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Exemestane and Its Active Metabolite 17-hydroexemestane Induce UDP-glucuronosyltransferase (UGT) 2B17 Expression in Breast Cancer Cells

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Running title: Upregulation of *UGT2B17* by exemestane

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Abbreviations:

17-HE, 17-hydroexemestane

AKR, aldo-keto reductase

ANOVA, analysis of variance.

AR, androgen receptor

BIC, bicalutamide

bp, base pair

ChIP, chromatin immunoprecipitation

CYP, cytochrome P450

DCC, dextran-coated charcoal-stripped media

DHT, dihydrotestosterone

E2, 17β-estradiol

ER, estrogen receptor

ERE, estrogen response element

EXE, exemestane

ERU, estrogen response unit

FBS, fetal bovine serum

FOXA1, Foxhead Box A1

FUL, fulvestrant

GAPDH, glyceraldehyde 3-phosphate dehydrogenase;

HSD, 17β-hydroxysteroid dehydrogenase

kb, kilobase pair;

MT, mutated construct;

qRT-PCR, quantitative reverse transcriptase polymerase chain reaction

SARM, selective androgen receptor modulator

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SEM, standard error of mean;

UGT, UDP-glucuronosyltransferase;

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Abstract

Exemestane (EXE) is an aromatase inhibitor indicated for endocrine therapy of breast cancer in post-menopausal women. The primary active metabolite of EXE, 17-hydroexemestane (17-HE), is inactivated via glucuronidation, mainly by UGT2B17. UGT2B17 also has a primary role in inactivation of endogenous androgens testosterone and dihydrotestosterone (DHT) and may play an important role in regulation of breast and prostate tumour intracrinology. We recently reported that *UGT2B17* could be induced by both estrogenic and androgenic ligands in breast cancer cells via binding of the estrogen receptor alpha (ERα) or the androgen receptor (AR) to a complex regulatory unit in the proximal *UGT2B17* promoter. In this study we show that both EXE and 17-HE increase UGT2B17 mRNA levels in breast cancer MCF-7 and MDA-MB-453 cells, and increase glucuronidation of UGT2B17 substrates, including 17-HE and androsterone. Using antagonists of ERα and AR, as well as siRNA-mediated inhibition, we demonstrate that EXE and 17-HE induce *UGT2B17* expression primarily via the AR. This result is consistent with previous reports that 17-HE can act as an AR ligand. *In vitro* studies suggest that multiple steroid-responsive DNA elements within the proximal promoter are involved in the response to 17-HE-liganded AR. The upregulation of *UGT2B17* by EXE and 17-HE in breast cancer cells might enhance the local metabolism of 17-HE as well as that of endogenous androgens, hence impacting potentially on treatment outcomes.

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Introduction

Breast cancer is the most commonly diagnosed cancer in women. Approximately 60% to 75% of breast cancers are estrogen receptor alpha positive ($ER\alpha^+$) (Barnes, 2004) and depend on estrogen for their proliferation (Miller et al., 2008). In postmenopausal women who do not produce ovarian estrogen, conversion of adrenal androgens to estrogens in peripheral tissues and/or locally in breast tissue by aromatase is a key driver of $ER\alpha^+$ breast cancer cell growth (Yue et al., 1998; Chetrite et al., 2000; Simpson et al., 2002). Aromatase inhibitors (AI) are indicated for these patients as first or second line therapies (Goss, 2003).

Exemestane is a steroidal (type I) aromatase inhibitor that is structurally related to the natural aromatase substrate 4-androstenedione. It is a mechanism based inactivator that binds irreversibly to the active site of aromatase. Postmenopausal patients with early stage $ER\alpha^+$ breast cancer typically receive two to three years of tamoxifen followed by two-three years of exemestane for a total of five years of hormonal therapy (Coombes et al., 2004; van de Velde et al., 2011). This regimen has been shown to improve relapse-free survival over continuing tamoxifen therapy (Miller et al., 2008; van de Velde et al., 2011). Exemestane may also be used as primary therapy for early stage invasive breast cancer (Ruddock and Molinari, 2006; Miller et al., 2008), and for advanced metastatic breast cancer that has progressed following tamoxifen therapy (Lonning and Geisler, 2008; Walker et al., 2013).

Exemestane is converted into an active metabolite 17-hydroexemestane (17-HE), which has comparable anti-aromatase activity to exemestane (Kamdem et al., 2011). In addition to binding to aromatase, 17-HE binds weakly to ERα and with moderate/high affinity to the androgen receptor (AR). The majority (67-88%) of $ER\alpha^+$ breast cancers express the AR (Moinfar et al., 2003; Hu et al., 2011; Loibl et al., 2011). 17-HE was reported to induce proliferation and gene activation at micromolar concentrations through ER in both MCF7 and T47D breast cancer cell lines, and via AR at low nanomolar concentrations

selectively in T47D cells (Ariazi et al., 2007). It has been suggested that circulating levels of 17-HE (representing approximately 10% of the parent compound in serum (Evans et al., 1992)) may be sufficient to regulate AR-dependent activities that decrease bone density loss (Ariazi et al., 2007). Moreover, 17-HE is reported to inhibit breast cancer cell survival by mechanisms different from those of exemestane (Amaral et al., 2015); it is possible that this involves AR-agonism.

UDP-glucuronosyltransferases (UGTs) constitute an enzyme superfamily responsible for the glucuronidation and inactivation of numerous therapeutic drugs and endobiotics including steroid hormones. The major exemestane metabolite found in human urine is the inactive glucuronide of 17-HE. *In vitro* studies demonstrate that UGT1A4, 2B17, 1A8 and 1A10 are all able to glucuronidate 17-HE, with UGT2B17 showing the highest activity (Sun et al., 2010). Supporting the primary role of UGT2B17 in exemestane metabolism, UGT2B17 expression levels correlate strongly with 17-HE-glucuronide production in liver microsomes (Sun et al., 2010). Moreover, patients with the *UGT2B17* null genotype showed lower levels of 17-HE-glucuronide in plasma (Chen et al., 2016). UGT1A4 and UGT2B17 are abundant in liver while UGT1A8 and UGT1A10 are predominantly expressed in the gastrointestinal tract. UGT2B17, UGT1A8 and UGT1A10 are also expressed at varying levels in normal and cancerous breast tissues (Thibaudeau et al., 2006; Nakamura et al., 2008; Starlard-Davenport et al., 2008; Zhu et al., 2016) and could be involved in intratumoural conjugation of 17-HE.

UGT2B17 and its paralog, UGT2B15, conjugate androgens including testosterone and dihydrotestosterone (DHT) (Chen et al., 1993; Beaulieu et al., 1996). *UGT2B17* and *UGT2B15* are widely expressed (Levesque et al., 1997; Tchernof et al., 1999; Nakamura et al., 2008; Ohno and Nakajin, 2009; Jones and Lazarus, 2014) including in sex hormone-sensitive tissues suggesting roles in maintaining intratissular hormone homeostasis. Indeed, UGT2B17 and UGT2B15 expression and function are linked to the risk of developing

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androgen-sensitive prostate diseases (Barbier and Belanger, 2008; Gauthier-Landry et al., 2015). We recently showed that high intratumoural *UGT2B17* and *UGT2B15* expression levels correlate with better survival outcomes in distinct genetic subgroups of breast cancer (Hu et al., 2016) although how this relates to altered intratumoural steroid metabolism remains to be determined.

