



Androgen Receptor Inhibition by Brominated Flame Retardants

Mary Jean See

U.S. EPA Office of Research and Development

Center for Computational Toxicology and Exposure



Outline

- Introduction
- Hypothesis
- Results
- Summary
- Next Steps

Disclaimer: Although this text was reviewed by EPA staff and approved for presentation, it does not necessarily reflect official EPA policy.



Flame Retardants

- Chemical mixtures added to consumer products after manufacturing.
- **Delay ignition and reduce combustion.**
- Commonly found in
 - Polyurethane foam and fabrics used in furniture
 - Plastics, insulation and electronics.



“Fire set in a modern room with many synthetic fibers spread much faster than in a comparable room using natural materials popular decades ago.”

<https://www.today.com/home/newer-homes-furniture-burn-faster-giving-you-less-time-escape-t65826>

- **California Technical Bulletin 117**

- 1975 California required all furniture filling materials to withstand a small open flame for at least 12 sec. without igniting.

2013 Test changed to require upholstery to resist igniting or smoldering from a lit cigarette.



California Technical Bulletin 117-2013



Flame Retardants

PBDEs

Penta-BDE, octo-BDE, deca-BDE

Cyclic Aliphatic Bromides

HBCD

Tetrabromobisphenol A

TBBPA

Brominated Phthalates

EH-TBB, BEH-TEBP

Chlorinated Phosphate Esters

TDCPP, TCEP

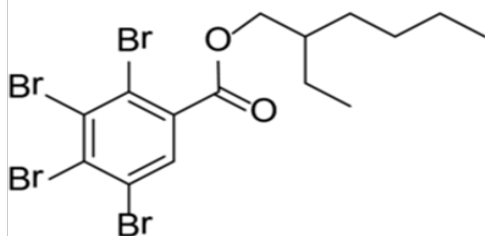
Organophosphates

ITP, TPP, TDCIPP



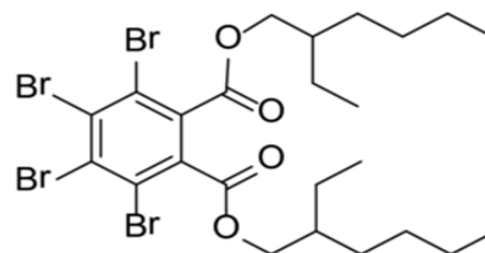
Firemaster 550[®] and BZ

EH-TBB



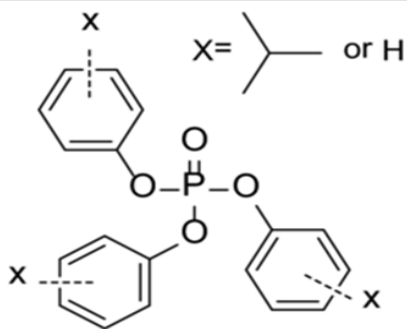
2-ethylhexyl-2,3,4,
5-tetrabromobenzoate

BEH-TEBP



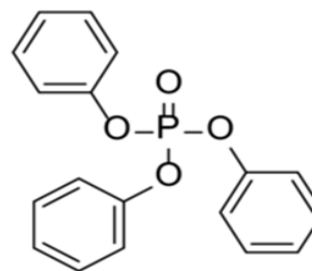
bis(2-ethylhexyl)-2,3,4,
5-tetrabromophthalate

ITPs



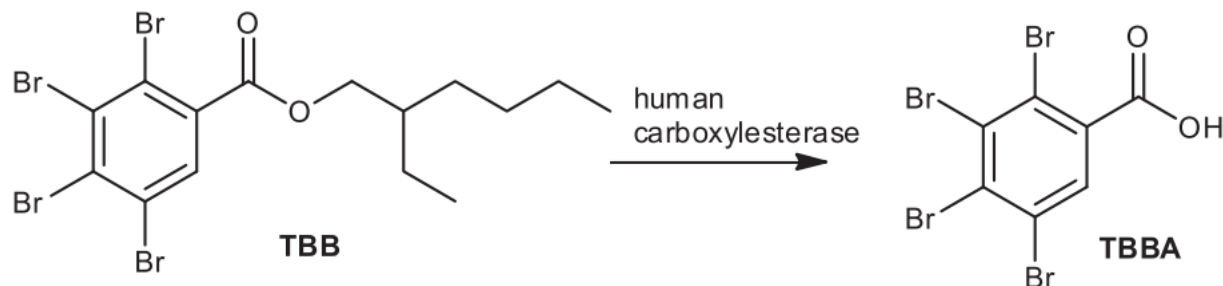
mixture of isopropylated
triphenylphosphate isomers

TPP



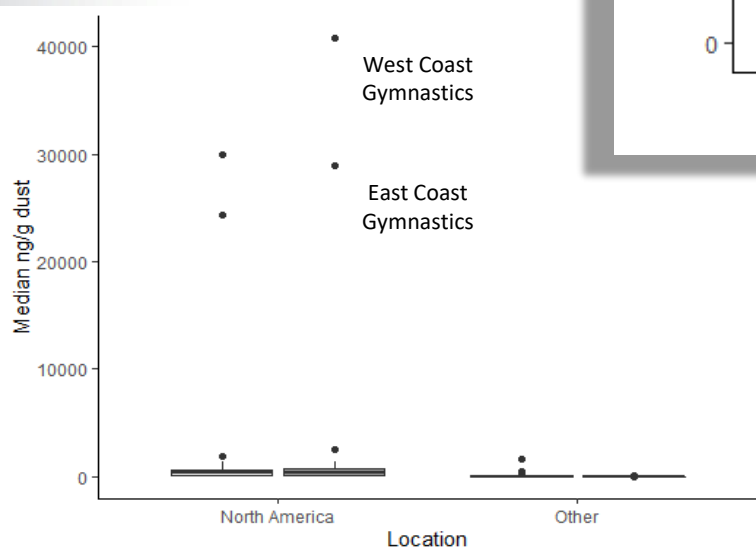
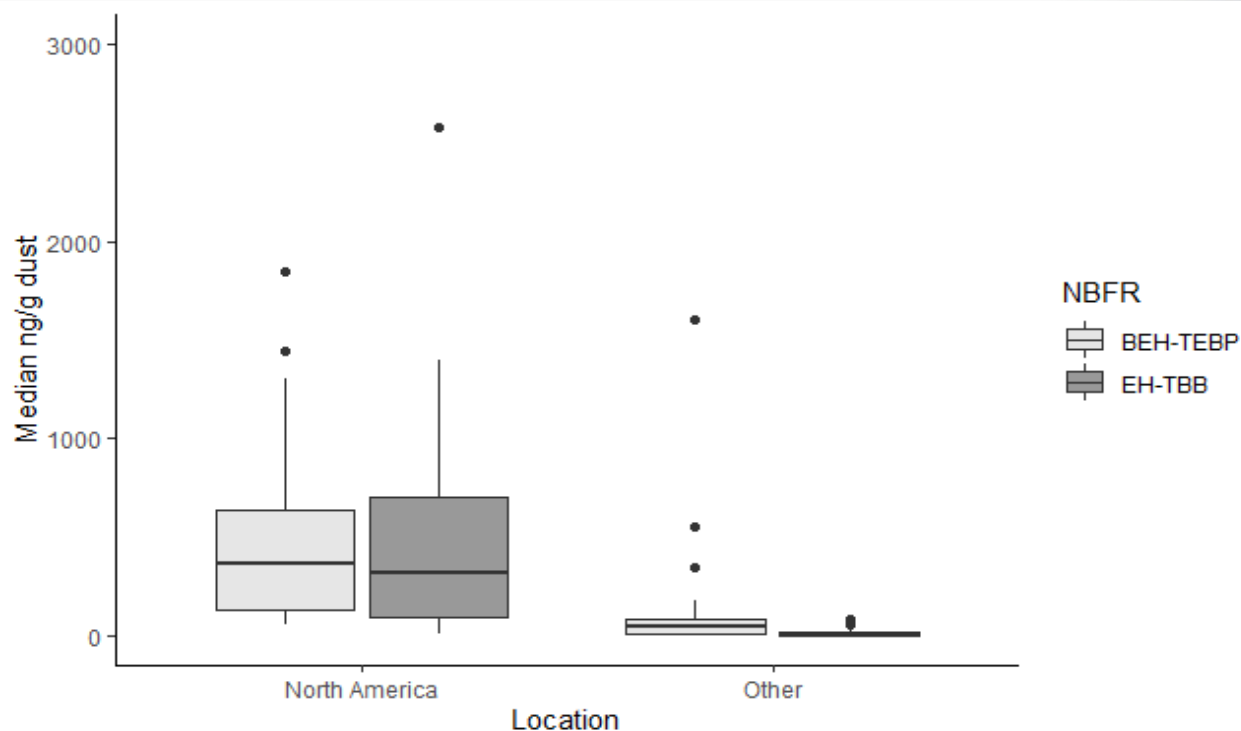
triphenyl phosphate

