The effects of different endocrine disruptors defining compound-specific alterations of gene expression profiles in the developing testis

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Abstract

Environmental contaminants considered endocrine disruptors have been shown to affect testis development and function but the mechanisms of action are not clear. We now have analyzed the effects on the transcriptome in testes of mice exposed to mono-(2-ethylhexyl)-phthalate (9.2; 46.3 or 92.7 mg/kg/d), zearalenone (1.3; 3.9 or 6.6 mg/kg/d), lindane (16.6; 32.2 or 64.4 mg/kg/d), bisphenol-A (0.16; 16 or 64 mg/kg/d) or 17β-estradiol (0.006; 0.012 or 0.048 mg/kg/d). The compounds were orally administered in the drinking water during distinct developmental periods: A) mothers were exposed only during the two weeks before mating; B) the exposure was continued during pregnancy until birth or C) exposure was continued for a further four weeks after birth. Testes were studied at four weeks of age. Mono-(2-ethylhexyl)-phthalate and zearalenone, both produced specific alterations of gene signatures. Interestingly, this was irrespective of the concentration of the toxicant or the developmental period during which exposure occurred.

Key Words

Endocrine disruptor; testis; 17β-estradiol; Lindane; Bisphenol-A; Mono-Ethylhexyl Phthalate; Zearalenone; DNA microarrays; gene expression profile.

Abbreviations

ED, Endocrine Disruptor. TDS, Testicular Dysgenesis Syndrome. MEHP, Mono-EthylHexyl Phthalate. DEHP, Di 2-EthylHexyl Phthalate. BPA, Bisphenol-A. LIN, Lindane. ZEA, Zearalenone. E2, 17-β-estradiol. pn, post-natal.

1. Introduction

There is significant documentation on the adverse effects of environmental pollutants on reproductive health [1-3]. Environmental toxicants that act as agonists or antagonists of natural hormones, generically considered as endocrine disruptors (EDs), can affect the development of the reproductive system and associated organs [4, 5]. There is some controversy as to the effects and mechanisms by which EDs act [6, 7], although the most accepted hypothesis holds that EDs interfere with steroid hormone action through disruption of steroid biosynthesis, the hormone balance, signaling pathways of downstream consequences. There is currently significant concern regarding the increase in male and female hormone-related disorders detected in epidemiological studies. In mammals, the male reproductive organs have been clearly identified as a target for the deleterious action of many environmental toxicants, and the Testicular Dysgenesis Syndrome (TDS) could be a consequence of developmental exposure to such compounds [8, 9]. TDS groups four clinical and etiologically related traits: hypospadias, cryptorchidism, low sperm counts and testicular tumors [10]. These dysfunctions could originate through changes in the microenvironment that affect different target cells during embryonic differentiation [11]. Nevertheless, the molecular mechanisms by which toxicants or potential EDs alter spermatogenesis and testicular function are yet to be fully established. Indeed, their effects cannot be simply explained by direct interactions with hormone pathways [12], and interference with gene expression regulation could occur at diverse levels during development, either as a direct or indirect consequence of exposure to these toxicants. The nature of the compounds, the dose and extent of exposure, as well as the developmental period at which exposure occurs are also factors that should be taken into account when considering the mechanisms causing the adverse effects on testicular development and function.

There is evidence that the origin of the adverse effects lies in fetal exposure [13]. Potential effects of endocrine disruptors via placental transport during pregnancy and via milk during lactation are well documented. Transplacental absorption and fetal detection of different environmental estrogenic compounds has been reported in experimental animals [14-16] and in humans [17, 18]. Similarly, persistence in breast milk was detected in different populations [19-21]. Nevertheless, the mechanisms underlying the influence of the different xeno-compounds are

not well established, especially since daily exposure usually involves contact with a mixture of contaminants.

Over 150 different contaminants have been reported in individuals in the US and some, such as phthalates, can be found in nearly 100% of the population [22]. In humans, basic studies associating chemical exposure and testis development disorders are very difficult to carry out [13]. Indeed, most of the biomarkers that could serve as endpoints, such as semen quality or hormone levels, cannot be considered as direct or adequate evaluation of toxicological exposure [23].

To date, studies into the environmental induction of changes in gene expression and regulation as part of disease etiology have mainly focused on specific genes or on the relevant genetic pathways related to particular diseases, including reproductive dysfunction [24]. However, most emphasis has been placed on altered gene expression mediated by estrogen receptors [25]. Some EDs can also act as anti-androgens [26, 27] producing specific alterations in gene expression [28]. Considering that developmental exposure to EDs interferes with gene expression in the testis, a comparison was performed here to evaluate whether specific EDs, with potential different molecular mechanisms of action, induce specific signatures at a global level of gene expression. In addition, we studied whether these compounds act in similar pathways of estrogen signaling and render similar signatures of gene expression, as well as whether specific patterns of gene modulation can be associated to developmental windows and/or doses of exposure and to cytological/histological changes in the seminiferous epithelium.

To address these questions, we used DNA microarrays that represent the global mouse transcriptome (31,769 printed 70-mer DNA probes corresponding to 24,878 expressed or predicted genes) to analyze the effects on gene expression of five environmental pollutants considered as EDs. Bisphenol-A (BPA) is one of the most intensely produced plasticizers worldwide that can leach into food and beverages [29]. BPA has been detected in blood samples and other fluids, and there is still considerable controversy regarding its potential effects [30]. Lindane (γ-hexachlorocyclohexane, LIN) is one of the oldest synthetic pesticides and despite being considered a persistent toxicant that adversely affects reproductive functions in animals [10, 31], it is still in use worldwide. Mono 2-ethylhexyl phthalate (MEHP) is the active metabolite of di 2-ethylhexyl phthalate (DEHP) and is widely used as plastic flexibilicizer despite

its reported estrogenic/antiandrogenic effect [11, 32, 33]. Zearalenone (ZEA) is a toxic substance considered a phytoestrogen that is produced by *Fusarium spp.*, a contaminant of grain, and is thought to cause male germ cell toxicity [34]. Finally, we assessed the effects of 17-β-estradiol (E2) as a natural estrogen.

The effects of each of these compounds on the transcriptome were compared after exposure during different developmental periods, and at different concentrations for each period. The objective was to evaluate the level of global gene expression modification in the testes of mice exposed to EDs and the signature of gene expression they provoked considering three factors: compound specificity, the developmental window and the dosage of exposure. Morphological effects on testicular development and spermatogenesis were also evaluated after the various experimental conditions. The parameters studied were: body and testis weight, the numbers of apoptotic cells in the testis, the percentage of tubule sections showing abnormalities such as missing generations of germ cells or abnormal cell associations, and the number of diploid spermatids as a measure of problems occurring at meiotic divisions.

2. Materials and methods

2.1. Ethics Statement

All animal care and the procedures for sacrificing the animals were in accordance with the regulations laid down by the CSIC Bioethics Committee and the relevant European Commission (EC) guidelines (directive 86/609/EEC). The present study was approved by the CSIC Bioethics Committee (ID number: CB/CIB-PI071007-2007).

2.2. Animal Exposure

CD-1 mice were supplied by our own animal facility, the CIB-CSIC bioterium. Breeding and production of the mice were carried out under specific pathogen-free (SPF), controlled temperature (22±1ºC) and regulated humidity (50-55%) conditions; periods of light/dark 12h and diet available *at libitum*.

CD-1 mice were exposed to different doses of EDs *in vivo* following a defined regimen, detailed in Figure 1. In all cases, females were mated with unexposed males. The day when the

vaginal plug was detected was recorded as day 0. The age-range of the parental mice used in this study was 2-3 months. At least, three adult females were exposed to each dose and ED during developmental exposure A, B and C. The male offspring were sacrificed at four weeks of age to obtain their testes for the different analysis. At least, three males, offspring from different mothers, including those from exposure C which were also exposed during four weeks after birth were used for RNA purification. For the histological and morphological analysis we proceeded using the same protocol of exposure and number of animals. The number of animals tested for histological analysis is explicitly indicated in the figures S2-S11. The control groups were comprised of the same number of animals and the exposure route for the appropriate vehicle was similar to that for mice that received an ED.

To emulate the regular intake of the environmental toxicants studied and to approximate the route of administration to whole body exposure, the compounds were administered orally in the drinking water at different dosages during different periods of development to reach the doses indicated in Table 1. As a result, 45 different experimental conditions were compared in this study. The estimated intakes were calculated on the basis of average of drinking and body weight as recorded in pilot experiments and in agreement with the data in the literature referred to these parameters. *In utero* and neonatal exposure was assumed to occur via placental transport and via milk, respectively. Ethanol was used as the vehicle for E2, BPA and ZEA, and DMSO for MEHP and LIN. Comparative control testes for microarray hybridizations were obtained from animals exposed to the vehicle alone, following the same exposure regimens as for EDs, detailed in Figure 1: ethanol at estimated intake of 0.060 g/kg/day and DMSO at 0.029 g/kg/day. The NOAEL (No-observed-adverse-effect-level) has been established at 2.4 g/kg/day for ethanol (OECD-SIDS, www.jetoc.or.jp/HP_SIDS/pdffiles/64-17-5.pdf) and at 2.5 g/kg/day for DMSO (http://www.epa.gov/oppt/chemrtk/pubs/summaries/dimthslf/c14721rr.pdf). All control mice were exposed to doses of vehicle equivalents to those for each ED.

2.3. Histological Parameters Studied

At least, 3 different mice were analyzed after every experimental condition. From each animal, body weight and testis weight were registered and relative testis weight were calculated (absolute testis weight proportioned to the body weight). Epithelial abnormalities of the

seminiferous tubules, namely vacuolisation, missing germ cell generations or abnormal germ cell associations were observed in PAS-hematoxylin stained sections and the numbers of tubules showing these abnormalities were counted and expressed per 100 tubule sections. In the same slides, the diameters of 25 cross sections of seminiferous tubules were measured per animal, and the average tubule diameter was calculated. To assess problems at the second meiotic division, tubules containing diploid spermatids were counted and the percentage of the tubules containing diploid spermatids was determined. The number of TUNEL positive cells was counted in all testes and calculated as numbers per 100 tubule cross sections. At least 100 tubules per animal were studied (all histological parameters studied are shown in Supplementary figure S12). Numbers were expressed as means ± SEM and statistical analysis was performed using the Univariate Analysis of Variance.

2.4. Apoptosis detection by way of Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate-biotin nick end labeling (TUNEL)

Five μm Bouins fixed, paraffin embedded testis sections were incubated for 5 min in 10mM citric buffer (pH6.0) at 98°C and slowly cooled to room temperature (RT). Endogenous peroxidase was blocked with 3% H_2O_2 in water for 5 min. Sections were washed three times with PBS before a 60 min incubation in TUNEL mix at 37°C. TUNEL mix consisted of 0.3 U/mL calf thymus terminal deoxynucleotidyl transferase (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), 6.66 mM/mL biotin dUTP (Roche Applied Science, Penzberg, Germany) in terminal transferase buffer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The TUNEL reaction was stopped by incubation in 300mM NaCl, 30mM Sodium citrate in water for 15 min at RT. After washing in PBS, sections were blocked with 2% BSA (Sigma-Aldrich, St. Gallen, Switzerland) in PBS at RT for 10 min. Sections were treated for 30 min at 37°C in a moist chamber with a 1:20 dilution of ExtrAvidin peroxidase antibody. After three washes in PBS, detection was performed with DAB+ (Dako, Glostrup, Denmark). Sections were counterstained with Mayer's hematoxylin, dehydrated and mounted with Pertex (Cellpath, Newtown, UK). No statistically significant differences in the evaluated parameters were seen between samples from animals exposed only to the vehicle of the compounds used as controls versus unexposed, i.e., EtOH or DMSO versus H2O.

