Transcriptional Regulatory Dynamics of the Hypothalamic-Pituitary-Gonadal Axis and its Peripheral Pathways as Impacted by the 3-Beta HSD inhibitor Trilostane in Zebrafish (*Danio rerio)*

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Abstract: To study mechanisms underlying generalized effects of 3β hydroxysteroid dehydrogenase (HSD3B) inhibition, reproductively mature zebrafish (*Danio rerio*) were exposed to trilostane at two dosages for 24, 48, or 96 hours and their gonadal RNA samples profiled with Agilent zebrafish microarrays. Trilostane had substantial impact on the transcriptional dynamics of zebrafish, as reflected by a number of differentially expressed genes (DEGs) including transcription factors (TFs), altered TF networks, signaling pathways, and Gene Ontology (GO) biological processes. Changes in gene expression between a treatment and its control were mostly moderate, ranging from 1.3 to 2.0 fold. Expression of genes coding for HSD3B and many of its transcriptional regulators remained unchanged, suggesting transcriptional up-regulation is not a primary compensatory mechanism for HSD3B enzyme inhibition. While some trilostane-responsive TFs appear to share cellular functions linked to endocrine disruption, there are also many other DEGs not directly linked to steroidogenesis. Of the 65 significant TF networks, little similarity, and therefore little cross-talk, existed between them and the hypothalamic-pituitary-gonadal (HPG) axis. The most enriched GO biological processes are regulations of transcription, phosphorylation, and protein kinase activity. Most of the impacted TFs and TF networks are involved in cellular proliferation, differentiation, migration, and apoptosis. While these functions are fairly broad, their underlying TF networks may be useful to development of generalized toxicological screening methods. These findings suggest that trilostane-induced effects on fish endocrine functions are not confined to the HPG-axis alone. Its impact on corticosteroid synthesis could also have contributed to some system wide transcriptional changes in zebrafish observed in this study.

Keywords

trilostane, HPG-axis, transcription networks, biological pathways

42

43 Abbreviations

44 Cy3, Cyanine 3; Cy5, Cyanine 5; DEG, differentially expressed gene; GSEA, gene set enrichment
45 analysis; E-GSEA, extended-GSEA; EDC, endocrine disrupting chemical; FDR, false discovery rate;
46 GO, gene ontology; HPG, hypothalamic-pituitary-gonadal; HSD3B, 3β hydroxysteroid dehydrogenase;
47 IPA, Ingenuity Pathway Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; TF,
48 transcription factor

49

1. Introduction

Over the past decade there has been an increasing emphasis on the potential harmful effects of endocrine disrupting chemicals (EDCs) on humans and wildlife (WHO 2002; Diamanti-Kandarakis et al., 2009). Investigation of the role and function of the hypothalamic-pituitary-gonadal axis (HPG-axis), and more specifically, receptors and enzymes involved in steroidogenesis, is critical to an improved mechanistic understanding of chemical effects on endocrine function (Ankley et al., 2009). While the identities of many HPG-axis components targeted by various chemicals are known, information concerning how these genes/proteins function in a wider biological context remains limited. A better understanding of the relationship between the HPG-axis and the transcription factor (TF) networks/signaling pathways it interacts with would facilitate the development of mechanistically-based indicators/endpoints and enhance the extrapolation of toxic effects across species and chemical structures. This should provide a basis for a more informative and efficient assessment of EDC exposures, adverse effects, and risks (Ankley et al., 2009).

Conceptually, perturbing the HPG-axis in a targeted manner over a series of experiments can help reveal its transcriptional regulatory dynamics (Ankley et al., 2009). One potential target for perturbation is 3β hydroxysteroid dehydrogenase (HSD3B, EC 1.1.1.145), a well-characterized enzyme catalyzing key steps in formation of corticosteroids and sex steroids (Simard et al., 2005). The HSD3B gene (family) is conserved across vertebrate species (Simard et al., 2005) and is typically present as multiple isozymes with tissue-specific expression. Inhibition of this enzyme activity should disrupt steroidogenesis, thereby affecting different biological pathways, including those within the HPG axis. Indeed, impaired spawning, vitellogenesis, and in vitro steroid production has been demonstrated in fathead minnows (and their tissues; *Pimephales promelas*) exposed to the HSD3B inhibitor, trilostane (Villeneuve et al., 2008). An elucidation of the genes and corresponding TF networks/signaling

pathways responsive to HSD3B inhibition could yield significant insights into the transcriptional regulatory control of steroidogenesis and the HPG-axis, and contribute to a better overall understanding of mechanisms of endocrine disruption.

While the impact of HSD3B inhibition on the overall transcriptional regulatory dynamics of steroidogenesis/HPG-axis is not well understood, considerable knowledge exists with regard to TFs and cytokine signaling molecules implicated in the regulation of HSD3B gene expression (Payne and Hales, 2004; Simard et al., 2005; Lavoie and King, 2009). These include interleukin (IL)-4, IL-13, insulin-like growth factor (IGF)-1, members of nuclear hormone receptor family NR4A, NR5A, DAX-1, STAT proteins (signal transducers and activators of transcription; STAT5, 6), epidermal growth factor (EGF), GATA protein family (GATA4, 6), and transforming growth factor TGF-β. A better biological context for understanding the role of these TFs and cytokines, relative to HSD3B specifically and the HPG-axis more broadly, could be established by systematically examining them, along with many other potential TFs and target genes, in a genome-wide framework of interacting TF networks/signaling pathways. Advances in "-omics" technologies and computational biology in the past decade have made construction of such a tentative framework possible in a model species like zebrafish (Wang et al., 2010). In the present study, networks/pathways involved in the functions of steroidogenesis/HPG-axis were explored by perturbing zebrafish via exposure to trilostane, followed by whole genome expression profiling. Trilostane is a relatively specific competitive inhibitor of HSD3B that was originally developed for treatment of Cushing's syndrome in humans (Komanicky et al., 1978; Potts et al., 1978; Touitou et al., 1984).

The data presented here are part of a larger integrated project investigating mechanisms of endocrine disruption using model chemicals with known or hypothesized impacts on HPG-axis function (Ankley et al., 2009). The specific objective of this investigation was, through studying the effect of the model compound trilostane on the transcriptional regulatory dynamics, to identify

97 candidate TFs, HPG-axis members, TF networks, and signaling pathways impacted by exposure to a
98 HSD3B inhibitor. Resulting insights can be used to formulate specific, testable hypotheses for future
99 studies of endocrine disruption and search for mechanistically-based molecular indicators. Using
100 previously reverse-engineered TF networks (Wang et al., 2010), the linkage between trilostane
101 exposure and TF networks/pathways impacted was examined by Gene Set Enrichment Analysis
102 (GSEA, Subramanian et al., 2005), Extended-GSEA (E-GSEA, Lim et al., 2009; Wang et al., 2010),
103 and IPA (Ingenuity Pathway Analysis, www.ingenuity.com).

2. Materials and Methods

All laboratory procedures involving animals were reviewed and approved by the Animal Care and Use Committee at the US EPA Mid-Continent Ecology Division (Duluth, MN) in accordance with Animal Welfare Act and Interagency Research Animal Committee guidelines. Details and rationale regarding the overall experimental design, zebrafish exposure to trilostane, and gene expression profiling, including microarray data preprocessing and analyses, are presented elsewhere (Ankley et al., 2009; Wang et al., 2010). Only a brief overview is provided here.