- Primary route of exposure is ingestion
- Detected with similar frequency in dust and external biological samples (hand wipes, nails and hair)
- Detected in human serum and milk
 - Uptake from ingestion
 - Transfer to infants
- EH-TBB metabolite (TBBA) detected in adult and child urine





Median dust concentration of BEH-TEBP and EH-TBB in United States and Canada compared to Africa, Asia and Europe





Estimated Daily Intake

$$EDI = \frac{Conc_{NBFR} * IR * AF}{body\ weight}$$

- EDI = Estimated Daily Intake (pg/kg/day)
- $Conc_{NBFR}$ = Dust concentration (pg/g)
- IR = Dust Ingestion Rate
 - Adult median $IR = 0.03$ g/day, for a
 - Child median $IR = 0.06$ g/day and the
 - Maximum IR for both adults and children = 0.10 g/day
- AF = Absorption factor
 - $AF_{EH-TBB} = 0.48$
 - $AF_{BEH-TEBP} = 0.25$
- Body weight is 70 kg for adult and 18.6 for child 3 – 6 years old

Fang et. al. 2014



Estimated Daily Intake (pg/kg/d)

		Median		Maximum	
		EH-TBB	BEH-TEBP	EH-TBB	BEH-TEBP
North America	Adults	13 - 1,011	22 - 410	10,650 - 26,008	4,039 - 7,554
	Children	96 - 7,607	166 - 3,085	40,081 - 97,879	15,201 - 28,431
Africa, Asia, Australia and Europe	Adults	2 - 4	3 - 25	0 - 1,805	350 - 1,581
	Children	12 - 30	25 - 187	0 - 6,793	1,317 - 5,949

90% confidence around the mean



Flame Retardant Risks

- Correlation with
 - Endocrine disruption
 - Cancer
 - Anxiety & ADHD
- EH-TBB + BEH-TEBP
 - Prenatal exposure increased anxiety behaviors in male mice (Patisaul et. al. 2013, Baldwin et. al., 2017)
 - Thyroid disruption (Dong et. al., 2021)
 - Disruption of steroid hormone production (Mankidy et. al., 2014, Saunders et. al., 2013)

PBDEs

Penta-BDE, octo-BDE, deca-BDE

Cyclic Aliphatic Bromides

HBCD

Tetrabromobisphenol A

TBBPA

Brominated Phthalates

TBB, TBPH

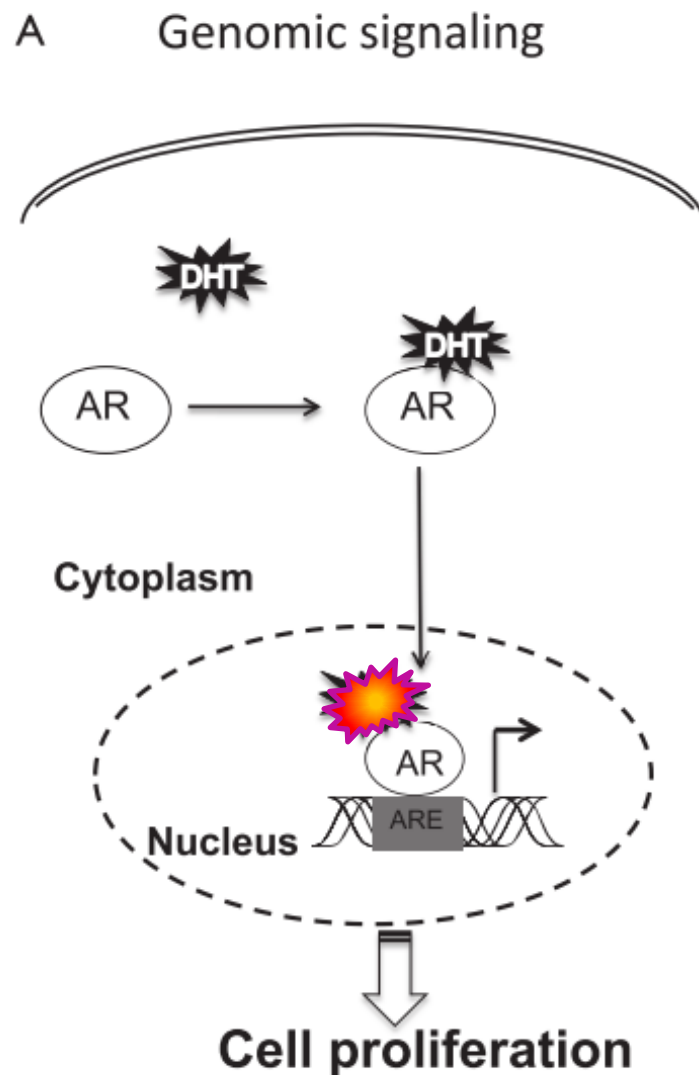
Chlorinated Phosphate Esters

TDCPP, TCEP

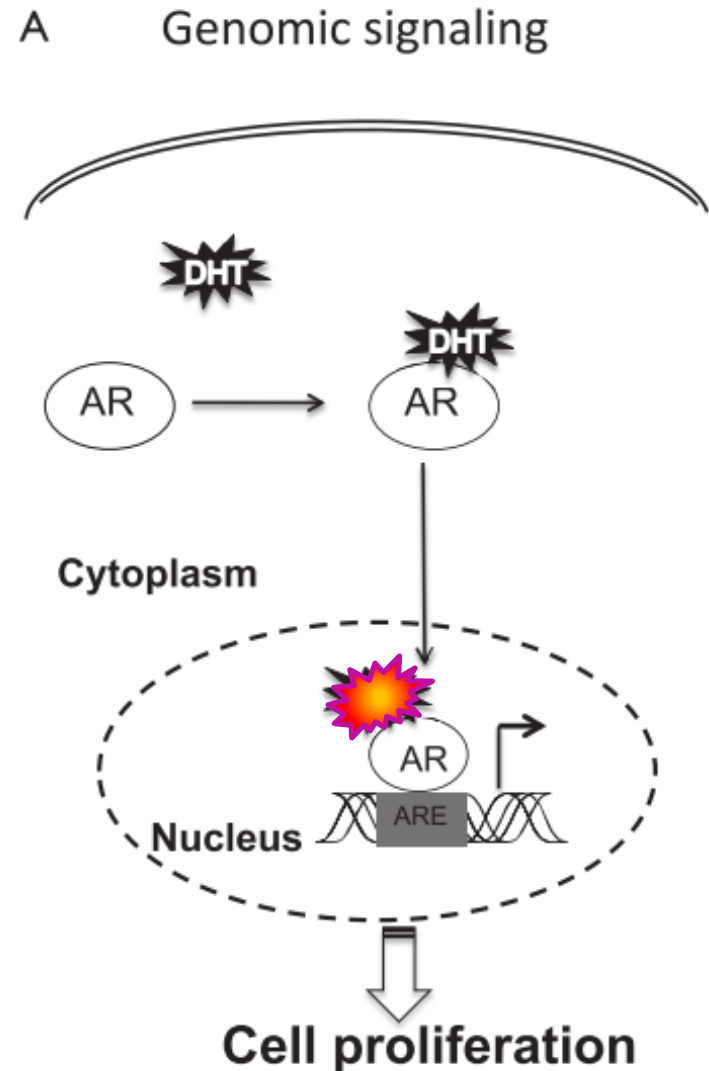
Organophosphates

ITP, TPP, TDCIPP

- *In vitro* AR inhibitor
 - YAS assay, Saunders et. al., 2013
 - YAS assay, Fic et. al., 2014
 - Luciferase assay in breast cancer reporter cell line, Klopčič et. al., 2016



Brominated phthalates (BP) inhibit AR function causing disruption of androgen mediated processes including AR dependent gene expression and cell proliferation in androgen-dependent prostate cancer cells.