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199 *2.5. RNA preparation*

200 Total RNA from the testes was extracted using TRIzol (Invitrogen, part of Life
201 Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and the RNA
202 was then purified with MegaClear (Ambion, brand of Life Technologies, Carlsbad, CA, USA).
203 To minimize the inter-individual variability and considering the broad number of variables (45)
204 in the global analysis, we used pools prepared from RNA samples from at least three individual
205 testes (5 μg total RNAs each) exposed to every experimental condition: compound, dose and
206 developmental stage and its respective controls. RNA quality was checked using the Experion
207 automated electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). Samples with an
208 rRNA ratio (28S/18S) higher than 1.4 were used for the experiments. Total RNA from samples
209 exposed to EDs and vehicles (DMSO or ETOH) (controls) (1.5 μg for each pool) was amplified
210 using the Amino Allyl MessageAmp aRNA kit (Ambion, brand of Life Technologies, Carlsbad,
211 CA, USA) and we obtained 15-60 μg of amino-allyl amplified RNA (aRNA). The mean aRNA
212 size was approximately 1500 nucleotides when measured in the Experion analyzer. For each
213 sample, 2.5 μg of aRNA was labeled with one aliquot of Cy3 or Cy5 mono NHS ester (CyDye
214 Post-labeling Reactive Dye, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and it was
215 purified with the Amino Allyl MessageAmp aRNA kit. Cy3 and Cy5 incorporation was measured
216 using 1 μl of probe in a Nanodrop spectrophotometer (Nanodrop Technologies, brand of Thermo
217 Fisher Scientific, Waltham, MA, USA). For hybridization, 110-150 pmol of the Cy3 and Cy5
218 probes were mixed, dried in a speed-vacuum and resuspended in 9 μl RNase-free water. Labeled
219 aRNA was fragmented by adding 1 μl of 10x fragmentation buffer (Ambion, brand of Life
220 Technologies, Carlsbad, CA, USA) and incubating for 15 min at 70ºC. The reaction was
221 terminated by adding 1 μl of the stop solution (Ambion, brand of Life Technologies, Carlsbad
222 CA, USA).

223 In order to minimize potential biological variations between individuals as effect of the
224 compounds, a pool of at least three individual RNA samples was used in each experimental
225 condition. Sample pooling, by mixing mRNAs from several biological-replicate samples, gives a
226 mixture of individual responses and the results have been statistically demonstrated to be valid
227 [35], [36, 37].

228

229 *2.6. Microarray processing*

230 Mouse Oligoset v3 (OPERON) arrays were used from the Genomics facility at the
231 University of Cincinnati (USA). The slides contained 31,769 spotted probes (70 mer
232 oligonucleotides) corresponding to 24,878 expressed or predicted mouse genes. Information on
233 the printing and the oligo set can be found at http://microarray.uc.edu. The slides were
234 prehybridized (42ºC, 45-60 min) in 6x SSC, 0.5% SDS and 1% BSA, and they were then rinsed
235 10 times with distilled water. Fragmented Cy5 and Cy3 aRNA probes were mixed (110-150 pmol
236 of each label) with 10 μg PolyA (Sigma-Aldrich, St. Gallen, Switzerland) and 5 μg Human Cot-
237 DNA (Invitrogen, part of Life Technologies, Carlsbad, CA, USA), and then dried in a speed-
238 vacuum. Each probe mix was resuspended in 60 μl of hybridization buffer (50% formamide, 6x
239 SSC, 0.5% SDS, 5x Denhardt's solution) and the probes were denatured at 95ºC for 5 min before
240 they were applied to the slide using a LifterSlip (Erie Scientific Company, Portsmouth, NH,
241 USA). The slides were incubated at 48ºC for 16 h in hybridization chambers (Array-It
242 Corporation, Sunnyvale, CA, USA); in a water bath. After incubation, the slides were washed
243 twice with 0.5x SSC, 0.1% SDS for 5 min each, three times with 0.5x SSC for 5 min, and finally
244 in 0.05x SSC for 5 min, before they were dried by centrifugation (1600 r.p.m., 1 min). Images
245 from the Cy3 and Cy5 channels were equilibrated and captured with an Axon 4000B scanner, and
246 the spots were quantified using GenePix 5.1 software. Dye swapping replicates were performed
247 for each particular hybridization, as described below (Data Analysis section).

248

249 *2.7. Data Analysis*

250 The raw data were processed using "AlmaZen" (Alma Bioinformatica, Tres Cantos, Spain)
251 software. The background was subtracted from each individual hybridization and they were
252 Lowess normalized [38]. For each condition, a technical replicate was performed by dye-
253 swapping (two hybridizations) in order to minimize a possible bias in the labeling process. The
254 mean of $\log_2$ ratio (exposed/control) from the merge of the two dye-swapping hybridizations
255 were calculated and p-values, standard deviations, and *z*-scores were obtained [39-41]. Raw and
256 processed data were included in the Gene Expression Omnibus database (GEO) [42] (accession
257 number GSE14774) following the Minimal Information About a Microarray Experiment

(MIAME) criteria [43].

2.8. Cluster analysis

To analyze transcriptome profiles in different experimental conditions, hierarchical clustering of array data has been used widely [44, 45]. Data were preprocessed previously to perform Hierarchical Clustering. Preprocessing included following criteria, using the "Preprocess DNA array data files" web tool (GEPAS v3.0) [46] [47]: merging values of gene replicates (by averaging), filtering missing values (70 % was the minimum percentage of missing values accepted), imputing missing values (using KNN impute) and filtering flat patterns by the numbers of peaks (a minimum of 3 peaks and a threshold of +0.7, in log scale, were considered). The distance matrix was calculated for both, genes and experimental conditions from the preprocessed datasets. The similarity metric used was a centered Pearson correlation. In addition, both genes and conditions were clustered by the Average Linkage method using Cluster 3.0 software [48], and the clustering results generated by Cluster 3.0 were visualized using Java Treeview [49].

We used this unsupervised hierarchical clustering analysis to organize the different conditions of exposure according to the similarity or dissimilarity of the gene expression profiles, situating the defined conditions of exposure with similar profiles together as neighboring columns in the clustergram. In addition, we analyzed the potential participation of gene signatures in molecular pathways as described in the results of the Ingenuity Pathway Analysis (http://www.ingenuity.com).

2.9. Confirmation of microarray expression data by TaqMan® Low Density Arrays (TLDA)

The expression of 23 genes randomly selected from the microarray analyses (Table S1 in supplemental material) was measured in custom TaqMan® Low Density Arrays (TLDA) (Applied Biosystems, part of Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. Expression was measured in the same RNA samples studied in the microarray experiments for each experimental condition.

From the entire data set corresponding to the expression of the 23 selected genes (Table S1) obtained from all analyzed microarrays, only 130 expression values filtered for statistically

significant p-value (>0.05) were used for qualifying purposes. These data were compared with the corresponding data obtained by qRT-PCR. Statistical analyses of Pearson correlation and p-value were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

3. Results

The first aim was to evaluate the global change in levels of gene expression as effect of exposure to EDs in the different experimental conditions. From the microarray data, we used the average of the log-ratio approach adopted in the MicroArray Quality Control (MAQC) project to compare the toxicogenomic effects of different compounds [50]. To assess inter-individual gene expression level as a baseline in the testis from the CD-1 mice used, we performed preliminary analyses comparing RNA from three mice (S#1 *vs* S#2, S#1 *vs* S#3 and S#2 *vs* S#3) and from pooled testes (S#Pool vs S#Pool) obtained from at least three four-weeks old mice, neither exposed to compounds nor solvents. Fig. 2 shows the level of the global gene expression variation found in this comparative analysis in terms of the average of log-ratio from genes with an average signal higher than 64 in MA plots. Only raw and processed data from dye swapping replicates coming from pooled samples were included in the Gene Expression Omnibus database (GEO, accession number GSE14774), named as "Testis_Control".

After the analysis of the signal distribution in MA plots (data not shown), we only considered spots with an average signal >64 in order to avoid false background corresponding to genes weakly or negligibly expressed in the testis. We found that MEHP induced the strongest degree of global modification of gene expression of all the compounds analyzed (Fig. 2). The maximum effects were detected in those mice that were exposed throughout the entire period of development until they were analyzed (Fig. 2, exposure C). However, the results obtained from mice whose mothers were only exposed during the preovulatory pre-mating period indicated that some of the EDs analyzed, such as ZEA and MEHP, still induced gene expression modifications in the testes of the offspring, suggesting a maternal developmental reprogramming effect (Fig. 2, exposure A). Interestingly, this effect was more pronounced at lower rather than at higher concentrations. This "low dosage" effect was also detected following prolonged exposure to all

the EDs analyzed except for E2 (Fig. 2 exposure C). Furthermore, E2 had a weaker effect, at the dosages used, than most of the other ED compounds assessed, except at the maximum exposure. We extended this first global estimation to assess the level of global gene expression changes determining the total number of genes differentially expressed in the different experimental conditions under common filtering criteria. To increase the stringency of the analysis [50], we filtered the data to eliminate the results corresponding to genes that displayed weak expression and large variability. Hence, in this case, we only considered and analyzed the data corresponding to genes that underwent a change >+2- or <-2-fold, with a p-value <0.01 and an average signal >64. These results indicated a similar pattern of global gene expression changes as effect of the different exposures as when the average of the signals was the criterion for analysis (Fig. 3).

To answer the question whether the profiles of expression, at the level of specific genes, were associated with specific EDs, the window of exposure during development and/or level of exposure, we carried out an analysis of the entire transcriptome data by unsupervised hierarchical clustering from dataset of all 45 different experimental conditions. The relationship between the exposure and the gene expression profile is depicted graphically as a dendrogram (Fig. 4). The filtering and pre-processing of the datasets indicated in the Materials and Methods defined specific gene expression signatures (involving 2,676 genes) for conditions corresponding to MEHP and ZEA exposures irrespective of the developmental stage or dose used (Fig. 4). Thus, for MEHP and ZEA the toxicogenomic effect in testis appears to be mediated by the nature of the compound rather than by the levels or the window of exposure during development. This also indicates that low dose exposure during the periconceptional period could result in deregulation of key genes in cellular functions of testicular cells in adulthood.

ZEA administration did not have a clear effect on morphological parameters other than a significant increase in the numbers of apoptotic cells after the middle dose group A, and the highest dose in exposure group B (Fig. S10 in supplemental material). However, MEHP did not cause any consistent morphological effect at any dose or exposure period (Fig. S11 in supplemental material).

By contrast, the effect of E2 on global gene expression was not consistent across different samples and did not segregate these samples hierarchically from those exposed to BPA and LIN exposures. These data suggested that developmental exposure to MEHP or ZEA modulated gene

348 expression in a manner distinct to E2 and the other compounds assessed. Hence, the effects of
349 these compounds on testicular development would appear to be mediated by mechanisms other
350 than those affected by E2. In this hierarchical clustering it was possible to identify groups of
351 genes preferentially expressed in specific cell types that might share putative functional
352 properties. Following previous gene classifications in the testis [51], different "signatures" could
353 be detected such as those expressed in round spermatids (*Rtds I-V* in grey bars, Fig. 4), as well as
354 the groups of genes preferentially expressed in spermatid elongation (Fig. 4, EI-III in orange
355 bars: Table S2 in supplemental material).