2.1. Exposure and tissue sampling

Reproductively mature male and female zebrafish were exposed to a continuous flow of trilostane (two exposure concentrations) dissolved in sand-filtered, UV-treated Lake Superior water (with no solvent), for 24, 48, or 96 hours (h). Concentrations of trilostane in these treatments (and a corresponding Lake Superior water control) were determined using high-pressure liquid chromatography with diode-array detection (Villeneueve et al., 2008) on each sampling day. Measured concentrations averaged 488 and 2367 µg trilostane/liter, and no trilostane was detected in the control tanks. At the end of each exposure period, fish were sacrificed in a buffered solution of tricaine methanesulfonate (MS-222; Finquel, Argent, Redmond WA, USA) and gonad, liver, and brain tissues were collected, snap frozen in liquid nitrogen, and stored at -80^{o} C until extracted for analysis. Total RNA isolated from gonadal tissue samples only was labeled with either Cyanine-5 (Cy5, treated) or Cy3 (control) and hybridized in pairs to individual Agilent two-color 4x44k zebrafish microarrays (G2519F, Agilent Technologies, Santa Clara, CA 95051, United States) by an Agilent certified contract laboratory (Cogenics, Morrisville, North Carolina 27560, USA). Microarray data were generated for a total of six treatment (time/exposure) conditions.

2.2. *Microarray data analysis*

Trilostane data from 30 microarrays, representing six conditions and five replicates each: 24, 48, and 96 h testis high dose, 96 h testis low dose, 96 h ovary high dose, and 96 h ovary low dose, were analyzed for the present investigation. Each treatment condition consisted of ten unique biological samples (five treated and five control), with a treated and control sample hybridized as a pair to a single Agilent two-color microarray. Unless specified otherwise, all expression data were analyzed in the form of $\log_2$ (Cy5/Cy3) ratios. After preprocessing, differentially expressed genes (DEGs) were identified for individual conditions by one class t-tests corrected for false discovery rate (FDR; Benjamini and Hochberg, 1995) in GeneSpring GX10 (Agilent Technologies). Where *N* is the total number of genes and *p(k)*the *k-th* smallest p value (out of *N* sorted from low to high), FDR for gene *k* is defined as

$$N \times p(k)/k, \quad k = 1 \text{ to } N$$

FDR could be interpreted as the expected fraction of false positives among the genes identified as significant (Benjamini and Hochberg, 1995). The concept of FDR is critical to microarray data analysis due to a large number of genes/tests involved. Due to the relatively small sample size (e.g., n=5) per condition (Pawitan et al., 2005), FDRs determined in the present study were widely variable and their cutoffs had to be set between 15-80% in order to identify a reasonable number of DEGs for individual treatment conditions. The DEGs with FDRs > 30% were dropped from further analysis. Given the excellent congruence in gene expression determination between quantitative-PCR and microarray in a pilot study (Wang et al., 2008), no additional independent validation of selected DEGs was repeated here.

To lower FDRs and increase confidence in the DEGs identified, DEGs were subsequently discovered by pooling the individual conditions to different degrees: trilostane (all six conditions), trilostane ovary (two conditions), and trilostane testis (four conditions). While this pooling approach

may obscure some condition-specific responses (i.e., those observed at a specific dose or time point), the increased statistical power enhanced the detection of genes modulated by trilostane treatment. For an examination of individual DEGs, only those conditions/pooled conditions with FDR cutoffs ≤ 30% and a fold change of treated/control ≥ 1.3 were considered. While selection of 30% FDR threshold was arbitrary, selection of 1.3 as a minimum fold-change criterion was based on the evaluation of the technical noise of the array platform and two-color design used for the present work (Wang et al., 2008). Mapping to orthologous HMR (human-mouse-rat) pathways by IPA was based on DEGs (FDR ≤ 5%, treated/control ≥ 1.3 fold) from pooled conditions of trilostane ovary and trilostane testis, at a P value threshold of ≤ 0.05. Probes (Agilent zebrafish annotation release on June 17, 2007 for designs 013223 and 015064) were first mapped to their human orthologs, which were then searched against Ingenuity Knowledge Base as a reference set for significant associations with HMR pathways.

Besides IPA mapping, several additional approaches were utilized to provide a biological context for the evaluation of the transcriptomic impact of trilostane on zebrafish gonad. First, GO terms associated with individual DEGs, as provided by Agilent in its zebrafish gene annotations, were examined. Second, an enrichment analysis of GO terms among groups of DEGs by various treatment conditions was conducted using GoMiner (http://discover.nci.nih.gov/gominer/htgm.jsp, Zeeberg et al., 2005) at a FDR ≤ 5%. And third, previously constructed TF networks, along with a group of compiled HPG-axis genes (Villeneuve et al., 2007) and publically available KEGG pathways (as of October, 2008; Kyoto Encyclopedia of Genes and Genomes, www.genome.jp/kegg), were treated as gene sets and associated with individual trilostane conditions by GSEA and its variant, E-GSEA at a FDR threshold of 25% (Wang et al., 2010). To assess the inter-relationships of selected networks, those determined to be significantly impacted by trilostane exposure were overlaid on a clustering dendrogram composed of the entire set of TF networks/pathways constructed based on their pairwise Jaccard distances (Jaccard, 1901). Trilostane-impacted TF networks were analyzed for their possible

enrichment of GO biological processes using GOMiner at a FDR ≤ 5%. Unless otherwise specified, all analyses in the current study involving probe annotations were based on the latest Agilent release on July 19, 2010 for its zebrafish microarray design 015064.

Given the variety of analyses conducted in this study, the exploratory nature of microarray data, and inherent limitations of the platform in terms of sample size relative to number of gene variables, setting up a consistent FDR cutoff for multiple testing corrections was difficult. The stringency of a test generally started high and was gradually relaxed if necessary, as in the case of identifying DEGs. In some procedures, such as GSEA/E-GSEA, a recommended FDR (i.e., 25%) was adopted. When additional uncertainties were introduced into an analysis, for example, in mapping DEGs from a treatment condition to HMR pathways across species by IPA, an attempt was made to minimize false positives by using only the DEGs with FDR ≤ 5%. In general, a high stringency was maintained for those statistical procedures dealing with gene groups (IPA mapping, GO term enrichment analysis by GOMiner). Where appropriate, an additional requirement of ≥ 1.3 fold for DEGs was also imposed based on our previous finding that changes less than 1.3 fold could not be easily resolved from technical noise (Wang et al., 2008).

3. Results

Transcriptional regulatory dynamics of steroidogenesis and the HPG-axis in response to trilostane exposure could be evaluated by examining individual expression of: HSD3B, transcriptional regulators of HSD3B, and a broad array of TFs as well as other DEGs in general. Several analytical approaches were applied in this study. First, the DEGs were examined individually and mapped to orthologous HMR pathways. Second, genome-wide transcription factor networks reverse-engineered via a relevance network approach and a number of publicly available biological pathways were statistically-linked to various trilostane treatment conditions. Third, for additional biological context, GO enrichment analyses were conducted for DEGs and selected TF networks. Since TF networks were constructed with three gene expression data sets prepared differently: OvaryCy5Cy3 (ovary tissue in single channel intensities), OvaryRatio (ovary tissue in Cy5/Cy3 ratios), and TestisCy5Cy3 (testis tissue in single channel intensities), the naming convention for a TF network in this study follows the format of DRTF (*D. rerio* transcription factor) – data set – Agilent probe ID for a hub TF, for example DRTFovaryRatio_A_15_P110418 (Wang et al., 2010).