AR dependent gene expression experimental design

10nM DHT	0.1% ethanol	10 μ M Bic.	20	2	0.2	0.02	0.002	0.0002	0.00002
Without DHT	0.1% ethanol	10 μ M Bic.	20	2	0.2	0.02	0.002	0.0002	0.00002

Decreasing concentration of EH-TBB (μ M)

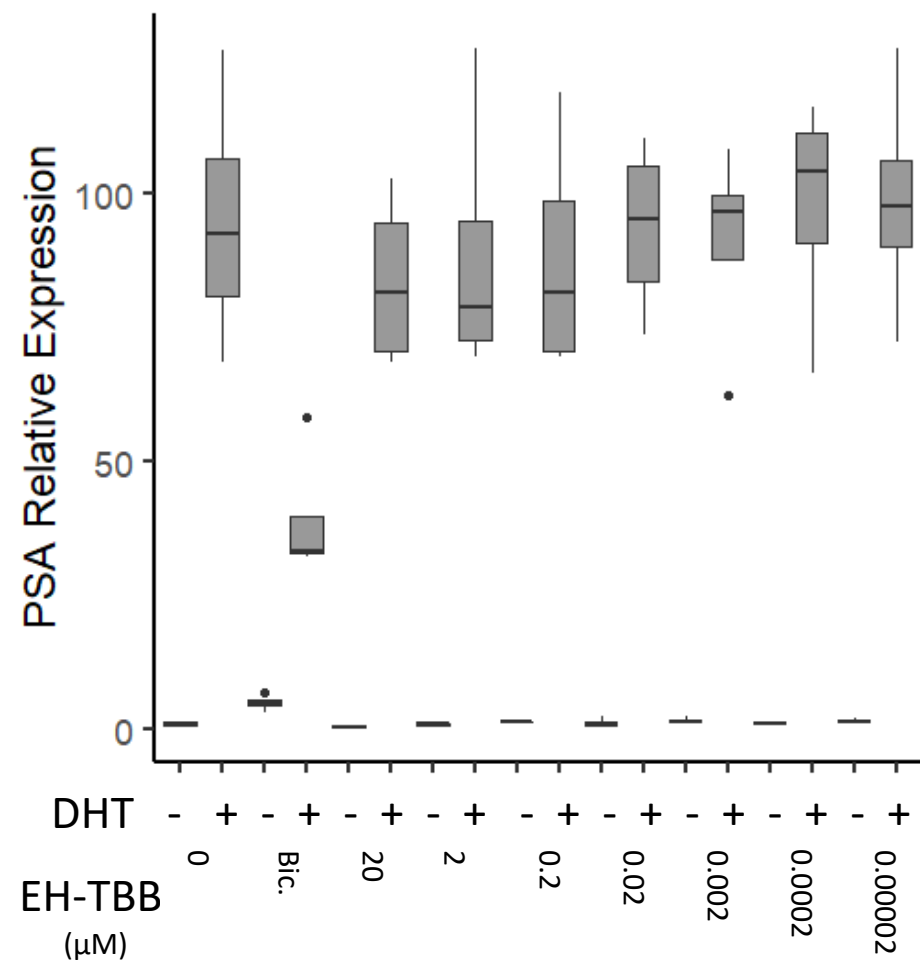
- **LNCaP cell line**
 - Derived from prostate cancer lymph node metastasis
 - Express AR (T877A)
 - Express high levels of the androgen-regulated gene prostate specific antigen (PSA)
- **LAPC4 cell line**
 - Derived from xenograft of prostate cancer lymph node metastasis
 - Express wild type AR
 - Express lower levels of PSA
- Target genes; **PSA (KLK3), GREB1, and NKX3-1**
- Reference genes; GAPDH, TBP and HPRT1



PSA mRNA expression is not significantly different in PCa cells treated with EH-TBB