We previously defined a conserved 282 bp region of the *UGT2B17* and *UGT2B15* proximal promoters as an estrogen response unit (ERU) that binds to ERα and mediates induction by estrogens in a FOXA1-dependent manner (Hu and Mackenzie, 2009). More recently we determined that the ERU can mediate both estrogenic and androgenic responses in breast cancer cells via binding to both ERα and AR in a FOXA1-dependent manner (Hu et al., 2016). In the present study, we demonstrate that both exemestane and 17-HE upregulate *UGT2B17* and *UGT2B15* expression in breast cancer cells. Analysis of the *UGT2B17* promoter indicates that induction by exemestane/17-HE is mediated by recruitment of AR to the ERU. We propose that induction of *UGT2B17* could facilitate intratumoural inactivation of 17-HE; moreover, induction of both *UGT2B17* and *UGT2B15* could alter tumour intracrinology, possibly impacting on cancer progression.

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Materials and Methods

Chemicals

Analytical grade chemicals including exemestane (EXE), bicalutamide (BIC), fluvestrant (FUL) were purchased from Sigma-Aldrich (St. Louis, MO). 17-Hydroexemestane (17β-hydroxy-6-methyleneandrosta-1,4dien-3-one) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada).

Cell Culture and Drug treatment, RNA extraction and Reverse Transcriptase Qualitative Real-time Polymerase Chain Reaction.

MCF-7 and MDA-MB-453 breast cancer cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Grand Island, NY) supplemented with 5% (v/v) fetal bovine serum (FBS) at 37 ºC and 5% CO_2. Cell lines were cultured in phenol-red free RPMI 1640 medium supplemented with 5% dextran-coated charcoal-stripped FBS (DCC-FBS) for 3 days prior to treatment with various drugs for 24 hours. Total RNA was isolated using TRIzol reagent (Life Technologies) and reverse transcribed using reagents from Invitrogen (Mulgrave, VIC, Australia) as previously described (Hu and Mackenzie, 2009). Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) was performed using the RotorGene 3000 (Corbett Research, NSW, Australia), and GoTaq qPCR master mix (Promega, Madison, WI). Sequences of PCR primers that amplify 18S rRNA, UGT1A4, UGT1A8, UGT1A10, UGT2B15, UGT2B17, and GAPDH were reported previously (Hu and Mackenzie, 2009; Hu et al., 2010). Data was analysed using the delta-delta cycle threshold method (Livak and Schmittgen, 2001), mRNA levels of target genes were normalized to the levels of 18S rRNA in the same sample and the fold change in mRNA levels after drug treatment was presented relative to levels in vehicle-treated cells (set as a value of 1). All treatments were performed in triplicate.

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Glucuronidation Assays

MCF-7 cells were treated in duplicate with vehicle (0.1% dimethyl sulfoxide, DMSO), 100 nM EXE, or 10 nM 17-HE for 72 hours. Whole-cell lysates were harvested in TE buffer (10 mM Tris-HCl , 1 mM EDTA, pH 7.6). Protein concentrations were determined using the Bradford protein Assay (Bio-rad, Hercules, CA). Androsterone glucuronidation assays were performed by incubating 100 µl of reaction mixture containing 0.2 mM UDP-glucuronic acid (Sigma-Aldrich), and 319 pmol of [^{14}C] UDP-glucuronic acid (PerkinElmer, Boston, MA), 0.2 mM androsterone, 100 mM potassium phosphate (pH 7.5) and 4 mM $MgCl_2$ with cell lysates containing 450 µg of protein at 37 ^{o}C for 2 hours. 17-HE glucuronidation assays were performed under the same conditions using 0.2 mM 17-hydroexemestane as substrate. Reactions were terminated by addition of 200 µl of ethanol and centrifugation at 12,000 rpm for 5 minutes. One-hundred microliters of supernatant from each sample was applied to a thin layer chromatography (TLC) plate (250 µm silica gel; TLC Uniplates; Analtech, Newalk, DE). Human UGT2B17 supersomes (In Vitro Technologies, Noble Park North, VIC, Australia) were included as positive controls in each assay. Chromatography and quantification of glucuronide band intensities were conducted as previously reported (Chanawong et al., 2015; Wijayakumara et al., 2015). Fold induction of glucuronidation activity in drug treated cells was calculated relative to vehicle treated cells.

Knockdown of gene expression using siRNA

ON-TARGETplus SMARTpools targeting ERα (siERα), AR (siAR), FOXA1 (siFOXA1), and a nontargeting control siRNA pool (siCtr), were purchased from Dharmacon RNAi technologies (Lafayette, CO). MCF7 cells (5 x 10^5 cells per well) were plated in 6-well plates and transiently transfected with either siCtr, siERα, siAR, or siFOXA1 at 100 nM

using 8 µl Lipofectamine 2000 (Invitrogen) as described previously (Hu and Mackenzie, 2009). Twenty-four hours post transfection, media were replaced with phenol-red free RPMI supplemented with 5% DCC–FBS. Forty-eight hours after transfection, cells were treated with EXE, 17-HE or vehicle in quadruplicate. Twenty-four hours post drug treatment three wells from each condition were harvested for RNA preparation and gene expression analysis by qRT-PCR. The cells of the fourth wells of each condition were cultured for an additional 24 hours and then cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer.

Western Blotting

Twenty micrograms of total protein from each sample was separated in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Biorad, Hercules, CA) for immunodetection. Membranes were probed with primary antibodies including anti-ER (HC-20), anti-AR (N-20) (Santa Cruz Biotechnology, Dallas, TX), anti-FOXA1 (Ab23738: Abcam, MA) followed by horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Neomarker; Fremont, CA). The same membranes were re-probed with GAPDH antibody (Sigma Aldrich) followed by secondary antibody as mentioned above. Immunosignals were detected with the SuperSignalWest Pico Chemiluminescent kit (Thermo-Fisher Scientific, Waltham, MA) and an ImageQuant LAS 4000 luminescent image analyzer (GE Healthcare, Chalfont St. Giles, United Kingdom). Quantitation of band intensity and background subtraction was performed using MultiGuge version 3.0 (Fujifilm Corporation, Tokyo, Japan) as described previously (Chanawong et al., 2015).

Transient transfection and Luciferase Reporter Assay.

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Two wild-type *UGT2B17* proximal promoter-pGL3 luciferase reporter constructs containing nucleotides - 2 to - 694 of *UGT2B17* and bearing either the common 'A' allele or the rarer 'G' allele within the polymorphic FOXA1 motif were described previously (Hu et al., 2010). The cloned promoter region contains one FOXA1 binding site, two estrogen response element (ERE) half sites, and one imperfect ERE site. The previously reported FOXA1 mutated *2B17-694/-2* promoter-reporter construct was also used in this study (Hu et al., 2010). As shown in Fig. 6A, this study generated seven additional mutated *2B17-694/-2* constructs using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), including mutation of a single ERE site [2B17 Mt-3'ERE half-site (2B17 Mt-S1-ERE), 2B17 Mt-5'ERE half-site (2B17 Mt-S2-ERE), and 2B17 Mt-Imperfect ERE (2B17 Mt-S3-ERE)], mutation of two ERE sites simultaneously [2B17 Mt-3'ERE half-site & 5'ERE half-site (2B17 Mt-D1-ERE), 2B17 Mt-3'ERE half-site & Imperfect ERE site (2B17 Mt-D2-ERE) and 2B17 Mt 5'ERE half-site & Imperfect ERE site (2B17 Mt-D3-ERE)], and mutation of three ERE sites simultaneously [2B17 Mt-3'ERE half-site, 5'ERE half-site and Imperfect ERE site (2B17 Mt-T1)]. The sequences of the wildtype and mutated ERE motifs are shown in Table 1 (motifs are underlined and boldfaced). The sequences of the all promoter-reporter constructs were confirmed by DNA sequencing.