3.1. Differentially expressed genes

One class t-tests with multiple test corrections identified a number of DEGs, most of which exhibited modest fold-changes relative to controls (Table 1). The FDRs at which a moderate number of putative DEGs were detected varied widely, largely because of limited sample size per treatment condition. Pooling conditions together, thereby increasing effective sample size, reduced FDRs considerably. For example, when all samples were combined approximately 10% of the entire collection of 21495 probes on the zebrafish microarray were identified as significantly impacted by trilostane at 5% FDR or less. However, given the strong impact on gene expression by tissue type/gender (Wang et al., 2008), DEGs are perhaps best examined by combining samples according to

either ovary or testis tissue type. In this case, there seemed to be a greater impact of trilostane on testis (1370 DEG; 523 at fold change ≥ 1.3) than ovary (306 DEG; 251 at fold change ≥ 1.3) based on DEG counts. Ranked by fold change, the top 10 DEGs, many of which did not have informative annotations, were presented individually for those trilostane treatment conditions with a FDR cutoff ≤ 30% (Table 2).

3.2. Mapping DEGs to GO terms

To facilitate interpretation, unique DEGs from conditions of ovary (96 h ovary low dose, ovary combined) and testis (48 h testis, 96 h testis low dose, testis combined) were examined using their latest functional (GO) annotations (Supplemental List 1). In both tissue types, genes annotated as associated with binding (GO:0005488, including GO:0003700, transcription factor activity; GO:0004872, receptor activity; GO:0046872, metal ion binding; GO:0005524, ATP binding; GO:0043565, sequence-specific DNA binding; GO:0003677, DNA binding; GO:0003676, nucleic acid binding; GO:0000166, nucleotide binding; GO:0005515, protein binding) were impacted by trilostane. The most common biological process associated with these DEGs was regulation of transcription (GO:0045449; also GO:0006355, regulation of transcription, DNA-dependent; GO:0006350, transcription). Genes associated with transport (GO:0006810) and multicellular organismal development (GO:0007275) were also impacted. However, overall, GO term enrichment analysis (at FDR < 5%) of the DEGs from these five individual conditions found no statistically enriched GO categories.

3.3. Ingenuity Pathway Analysis

Mapping DEGs for various trilostane treatment conditions to orthologous HMR pathways could uncover potential signaling pathways linked to steroidogenesis and other HPG-axis functions. The

239 DEGs from pooled conditions of trilostane ovary and trilostane testis were used in this mapping to
240 minimize the impact of highly variable FDRs (Table 3, Supplemental List 2, 3). Besides a number of
241 amino acid metabolic pathways, Wnt and Ephrin pathways, both involved in signaling transduction,
242 were affected in ovary. Several other pathways involved in disease-related signaling and basic
243 metabolic functions were also linked to trilostane effects in testis. These signaling pathways appeared
244 to play roles in cellular growth, proliferation, apoptosis, and cell to cell communication.
245
246 *3.4. GSEA and E-GSEA*
247 A more direct (species specific) approach to explore the regulatory pathways modulated by
248 trilostane is through linking zebrafish-specific KEGG pathways and reverse-engineered TF networks,
249 to individual trilostane treatment conditions by GSEA and E-GSEA (Table 4, Supplemental List 4, 5).
250 Among a total of 1707 size eligible networks/pathways, 550 (including three KEGG pathways) were
251 significantly affected by trilostane. The bulk of those (540) were associated with the trilostane 96 h
252 ovary high dose condition. For simplicity, only the top 30 networks/pathways for this condition from
253 GSEA and E-GSEA, as ranked by their FDRs, were presented, resulting in a total of 65 trilostane-
254 impacted TF networks to be evaluated (Table 4). These 65 networks were examined in the context of
255 their distributions throughout a genome-wide clustering dendrogram, which is based on pairwise
256 Jaccard distances among 1932 TF networks/KEGG pathways (Wang et al., 2010). The
257 networks/pathways modulated by trilostane were distributed throughout the dendrogram, and none
258 were in close proximity to the HPG-axis-associated gene set (Supplemental Figure 1). About half of
259 them formed 11 relatively tight clusters (Table 4) indicative of substantially overlapping gene
260 membership across networks within these individual clusters. However, an examination of the genes
261 shared among networks within each cluster revealed no significant enrichment of any GO biological
262 processes.

3.5. GO enrichment analysis

Among the 547 trilostane-impacted TF networks, 37 were enriched with various numbers of biological processes (Table 5). The most frequently enriched are cellular functions such as regulation of phosphorylation, regulation of protein kinase activity, cell migration, and cellular localization. Trilostane also appeared to impact the transcription of some of the genes known to be involved in development of eye and neural system. The functional significance of these genes in zebrafish gonadal tissue is not clear. However, the observation was not without precedent as genes with similar function have been impacted by exposure to other EDCs as well as complex effluents (unpublished data).

3.6. Trilostane and expression of HSD3B and its transcriptional regulators

In addition to the unsupervised analyses of DEG and associated functions and pathways, we also took a more supervised look at the HSD3B genes themselves (probe A_15_P112736 and A_15_P120126) and their regulators. Neither HSD3B probe on the microarray was differentially expressed under any trilostane conditions, individual or pooled. The A_15_P112736 probe had an average fold change (treatment/control) of 1.07, 1.09, and 1.06 for pooled conditions of trilostane, trilostane ovary, and trilostane testis, respectively, none of which met the FDR < 5% threshold for significance. Similarly, the A_15_120126 probe had a fold change of 1.09, 1.58, and 1.43 and FDRs > 24%. Furthermore, no differential expression was observed for zebrafish orthologs of a number of human/murine genes known to regulate HSD3B expression in various cell types, including AP2B1, AP2M1, BMP-2, -4, -6, DLX3B, EGF, IGF1, ENO1, FXR beta, GATA4, GATA6, HIF1A, NR4A1, NR4A2, NR5A1A , NR5A1B, NR5A2, NR5A5, POMCA, STAT5.1, STAT5.2, TGF-beta-1, -2, -3, and TNF-alpha (data not shown; Lavoie and King, 2009). An analysis of these HSD3B regulators as a gene set by GSEA found no association to various trilostane conditions. Interestingly though, as a group,

these HSD3B regulators were significantly impacted by two chemical/conditions in a different study using the same experimental design as here: 17α-ethynyl estradiol 48 h testis (FDR 23%) and prochloraz 48 h testis (FDR 1%), out of the 58 conditions tested (Wang et al., 2010), suggesting that HSD3B transcription may be regulated by multiple distinct mechanisms.

3.7. Trilostane and expression of transcription factors

Finally, even moderate changes in TF expression after chemical exposures could have far-reaching impacts on downstream apical endpoints because of amplification effects through target genes (Vaquerizas et al., 2009). To assess how zebrafish TFs responded to trilostane, we assembled the entire collection of annotated zebrafish TFs and analyzed their transcriptional profiles under the two trilostane pooled conditions (Table 6). Among a total of 951 TF probes, 35 were differentially expressed at a FDR cutoff of 5%, 26 from trilostane testis and 11 from trilostane ovary. Only two TF probes, A_15_P121158 (unannotated) and A_15_P107503 (POUC, POU domain gene C), were determined to be DEGs from both pooled conditions. In general, expression changes of these TFs were fairly modest, less than two fold for the majority. Ranked by absolute fold change, the top five for trilostane ovary were JUNB, ESR2B, RARG, MYSM1, and POUC and for trilostane testis they were ZBTB16, OLIG2, PEA3, TWIST1B, and MEIS2.2. The networks anchored by 18 out of these 35 TF probes were also significantly impacted according to GSEA/E-GSEA, all under the condition of trilostane 96 h ovary.