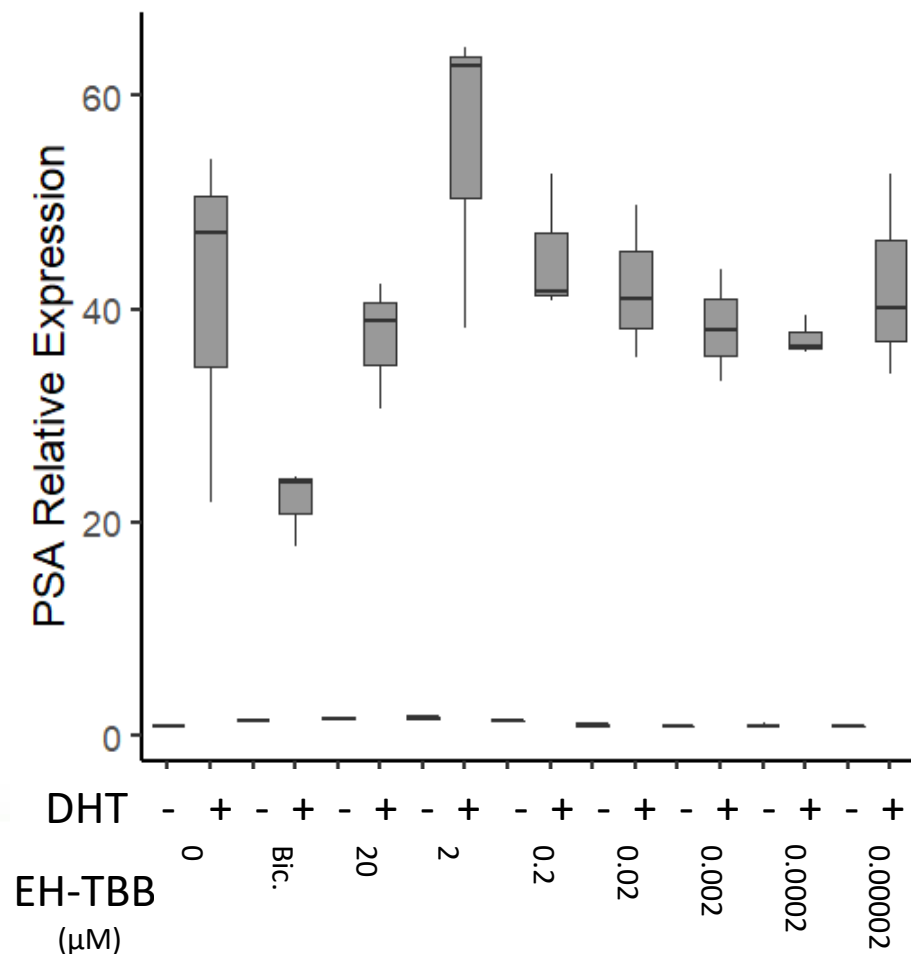
LNCaP

n=4



LAPC4

n=3

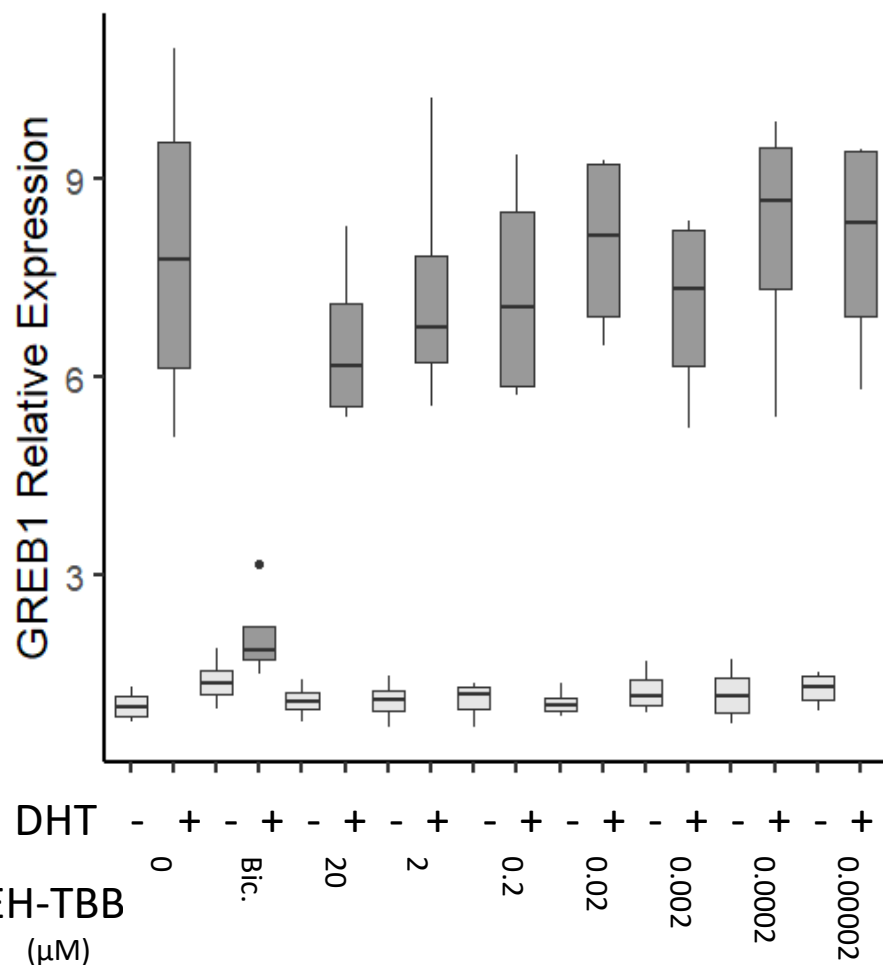




GREB1 mRNA expression has different patterns in different cell types

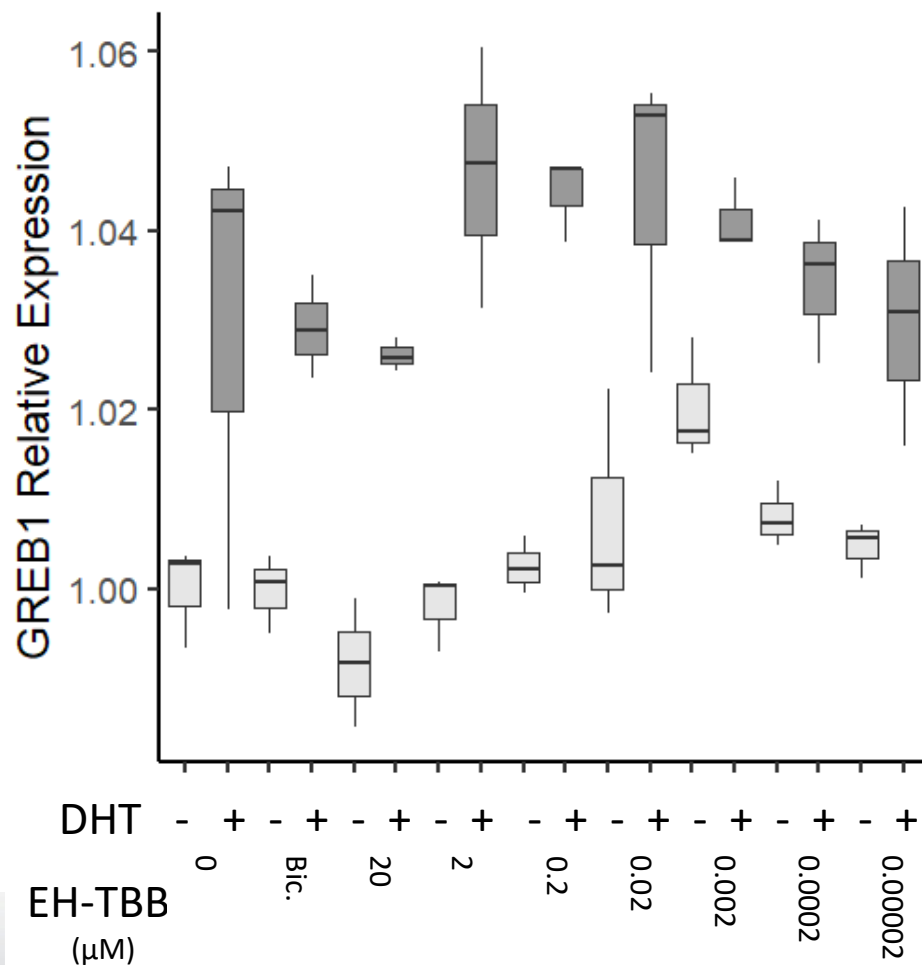
LNCaP

n=4



LAPC4

n=3

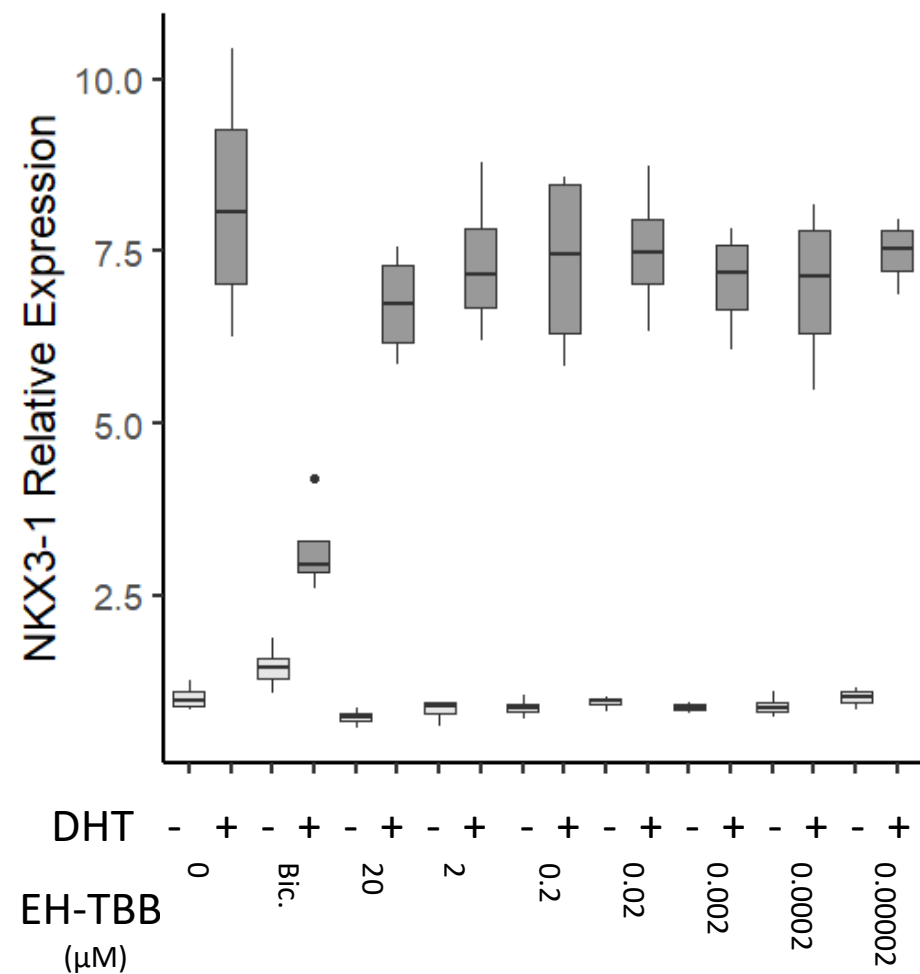