MCF-7 cells were seeded into 96-well plates at a density of 3 x 10^4 cells/well in phenol red-free RPMI supplemented with 5% DCC-FBS. After culture overnight, cells were transfected with 100 ng of wild-type or mutated promoter-reporter constructs combined with 2 ng of the control pRL-null (*Renilla*) vector using 0.4 ul of Lipofectamine 2000 (Invitrogen). Six hours post transfection, transfection medium was replaced with fresh medium containing 100 nM EXE, 10 nM 17-HE, or vehicle (0.1% DMSO). Cells were harvested after 48 hours in passive lysis buffer and firefly luciferase and *Renilla* luciferase activities analysed using the Dual-Glo Luciferase Reporter Assay System (Promega, Madison, WI) as described

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previously (Hu and Mackenzie, 2010). Firefly activities were normalized to *Renilla* activities, and then the activity of each promoter-reporter construct was normalized to that of the empty pGL3 basic vector within each treatment group. Fold induction of promoter activities after drug-treatment was calculated by normalization to the vehicle-treatment for each promoter-reporter construct.

Statistical Analysis

Statistical analysis was performed using one-way or two-way analysis of variance followed by Tukey's posthoc test as indicated in the figures legends. A p-value ≤ 0.05 was considered to be statistically significant.

Results

Exemestane induces expression of UGT2B17 in MCF7 breast cancer cells

Previous work shows that UGT2B17, UGT1A4, UGT1A8 and UGT1A10 conjugate the main active metabolite of exemestane, 17-hydroexemestane (17-HE), with UGT2B17 having the highest catalytic activity (Sun et al., 2010) (Figure 1A). UGTs are frequently induced by molecules that they metabolize as part of a regulatory feedback loop; hence we considered the possibility that some of these UGTs might also be induced by exemestane. To test this hypothesis, MCF-7 cells maintained in either standard media (Figure 1B) or steroid-reduced media (Figure 1C) were treated with exemestane (100 nM), and mRNA levels of the four UGTs responsible for exemestane-17-O-glucuronide formation, UGT2B17, UGT1A4, UGT1A8, and UGT1A10, were assessed. Exemestane significantly increased the level of UGT2B17 mRNA but not that of UGT1A4, UGT1A8 and UGT1A10 (Figure 1). Interestingly we recently showed that UGT2B17, which in addition to liver is expressed in steroid responsive tissues and tumours, is induced by both estrogenic and androgenic signalling cascades in breast cancer cells (Hu et al., 2016). In contrast, the other UGTs discussed here have not shown significant induction by steroid signalling in previous work. The active exemestane metabolite 17-HE can act as both a weak estrogenic and strong androgenic ligand, suggesting that this metabolite might induce UGT2B17 via either ER or AR signalling.

To begin to assess whether the estrogenic or androgenic function of the exemestane metabolite 17-HE might be responsible for induction of UGT2B17, we treated both ER+/AR+ MCF7 and ER-/AR+ MDA-MB-453 breast cancer cells with exemestane (100 nM) or 17-HE (10nM) (17-HE). Both drugs elevated UGT2B17 mRNA levels in both cell lines in a dose-dependent manner (Figure 2). Moreover, both exemestane and 17-HE increased androsterone and 17-HE glucuronidation activities in MCF7 cells, indicating an increase in functional UGT2B17 protein (Figure 3). The ability of exemestane and 17-HE to induce

UGT2B17 expression in ER-/AR+ MDA-MB-453 cells suggests a primary role for the androgen receptor signalling pathway.

The AR antagonist, bicalutamide (BIC) and the ERα antagonist, fulvestrant (FUL) were used to assess the relative role of ER and AR in induction of UGT2B17 expression by exemestane and 17-HE. In MCF-7 cells BIC (10 μM) but not FUL (1 μM) blocked induction of UGT2B17 by both drugs, suggesting the specific involvement of AR (Figure 4A and 4B). Consistent with this interpretation, knock-down of AR with siRNA (Figure 4C) led to almost complete inhibition of exemestane and 17-HE mediated induction of UGT2B17 mRNA (Figure 4D).

Our recent work showed a critical role for FOXA1 in the induction of UGT2B17 by estrogenic and androgenic ligands (estradiol, tamoxifen, dihydrotestosterone, enobosarm) (Hu et al., 2016), hence we examined whether FOXA1 may play a role in induction of UGT2B17 by exemestane/17-HE. FOXA1 siRNA dramatically reduced but did not abolish induction of UGT2B17 by both drugs (Figure 5B); the residual activation may be due to incomplete inhibition of FOXA1 expression (Figure 5A).

To define the region of the UGT2B17 promoter that is involved in induction by exemestane/17-HE, we used UGT2B17 promoter-reporter constructs containing 694 bp of the proximal promoter including the previously defined ERU (Hu et al., 2016). Mutated (Mt) reporter constructs were used that contain deletions in the FOXA1 binding motif and the three ER/AR binding motifs (imperfect ERE, 5' ERE half-site, 3' ERE-half site) (Figure 6A). In addition, we used a UGT2B17 promoter construct that contains a naturally occurring SNP in the FOXA1 motif that is referred to as the 'G' allele (the most common allele has an 'A' residue at this position). Previous work showed that the presence of the 'G' residue in the motif reduced binding of FOXA1 (Hu et al., 2010). The wild-type promoter construct was activated 4-5 fold by both ligands (Figure 6B, 6C). The promoter containing the FOXA1 'G'

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allele was not activated by exemestane/17-HE, neither was the construct with the mutated FOXA1 motif. Deletion of single ERE motifs modestly reduced activation; double mutations of the EREs reduced activation further, and the triple ERE mutation abolished activation (Figure 6B, 6C).

Our previous work showed that *UGT2B17* and *UGT2B15* are regulated similarly by estrogens and androgens via the highly conserved ERU (Hu et al., 2016). To assess whether exemestane and 17-HE also induce *UGT2B15* and whether the mechanism may be the same for *UGT2B15* and *UGT2B17*, we treated MCF-7 (Figure 7A) and MDA-MB-453 (Figure 7B) breast cancer cells with exemestane (100 nM) or 17-HE (10 nM), with or without BIC or FUL. *UGT2B15* mRNA expression was induced by both drugs and this was suppressed by BIC, but not FUL (Figure 7C). Similarly, siRNA targeting AR (siAR) but not siRNA targeting ERα (si-ERα) inhibited induction (Figure 7D). siRNA targeting FOXA1 also inhibited induction by EXE and 17-HE, suggesting that induction of *UGT2B17* and *UGT2B15* by these drugs involves very similar molecular mechanisms.