4. Discussion

The present study examined transcriptional regulatory dynamics in the gonads of reproductively-mature zebrafish following exposure to trilostane. The direct effects of trilostane exposure on HSD3B enzyme activity and/or gonadal or anterior kidney steroid production were not determined for zebrafish. However, trilostane's effectiveness in inhibiting HSD3B and inducing phenotypic responses in a small fish was demonstrated in previous work with fathead minnows exposed to concentrations similar to or less than those administered to zebrafish in the present study (Villenevue et al., 2008). Given substantial conservation of HPG functions across vertebrates (Ankley and Johnson, 2004), we assume that the transcriptional dynamics observed in this study were linked, at least in part, to inhibition of HSD3B by trilostane. However, since that has not been verified in this species, direct linkage of the results of this study with HSD3B inhibition should be made with caution. Our intent in the present study was to develop hypotheses for later investigation. Furthermore, with the large number of genes, functions, pathways, and networks putatively altered under the various exposure conditions tested, our discussion is necessarily focused on well annotated genes with functions and/or pathways readily connected to the HPG-axis function. We also focus some attention on a selected few targets exhibiting particularly large fold changes. While this potentially ignores a variety of novel associations that may ultimately prove informative and/or important, detailed consideration of poorly annotated features responding to trilostane is outside the scope of our current analysis.

4.1. Trilostane, steroidogenesis, and endocrine disruption

Reports on effects of trilostane or similar inhibitors at a whole transcriptome level are scarce. Co-treatment of human breast cancer cells with 17β-estradiol and trilostane resulted in a significant change in expression of a number of genes involved in chromatin modification, cell cycle control,

329 apoptosis, cell adhesion, and signal transduction pathways (Barker et al., 2006). These DEGs,
330 however, have little overlap with those from the current study, except for up-regulation of estrogen
331 receptor ESR2B. Similar to results from a previous study examining HSD3B transcription in liver and
332 adrenal glands of rat treated with trilostane (Malouitre et al., 2006), zebrafish HSD3B gene expression
333 remained unchanged in ovary and testis under various trilostane conditions. Likewise, Villeneuve et al.
334 (2008) reported no significant effects on HSD3B expression in the gonads or brains of fathead
335 minnows exposed to trilostane for 21 d. The lack of HSD3B modulation was consistent with the
336 observation that expression of many previously identified transcriptional regulators of HSD3B were
337 also unchanged in the present study. Overall, results of our supervised analysis of HSD3B, and its
338 known transcriptional modulators in other vertebrates, suggest that up-regulation of HSD3B expression
339 is not a primary compensatory response to inhibition of this enzyme, at least at the time scale
340 considered in the present study. This is in contrast to inhibition of other steroidogenic enzymes such as
341 aromatase (CYP19) where significant up-regulation of transcripts for the impacted enzyme have been
342 consistently observed as part of an apparent feedback response (Villeneuve et al., 2006, 2009a, 2009b).
343 Indeed, many genes impacted by trilostane in the zebrafish were not directly linked to
344 steroidogenesis. Among all of the DEGs identified in the present study, the SI:CH211-240L19.8 gene,
345 whose expression was altered in the trilostane ovary group, had the greatest fold change (10.72).
346 Although it is not annotated, this feature shares substantial homology to ECOC2, an ovary-specific
347 gene implicated in regulating oocyte maturation and ovulation in fish (Ji et al., 2006). SLC4A4A, a
348 membrane transporter, was down regulated 2.33 fold in this group. In testis, ZGC:77041, a trans-
349 membrane protein highly conserved across eukaryotes, exhibited the greatest fold change (9.35), while
350 SLC39A13, another membrane protein, was down regulated 3.76-fold. Transcripts for CRABP1B, a
351 retinoic acid binding protein, were up-regulated 2.15 fold in testis. This protein mediates access to
352 retinoic acid receptor which regulates cell growth and differentiation along with the TGF-β signaling

pathway (Pendaries et al., 2003). PLA2G6, a phospholipase, involved in several signaling pathways regulating cell growth (Hooks and Cummings, 2008), was the most down-regulated gene (-2.57 fold) in the 48 h testis treatment. While not exhaustive, these examples highlight the fact that trilostane exposure appears to have effects on diverse cellular functions beyond steroidogenesis.

Supervised investigation of differential expression of TF probes themselves revealed that many trilostane-responsive TFs appear to share cellular functions putatively linked to endocrine disruption. For example, MEIS2.2 serves as a cofactor to HOX (Moens and Selleri, 2006), a family of TFs regulated by several hormones and their receptors, which can be impacted by other classes of endocrine active chemicals (Daftary and Taylor, 2006). YY1 and NR4A2 also appear to be directly involved in transcriptional regulation of steroidogenic genes, although no such effects were observed in the present study (Lavoie and King, 2009). NR2F1 (COUP-TF) competes with other nuclear receptors including RAR and ESR to bind target genes (Zhang and Dufau, 2004). It may inhibit STARD1 as well, the gene coding for StAR (steroidogenic acute regulatory), a protein responsible for a key rate-limiting step in sex steroid production (Lavoie and King, 2009). ESR2B, JUNB, RARG are all well-known partners to SMADs, the cellular effectors critical to TGF-β signaling and functions such as cell proliferation, differentiation, migration, and apoptosis (Kang et al., 2009). Given the key roles the TGF-β signaling pathway plays in these cellular functions, it is not surprising to find that many of TFs impacted by trilostane, and their associated networks, including YY1, JARID1C, E2F5, MEF2D, RARG, MAX, HIF1AL, and ZNF216 (through NfkB) (reviewed in Feng and Derynck, 2005; Kim et al., 2008), interact with SMAD proteins. Not coincidentally, both androgen and estrogen receptors interact with SMADs as well (Chipuk et al., 2002; Matsuda et al., 2001). Thus, not surprisingly, functions related to cell cycle control and cell fate appear closely tied with endocrine functions and subject to influence by EDCs.

4.2. Trilostane impact on biological pathways/TF networks

We employed IPA of pathways for human, mouse and rat, and GSEA and E-GSEA analyses of de novo, inferred, transcription factor networks for zebrafish (Wang et al., 2010) in an effort to extend our understanding of the biology being impacted by trilostane perturbation of HSD3B activity. Two signaling pathways mapped by IPA to trilostane effects in ovary, Wnt and Ephrin, are known to be involved in cellular growth, proliferation, and cancer. GABA receptor and two other disease-related signaling pathways linked to trilostane effects in testis have roles in apoptosis, neural system development and function, and cell to cell interactions. Among the 65 de novo zebrafish TF networks impacted following trilostane exposure, about half form 11 closely related clusters, while the remaining ones are fairly distinctive and scattered throughout a 1932-node dendrogram (Wang et al., 2010). Since the clustering is based on a Jaccard distance matrix, TF networks within a cluster tend to share more gene members and are more likely to interact with one another. The biological functions of the hub TFs anchoring the 65 trilostane-significant networks are also significantly oriented toward cell proliferation, differentiation, and apoptosis. Selected examples in this regard include some of the better studied hub TFs from the 11 identified clusters such as YY1, ARID2, E2F5, MEF2D, MYBL2, and STAT4 (Gordon et al., 2006; Wilsker et al., 2005; Dimova and Dyson, 2005; Potffhoff and Olson, 2007; Sala, 2005; Rawlings et al., 2004). Similar cellular functions are also associated with additional hub TFs distributed outside the 11 clusters including TFDP1, DPF2, MAX, MXI1, and HOXB1B (Hitchens and Robbins, 2003; Gabig et al., 1998; Hurlin and Huang, 2006; Delpuech et al., 2007; Kataoka et al., 2001).