NKX3-1 mRNA expression has different patterns in different cell types

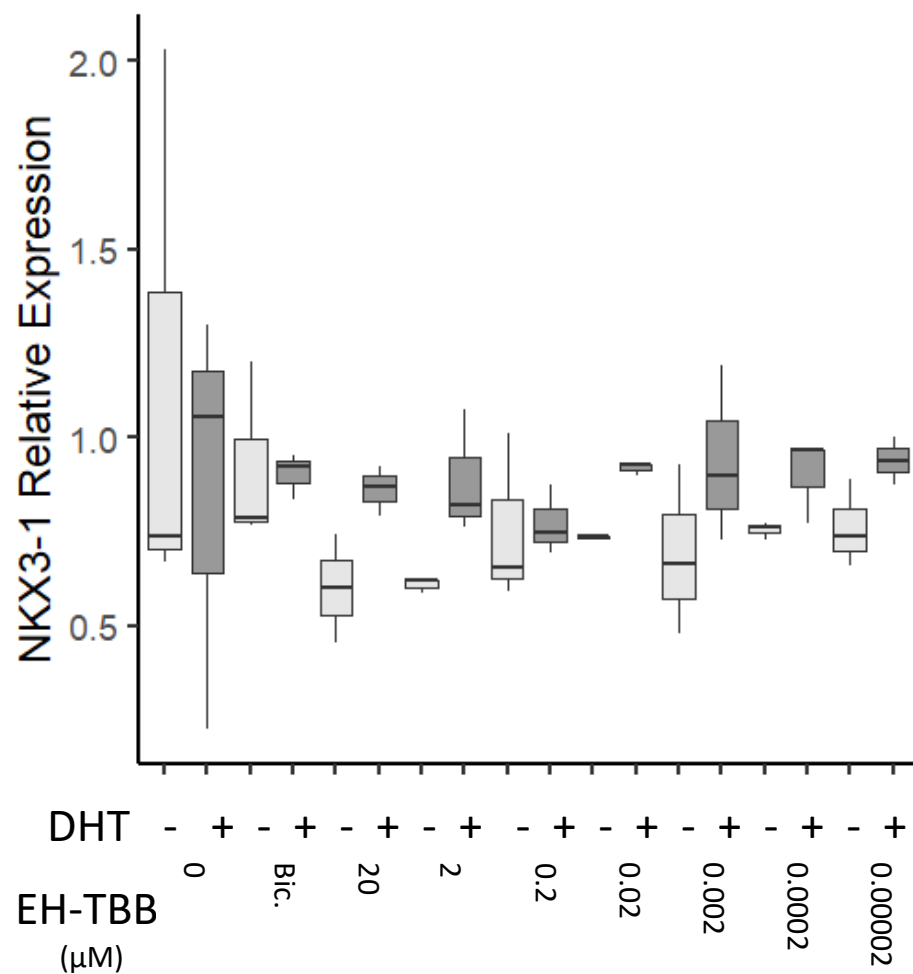
LNCaP

n=4



LAPC4

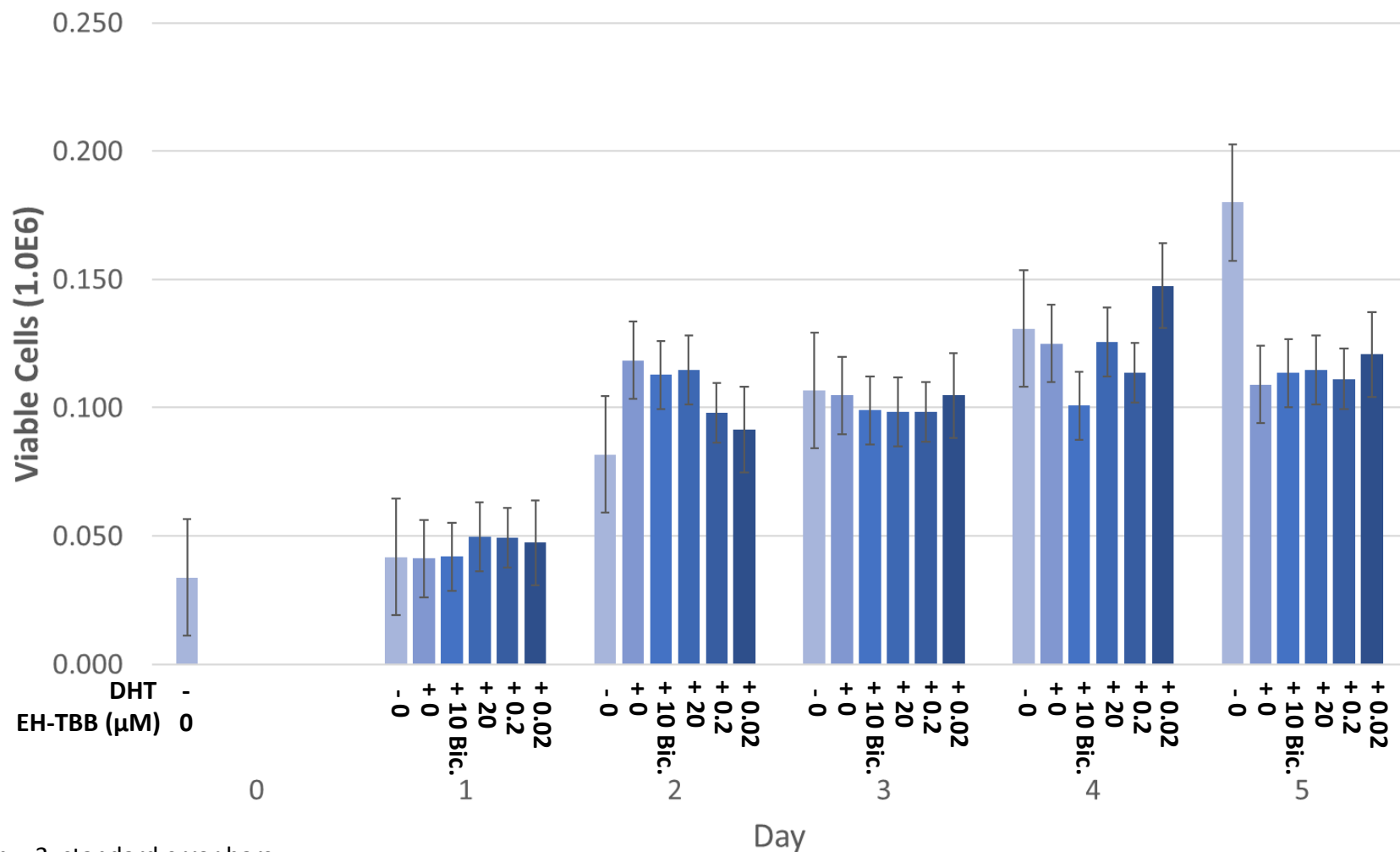
n=3





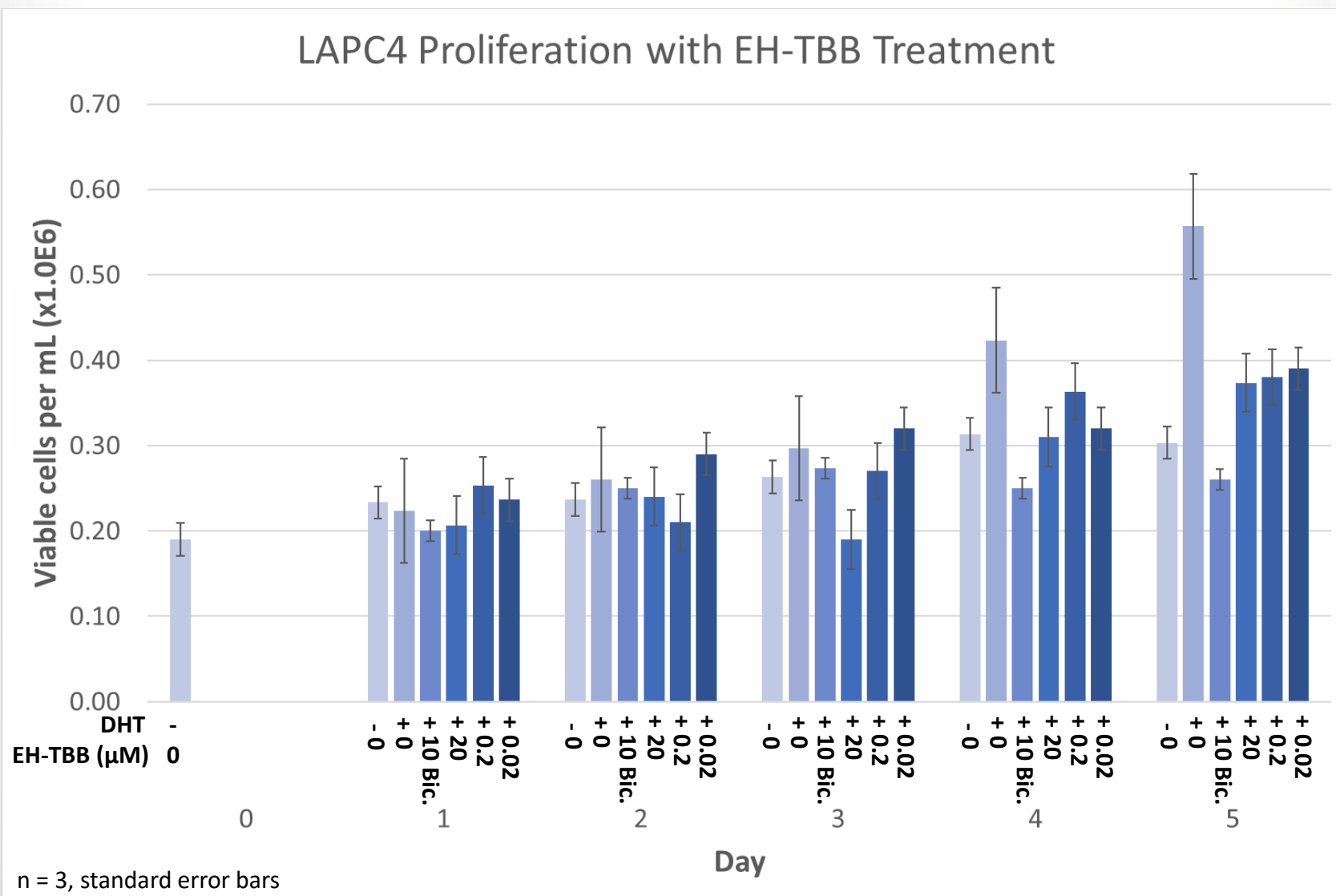
LNCaP Proliferation was not altered with EH-TBB treatment