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Discussion

We recently showed that *UGT2B17* and *UGT2B15* are regulated in ER^+/AR^+ breast cancer cells by estrogenic and androgenic ligands via binding of ERα and AR to a response unit in the proximal promoters of both genes that we named the ERU (Hu and Mackenzie, 2009; Hu et al., 2016). In the current study, we found that *UGT2B17* (and *UGT2B15*) could be induced by exemestane and its active metabolite, 17-HE. Consistent with the previous observation that 17-HE could act as an AR agonist with high nanomolar affinity, 17-HE induced these genes specifically through the AR. The affinity of exemestane for AR (measured by competitive binding assays) is approximately 100-fold lower than that of 17-HE (Ariazi et al., 2007); hence the ability of exemestane to potently induce *UGT2B17/UGT2B15* at only 10-fold higher concentration than 17-HE, suggests that it is at least partially converted to 17-HE within the breast cancer cell lines. 17-HE is known to be formed in liver predominantly by CYP1A1/2 (Kamdem et al., 2011) and aldo-keto reductases (AKRs) (Sun et al., 2011). The conversion of exemestane to 17-HE is likely to also occur in breast tissue and breast cancer cell lines where these enzymes are expressed (Goth-Goldstein et al., 2000; Penning and Byrns, 2009; Rodriguez and Potter, 2013; Yin et al., 2014). The relative roles of CYP and AKR enzymes in the production of 17-HE in the breast cancer cell lines used in this study is a subject for further investigation.

The *UGT2B17/UGT2B15* ERU contains three ERE-like motifs that we previously named the imperfect ERE, 5' ERE half site, and 3' ERE half site, as well as a FOXA1 binding site. We previously showed that the FOXA1 motif was important for AR activity (Hu et al., 2016) and that the 'G' allele SNP in the FOXA1 motif impaired FOXA1 binding. Consistent with this, a UGT2B17 promoter construct containing the 'G' allele sequence was not induced by 17-HE. Of note, both MCF7 and MDA-MB-453 cells are homozygous for the more common 'A' allele that confers efficient binding of FOXA1 (not shown). These data

suggest that individuals homozygous for the 'G' allele may not induce UGT2B17 in response to exemestane; however the therapeutic consequence of this, if any, will require further study as discussed below.

In recent work we used high resolution ChIP analysis and mutational analysis of the *UGT2B15* promoter in MCF7 cells to show that the imperfect ERE may bind predominantly to ERα, whereas the 3' ERE might be the predominant binding site for AR (Hu et al., 2016). These previous studies used DHT and the selective androgen receptor modulator (SARM) enobosarm as AR ligands. In contrast, in the current study, mutational analysis of the *UGT2B17* promoter in MCF7 cells suggested that all three ERE-like motifs may play equal and additive roles in the response to 17-HE-liganded AR. Moreover, the motifs are at least partially redundant, as deletion of all three motifs was required to completely eliminate the response to these drugs.

It is also notable that in our previous study, induction of *UGT2B15* mRNA by DHT was only observed in ER^+/AR^+ breast cancer cell lines (e.g. MCF7) and not in ER^-/AR^+ (e.g. MDA-MB-453) cells; this together with other findings, suggested that activation required the presence of both AR and ER and implied functional interaction of these receptors (Hu et al., 2016). In contrast to these previous findings, in the present study both *UGT2B15* and *UGT2B17* mRNA could be induced by 17-HE in both MCF7 and MDA-MB-453 cells. The differences in the ability of different AR ligands to induce *UGT2B15* and *UGT2B17* in an ER^- context, suggests that 17-HE and DHT may induce different conformations of the AR, which may in turn mediate different functional interactions with co-factors. This hypothesis will require further investigation. However, it is also likely that the *UGT2B17* promoter has inherent differences in sequence/structure that allow it to respond differently to the *UGT2B15* promoter. In support of this we recently found that unlike *UGT2B15*, *UGT2B17* can be weakly induced by DHT in MDA-MB-453 cells (not shown), albeit to a much lesser extent

than by 17-HE. This suggests that indeed the promoters can respond differently even to the same ligand.

We recently showed that *UGT2B15* can be induced by tamoxifen and its active metabolite 4-OH tamoxifen via the ERU (Chanawong et al., 2015). Because UGT2B15 glucuronidates and inactivates 4-OH-tamoxifen, its intratumoural induction by tamoxifen generates a potential regulatory feedback loop that might impact on therapeutic efficacy and promote the acquisition of drug resistance (Chanawong et al., 2015). The ability of UGT2B17 to conjugate 17-HE suggests the possibility of a similar regulatory feedback loop in which exemestane/17-HE-mediated induction of UGT2B17 increases local inactivation of 17-HE. However, the relevance of this feedback loop to exemestane efficacy *in vivo* depends upon whether aromatase and UGT2B17 are co-expressed. *In situ* aromatization of androgens is an important mechanism for promoting growth of ER^+ breast cancer. While this is considered primarily a paracrine effect with aromatase expressed in breast stromal fibroblasts and preadipocytes/adipocytes (Zhou et al., 2001; Catalano et al., 2014; Wang et al., 2015), there is also evidence for aromatase expression in some carcinoma cells within invasive ductal carcinoma as well as in some breast cancer cell lines (Chetrite et al., 2000; Rajhans et al., 2008; Sasano et al., 2009; McNamara and Sasano, 2015; Takagi et al., 2016). Moreover, co-culture of MCF7 cells with breast cancer stromal cells was able to induce aromatase expression in the former. (Sasano et al., 2009). Hence a small subset of tumours may have functionally relevant autocrine ER signalling mediated by aromatization within carcinoma cells, in addition to stromal estrogen synthesis. In such tumours, induction of UGT2B17 by exemestane/17-HE in carcinoma cells might lead to reduction in exemestane efficacy.

It is also possible that UGT2B17 is expressed in breast tumour stromal tissue, and this requires further investigation. In support of the latter idea, UGT2B17 was found to be highly expressed in stromal fibroblasts of normal prostate (Barbier et al., 2000). Of note, AR is also

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expressed in breast cancer stromal cells (Lanzino et al., 2016). To date studies of exemestane metabolism in breast cancer patients with the *UGT2B17* null genotype (~12% of the population) have been restricted to measuring altered levels of 17-HE-glucuronide in plasma (Chen et al., 2016). The effect of the *UGT2B17* null genotype (and indeed the effect of *UGT2B17*-FOXA1 motif polymorphism) on intratumoural drug levels, and whether this may impact on exemestane efficacy, remains to be examined.

The role of androgens in $ER\alpha^+/AR^+$ breast cancer remains poorly defined, although AR expression has been associated with reduced mortality (Castellano et al., 2010; Hu et al., 2011), and androgens may antagonize estrogenic signalling by a variety of mechanisms (Hickey et al., 2012). In contrast, in $ER\alpha^-/AR^+$ breast cancers, or those in which ERα has been functionally ablated by long term anti-estrogen therapy, androgen signalling may be pro-proliferative (Cochrane et al., 2014; Barton et al., 2015; Zhu et al., 2016). Aromatase activity reduces local and circulating androgen levels by conversion to estrogens; exemestane has been shown to significantly increase intratumoural levels of DHT *in vivo* (Takagi et al., 2010). Exemestane also increases expression of 17β-hydroxysteroid dehydrogenase (HSD)2 that converts estradiol to the weak estrogen estrone, hence further reducing estrogenic signalling (Takagi et al., 2010). The combined effect of the increase in DHT, decrease in estrogen synthesis, and increased conversion of estradiol to estrone, may all contribute to reduced breast cancer growth through lowering the estradiol:DHT ratio (Takagi et al., 2010).