356 Considering the results for E2, BPA and LIN, we studied whether we could define,
357 through gene expression profiles, a pattern of response for these three EDs that could clarify
358 similarities in their mechanism of action in testis development. For this reason, only data of the
359 three compounds were reclustered. The profile of the dendogram was more tightly defined for the
360 exposure to lindane up until birth (LB in the dendogram Fig. 5) or throughout the entire
361 developmental period (LC: Fig. 5). It is noteworthy that there was a close linkage between the
362 lowest level of exposure to LIN (LC+) and the highest level of E2 (EC+++) after prolonged
363 exposure during development. However, the gene expression profiles for BPA and E2 were more
364 interspersed, suggesting that BPA and E2 could act through similar mechanisms that differ from
365 those used by the other compounds analyzed.

366 The exposure to LIN had the strongest cytopathological effects on the seminiferous
367 epithelium. Germ cell apoptosis strongly increased in all dose groups after exposure schedules B
368 and C. In all these groups of B and C exposures the differences were statistically significant and
369 moreover all three dose groups in exposure C showed decreased tubule diameters. In addition,
370 significant changes in the frequency of epithelial abnormalities were seen for the two highest
371 doses in group C. The percentages of tubules with diploid spermatids tended to increase parallel
372 to the height of applied dose in exposure C, but the difference was only significant at the highest
373 dose (Fig. S12 in supplemental material).

374 Generally, exposure to the various compounds did not cause clear effects on body and
375 relative testis weights. However, at the longest exposure period (group C) E2 did cause a
376 decrease of about 30 % in body weight at all dose levels and an about 15% decrease in relative
377 testis weight, was seen after exposure to LIN (Fig. S3-S7 in supplemental material).

Exposure to E2 caused some particular effects on spermatogenic cells. Significant increases in numbers of apoptotic germ cells were detected in mice exposed according to experimental conditions B and C and receiving the highest doses of E2. Furthermore, clear decreases in tubule diameter and increased numbers of tubules with epithelial abnormalities were found with the high doses of E2 following exposure C. Remarkably, following exposure C diploid spermatids increased after all doses of E2 (Fig. S8 in supplemental material).

The effects of BPA administration were less than after E2 exposure. Only increases in the numbers of apoptotic germ cells were seen and only after exposure C and highest doses of BPA. No significant effects on the other parameters were seen after BPA administration (Fig. S9 in supplemental material).

The data from the arrays were qualified by qRT-PCR (TaqMan probes). On the basis of the expression data in all experimental conditions from 23 genes randomly selected from those annotated in the clustering analysis, we considered eligible those data that showed statistical significance ($p<0.05$) in the microarray. A total of 130 expression values, fitting the mentioned statistical stringency, were compared with the data of expression by qRT-PCR. We found an expected correlation in this type of replication analysis between gene expression from the microarray analyses and the qRT-PCR results [52] ($r=0.535$ $p< 0.0001$; Fig. S2 in supplemental material).

By analyzing the genes that define the clusters using Ingenuity Pathway Analysis software (IPA), the highest scoring gene networks could be identified, those that were relevant to disorders related to the pathologies supposedly induced by EDs. Among the most relevant are diseases of the reproductive system: cancer, developmental and endocrine system disorders (Table 2). The canonical pathways with the highest values identified were the oxidative stress response mediated by *Nrf2*, protein ubiquitination, oxidative phosphorylation and mitochondrial dysfunction (Table 3). These pathways can also be considered as relevant to the mechanisms that potentially cause cell and developmental disorders related to the proposed effects of EDs on mammalian testis.

The analysis of the networks of interacting genes was carried out only considering the genes defined in the hierarchical clustering, comparing all EDs and conditions. This means that the genes detected in each network are deregulated in at least three different conditions

(considering the criteria of 3 peaks of the clustering), and consequently, this does not mean that the changes in expression affect them equally with all EDs or in all conditions tested. However, these genes could be indicators of the pathways that may be more affected by individual ED or mixtures of EDs. The most representative network described a core of interacting genes that encode proteins involved in the global regulation of translation (Fig. 6 and Table S3 in supplemental material), as represented by the eukaryotic translation initiation factors (EIFS) and the cytoplasmic poly(A) binding protein (PABPC), critical post-transcriptional regulators. In addition members of the CCR4-NOT complex, such as *Cnot1*, *Cnot7 and Cnot6*, which interacts with PabpC1, were also detected in this most prominently affected network. Through its interaction with this network, the gene encoding the breast cancer anti-estrogen resistance 3 protein (*Bcar3*) was also seen to be modified expression by all the ED's analyzed.

4. Discussion

In this study we have compared global gene expression in testes of mice exposed to five endocrine disruptors, using three different doses and studying three distinct periods of developmental exposure. The main findings point out that Mono-(2-ethylhexyl)-phthalate and Zearalenone, more than 17β-Estradiol, Lindane or Bisphenol-A, produce specific gene signatures during testis development, irrespective of the concentration of the toxicant or the developmental period during which exposure takes place. These data suggest that prevalent alterations to defined networks can induce the disequilibrium in gene expression programs involved in correct testicular development and spermatogenesis, that can potentially be transmitted to male offspring in an epigenetic-like manner. However, histopathological study only showed moderate morphological abnormalities.

In planning the dose range of each compound, we did not consider doses which cause acute toxicity but selected doses above the NOAEL and as the exposures were initiated in the mothers, we used doses that did not induce fetal loss. For some compounds such as E2 or BPA the doses used were below those that were considered to affect the number of births. In pilot experiments, for each compound we evaluated the rates of birth at different doses and developmental periods of exposure. In this way, a dietary concentration of intake of 100

μg/kg/day of E2 in CD1 mice was found to highly affect the number of successful pregnancies [53]. We confirmed this result and established the maximum dose at 48 μg/kg/day. Similarly, exposure of laboratory animals to BPA was reported to influence fertility [54] [7, 55]. To this respect, considering our scheme of experimental exposure during development, higher doses of compounds as E2 and BPA that potentially can induce severe effects on testis, were not be applied because the pregnancy of exposed mice was also clearly affected. This could explain the relative small effect detected in our study for E2 and BPA with respect to the level of gene deregulation in comparison to the other EDs evaluated.

The tolerable daily intake (TDI) for lindane was originally estimated at 12.5 μg/kg bw/day [56]. However, lindane is a persistent contaminant and bioaccumulates. Traina et al. [57] using a similar dosage and method of administration as we used, reported long-lasting effects of lindane on mouse spermatogenesis without maternal toxicity or delayed growth and development of pups.

With respect to MEHP, the ED with ZEA showing the most conspicuous effect of the compounds studied, a wide spectrum of human exposures to phthalates has been recently reviewed with estimations of intakes of DEHP varying between 7.3 μg to 409 μg/kg bw/day [58]. However, in higher exposed groups at risk such as premature neonates in a neonatal intensive-care unit the estimated level can reach up to 6 mg/kg bw/day [59]. Maximal DEHP exposures up to 22 mg/kg bw/day have also been estimated for newborns infants following blood transfusion procedures [60]. These doses are equivalent to 45 mg/kg bw/day and 165 mg/kg bw/day respectively in the mouse, which is in the range of the median dose (46.3 mg/kg bw/day) or over maximum dose (92.7 mg/kg bw/day) used in the present study. This indicates that the defined pattern of altered gene expression detected in this study may be representative of the action of phthalates in exposed humans. Relevant expression patterns, with hundreds of deregulated genes, might not be associated to dramatic changes at the morphological or histological level in the testis, as it has been shown in this work. Previous studies reported that MEHP exposures cause an increase in apoptosis of germ cells in adult mice mediated by Sertoli cell injuries at 10 fold [50] or 20 fold the doses used in our study [51].

Worldwide, zearalenone is found in a number of cereal crops and derivatives. The concentrations of zearalenone in cereals vary over a wide range between 3 to 8000 μg/kg

(International Programme on Chemical Safety. WHO food additives series: 44, Geneva 2000 http://www.inchem.org/documents/jecfa/jecmono/v44jec14.htm). However, high concentrations have been reported. For example: as zearalenone may be transmitted from contaminated grains into beer a very high concentration of zearalenone was found in beers brewed in Africa at levels of 53 mg/L [61]. Assuming an intake of 500 ml of beer per day, this is equivalent to 0.44 mg/kg bw/day. Applying the metabolic factor, this can be calculated to be 3.3 mg/kg bw/day for a mouse which is in the range of the medium dosage used in our study. Similar to MEHP, the specific profile of gene deregulation in testes from mice exposed to ZEA are not directly correlated with morphological alterations neither modifications of cellular composition in the seminifereous epithelium. However, the exposure to E2, BPA and LIN increased the numbers of apoptotic cells after the longest period of exposure but also when the treatment was stopped at birth, indicating long-term effects of these compounds. Epithelial abnormalities and modifications of tubule diameter showed increases at the highest dose of E2 and BPA or LIN after long-term exposure.

Studies of the environmental induction of changes in gene regulation as disease etiology have focused on defined genes or genetic pathways related to particular diseases, including reproductive dysfunctions [24]. Estrogen has a crucial role during spermatogenesis [62] and hence, estrogen–like compounds can interfere with the activity of estrogen and its binding to estrogen receptors that mediates the transcription of target genes. However, the mechanisms by which estrogen regulates gene expression are known to be more complex than originally thought [63, 64]. The extensive alterations in gene expression observed in the present work can be explained by a cascade of effects triggered by environmental pollutants with the capacity to interfere with hormonal pathways (acting as EDs) and to lead to gene deregulation.

Our results of global gene expression and the hierarchical clustering analysis suggest that some compounds considered as EDs could act via different mechanisms to estrogen. While MEHP [65] and ZEA [66] are considered to have estrogenic activity *in vitro*, they displayed distinct effects on gene expression that could not be considered to mimic those of estrogen, suggesting another mode of action. Indeed, it was recently reported that MEHP alters both steroidogenesis and germ cell number in mice, without involving either the estrogen or androgen receptor [33].

In the hierarchical clustering, different signatures included the upregulation or downregulation of the same genes depending on the compound, dose or developmental period of exposure. Hence, it would appear that the effect of EDs cannot solely be attributed the reduced expression of genes specific to spermatids that might produce a lack or decrease in the number of these cell types (round or elongated), as suggested previously [67]. Indeed, alterations of the cytological structure of the seminiferous epithelium that could compromise spermatogenesis were not found in our cytological/histological analysis. Moreover, the results indicate that acute effects of the toxicants may not explain the altered gene expression since low doses and pre-mating exposure of mothers induced the modulations of expression of genes in a clustered manner.

In the present study, when the mothers were only exposed to EDs during the pre-mating period (exposure A), the level and type of gene modifications of gene expression observed in the offspring can only be attributed to epigenetic modifications in the oocyte/zygote, or during early embryonic development prior to gonadal sex differentiation if traces of the compound remained in the maternal metabolism. No epigenetic transgenerational effect of ZEA, MEHP or LIN has been reported previously. In rats exposed to vinclozolin, a fungicide considered to be an ED, effects on the male reproductive system were evident until the F4 generation [68] and they were attributed to epigenetic effects. Although, the transgenerational effects of orally administered vinclozolin were recently questioned [69], alterations to the transcriptome have been demonstrated in rat embryonic testis in the F1 to F3 generations when the F0 generation was exposed to vinclozolin from day 8 to 14 of gestation [70]. This exposure coincides with a period when epigenetic reprogramming occurs in the transition of primordial germ cells to sex-differentiated germ cells [71]. The transgenerational effect of some of the EDs that we have analyzed should also be evaluated further at the promoter level on the basis of specific potential epigenetic alterations.