LNCaP Proliferation with EH-TBB Exposure





LAPC4 Proliferation had a downward trend with EH-TBB treatment





LNCaP global mRNA expression after EH-TBB treatment

No DHT	10 nM DHT
0.1% Ethanol	0.1% Ethanol
10 μ M bicalutamide	10 μ M bicalutamide
20 μ M EH-TBB	20 μ M EH-TBB
0.02 μ M EH-TBB	0.02 μ M EH-TBB
0.00002 μ M EH-TBB	0.00002 μ M EH-TBB

- LNCaP exposed to treatment for 48 hours.
- RNASeq on mRNA
- DEGs identified compared to vehicle control and DHT control for DHT + treatment



Ingenuity Pathway Analysis

20 μ M TBB + DHT

Ingenuity Toxicity Lists	$-\log(\text{p-value})$	Ratio
Cholesterol Biosynthesis	2.96	75%
Cell Cycle: G1/S Checkpoint Regulation	2.26	47%
Decreases Permeability Transition of Mitochondria and Mitochondrial Membrane	2.07	83%
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.86	46%
p53 Signaling	1.70	40%

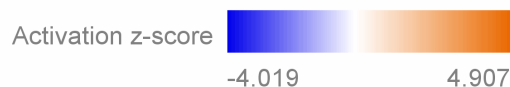
10 μ M Bic. + DHT

Ingenuity Toxicity Lists	$-\log(\text{p-value})$	Ratio
Cholesterol Biosynthesis	4.07	92%
p53 Signaling	3.60	55%
Renal Necrosis/Cell Death	2.35	42%
Primary Glomerulonephritis Biomarker Panel (Human)	2.26	100%
Pro-Apoptosis	1.83	57%



DHT Driven Gene Expression

BD comparison taD



Upstream Regulat...

20 μ M TBB + DHT
10 μ M Bic. + DHT

→ UXT
KDM1A
PRKAA1
→ AR
→ TP53
→ EP300



BD comparison taD



Canonical Pathways

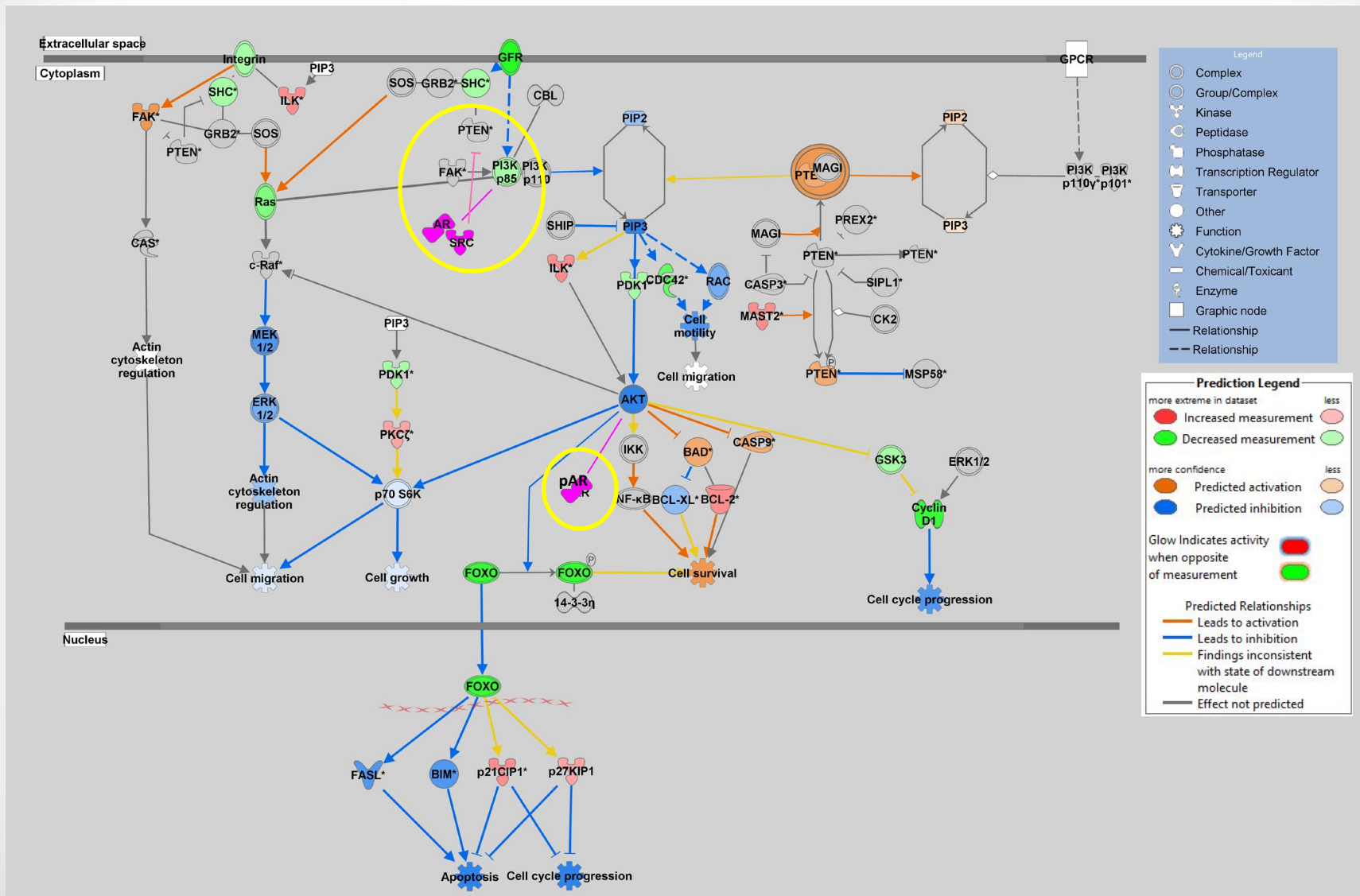
- Superpathway of Cholesterol Biosynthesis
- Superpathway of Inositol Phosphate Compounds
- Cyclins and Cell Cycle Regulation
- Signaling by Rho Family GTPases
- Ephrin B Signaling
- Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via M...
- 3-phosphoinositide Biosynthesis
- Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism
- Cholesterol Biosynthesis III (via Desmosterol)
- Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)
- Cholesterol Biosynthesis I