4.3. Trilostane and HPG-axis

Although rather broad and non-specific, the preponderance of associations with cell proliferation, differentiation, apoptosis, migration, and morphology is interesting in light of the

401 increase in testis mass, relative to body mass (i.e., gonadal somatic index; GSI), observed in male
402 fathead minnows exposed to trilostane for 21 d (Villeneuve et al., 2008). Effects on GSI have also
403 been reported in fathead minnows exposed to the steroidogenesis inhibitor, ketoconazole (Ankley et al.,
404 2007), and were hypothesized to be part of a compensatory feedback response. These observations
405 raise the possibility that depending on the nature and specificity of the inhibition, the dominant
406 compensatory feedback response to some types of steroidogenesis inhibition (e.g., inhibition of
407 HSD3B by trilostane) may be proliferation and remodeling of the steroid synthesizing cell types within
408 the gonad. In other cases, such as following exposure to the aromatase inhibitor, fadrozole, up-
409 regulation of genes coding for particular steroidogenic enzymes may be the dominant response. A
410 combination of both types of responses is another possibility (e.g., steroidogenesis inhibition with
411 ketoconazole). Detailed comparison of the TF network responses to trilostane and other
412 steroidogenesis inhibitors may be fruitful in evaluating the nature of compensation in the HPG axis in
413 response to different types of chemical stressors.
414 The current study indicates that, although trilostane clearly can impact HPG function in fish
415 (e.g., Villeneuve et al., 2008), effects of the drug are not confined solely to the HPG-axis. Specifically,
416 the number of genes (including TFs) impacted by trilostane in zebrafish is substantial, and many of
417 them lack an apparent association with the HPG-axis. Consistent with this, in a dendrogram capturing
418 genome-wide interactive relationships among TF networks and biological pathways, those altered by
419 trilostane are scattered throughout the tree with none of them in close proximity to the HPG-axis.
420 Further, according to GO enrichment analysis of trilostane-impacted TF networks and GO terms linked
421 to its DEGs, regulation of transcription, phosphorylation, and protein kinase activity appear to be
422 biological processes most closely linked to exposure to trilostane. These cellular functions are
423 involved in many basic gene-regulatory and signal transduction pathways with potentially widespread
424 impact on an organism's growth and development, so their disruption could have diverse and far

reaching phenotypic consequences to an organism. In this regard, it is also important to note that inhibition of HSD3B activity can not only affect sex steroid production, but also corticosteroid synthesis (Potts et al., 1978), which could possibly lead to several of the more-system wide changes in gene expression we observed in the zebrafish.

5. Conclusions

In conclusion, transcriptional regulatory dynamics in the zebrafish gonad appear to be significantly altered by trilostane. Most of the impacted TFs and TF networks are broadly involved in cell proliferation, differentiation, migration, and apoptosis. These findings are largely supported by the HMR signaling pathways identified through IPA mapping of trilostane-responsive DEGs, perhaps indicative of critical roles played by these cellular functions in feedback responses to specific mechanisms of endocrine perturbation. While these cellular responses are fairly basic, their underlying pathways and TF networks may be useful to development of generalized toxicological screening methods (Simmons et al., 2009). Additionally, a greater scope of impact by trilostane beyond HPG-axis as focused on a priori and effects of reproductive endocrine disrupting chemicals speaks to the potential value of the type of unsupervised analysis employed in the present study in ultimately informing a more systems-oriented understanding of biological responses to stressors that is less constrained by a historically modular view of biological systems. Future studies targeting the TFs and their networks identified here could bridge the knowledge gap between the HPG-axis and existing canonical signaling pathways in their contributions to endocrine responses in fish and other vertebrates.

Acknowledgements

This work was supported in part by an award from the National Center for Computational Toxicology of the US Environmental Protection Agency (US EPA) to the Ecological Exposure

Research and Ecosystem Research Divisions (National Exposure Research Laboratory) in Cincinnati, Ohio, and Athens, Georgia, USA, respectively, and the Mid-Continent Ecology Division (National Health and Environmental Effects Research Laboratory) in Duluth, MN, USA. The paper has been subjected to Agency's administrative review and approved for publication as a U.S. EPA document.

Appendix A. Supplementary Data

Supplemental List 1-5: The List 1 contains the DEGs and their annotations for five trilostane treatment/pooled conditions. The List 2 and 3 contain human-mouse-rat biological pathways mapped with the DEGs for trilostane ovary and testis by Ingenuity Pathway Analysis. List 4 and 5 show TF networks linked to various trilostane treatment conditions by GSEA or E-GSEA.

Supplemental Figure 1: The distributions of 65 trilostane-impacted TF networks in a genome-wide dendrogram of 1932 TF networks as related to the HPG-axis.

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Table 1. A summary of differentially expressed genes (DEGs; counted as unique probes) for various individual/pooled trilostane conditions (Trt). Those with a false discovery rate (FDR) > 30% were not considered further. A fold change is based on treated/control in a given condition.

Trt	Trilostane treatment	DEGs (FDR)	And ≥ 1.3 fold	And ≥ 2 fold
1	96 h testis low	141 (20%)	95	3
2	96 h testis high	141 (55%)	83	14
3	96 h ovary high	494 (55%)	418	169
4	96 h ovary low	156 (15%)	88	5
5	24 h testis high	98 (80%)	65	7
6	48 h testis high	104 (30%)	54	1
	Testis combined	1370 (5%)	523	12
	Ovary combined	306 (5%)	251	39
	All combined	2139 (5%)	808	14

650 Table 2. The top 10 differentially expressed genes (DEGs) by fold change for various trilostane treatment conditions with a
651 false discovery rate (FDR) ≤ 30% and fold change ≥ 1.3. The down-regulated genes are indicated by negative values, and
652 up-regulated by positive values in the "Fold change" column. A fold change is based on treated/control in a given
653 condition. The "trilostane testis" groups together 4 individual testis treatment conditions (24, 48, and 96 h in two dosages).
654 The "trilostane ovary" includes two treatments (96 h in two dosages). The probe annotations are based on the Agilent
655 release of July 19, 2010 for design 015064. Probes without annotations are marked by "---". The entire collection of
656 trilostane DEGs is available in Supplemental List 1.