By analyzing gene expression signatures in relation to their biological pathways, oxidative stress response mediated by NF-E2-related factor 2 (*Nrf2*) was detected as a particularly relevant pathway that could be altered as response to the different EDs. Nrf2 is a transcription factor that acts through the antioxidant response element [72]. The expression of mitochondrial and nuclear-encoded subunits of respiratory chain complexes must be closely

528 coordinated, and *Nrf1* and *Nrf2* are the main genes responsible for this coordination. Testicular
529 oxidative stress appears to be a common feature of male infertility and indeed, exposure to
530 toxicants has been correlated with an increase in oxidative stress in the testis [73]. These data
531 suggest that in the testis, EDs modify the expression of genes involved in pathways common to
532 other xenobiotics described in the literature that clearly affect reproduction, even when the
533 compound is administered to the mother prior to fertilization.

534 In the most relevant networks of genes involved in post-transcriptional regulation,
535 regulators such as EIF and poly-A binding protein occupy a central position. Five genes
536 encoding different isoforms of EIF4 (*Eif4A1*, *Eif4A2*, *Eif4E*, *Eif4G1*, and *Eif4G2*) and three
537 encoding EIF3 isoforms (*Eif3B*, *Eif3E*, *Eif3H*), together with *Pabpc*, participate in the core of the
538 43S pre-initiation ribosomal complex, binding mRNAs prior to translation [74]. These nine
539 genes showed different altered expression under different experimental conditions. The EIF4F
540 complex is composed of EIF4E, EIF4G and EIF4A, and it associates with the 5' cap structure of
541 the mRNA as part of the 43S preribosomal complex. This EIF4F complex is largely dependent
542 on the availability of EIF4E, which thereby limits the rate at which translation is initiated [75].
543 *Eif4E* overexpression has long been associated with oncogenesis [76] and there is increasing
544 evidence correlating it with cellular transformation, tumorigenesis and metastatic progression in
545 human cancers [77]. However, experimental downregulation of *Eif4E* induces apoptosis [75].
546 The modification of *Eif* and *Pabpc* expression after exposure to EDs may involve
547 downregulation or upregulation depending on the compound used and the period of
548 developmental exposure. Although, the general tendency was a diminution of their expression,
549 compared to the controls, as seen for *Eif4E*, *Eif3E*, *Eif4A2,* after MEHP exposure we detected an
550 increase in the expression of *Eif4A1*.

551 Post-transcriptional regulation of most mRNAs is mediated by the length of their 3'
552 poly[A] tails. PABP acts by promoting mRNA translation and several deadenylases serve as
553 antagonists, facilitating the degradation of the mRNA [78]. PABPC1 interacts with subunits of
554 the CCR4-NOT complex. Mammalian CCR4-NOT represents a complex of several subunits that
555 acts as a deadenylase for mRNAs [79]. A recent study indicated that CCR4-NOT participates in
556 the regulation of certain endogenous retinoic acid receptors that are essential for normal
557 spermatogenesis [80]. The association and combination of different subunits in this complex

558 modulate different specific cell functions [81]. Three components of this complex in the
559 network: *Cnot1*, *Cnot6* and *Cnot7* were deregulated in different ways. CNOT1 represses the
560 ligand–dependent transcriptional activity of the estrogen receptor (ER)α receptor [82]. Indeed, in
561 all conditions both E2 and MEHP exposure diminishes the amount of *Cnot1* mRNA compared to
562 the controls. CNOT7 acts as a co-regulator of retinoid X receptor beta (Rxrb) and significantly,
563 male null mutant mice (*Cnot7*$^{-/-}$) are sterile due to oligo-astheno-teratozoospermia resulting from
564 Sertoli cell defects [83]. Moreover, the expression of *Cnot7* was downregulated following
565 exposure to all compounds and conditions, except ZEA.

566 The data obtained suggest that global translation of cap-dependent mRNAs, mediated by
567 the initiation complexes, may be deregulated through the disequilibrium in the availability of the
568 components of these complexes. Accordingly, phenotypic alterations may be promoted that
569 range from apoptosis to cellular transformation and cancer, depending on the cell type and
570 developmental stage. In addition, modification of expression of genes encoding proteins that
571 interact with hormone receptors, such as *Cnot*, can have a variety of pathological consequences
572 during testicular development, already proposed to be effects of ED exposure. We speculate that
573 the disequilibrium in different elements of these complexes and associated genes/proteins
574 provoke different pathologies during testes development that can be attributed to different
575 nosological entities.

576 It is interesting to note that in the network the *Bcar3* gene is connected to *Pabpc1* and its
577 level of expression was also altered. The genes of the *Bcar* family contribute to cell proliferation
578 in estrogen-independent breast cancer and hence, these cells are resistant to anti-estrogen
579 endocrine therapy [84]. We speculate that this gene may also participate in some alterations of
580 the endocrine response in germ cells.

581 Besides emphasizing the comparative results of the effect of different compounds, doses
582 and exposure periods, the data points of more than 3 million quantitative values of expression
583 levels of genes expressed in testis that we have included in GEO (Accession number GSE14774)
584 may be of significant value in further comparative studies to determine potential mechanisms of
585 action and biomarkers of the effects of endocrine disrupters.

586

587 **Ackowledgements**

588 This work was supported by the EC (grant QLK4-CT-2002-02403), CEFIC-LRi, MICINN (FIS
589 PI071007) and PIE (201020E016). LALF was supported by a Miguel Servet grant from Instituto
590 de Salud Carlos III (CP06 /00267).
591 We thank F. Escolar, E. Gonzalez, M. Moreno and M.I. García for their technical assistance and
592 Mark Sefton for English revision.

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808 Breast Cancer Res Treat. 2009;114:23-30.

809
810

FIGURE LEGENDS:

Figure 1. Schedule of ED exposures and their controls. Three exposure regimes were employed: mothers were exposed for two weeks before mating only (exposure A) or the exposure was maintained during embryonic and fetal development, ceasing at birth (exposure B) or at the end of the prepubertal stage (exposure C). Three ED concentrations were used for each developmental period of exposure. All male offspring were orchidectomized at four weeks of age to obtain the total RNA. The solvents of each ED (DMSO or ethanol) were used as controls.

Figure 2. Assessment of global gene expression changes. Graph representing the calculated average of the $\log_2$ (R/G) obtained from the microarray expression data. Basal Gene Expression Average (BGEA) from testis was obtained by hybridization of individual and pooled RNA samples from testes neither exposed to compounds nor solvents (S#1 *vs* S#2 = individual sample 1 *versus* individual sample 2; S#1 *vs* S#3 = individual sample 1 *versus* individual sample 3; S#2 *vs* S#3 = individual sample 2 *versus* individual sample 3; S#pool =pooled sample *versus* itself). Only the expression data from genes with average signals higher than 64 were considered. Grey triangles indicate the increasing doses of the compounds.

Figure 3. Total number of genes with altered gene expression. The total number of genes induced and repressed (fold change >+2 and <-2, p-value <0.01 and an average signal >64) in all the conditions analyzed.

Figure 4. Hierarchical clustering. Dendogram of the unsupervised hierarchical clustering performed with filtered and pre-processed (3 peaks and a threshold of 0.7) expression data from each experimental condition (see methods). After processing the datasets, 2,676 genes were considered for clustering. Each column represents a specific exposure regime. The codes are made up of the first letter of the compound (L-LIN; E-E2; Z-ZEA; M-MEHP and B-BPA), a second letter representing the developmental period of exposure (pre-mating = A; embryonic and fetal = B; postnatal = C), followed by the dosage in brackets (see Fig. 1). Each experimental compound is depicted with a specific color to readily visualize the clustered conditions (blue for

LIN, magenta for E2, yellow for ZEA, light green for MEHP, brown for BPA). Orange and grey bars denote gene clusters that include the genes preferentially expressed during spermiogenesis or in round spermatids, respectively. The color scale of expression (log_2 ratio) is shown.

Figure 5. Clustering of LIN, BPA and E2 conditions. Unsupervised hierarchical clustering performed with filtered and pre-processed expression data (3 peaks and a threshold of 0.7) after exposure to LIN, BPA and E2 (all exposures excluding MEHP and ZEA). After processing the datasets, 666 genes were considered for clustering. The color scale of expression (log_2 ratio) is shown.

Figure 6. Identified gene networks. The top ranked networks identified by Ingenuity Pathway Analysis (IPA) software from the genes selected for hierarchical clustering considering all the experimental regimes of exposure. Shaded genes represent those that were included in the geneset analyzed by IPA.

Table 1. Compound dosages administrated to mice in the drinking water (estimated intake in mg/kg/day).

Escalation	Estradiol (E2)	Bisphenol-A (BPA)	Zearalenone (ZEA)	Mono (2-EthylHexyl) Phthalate (MEHP)	Lindane (LIN)
Dose (+)	0.006	0.16	1.3	9.2	16.6
Dose (++)	0.012	16	3.9	46.3	32.2
Dose (+++)	0.048	64	6.6	92.7	64.4

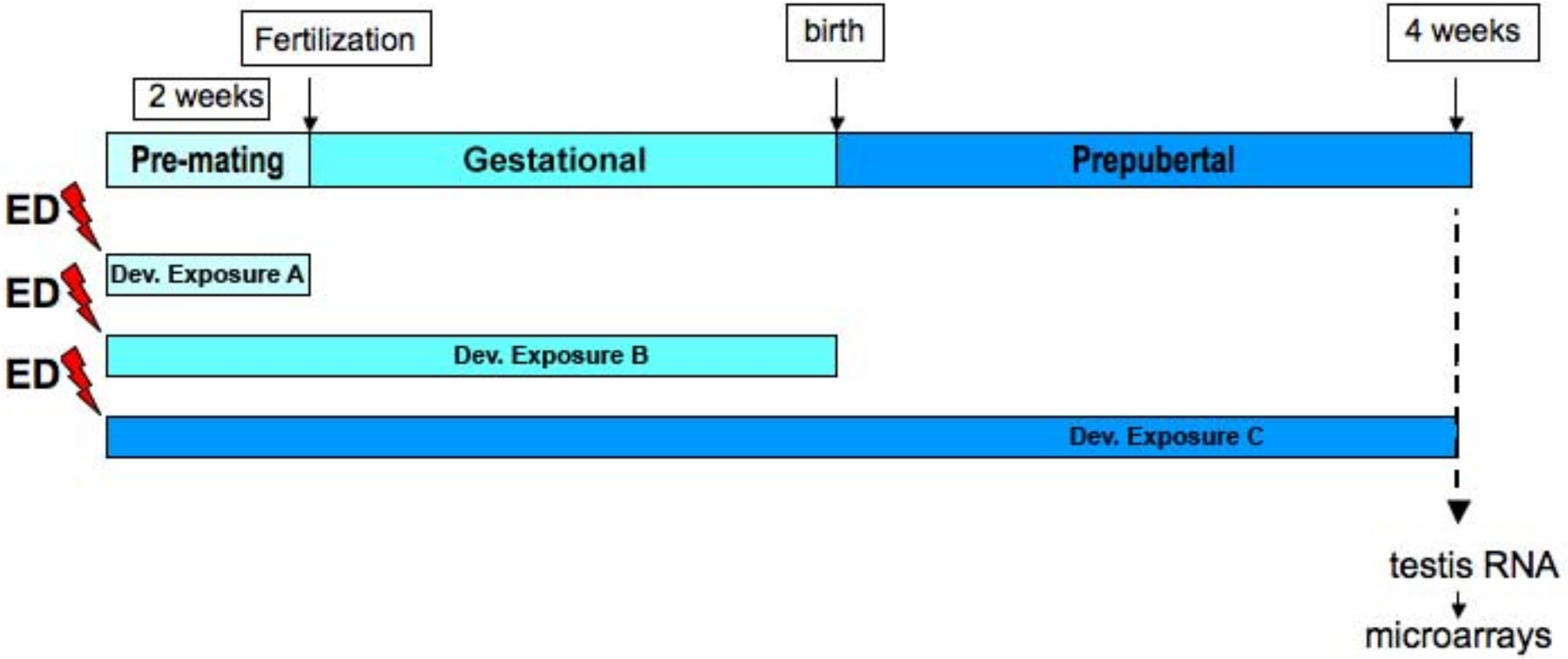
Table 2. Top associated network functions identified by Ingenuity Pathway Analysis considering genes selected by hierarchical clustering of all experimental conditions.