As DHT and testosterone are primarily inactivated by UGT2B17 and UGT2B15 (Chouinard et al., 2008), the induction of these UGTs by exemestane/17-HE could have an anti-androgenic effect in breast cancer cells. In particular, a reduction in DHT levels due to UGT2B17 metabolism may reduce the amount of substrate for aromatase (if these enzymes are expressed in the same cells as discussed above) and reduce estrogen production by this pathway. This could potentially reduce the growth of ER^+ breast cancers (McNamara et al.,

2014), and this might even offset the potentially deleterious consequences of increased UGT2B17-mediated 17-HE clearance from tumours. However as mentioned above, AR appears to have context-dependent roles in breast cancer, and may be influenced by ER:AR ratios and prior hormonal therapy. In particular, androgens have been shown to promote growth of some ER^+/AR^+ breast cancer cells *in vitro* and xenografts which has been correlated to higher AR:ER expression ratios (Cochrane et al., 2014), and high AR expression has also been linked to acquired tamoxifen resistance (De Amicis et al., 2010). In such contexts the upregulation of UGT2B17 and UGT2B15 by exemestane might be directly beneficial in inhibiting androgen-driven growth pathways. However, given the complexities of androgen and AR functions in breast cancer, it is clear that that further work, including UGT perturbation *in vitro* and in *in vivo*, will be required to determine whether UGT-mediated androgen inhibition is a functionally important consequence of exemestane therapy, and if so by what mechanisms.

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Authorship Contributions

Participated in research design: Meech, Hu, Mackenzie, Chanawong,, and McKinnon

Conducted experiments: Chanawong, Hu

Performed data analysis: Chanawong, Meech, Hu, and Mackenzie

Wrote or contributed to the writing of the manuscript: Meech, Chanawong, Hu, Mackenzie, and McKinnon

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Footnotes

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Figure Legends

Fig 1

A. The structure of exemestane (EXE) and its primary active metabolite 17-hydroexemestane (17-HE) and the identities of enzymes involved in their metabolism. **B, C.** Effects of EXE on expression of UGTs that inactivate 17-HE in MCF7 cells. MCF-7 cells maintained in **(B)** RPMI medium supplemented with 5% FBS (V/V) or **(C)** RPMI medium supplemented with 5% DCC FBS were treated in triplicate for 24 hours with 100 nM EXE or vehicle (Veh, 0.1% dimethylsulfoxide) and gene expression assessed by quantitative real-time PCR as described in *Materials and Methods*. Following normalizing to 18S rRNA, mRNA levels of target genes in EXE-treated cells are presented as fold change relative to the mRNA levels of the respective genes in the vehicle-treated cells (set as a value of 1) from two independent experiments. (* indicates statistical significant differences between vehicle and EXE treatment, using Two-way ANOVA, Tukey post-hoc analysis, ****p-value ≤ 0.0001).

Fig 2

Both exemestane (EXE) and 17-hydroexemestane (17-HE) elevate UGT2B17 mRNA levels in MCF-7 and MDA-MB-453 breast cancer cells. MCF-7 cells **(A)** and MDA-MB453 cells **(B)** were maintained in RPMI medium supplemented with 5% DCCFBS and then treated with 100 nM EXE, 10 nM 17HE or vehicle (Veh, 0.1% dimethysulfide) for 24 hours; gene expression was assessed by quantitative real-time RT-PCR. The dose dependence of *UGT2B17* mRNA induction in MCF7 cells by EXE **(C)** and 17HE **(D)** was assessed over a 4-fold log range. Data shown are fold induction (mean ± SEM) of target mRNA levels from drug-treated MCF7 cells over vehicle-treated performed in triplicate from two independent experiments. Statistical analyses used one-way ANOVA followed by Tukey's post hoc. p-value ≤ 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001, ****p-value ≤ 0.0001).

Fig. 3

Both exemestane (EXE) and 17-hydroexemestane (17-HE) increase androsterone and 17-HE glucuronidation activities in breast cancer cells. MCF-7 cells were treated with 100 nM EXE,

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10 nM 17-HE, or vehicle (Veh, 0.1% dimethysulfide) for 72 hours then subjected to glucuronidation assays as described in *Materials and Methods*. **(A)** Androsterone and **(B)** 17-HE glucuronidation activity in cells treated with EXE or 17-HE is shown relative to glucuronidation activity in vehicle-treated cells (set to 1). Data are the average of two independent experiments were performed in duplicate. (* indicates statistical significant differences between vehicle and ligands treatment, using One-way ANOVA, Tukey post-hoc, *p-value ≤ 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001, ****p-value ≤ 0.0001).

Fig.4

Exemestane (EXE) and 17-hydroexemestane (17-HE) induce UGT2B17 expression through the androgen receptor (AR) signalling pathway. AR antagonist; bicalutamide (BIC) but not estrogen receptor antagonist; fulvestrant (FUL) suppresses the induction of UGT2B17 expression by EXE and 17-HE. MCF-7 cells were treated with **(A)** 100 nM EXE, **(B)** 10 nM 17-HE, or vehicle, alone or together with 10 μM BIC or 1 μM FUL for 24 hours. Data shown are fold induction (mean ± SEM) of target mRNA levels from drug-treated cells over vehicle-treated cells from three independent experiments performed in triplicate. AR knock down **(C, D)**. MCF-7 cells were transfected with scrambled negative control siRNA (siCtr) or siRNA targeting AR (siAR) as described in *Materials and Methods***. (C)** Western Blot analysis showing knock-down of AR protein expression in cells transfected with siAR or siCtr and treated with EXE or 17-HE for 48 hours. **(D)** Fold changes (mean ± SEM) of target mRNA levels in cells transfected with siAR or siCtr and treated with EXE or 17-HE. Data are shown relative to vehicle treatment and are the average of three independent experiments performed in triplicate. (*indicates statistically significant differences, using Two-way ANOVA, Tukey post-hoc at ****p-value ≤ 0.0001).

Fig 5

FOXA-1 is required for exemestane (EXE)- and 17-hydroexemestane (17-HE)-mediated induction of *UGT2B17*. MCF-7 cells were transfected with scrambled siRNA (siCtr) or siRNA targeting FOXA1 (siFOXA1) as described in *Materials and Methods***.** (**A)** Western Blot analysis showing knock-down of FOXA1 protein expression after transfection of siCtr or siFOXA1 and treatment with EXE, 17HE, or vehicle for 48 hours. **(B)** Fold changes

(mean ± SEM) of target mRNA levels after transfection of siCtr or siFOXA1 and treatment with EXE or 17-HE. Data are shown relative to vehicle control and are the average of three independent experiments performed in triplicate. (* indicates statistical significant differences, using Two-way ANOVA, Tukey post-hoc at ***p-value ≤ 0.001, ****p-value ≤ 0.0001).