Probe ID	Fold change	Gene symbols / description
Trilostane 48 h testis high (FDR ≤ 30%)		
A_15_P115111	-2.57	PLA2G6, phospholipase A2, group VI (cytosolic, calcium-independent)
A_15_P100586	-1.87	DPYSL5A, dihydropyrimidinase-like 5a
A_15_P110881	-1.83	---
A_15_P112115	-1.75	SCRT1A, scratch homolog 1, zinc finger protein a
A_15_P115933	-1.72	UNK, unkempt homolog (Drosophila)
A_15_P117718	1.64	---
A_15_P119269	-1.64	wu:fd15f08
A_15_P101596	1.63	---
A_15_P111886	1.62	FKBP9, FK506 binding protein 9
A_15_P120649	1.56	---
Trilostane 96 h testis low (FDR ≤ 20%)		
A_15_P101015	-2.22	ZGC:55888, ovochymase 1
A_15_P115284	-2.05	---
A_15_P100973	-2.00	ASS1, argininosuccinate synthetase 1
A_15_P101688	-1.98	---
A_15_P107609	-1.96	---
A_15_P110263	-1.95	---
A_15_P116370	1.94	---
A_15_P111077	-1.93	---
A_15_P116740	1.92	hypothetical protein LOC793937
A_15_P100683	1.89	wu:fc30e02
Trilostane testis (FDR5%)		
A_15_P101703	9.35	ZGC:77041, transmembrane protein 208
A_15_P115339	-3.76	SLC39A13, solute carrier family 39 (zinc transporter), member 13

A_15_P120918	-3.75	ZGC:103438
A_15_P116701	3.07	id:ibd5024
A_15_P103533	-2.58	LOC555344, similar to C1GALT1
A_15_P101338	2.15	CRABP1B, cellular retinoic acid binding protein 1b
A_15_P118171	2.08	---
A_15_P119458	2.07	---
A_15_P115371	2.06	---
A_15_P101484	-2.04	LRATA, lecithin retinol acyltransferase a
Trilostane 96 h ovary low (FDR ≤ 15%)		
A_15_P101996	-3.64	---
A_15_P104924	-2.33	SLC4A4A, solute carrier family 4, member 4a
A_15_P112106	2.19	LDB1A, LIM-domain binding factor 1a
A_15_P120411	2.17	---
A_15_P100864	-2.01	MARCKSA, myristoylated alanine rich protein kinase C substrate a
A_15_P120731	1.98	IM:7153990
A_15_P108806	-1.97	ZGC:136374
A_15_P106668	-1.87	---
A_15_P116718	1.86	---
A_15_P105561	1.85	GREM1A, gremlin 1 homolog a, cysteine knot superfamily (Xenopus laevis)
Trilostane ovary (FDR ≤ 5%)		
A_15_P120956	10.72	SI:CH211-240L19.8, hypothetical protein LOC799298
A_15_P117758	4.43	JUNB , jun B proto-oncogene
A_15_P115095	3.28	ESR2B, estrogen receptor 2b
A_15_P118979	3.27	TNFRSF19, tumor necrosis factor receptor superfamily, member 19
A_15_P102436	-2.93	---
A_15_P111880	-2.92	GORASP1, golgi reassembly stacking protein 1
A_15_P115312	2.89	GADD45AB, growth arrest and DNA-damage-inducible, alpha, b
A_15_P120411	2.73	---
A_15_P113562	-2.65	CARS, cysteinyl-tRNA synthetase
A_15_P102278	2.63	---

Table 3. Ingenuity Pathway Analysis (IPA) mapping (P value ≤ 0.05) of the DEGs (false discovery rate, FDR, ≤ 5% and treated/control ≥ 1.3 fold) from trilostane (TRI) ovary and testis to human-mouse-rat (HMR) pathways, based on human orthologs of the zebrafish genes (Agilent release of zebrafish microarray annotations of June 17, 2007 for designs 013223 and 015064) and Ingenuity Knowledge Base as a reference set. TRI ovary: 96 h and 96 h low dose; TRI testis: 24, 48, 96 h, and 96 h low dose.

Significant HMR Pathways	-Log (P value)	Top cellular functions according to IPA
TRI ovary (251 DEGs)		
Aminoacyl-tRNA Biosynthesis	1.72	Amino Acid Metabolism; Molecular Transport; Small Molecule Biochemistry
Methionine Metabolism	1.68	Amino Acid Metabolism; Molecular Transport; Small Molecule Biochemistry
Wnt/beta-catenin Signaling	1.58	Gene Expression; Cancer; Cellular Growth and Proliferation
Arginine and Proline Metabolism	1.46	Amino Acid Metabolism; Molecular Transport; Small Molecule Biochemistry
Ephrin Receptor Signaling	1.40	Cellular Movement; Cancer; Cell Morphology
Alanine and Aspartate Metabolism	1.37	Cell Cycle; Hepatic System Development and Function; Amino Acid Metabolism
TRI testis (523 DEGs)		
Oxidative Phosphorylation	2.44	Molecular Transport; Lipid Metabolism; Small Molecule Biochemistry
Virus Entry via Endocytic Pathways	2.33	Cellular Function and Maintenance; Cellular Movement; Cell Death
Inositol Metabolism	2.26	Lipid Metabolism; Molecular Transport; Nucleic Acid Metabolism
Amyotrophic Lateral Sclerosis Signaling	2.26	Cell Death; DNA Replication, Recombination, and Repair; Cellular Compromise
Huntington's Disease Signaling	1.9	Cell Death; Nervous System Development and Function; Genetic Disorder

Glutamate Metabolism	1.62	Nucleic Acid Metabolism; Small Molecule Biochemistry; Amino Acid Metabolism
Valine, Leucine and Isoleucine Degradation	1.46	Lipid Metabolism; Molecular Transport; Nucleic Acid Metabolism
Galactose Metabolism	1.41	Lipid Metabolism; Small Molecule Biochemistry; Endocrine System Disorders
Fructose and Mannose Metabolism	1.33	Cell Morphology; Cellular Compromise; Cell-To-Cell Signaling and Interaction
Lipid Antigen Presentation by CD1	1.33	Lipid Metabolism; Small Molecule Biochemistry; Cell-To-Cell Signaling and Interaction
GABA Receptor Signaling	1.31	Cell-To-Cell Signaling and Interaction; Nervous System Development and Function; Amino Acid Metabolism

Table 4. Selected pathways and transcription factor (TF) networks significant for various trilostane conditions (Trt) as determined by Gene Set Enrichment Analysis (GSEA) or Extended-GSEA. Networks/pathways are listed sequentially according to their positional order in a dendrogram consisting of 1932 zebrafish TF network/canonical pathways (Wang et al., 2010), with those closely clustered together marked by common numeric identifiers. Gene Ontology enrichment analysis of biological processes for individual TF networks was conducted through GoMiner (http://discover.nci.nih.gov/gominer/htgm.jsp) and those significant (false discovery rate, FDR, ≤ 5%) were marked by '*'. For the treatment of TRI 96 h female ovary, only the top 30 networks (as ranked by FDRs) were listed among those significant at FDR ≤ 25%, totaling 514 in GSEA and 133 in E-GSEA. The probe annotations are based on the Agilent release of July 19, 2010 for design 015064. Probes without annotations are marked by "---". The entire sets of GSEA and E-GSEA significant TF networks are available in Supplemental List 4 and 5.