Associated network functions	**Score**
Cancer, cell cycle, reproductive system disease	43
Cellular functions and maintenance, developmental disorders , genetic disorders	43
Molecular transport, protein trafficking, endocrine system development and function	41
Drug metabolism, small molecule biochemistry, cell cycle	38
RNA post-transcriptional modification, protein synthesis, gene expression	36

871 **Table 3.** Top canonical pathways identified by Ingenuity Pathway Analysis considering genes
872 selected by hierarchical clustering of all experimental conditions.

Pathways	p-value
Nrf2-mediated oxidative stress response	0.0000000165
Protein ubiquitination pathway	0.00000011
Oxidative phosphorylation	0.000000464
Mitochondrial dysfunction	0.00000118
Ubiquinone biosynthesis	0.000142

Figure 1


Fertilization
birth
4 weeks
2 weeks
Pre-mating
Gestational
Prepubertal
ED
ED
ED
Dev. Exposure A
Dev. Exposure B
Dev. Exposure C
testis RNA
microarrays

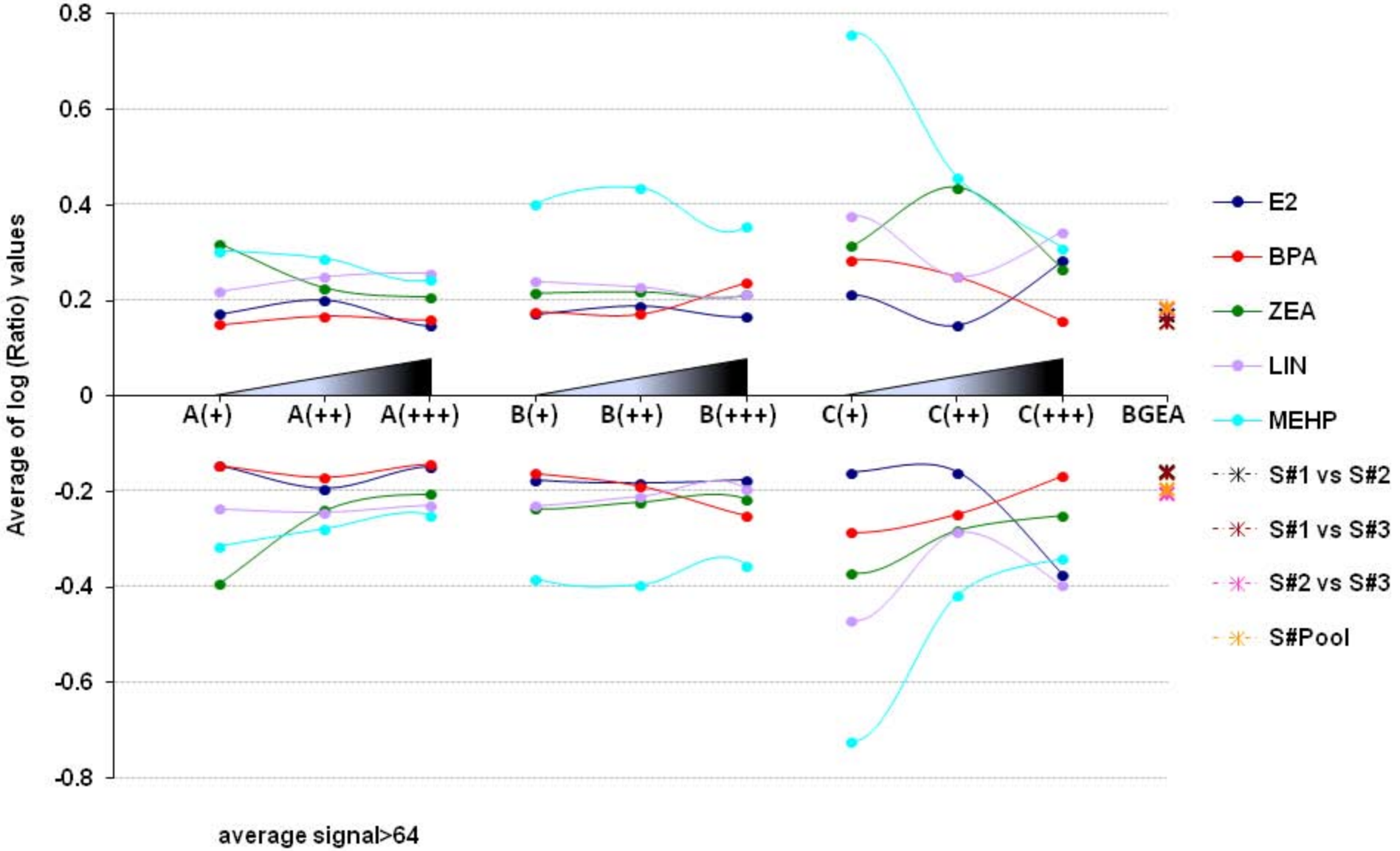

0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
Average of log (Ratio) values
A(+)
A(++)
A(+++)
B(+)
B(++)
B(+++)
C(+)
C(++)
C(+++)
BGEA
E2
BPA
ZEA
LIN
MEHP
S#1 vs S#2
S#1 vs S#3
S#2 vs S#3
S#Pool
average signal>64

Figure 3.

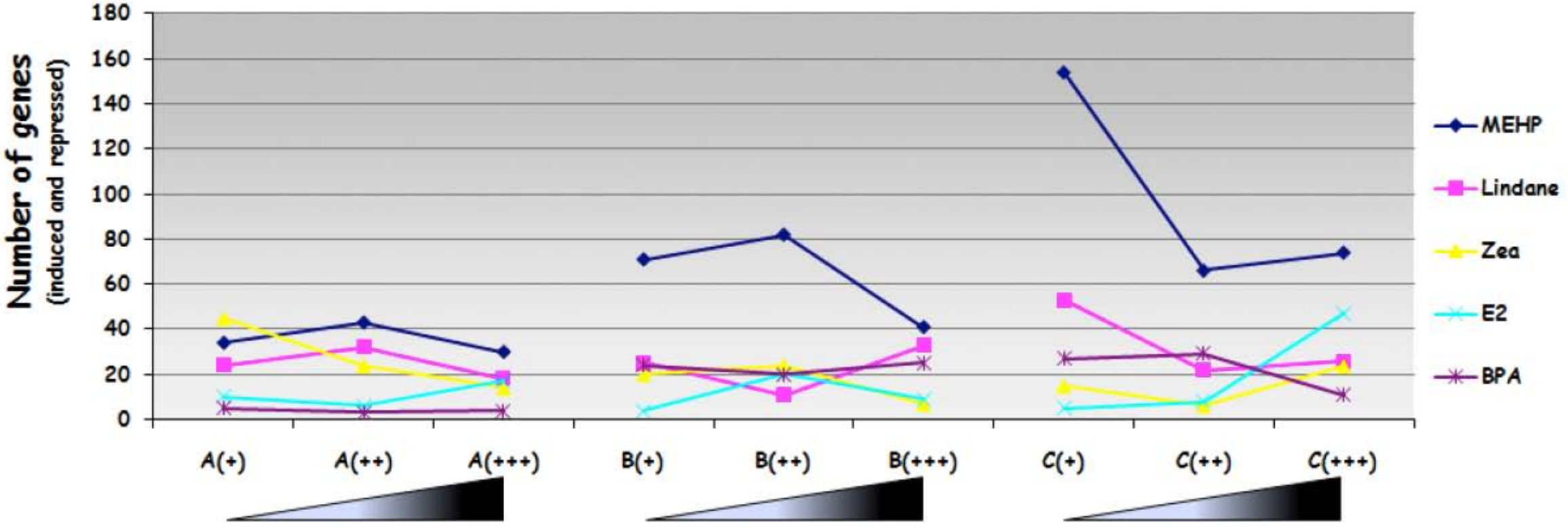

Number of genes
(induced and repressed)
180
160
140
120
100
80
60
40
20
0
A(+)
A(++)
A(+++)
B(+)
B(++)
B(+++)
C(+)
C(++)
C(+++)
MEHP
Lindane
Zea
E2
BPA

Figure 4

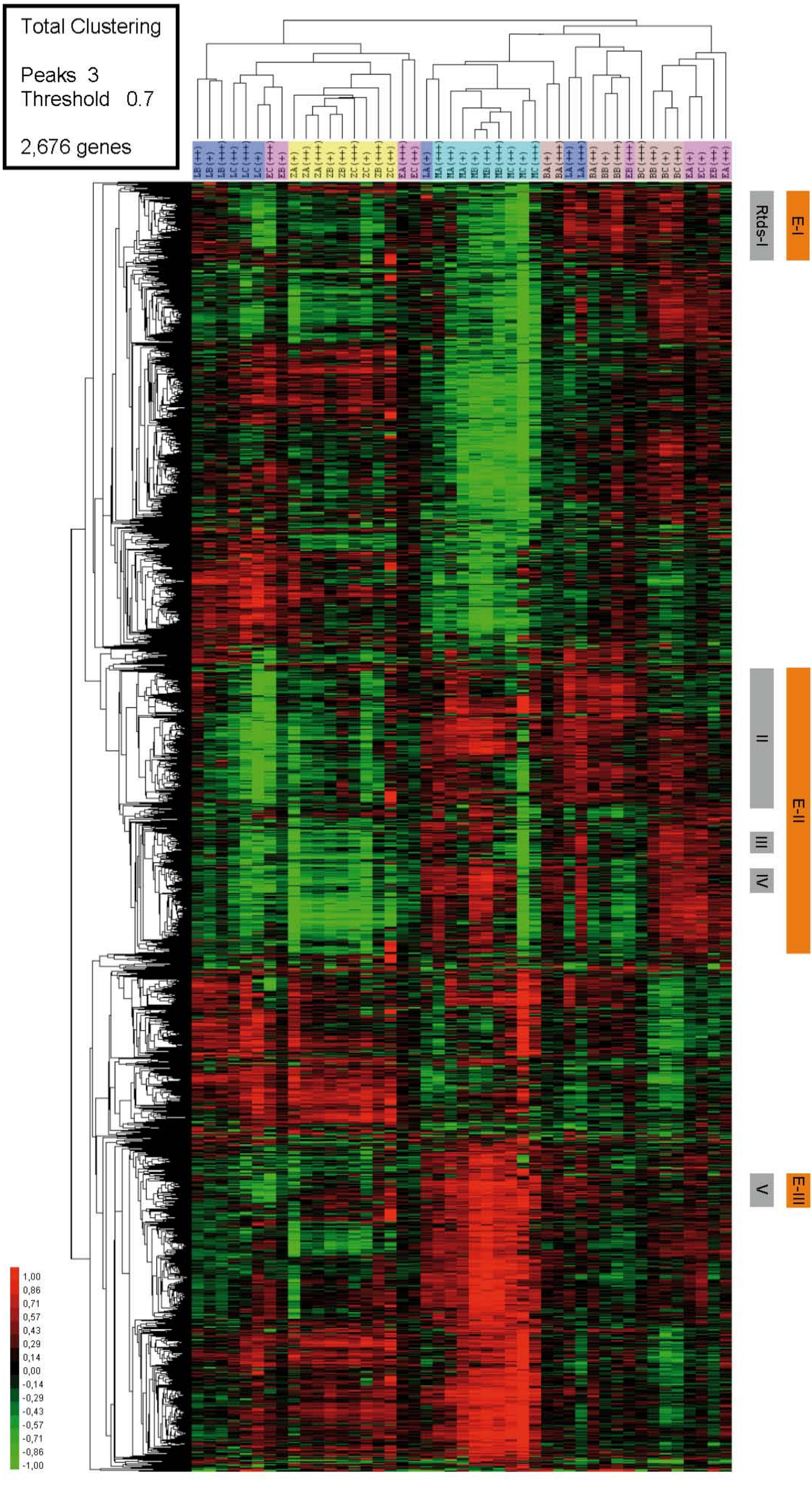

Total Clustering
Peaks 3
Threshold 0.7
2,676 genes
Rtds-I
E-I
II
III
IV
E-II
V
E-III
1,00
0,86
0,71
0,57
0,43
0,29
0,14
0,00
-0,14
-0,29
-0,43
-0,57
-0,71
-0,86
-1,00