20 μ M TBB + DHT
10 μ M Bic. + DHT



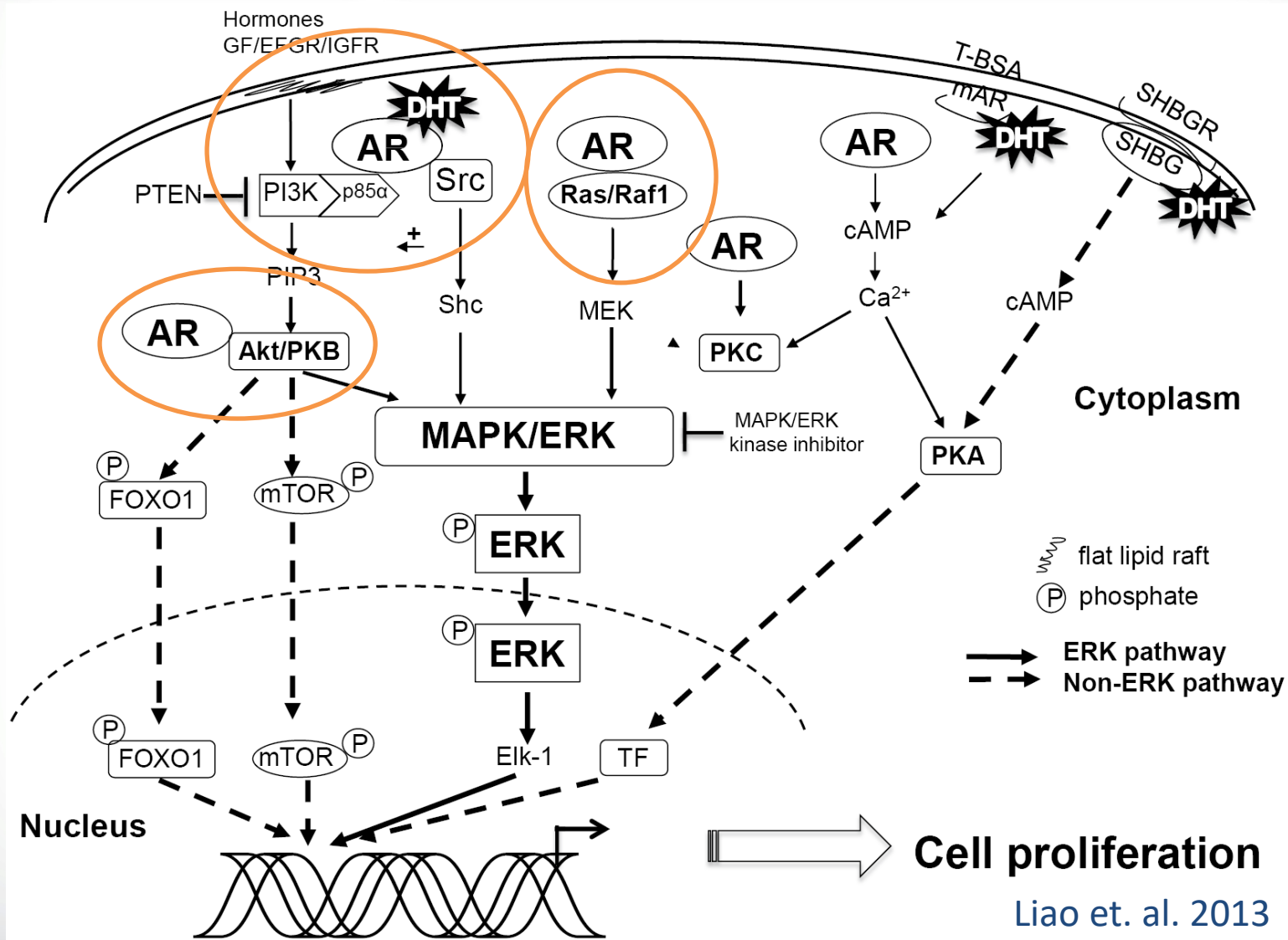


Non-genomic AR signaling ; TBB + DHT

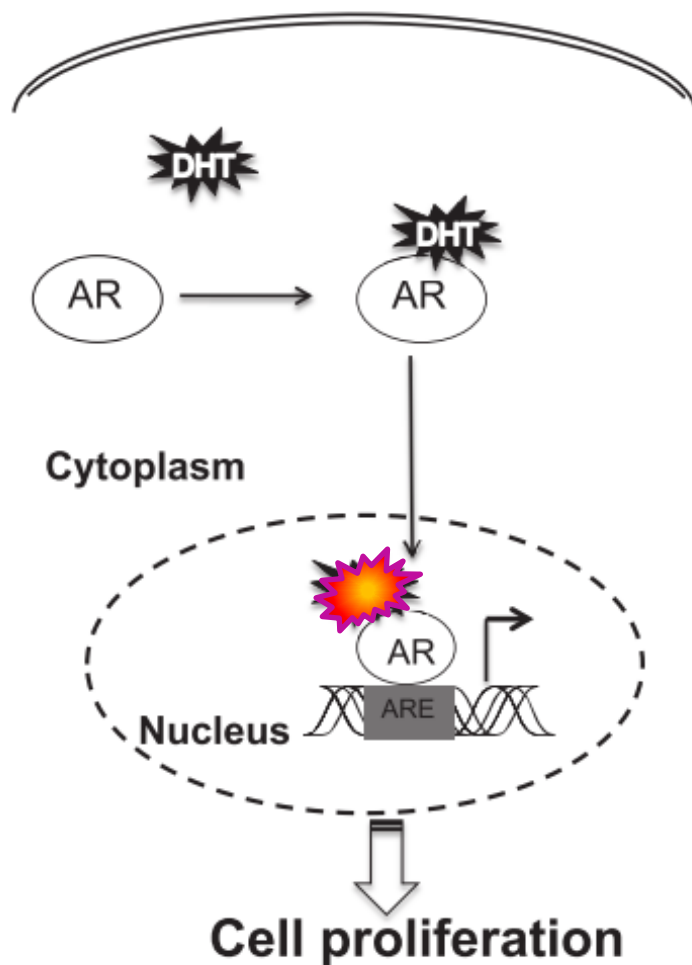




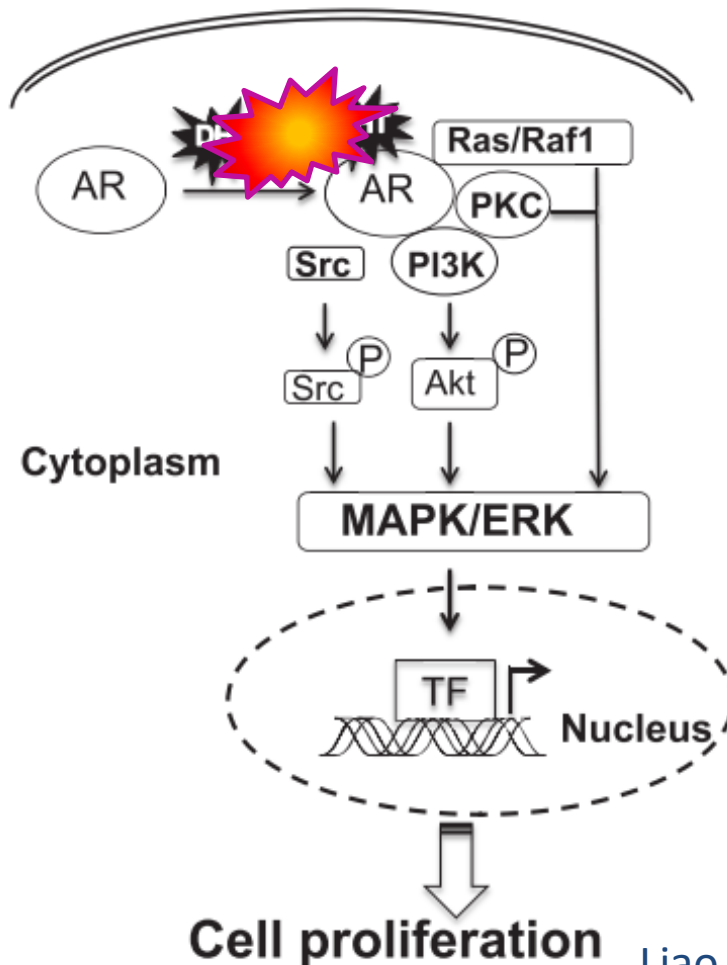
Non-genomic Androgen Receptor Signaling



A Genomic signaling



B Non-genomic signaling



- EH-TBB does not inhibit AR regulated gene expression
 - PSA, GREB1, NKX3-I
- EH-TBB may inhibit prostate cancer cell proliferation
- RNASeq suggests EH-TBB inhibits PI3K/Akt signaling, MAPK/ERK signaling, cell cycle progression and cell growth in LNCaP.

