3α-diol
3β-diol
R1881

Figure 5

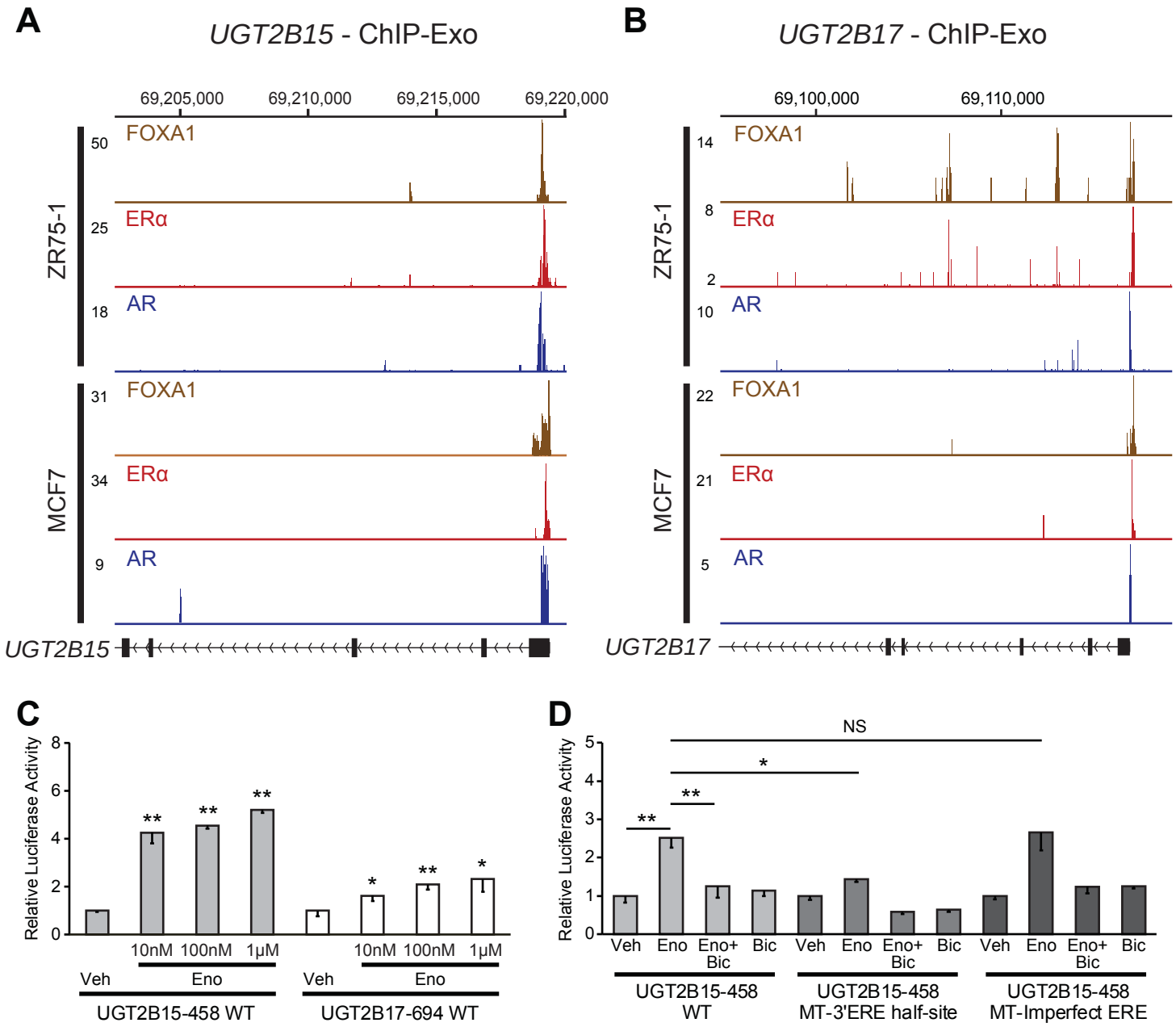