Fig 6

Exemestane (EXE) and 17-hydroexemestane (17HE) activate the *UGT2B17* proximal promoter via FOXA1 and ERE motifs. **A.** Schematic diagram of the *UGT2B17* promoter from nucleotide -2 to -694 showing one FOXA-1 binding site, two ERE-like half sites (3'ERE half-site and 5'ERE half-site), and an imperfect ERE-like inverted repeat. Mutations generated in these motifs within promoter constructs are indicated below. **B, C.** MCF-7 cells were transiently transfected with 2B17-694 wild-type (WT) or mutated (Mt) reporter constructs, as well as a version of the promoter containing a naturally occurring SNP in the FOXA1 site ('G' allele). Cells were then treated with **(B)** 100 nM EXE, **(C)** 10 nM 17-HE or vehicle for 48 hours. Relative luciferase activity was calculated as described in *Materials and Methods* and is presented as fold induction over empty vector and vehicle control. The statistical significance of differences between vehicle and ligand treatments for each reporter construct, and for differences between the activities of WT, SNP variant, and Mt constructs when treated with same ligand were assessed using Two-way ANOVA and Tukey post-hoc test. **p-value ≤ 0.01, ****p-value ≤ 0.0001.

Fig 7

Exemestane (EXE) and 17-hydroexemestane (17HE) induce UGT2B15 expression in an AR- and FOXA1-dependent manner. UGT2B15 mRNA levels were measured in MCF-7 **(A)** and MDA-MB-453 **(B)** breast cancer cells after treatment EXE (100 nM) or 17-HE (10 nM) for 24 hours. **(C)** Androgen receptor antagonist (10 μM BIC) but not estrogen receptor antagonist (1 μM FUL) suppresses the induction of UGT2B15 mRNA expression by EXE and 17HE in MCF7 cells. **(D)** siRNA targeting AR (siAR) but not siRNA targeting ERα (si-ERα) inhibit the induction of UGT2B15 mRNA by EXE and 17-HE in MCF7 cells. **(E)** siRNA targeting FOXA1 also inhibits the induction of UGT2B15 mRNA by EXE and 17-HE in MCF7 cells. Data shown are fold induction (mean ± SEM) of target mRNA levels from EXE- or 17-HE-treated cells relative to vehicle treated cells and are derived from two

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independent experiments performed in triplicate. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test. p-value ≤ 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001, ****p-value ≤ 0.0001).

Table 1 Wildtype and mutated ERE sequences of UGT2B17 promoter constructs used in this study.

	Nucleotide sequence (5' to 3')
WT 3'ERE	CTAAAATAAATATGA**GGTCA**ACTCAAATTTTAGCAG
Mt 3'ERE	CTAAAATAAATATGA**CTCGC**ACTCAAATTTTAGCAG
WT 5'ERE	TACTTACATATTCTA**GGTCA**TAAAAATTATTGCT
Mt 5'ERE	TACTTACATATTCTA**CAGCG**TAAAAATTATTGCT
WT Imperfect ERE	TACTTCTTCTCTCTA**TGTCA**AGGGCACCGAACAGG
Mt Imperfect ERE	TACTTCTTCTCTCTA**ATTAC**AGGGCACCGAACAGG