Future work

- Nuclear translocation of AR
 - Immunofluorescent cell imaging
- Western blot of PSA and 3 β HSD



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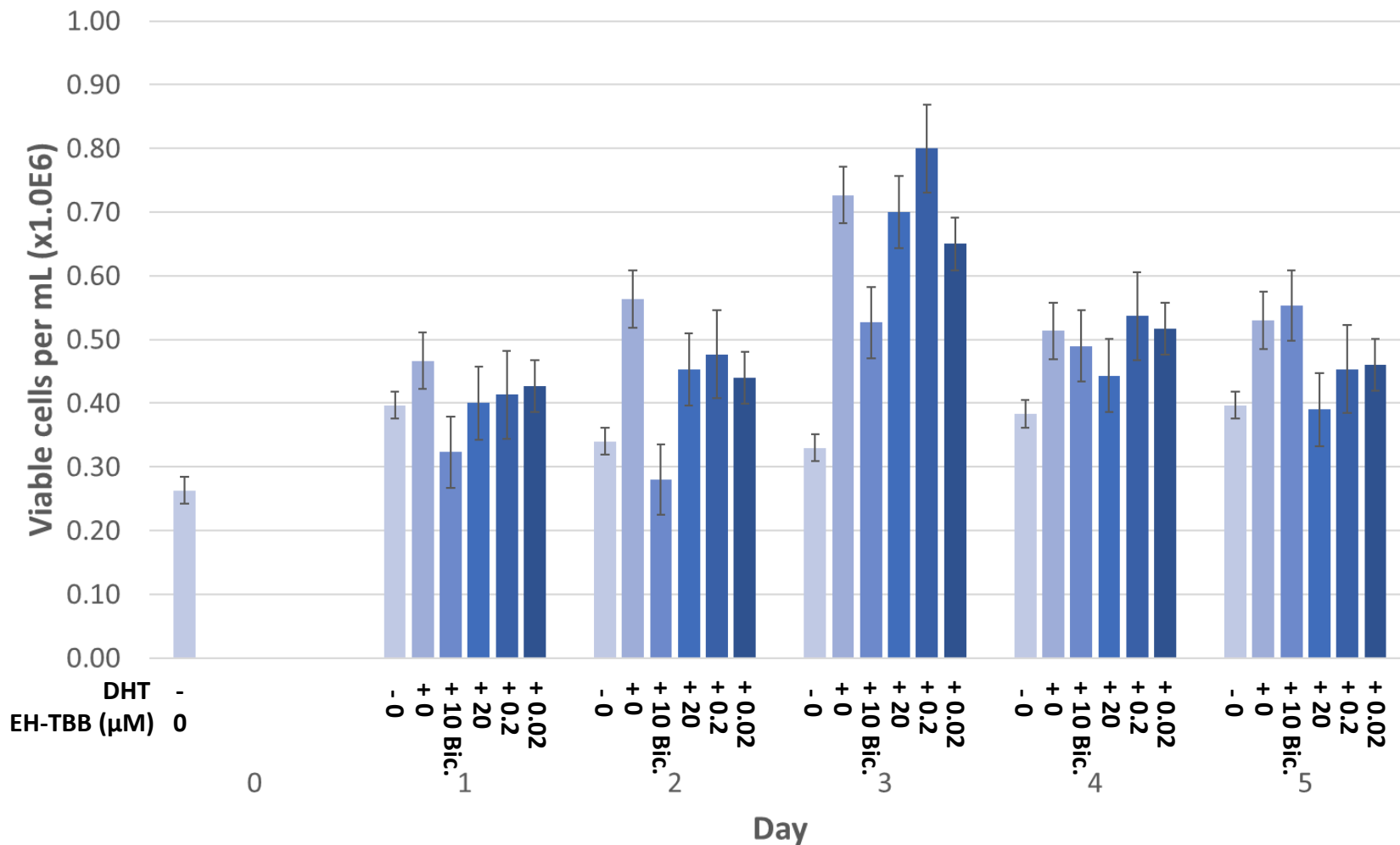
U.S. EPA Chemical Safety and Sustainability National Research Program





LNCaP Proliferation

Proliferation of LNCaP exposed to EH-TBB



- **RNASeq libraries** were prepared with Illumina TruSeq stranded mRNA kits from RNA samples paired to the luciferase samples (n=4 per treatment). Sequencing was performed on an Illumina HiSeq 4000 with single-end 50bp reads.
- **Differentially expressed gene (DEG) lists** were generated for each treatment compared to control (ethanol, no DHT) or compared to ethanol + DHT (positive control). Analysis in R (v. 3.6.0 (12)). Normalization (TMM (15)) and feature selection with edgeR (v. 3.26.8, (14)) observation weights and limma/voom (v. 3.40.6, (13)) hypothesis testing produced lists of differentially expressed genes (DEGs) ranked by false discovery rate (FDR, (1)).
- **Ingenuity Pathway Analysis (IPA)** core analysis was performed on the DEG lists (Expression cutoff -5 to 5, p-value cutoff ≤ 0.05). Comparison analysis was done to identify similarities/differences between treatment groups.



BP Inhibition of AR target genes

Treatment + 10 nM DHT		0.4% Ethanol		0.2 nM TBB		20 nM TBB		20,000 nM TBB		10,000 nM Bic.		
Symbol	Entrez Gene Name	Expr	p-value	Expr	p-value	Expr	p-value	Expr	p-value	Expr	p-value	Symbol
AR	androgen receptor	-1.469	3.02E-01	-2.551	9.33E-02	-1.734	2.83E-01	-5.730	1.95E-03	-4.670	1.60E-02	AR
GREB1	growth regulating estrogen receptor binding 1	33.666	2.48E-11	26.089	1.85E-08	28.994	2.32E-08	18.922	1.05E-06	17.872	4.66E-06	GREB1
KLK3	kallikrein related peptidase 3	32.620	3.42E-11	35.692	1.94E-09	33.248	9.56E-09	15.029	4.38E-06	24.428	6.08E-07	KLK3
NDRG1	N-myc downstream regulated 1	37.642	8.21E-12	31.735	4.69E-09	30.960	1.51E-08	12.233	1.64E-05	8.684	4.35E-04	NDRG1
NKX3-1	NK3 homeobox 1	42.561	2.43E-12	37.247	1.45E-09	36.829	4.56E-09	31.507	4.01E-08	30.367	1.65E-07	NKX3-1
TMPRSS2	transmembrane serine protease 2	40.467	3.99E-12	38.978	1.06E-09	36.804	4.57E-09	31.041	4.37E-08	24.198	6.20E-07	TMPRSS2

Dose dependent decrease in AR-ligand dependent gene expression

No DHT		20 nM TBB		20,000 nM TBB		10,000 nM Bic.		
Symbol	Entrez Gene Name	Expr	p-value	Expr	p-value	Expr	p-value	Symbol
AR	androgen receptor	-1.775	7.03E-01	-4.940	2.36E-02	-5.942	9.16E-03	AR
GREB1	growth regulating estrogen receptor binding 1	-2.177	7.03E-01	-6.525	7.43E-03	2.955	1.54E-01	GREB1
KLK3	kallikrein related peptidase 3	1.012	7.03E-01	-9.990	1.39E-03	8.456	1.85E-03	KLK3
NDRG1	N-myc downstream regulated 1	4.191	7.03E-01	0.451	9.10E-01	2.094	3.48E-01	NDRG1
NKX3-1	NK3 homeobox 1	0.148	9.71E-01	-21.467	3.67E-05	1.800	4.48E-01	NKX3-1
TMPRSS2	transmembrane serine protease 2	-1.398	7.03E-01	-6.276	8.69E-03	1.804	4.47E-01	TMPRSS2

Decrease in AR mediated gene expression in cells exposed to high dose TBB. Contrary to expression from competitive AR inhibitor suggesting TBB action is not in the ligand binding domain of AR.