Figure 5

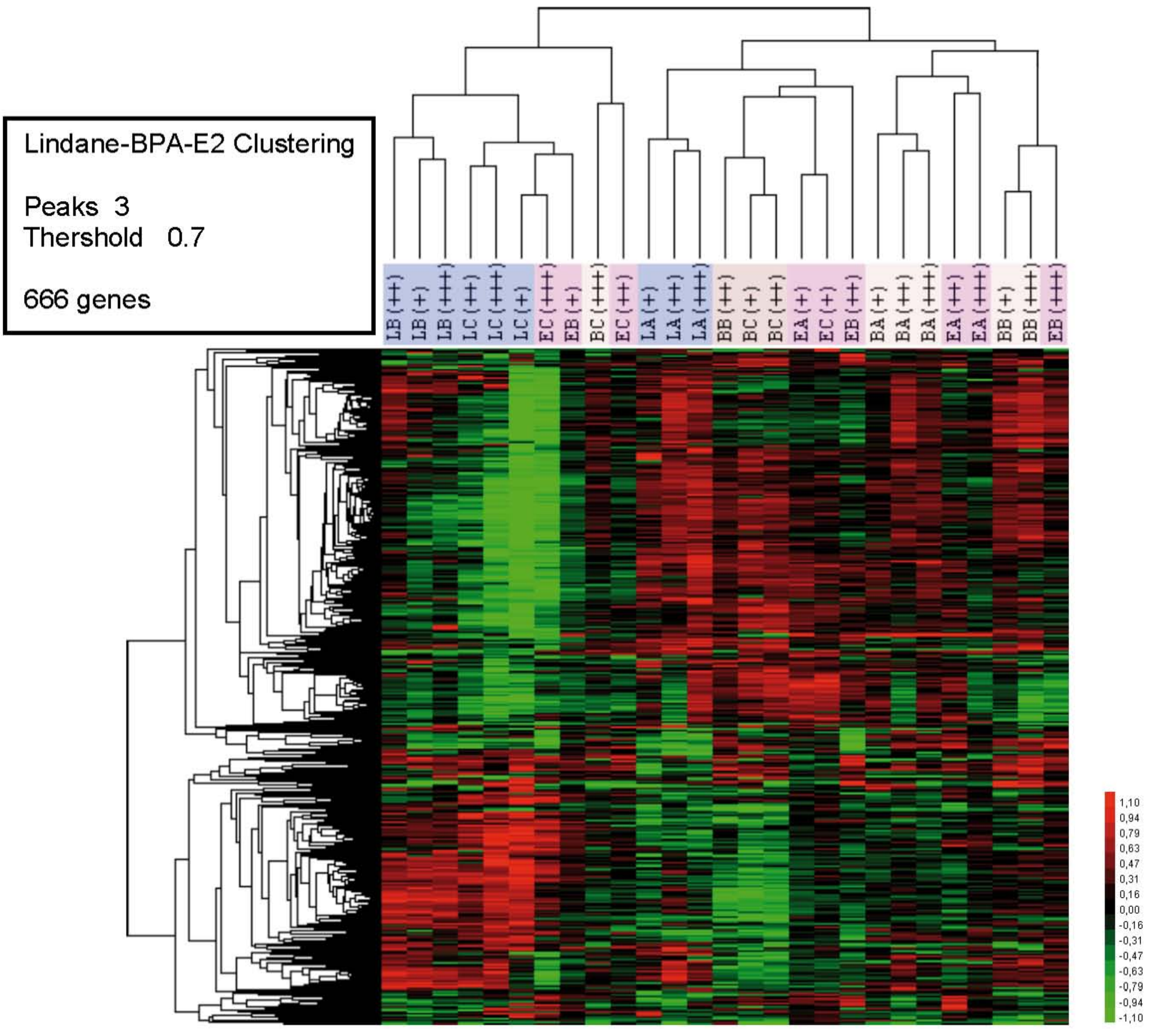
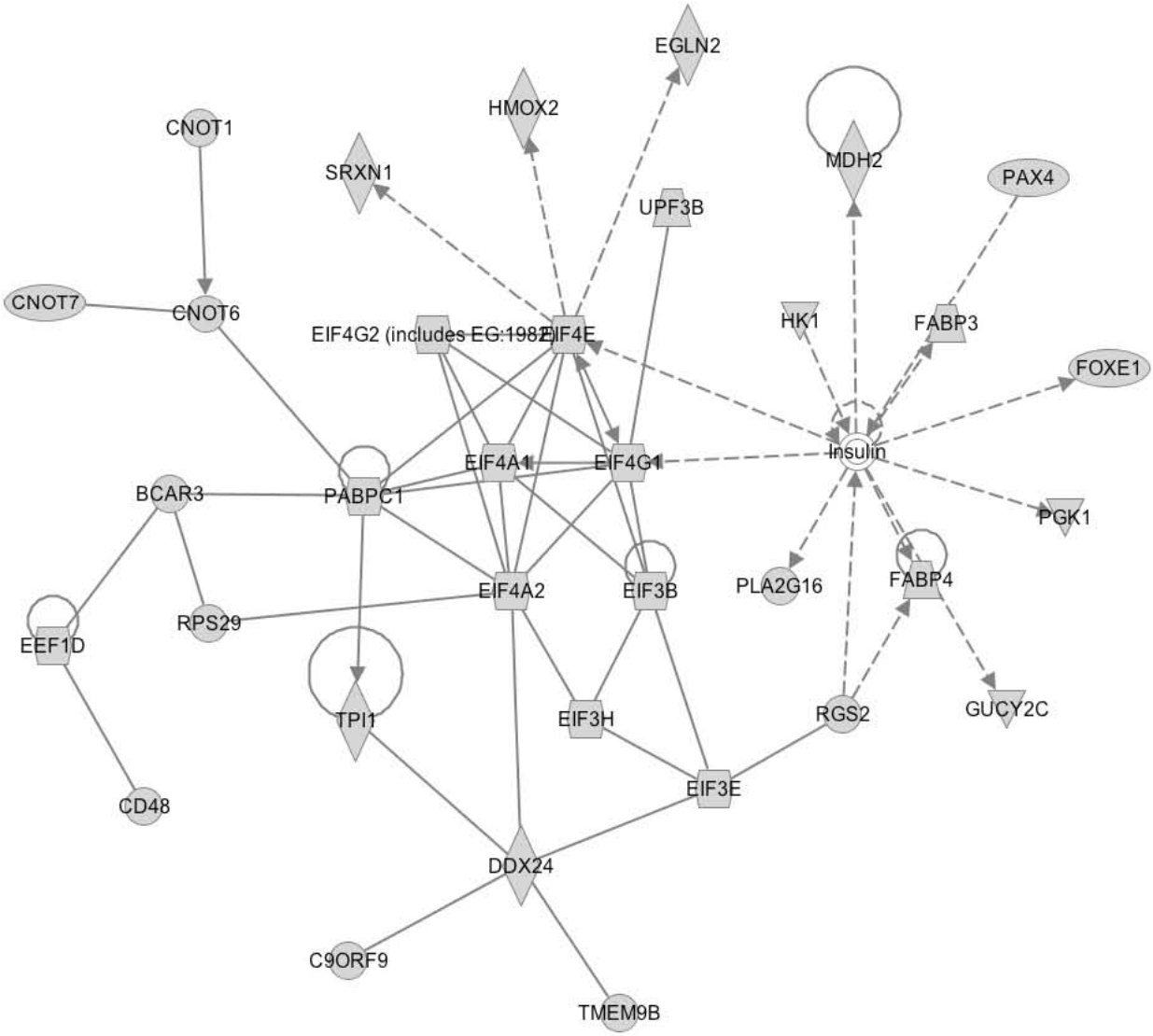
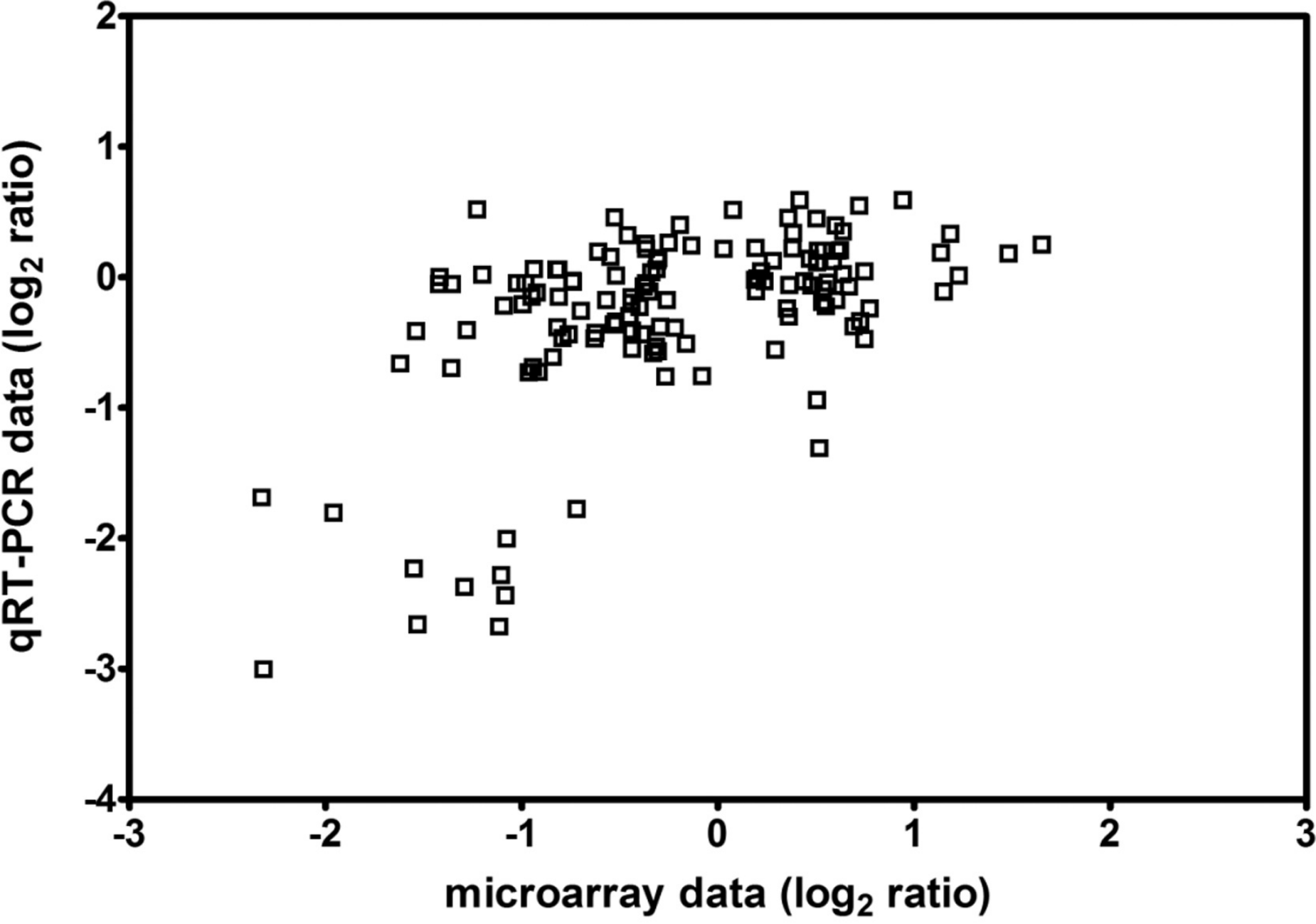
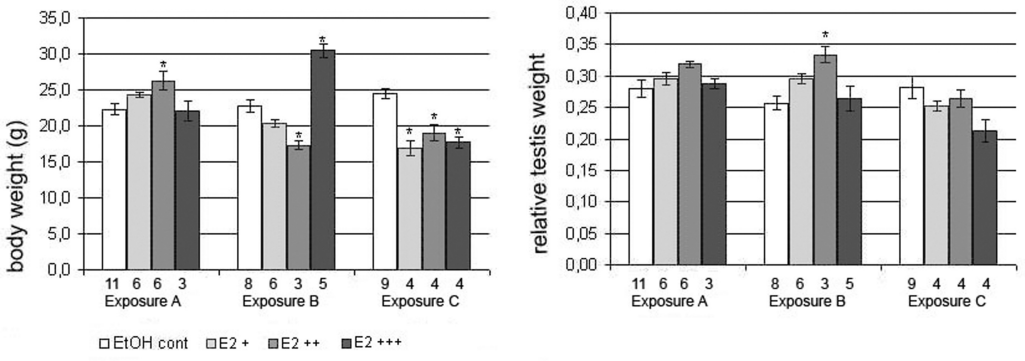