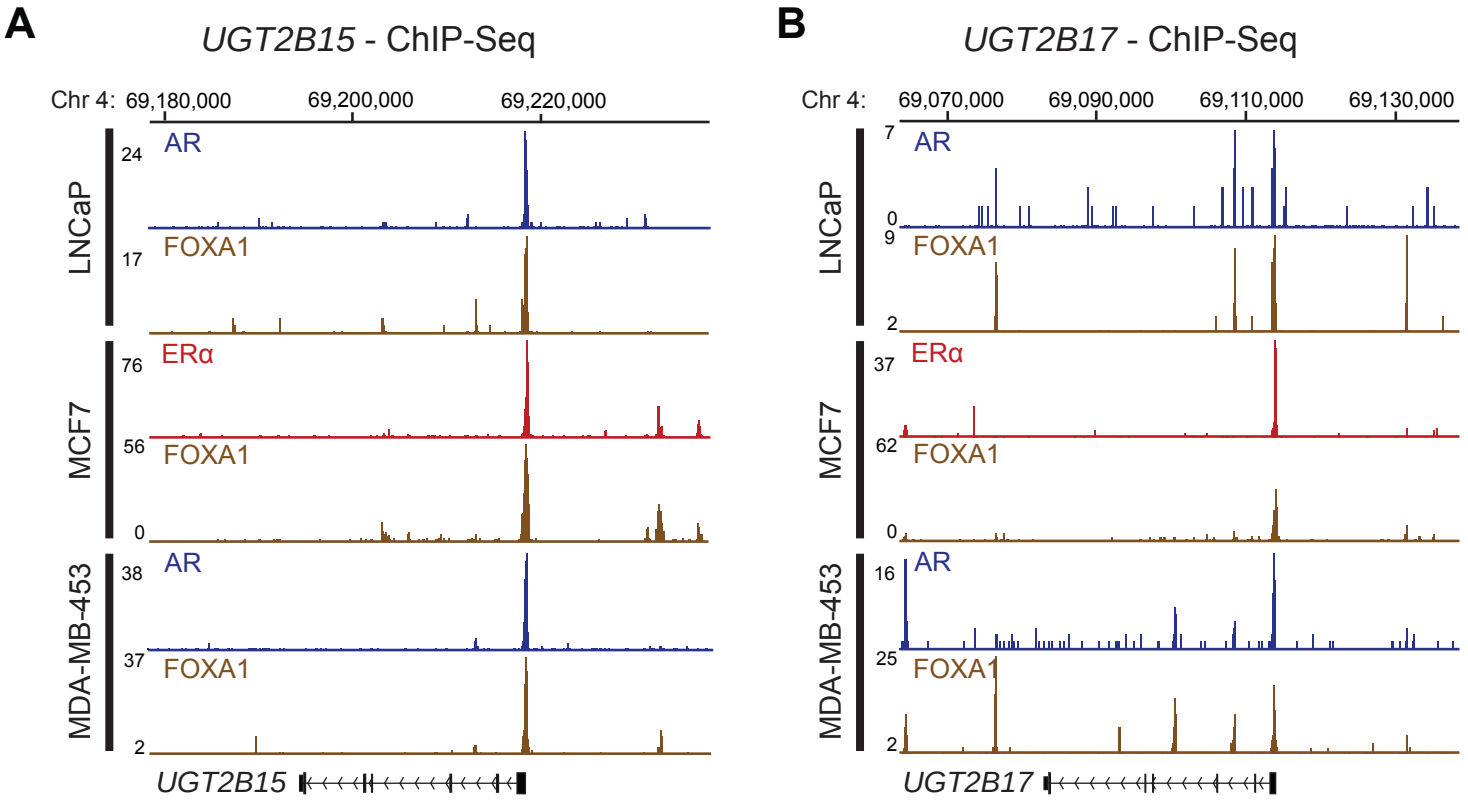