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Fig. 1

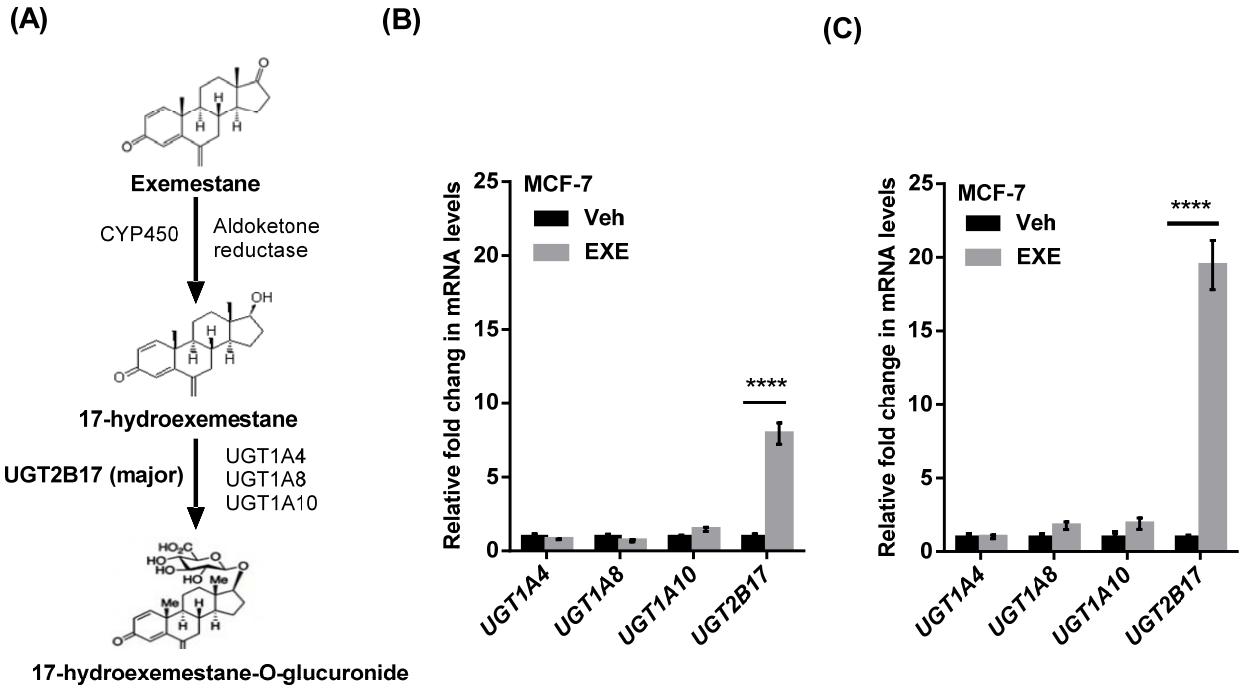

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Fig. 2

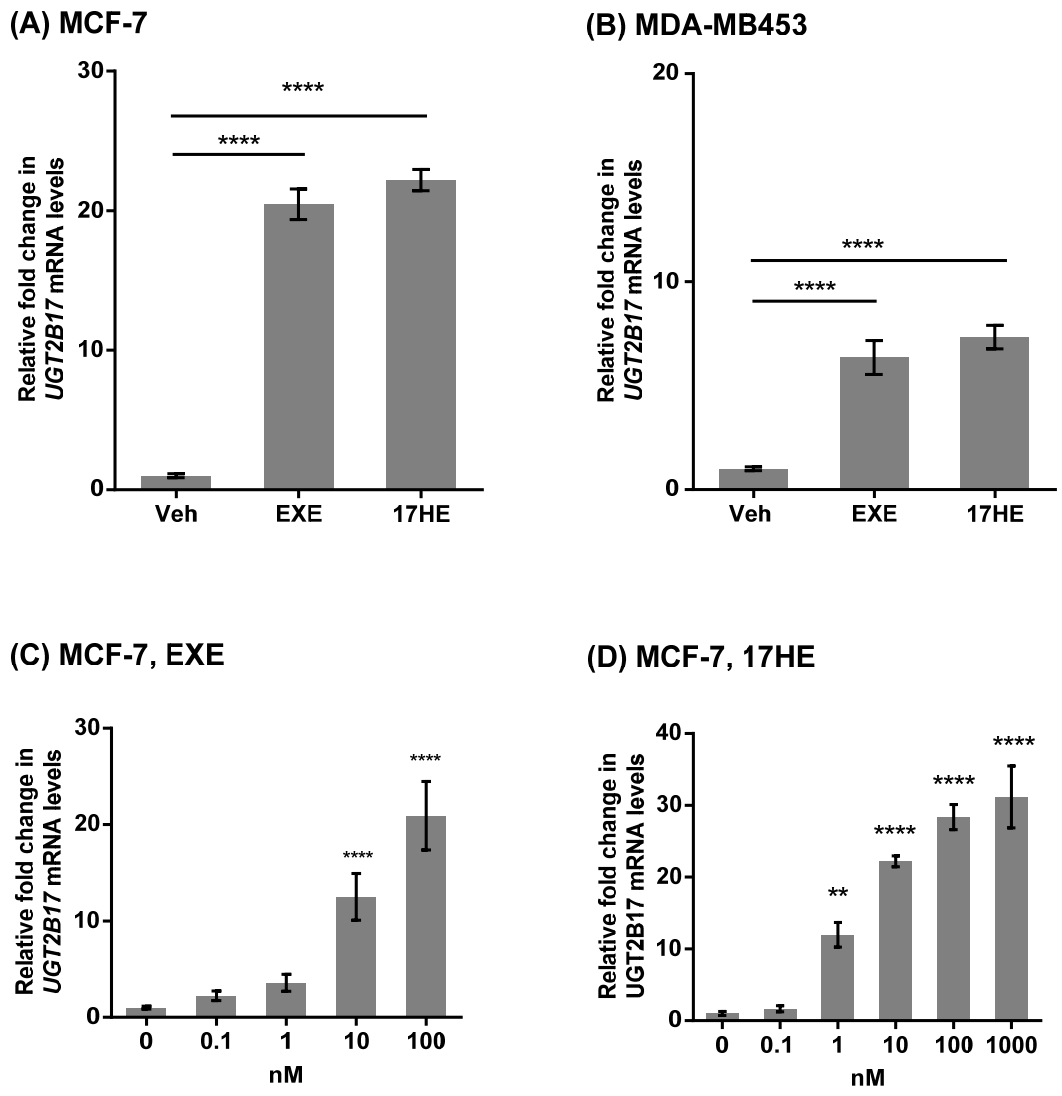

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Fig. 3

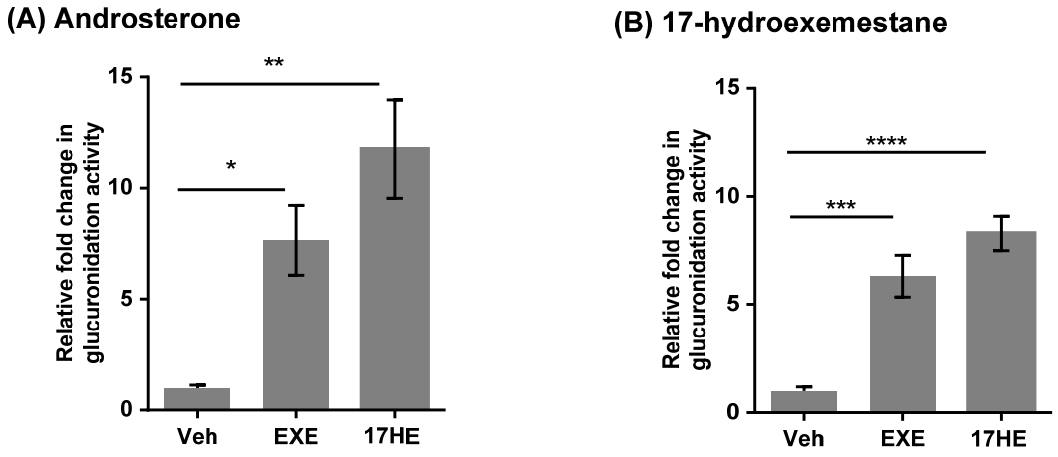