Lindane-BPA-E2 Clustering
Peaks 3
Thershold 0.7
666 genes
LB(+)
LB(+)
LB(+)
LC(+)
LC(+)
LC(+)
EC(+)
EB(+)
BC(+)
EC(+)
LA(+)
LA(+)
LA(+)
BB(+)
BC(+)
BC(+)
EA(+)
EC(+)
EB(+)
BA(+)
BA(+)
BA(+)
EA(+)
EA(+)
BB(+)
BB(+)
EB(+)
1,10
0,94
0,79
0,63
0,47
0,31
0,16
0,00
-0,16
-0,31
-0,47
-0,63
-0,79
-0,94
-1,10

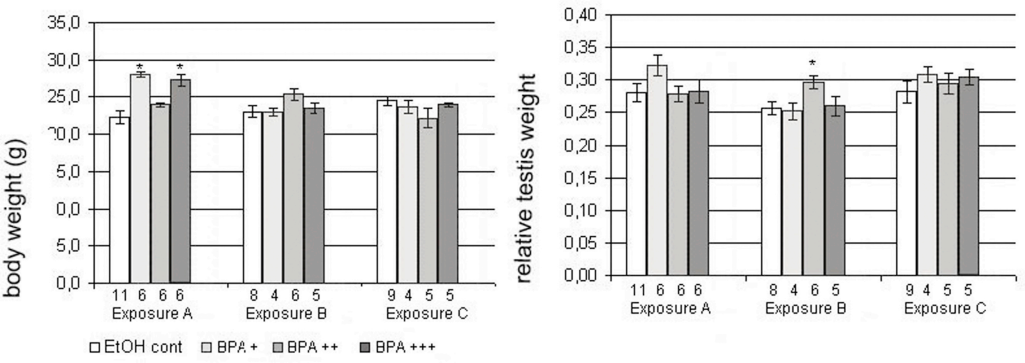
Figure 6

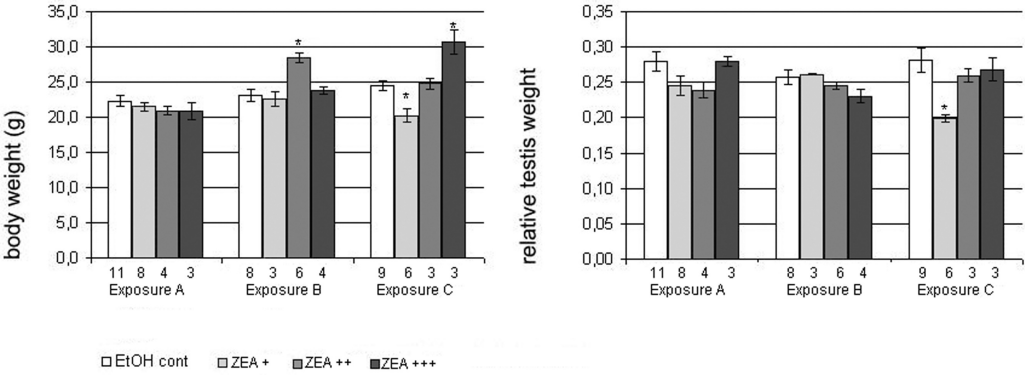

EGLN2
HMOX2
CNOT1
SRXN1
MDH2
PAX4
UPF3B
CNOT7
CNOT6
EIF4G2 (includes EG:1982)
EIF4E
HK1
FABP3
FOXE1
Insulin
EIF4A1
EIF4G1
BCAR3
PABPC1
PGK1
PLA2G16
FABP4
EIF4A2
EIF3B
RPS29
EEF1D
TPI1
EIF3H
RGS2
GUCY2C
EIF3E
CD48
DDX24
C9ORF9
TMEM9B

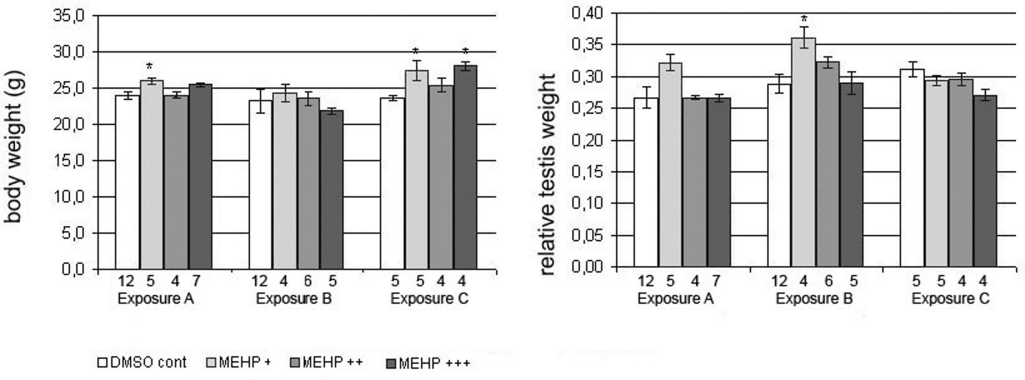


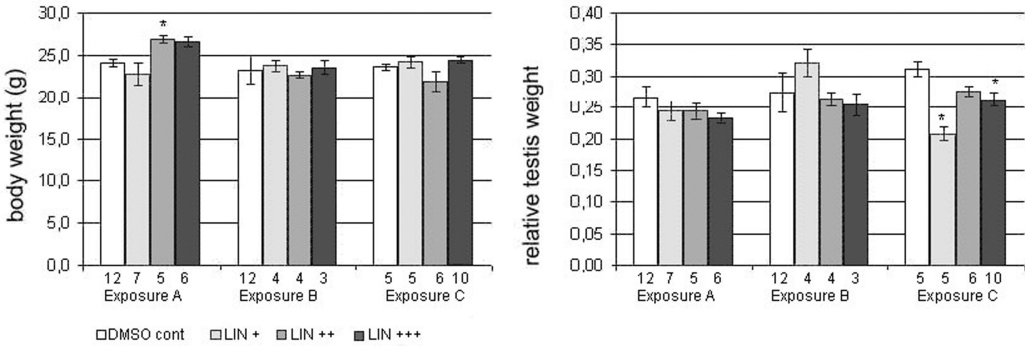
Variable X: microarray data (log_2 ratio)
Variable Y: qRT-PCR (log_2 ratio)
Number of XY Pairs= 130
Pearson r = 0.5346
95% confidence interval for r= 0.3991 to 0.6472
P-value (two-tailed): p<0.0001

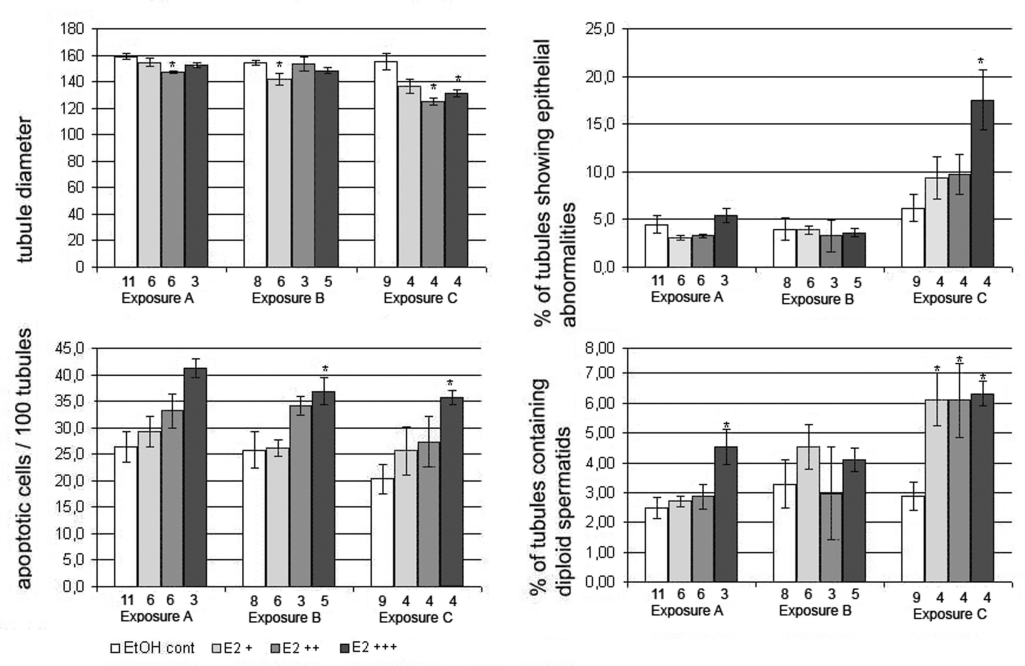

body weight (g)
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
11 6 6 3
Exposure A
8 6 3 5
Exposure B
9 4 4 4
Exposure C
EtOH cont
E2 +
E2 ++
E2 +++
relative testis weight
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
11 6 6 3
Exposure A
8 6 3 5
Exposure B
9 4 4 4
Exposure C