A
UGT2B15 - ChIP-Exo
69,205,000
69,210,000
69,215,000
69,220,000
ZR75-1
50
FOXA1
25
ERα
18
AR
MCF7
31
FOXA1
34
ERα
9
AR
UGT2B15
B
UGT2B17 - ChIP-Exo
69,100,000
69,110,000
ZR75-1
14
FOXA1
8
ERα
2
10
AR
MCF7
22
FOXA1
21
ERα
5
AR
UGT2B17
C
Relative Luciferase Activity
0
2
4
6
8
**
**
**
*
**
*
10nM 100nM 1μM
10nM 100nM 1μM
Veh
Eno
Veh
Eno
UGT2B15-458 WT
UGT2B17-694 WT
D
Relative Luciferase Activity
0
1
2
3
4
5
NS
*
**
**
Veh
Eno
Eno+ Bic
Bic
Veh
Eno
Eno+ Bic
Bic
Veh
Eno
Eno+ Bic
Bic
UGT2B15-458 WT
UGT2B15-458 MT-3'ERE half-site
UGT2B15-458 MT-Imperfect ERE

Figure 6


A
UGT2B15 - ChIP-Seq
Chr 4: 69,180,000
69,200,000
69,220,000
LNCaP
AR
FOXA1
MCF7
ERα
FOXA1
MDA-MB-453
AR
FOXA1
UGT2B15
B
UGT2B17 - ChIP-Seq
Chr 4: 69,070,000
69,090,000
69,110,000
69,130,000
LNCaP
AR
FOXA1
MCF7
ERα
FOXA1
MDA-MB-453
AR
FOXA1
UGT2B17