Trt	Method	Networks/pathways	Cluster	FDR (%)	Gene Symbol and annotation
1	E-GSEA	DRTFovaryCy5Cy3_A_15_P119495		8.5	VED ventrally expressed dharma/bozozok antagonist
1	E-GSEA	DRTFovaryCy5Cy3_A_15_P109988		12.0	MBD3B methyl-CpG binding domain protein 3b
1	E-GSEA	DRTFovaryCy5Cy3_A_15_P112149		22.7	GATAD2A GATA zinc finger domain containing 2A
2	E-GSEA	DRTFtestisCy5Cy3_A_15_P119788		19.0	Zgc:101606 PRDM12 PR domain containing 12
3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P117096**	1	6.8	Zgc:154057 TADA2L transcriptional adaptor 2-like
3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P111976**	1	8.3	YY1L YY1 transcription factor, like
3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P106037**	1	8.3	ARID2 AT rich interactive domain 2
4	E-GSEA	DRTFovaryCy5Cy3_A_15_P117603		0.95	TFDP1 transcription factor Dp-1
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P104909		8.2	DPF2 D4, zinc and double PHD fingers family 2
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P111423		7.8	XBP1 X-box binding protein 1
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P100238		8.1	si:ch211-221n23.1 si:ch211-221n23.1
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P121251		8.5	FOXP1B forkhead box P1b
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P101062		8.3	HIF1AL hypoxia-inducible factor 1, alpha subunit, like
3	GSEA	DRTFtestisCy5Cy3_A_15_P101536		1.1	---
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P106117		7.0	ZNF513 zinc finger protein 513
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P101993		8.5	TSHZ1 teashirt family zinc finger 1

3	GSEA	DRTFovaryRatio_A_15_P120158		1.6	MYSM1 Myb-like, SWIRM and MPN domains 1
3	GSEA	DRTFovaryRatio_A_15_P102602		1.6	HEY1 hairy/enhancer-of-split related with YRPW motif 1
4	GSEA	**DRTFovaryRatio_A_15_P103430**	2	25.0	FAM60AL family with sequence similarity 60, member A, like
3	GSEA	**DRTFovaryRatio_A_15_P102642**	2	1.5	MYSM1 Myb-like, SWIRM and MPN domains 1
3	GSEA	**DRTFovaryRatio_A_15_P113870**	2	1.3	zgc:110075 zgc:110075
3	GSEA	DRTFovaryRatio_A_15_P103227		2.0	SETDB1B SET domain, bifurcated 1b
3	GSEA	**DRTFovaryRatio_A_15_P113313**	3	1.7	FOXP1A forkhead box P1a
3	GSEA	**DRTFovaryRatio_A_15_P121011**	3	1.6	ZNF513 zinc finger protein 513
3	GSEA	DRTFovaryRatio_A_15_P106336		1.7	MAX myc-associated factor X
3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P100335**	4	8.1	KDM5C, lysine (K)-specific demethylase 5C
3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P112272**	4	8.1	si:dkey-7l12.4 si:dkey-7l12.4
3	GSEA, E-GSEA	**DRTFtestisCy5Cy3_A_15_P103227**	4	1.8, 5.2	SETDB1B SET domain, bifurcated 1b
3	GSEA, E-GSEA	**DRTFtestisCy5Cy3_A_15_P106248**	4	1.7, 7.5	SETDB1B SET domain, bifurcated 1b
3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P117380**	5	7.8	IKZF5 IKAROS family zinc finger 5
3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P111031**	5	8.6	---
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P116104		7.5	MAFK v-maf musculoaponeurotic fibrosarcoma oncogene homolog K (avian)
3	GSEA, E-GSEA	**DRTFtestisCy5Cy3_A_15_P109916**	6	1.7, 6.1	CLOCK3 clock homolog 3 (mouse)
3	GSEA	**DRTFtestisCy5Cy3_A_15_P121053**	6	2.1	---
3	GSEA	DRTFtestisCy5Cy3_A_15_P118438		1.9	NR4A2B, nuclear receptor subfamily 4, group A, member 2b
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P101003		8.6	---
5	E-GSEA	DRTFtestisCy5Cy3_A_15_P118821		23.3	---
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P101193*		6.2	MXI1 max interacting protein
6	E-GSEA	DRTFtestisCy5Cy3_A_15_P104405*		14.6	ZNF277 zinc finger protein 277
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P117115		8.0	XBP1 X-box binding protein 1

3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P107038**	7	5.5	XBP1 X-box binding protein 1
3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P114302**	7	4.2	ZFAND5A, Danio rerio zinc finger, AN1-type domain 5a
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P120416		8.6	---
3	GSEA	**DRTFovaryRatio_A_15_P115074**	8	1.6	---
3	GSEA	**DRTFovaryRatio_A_15_P118373**	8	1.2	---
3	GSEA	**DRTFovaryRatio_A_15_P110705**	8	1.6	zgc:66448 zgc:66448
3	GSEA	**DRTFovaryRatio_A_15_P107988**	8	0.8	GLI3 GLI-Kruppel family member GLI3
3	GSEA	**DRTFovaryRatio_A_15_P102184**	9	1.6	si:ch211-262e15.1
3	GSEA	**DRTFovaryRatio_A_15_P121463**	9	0.2	MEF2D myocyte enhancer factor 2d
3	GSEA	**DRTFovaryRatio_A_15_P104270**	9	1.5	RARGA retinoic acid receptor gamma a
3	GSEA	**DRTFovaryRatio_A_15_P109916**	9	1.5	CLOCK3 clock homolog 3 (mouse)
3	GSEA	**DRTFovaryRatio_A_15_P113803**	9	2.0	ZNF384L zinc finger protein 384 like
3	GSEA	DRTFovaryRatio_A_15_P107486		1.6	MEF2D myocyte enhancer factor 2d
3	GSEA	DRTFtestisCy5Cy3_A_15_P104270		0.7	RARGA retinoic acid receptor gamma a
3	GSEA, E-GSEA	DRTFtestisCy5Cy3_A_15_P115983		0.6, 8.5	VEZF1 vascular endothelial zinc finger 1
3	GSEA	DRTFtestisCy5Cy3_A_15_P113142		0.5	TCF12 transcription factor 12
3	GSEA	**DRTFtestisCy5Cy3_A_15_P105566**	10	1.0	MYBL2 myeloblastosis oncogene-like 2
3	GSEA	**DRTFtestisCy5Cy3_A_15_P118392**	10	0.5	RBPJA recombination signal binding protein for immunoglobulin kappa J region a
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P114243		7.4	XBP1 X-box binding protein 1
3	GSEA	DRTFovaryRatio_A_15_P101342		1.8	HOXB1B homeo box B1b
		…			
		HPG-axis			
		…			
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P104642		5.7	GBX2 gastrulation brain homeo box 2
3	E-GSEA	**DRTFtestisCy5Cy3_A_15_P101487**	11	8.4	IRX4A iroquois homeobox protein 4a

5	E-GSEA	**DRTFtestisCy5Cy3_A_15_P113139**	11	24.9	PRDM8 PR domain containing 8
3	E-GSEA	DRTFtestisCy5Cy3_A_15_P101871		8.3	KDM5BA, lysine (K)-specific demethylase 5Ba
3	E-GSEA	DRTFovaryRatio_A_15_P102654		3.1	STAT4 signal transducer and activator of transcription 4
1	GSEA	KEGG_PATHWAY_DRE04120		20.7	Ubiquitin mediated proteolysis
6	GSEA	KEGG_PATHWAY_DRE04630		18.9	Jak-STAT signaling pathway
6	GSEA	KEGG_PATHWAY_DRE00271		12.5	Methionine metabolism

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674 Table 5. Gene Ontology (GO) biological processes enriched in the individual trilostane-impacted transcription factor (TF)
675 networks at a false discovery rate (FDR) ≤ 5% according to GoMiner (http://discover.nci.nih.gov/gominer/htgm.jsp), based
676 on the entire set of trilostane-impacted TF networks.