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Fig. 4

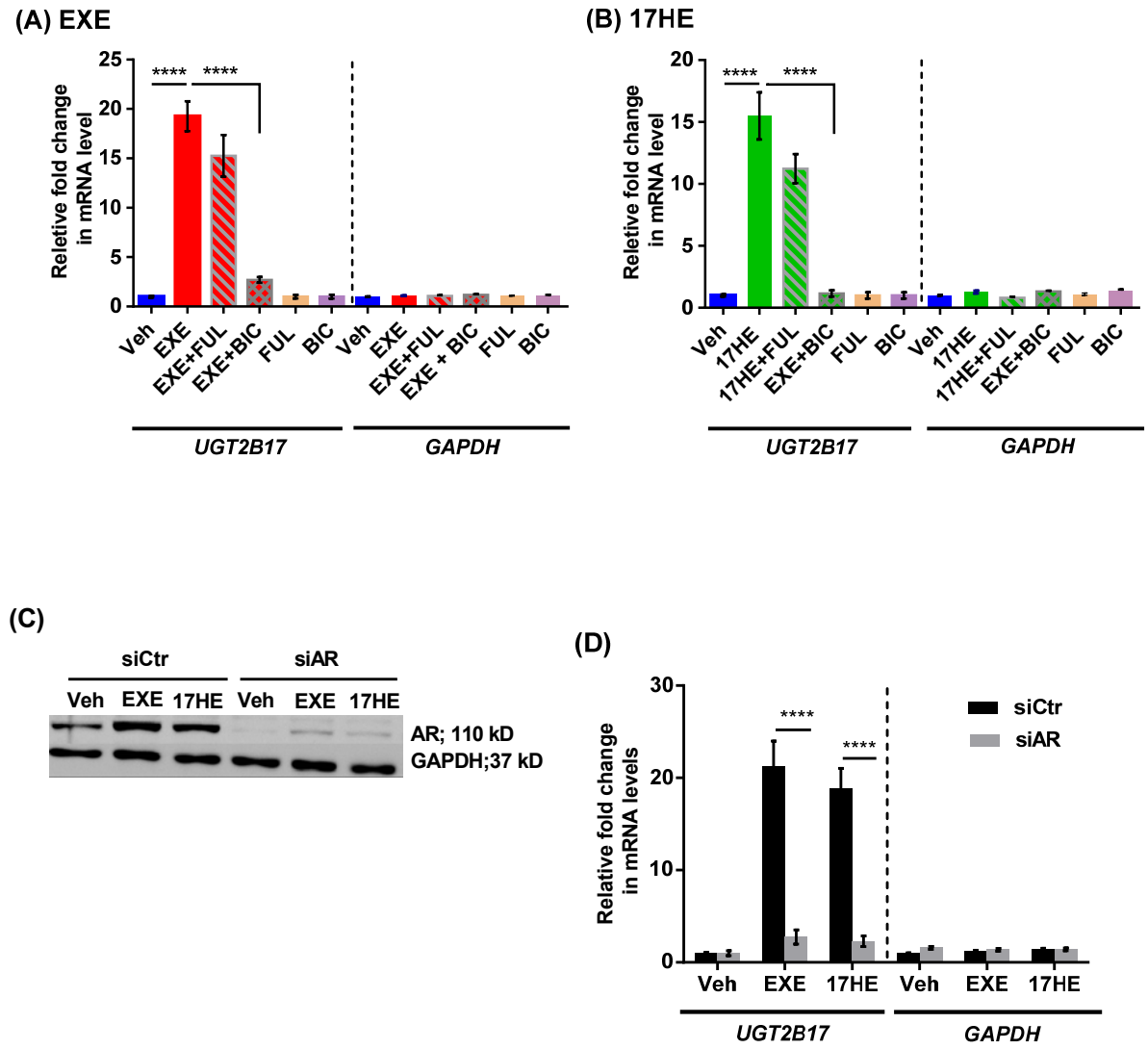

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Fig. 5

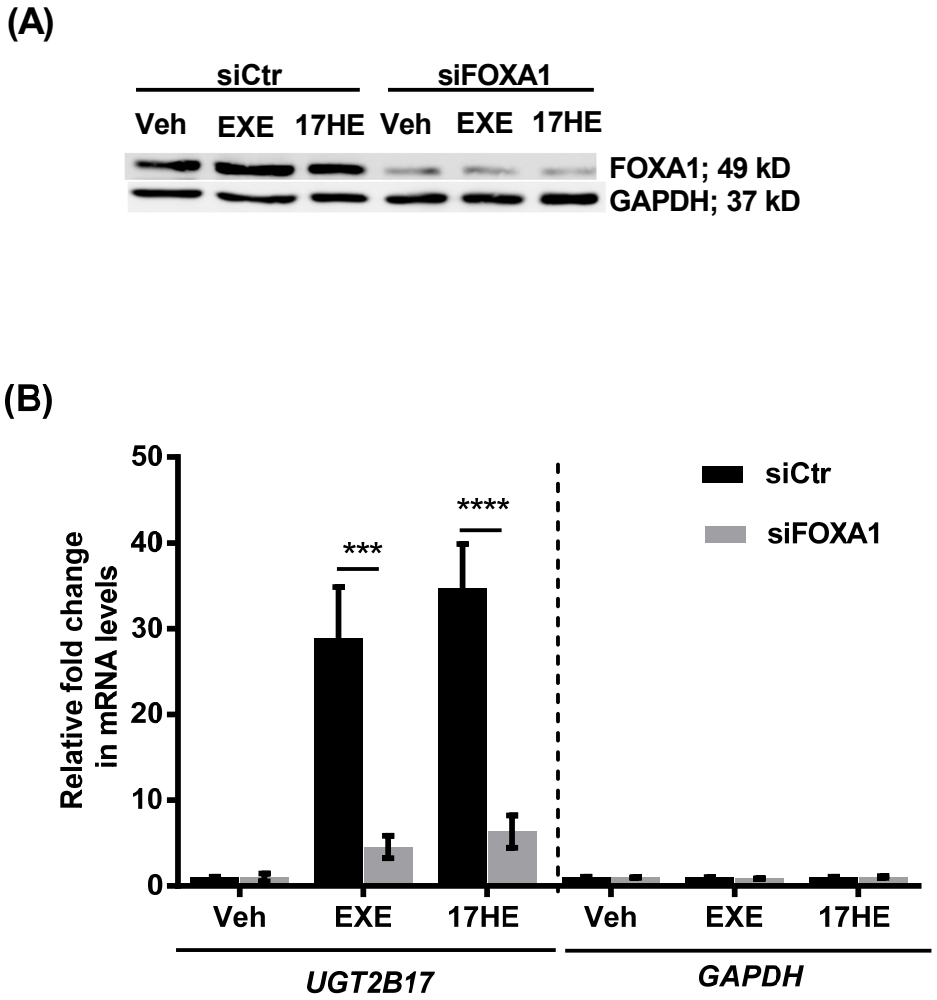

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Fig.6

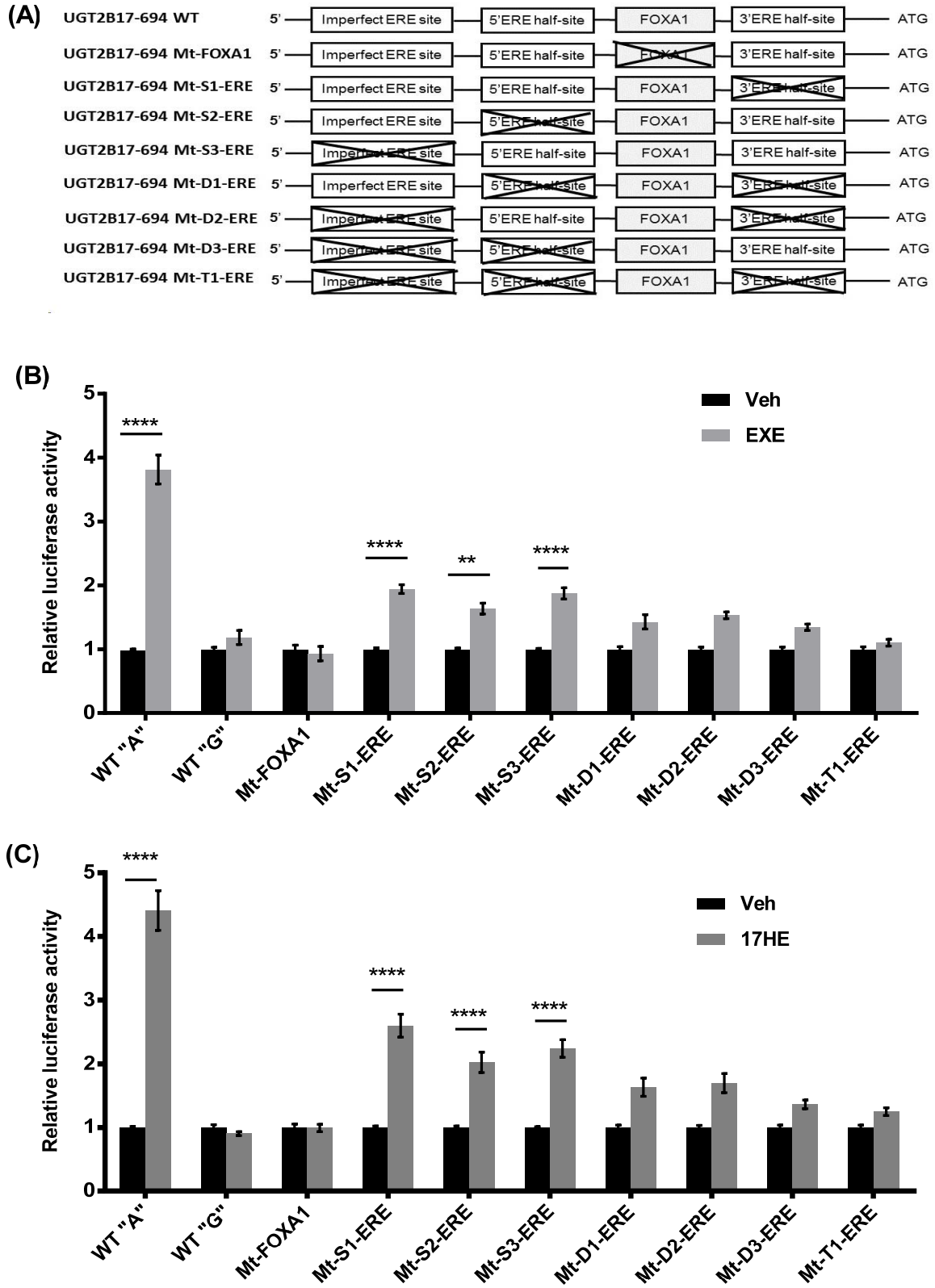

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Fig. 7