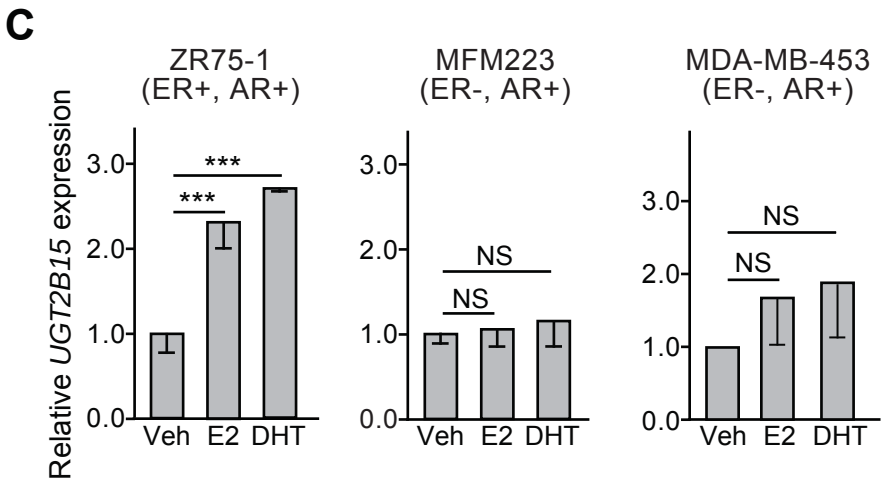
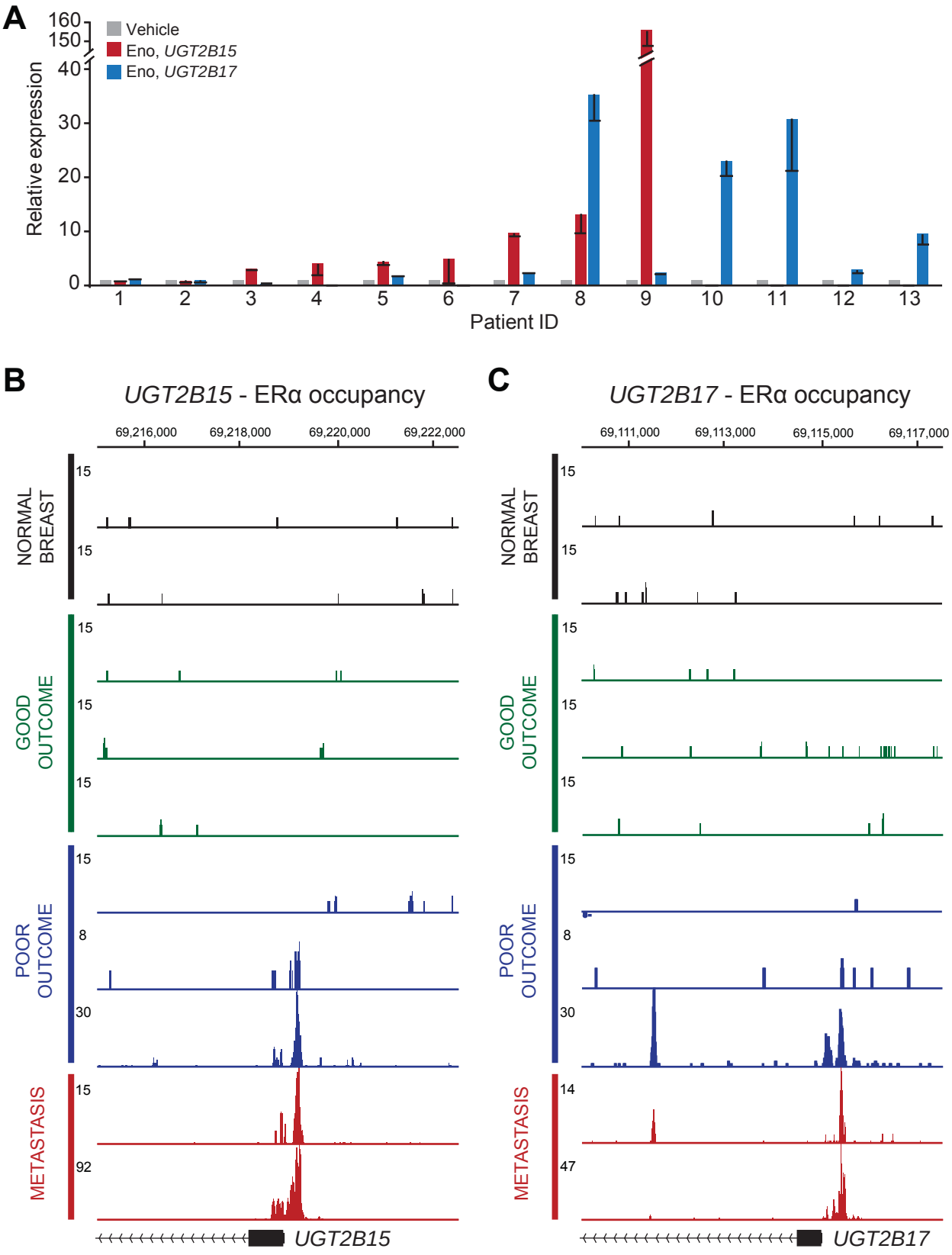

C
ZR75-1
(ER+, AR+)
MFM223
(ER-, AR+)
MDA-MB-453
(ER-, AR+)
Relative UGT2B15 expression
3.0
2.0
1.0
0.0
***
***
NS
NS
NS
NS
Veh
E2
DHT

Figure 7


A
Vehicle
Eno, UGT2B15
Eno, UGT2B17
Relative expression
160
150
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Patient ID
B
UGT2B15 - ERα occupancy
69,216,000
69,218,000
69,220,000
69,222,000
NORMAL BREAST
GOOD OUTCOME
POOR OUTCOME
METASTASIS
UGT2B15
C
UGT2B17 - ERα occupancy
69,111,000
69,113,000
69,115,000
69,117,000
NORMAL BREAST
GOOD OUTCOME
POOR OUTCOME
METASTASIS
UGT2B17