TF networks	Hub TF Gene symbols	No. GO processes enriched	Summary of GO biological processes enriched
DRTFtestisCy5Cy3_A_15_P104723	RARGA	29	Organelle localization, cilium assembly, regulation of protein kinase cascade, regulation of stress response, body pattern specification
DRTFtestisCy5Cy3_A_15_P101193	MXI1	22	Organelle and protein localization, cilium assembly
DRTFovaryCy5Cy3_A_15_P105179		20	Regulation of phosphorylation, kinase activity; convergent extension in gastrulation, protein homooligomerization, protein kinase cascade
DRTFtestisCy5Cy3_A_15_P119594	MBD1	17	Organelle and protein localization, cilium assembly, cell division
DRTFovaryCy5Cy3_A_15_P104878	NR2F6B	15	Tissue regeneration, cell migration in gastrulation, stress response, cell polarity establishment
DRTFovaryRatio_A_15_P105179		14	Regulation of phosphorylation, kinase activity, protein homooligomerization
DRTFtestisCy5Cy3_A_15_P111139		13	Celluar metabolic process, circadian rhythm
DRTFovaryCy5Cy3_A_15_P101643	NR2F1A	9	Regulation of neurogenesis, hemopoiesis, cell development; immune system
DRTFovaryCy5Cy3_A_15_P107503	POU6F1	8	Regulation of phosphorylation, kinase activity
DRTFovaryCy5Cy3_A_15_P120158	MYSM1	8	Retina development in camera-type eye
DRTFtestisCy5Cy3_A_15_P100890	TGIF1	8	Neural tube patterning
DRTFtestisCy5Cy3_A_15_P109417	CEBPG	8	Organelle localization
DRTFtestisCy5Cy3_A_15_P113550	AR	8	Eye photoreceptor development, neuron development
DRTFtestisCy5Cy3_A_15_P117912	DMRT1	8	Neuron differentiation, cell projection morphogenesis
DRTFtestisCy5Cy3_A_15_P118509	SNX3	8	Regulation of phosphorylation, kinase activity, fin regeneration

DRTFtestisCy5Cy3_A_15_P115074		6	Regulation of phosporylation, kinase activity
DRTFtestisCy5Cy3_A_15_P115985	SI:DKEY-211G8.3	6	Cellular localization and transport
DRTFtestisCy5Cy3_A_15_P103430	FAM60AL	5	Cell cycle phase, nuclear division, organelle fission
DRTFtestisCy5Cy3_A_15_P118261	ZFAND5A	5	Negative regulation of gene expression and macromolecule biosynthesis
DRTFovaryCy5Cy3_A_15_P106612	ZGC:112083	4	Retina development in camera-type eye, vesicle mediated transport
DRTFtestisCy5Cy3_A_15_P113375	ZGC:66448	4	Immune system development, hemopoiesis
DRTFtestisCy5Cy3_A_15_P115134	NEUROG1	4	Neuromast development
DRTFtestisCy5Cy3_A_15_P107872	MSXD	3	Hindbrain development, peripheral nervous system development
DRTFovaryRatio_A_15_P119732	SMAD2	2	Retina development in camera-type eye
DRTFtestisCy5Cy3_A_15_P104405	ZNF277	2	Circadian rhythm
DRTFtestisCy5Cy3_A_15_P107467	RBPJA	2	Somite specification
DRTFtestisCy5Cy3_A_15_P112687	ZGC:154057	2	Cellular response to DNA damage stimulus
DRTFovaryCy5Cy3_A_15_P101177	CEBPG	1	Retina development in camera-type eye
DRTFovaryCy5Cy3_A_15_P111700	CHURC1	1	Retina development in camera-type eye
DRTFovaryCy5Cy3_A_15_P113640	SMAD9	1	Regulation of multicellular organismal process
DRTFovaryCy5Cy3_A_15_P119732	SMAD2	1	Vesicle mediated transport
DRTFovaryCy5Cy3_A_15_P120536	TP53	1	Cell cycle
DRTFovaryRatio_A_15_P105718	SMARCC1	1	Cell migration in hind brain
DRTFovaryRatio_A_15_P107347	MEF2D	1	Cell migration in hind brain
DRTFovaryRatio_A_15_P113926	RARGA	1	Cell migration in hind brain
DRTFtestisCy5Cy3_A_15_P107228	STAT1A	1	Responses to virus
DRTFtestisCy5Cy3_A_15_P117549	NEIL3	1	Retina development in camera-type eye

677 Table 6. Among a total of 951 TF probes, 35 were differentially expressed under trilostane ovary and/or trilostane testis at
678 false discovery rate (FDR) ≤ 5% with treatment/control ≥ 1.3. Fold changes up or down are indicated by '+/-'. A fold
679 change is based on treated/control in a given condition. Highlighted in bold and/or italicized are those TFs whose networks
680 were also significantly impacted under trilostane 96 h ovary condition according to either GSEA or E-GSEA. The probe
681 annotations are based on the Agilent release of July 19, 2010 for design 015064. Probes without annotations are marked by
682 "---".

TFs differentially expressed	Trilostane ovary (fold change)	Trilostane testis (fold change)	Gene symbol and annotation
A_15_P101004		-1.59	EPAS1A, endothelial PAS domain protein 1a
A_15_P101208		1.65	PEA3 ETS-domain transcription factor
A_15_P101489		1.3	RUNX2B runt-related transcription factor 2b
A_15_P101643		1.38	NR2F1A nuclear receptor subfamily 2, group F, member 1a
A_15_P102513		-1.31	ZGC:136874 zgc:136874
A_15_P102642	-2.51		MYSM1, Myb-like, SWIRM and MPN domains 1
A_15_P102915		1.37	---
A_15_P104258		1.37	---
A_15_P104270	-2.52		RARGA, retinoic acid receptor gamma a
A_15_P105367		-1.66	TWIST1B twist1b
A_15_P105886		-1.59	TWIST1B twist1b
A_15_P106248		-1.3	SETDB1B SET domain, bifurcated 1b
A_15_P107124		1.73	OLIG2 oligodendrocyte lineage transcription factor 2
A_15_P107503	-1.74	-1.34	POU6F1, POU class 6 homeobox 1
A_15_P108960		1.54	---
A_15_P109209		1.63	MEIS2.2 myeloid ecotropic viral integration site 2.2
A_15_P110682		1.68	PEA3, ETS-domain transcription factor
A_15_P111881		1.34	HER13 hairy-related 13

A_15_P112112		-1.89	ZBTB16 zinc finger and BTB domain containing 16
A_15_P113142		-1.32	TCF12 transcription factor 12
A_15_P113375		-1.35	ZGC:66448 zgc:66448
A_15_P113397		1.37	---
A_15_P113870		-1.41	ZGC:110075 zgc:110075
A_15_P114960	-1.66		CLOCK clock
A_15_P115074	-1.52		---
A_15_P115095	3.28		ESR2B estrogen receptor 2b
A_15_P115367		1.31	LHX6 LIM homeobox 6
A_15_P117172		1.34	---
A_15_P117758	4.43		JUNB jun B proto-oncogene
A_15_P118373	-1.55		---
A_15_P118392	-1.52		RBPJA recombination signal binding protein for immunoglobulin kappa J region a
A_15_P119739	-1.48		---
A_15_P120383		1.32	NR2F1A nuclear receptor subfamily 2, group F, member 1a
A_15_P120814		-1.3	TCF12 transcription factor 12
A_15_P121158	1.3	1.36	---

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