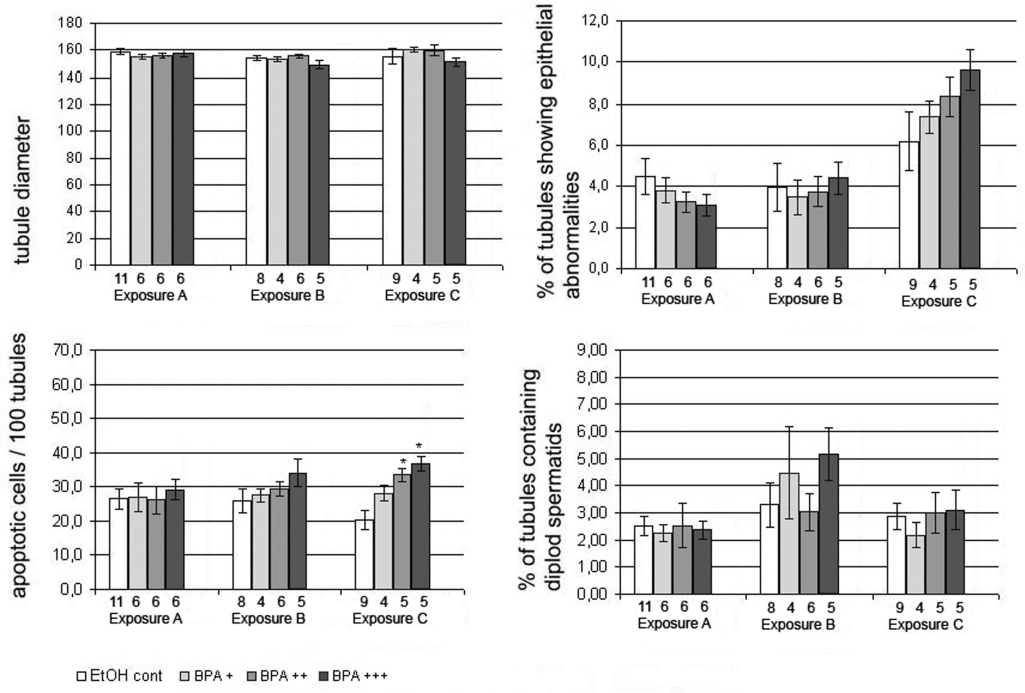

body weight (g)
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
*
*
11 6 6 6
Exposure A
8 4 6 5
Exposure B
9 4 5 5
Exposure C
EtOH cont
BPA +
BPA ++
BPA +++
relative testis weight
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
*
11 6 6 6
Exposure A
8 4 6 5
Exposure B
9 4 5 5
Exposure C

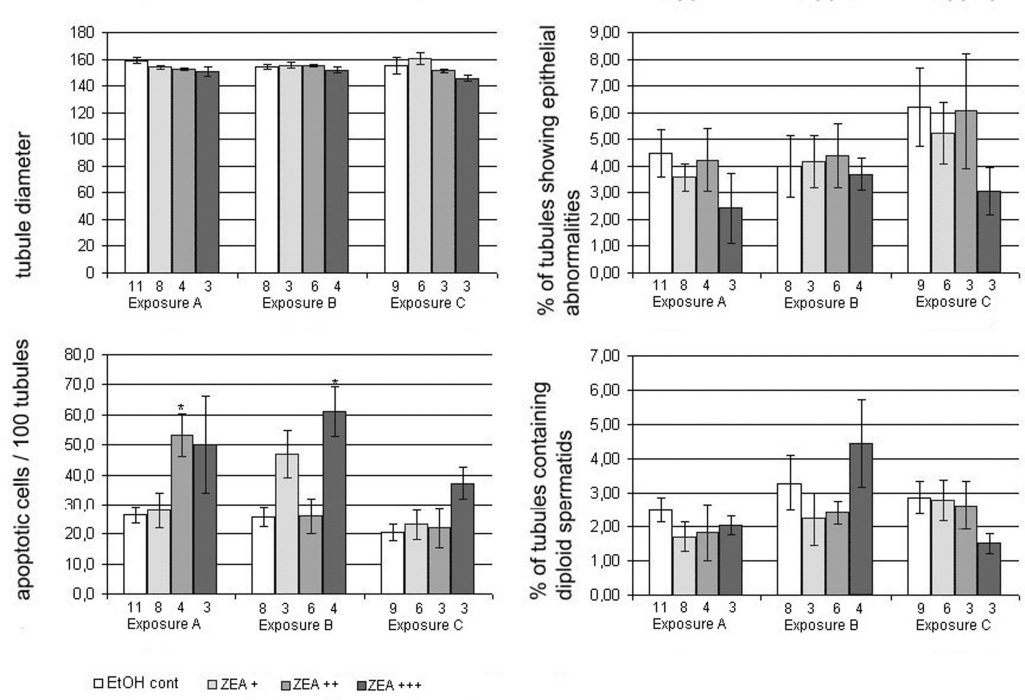

body weight (g)
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
11 8 4 3
8 3 6 4
9 6 3 3
Exposure A
Exposure B
Exposure C
relative testis weight
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
11 8 4 3
8 3 6 4
9 6 3 3
Exposure A
Exposure B
Exposure C
EtOH cont
ZEA +
ZEA ++
ZEA +++

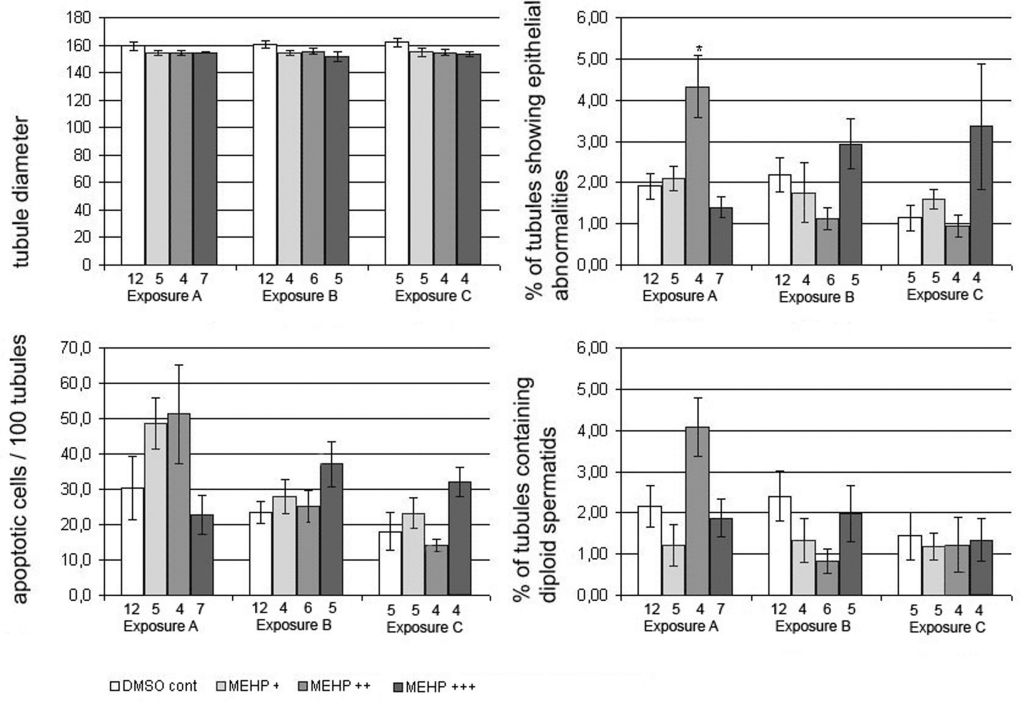

body weight (g)
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
*
*
*
12 5 4 7
Exposure A
12 4 6 5
Exposure B
5 5 4 4
Exposure C
relative testis weight
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
*
12 5 4 7
Exposure A
12 4 6 5
Exposure B
5 5 4 4
Exposure C
DMSO cont
MEHP +
MEHP ++
MEHP +++

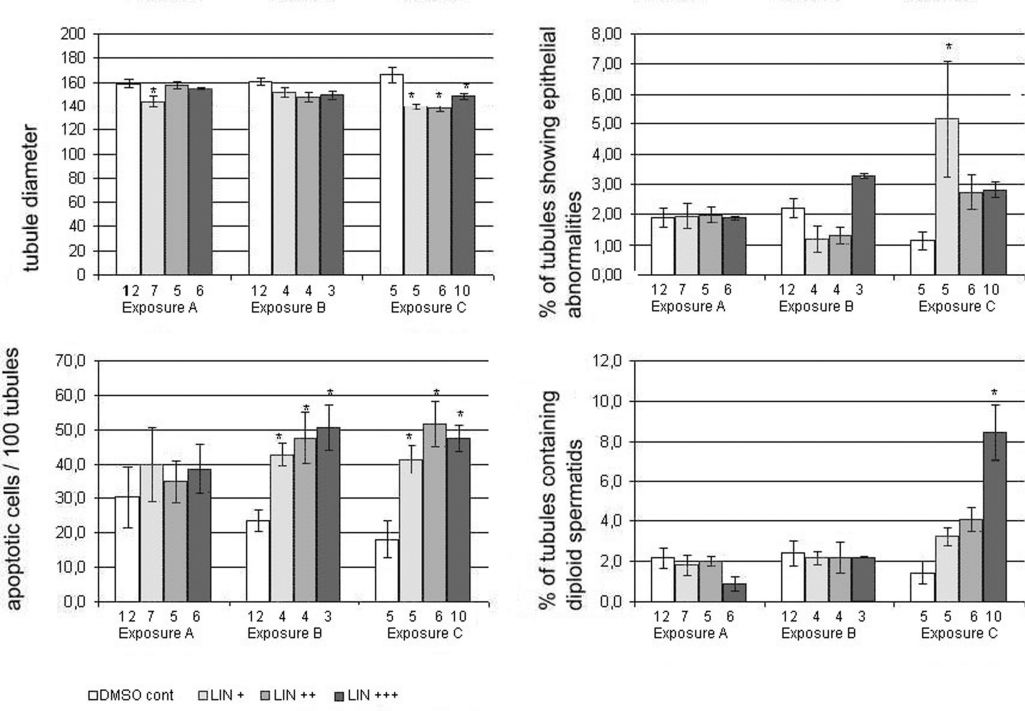

body weight (g)
30,0
25,0
20,0
15,0
10,0
5,0
0,0
*
12 7 5 6
Exposure A
12 4 4 3
Exposure B
5 5 6 10
Exposure C
DMSO cont
LIN +
LIN ++
LIN +++
relative testis weight
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
12 7 5 6
Exposure A
12 4 4 3
Exposure B
5 5 6 10
Exposure C


tubule diameter
180
160
140
120
100
80
60
40
20
0
11 6 6 3
Exposure A
8 6 3 5
Exposure B
9 4 4 4
Exposure C
% of tubules showing epithelial abnormalities
25,0
20,0
15,0
10,0
5,0
0,0
apoptotic cells / 100 tubules
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
% of tubules containing diploid spermatids
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
EtOH cont
E2 +
E2 ++
E2 +++


tubule diameter
180
160
140
120
100
80
60
40
20
0
11 6 6 6
Exposure A
8 4 6 5
Exposure B
9 4 5 5
Exposure C
% of tubules showing epithelial abnormalities
12,0
10,0
8,0
6,0
4,0
2,0
0,0
11 6 6 6
Exposure A
8 4 6 5
Exposure B
9 4 5 5
Exposure C
apoptotic cells / 100 tubules
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
*
*
11 6 6 6
Exposure A
8 4 6 5
Exposure B
9 4 5 5
Exposure C
% of tubules containing diplod spermatids
9,00
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
11 6 6 6
Exposure A
8 4 6 5
Exposure B
9 4 5 5
Exposure C
EtOH cont
BPA +
BPA ++
BPA +++


tubule diameter
% of tubules showing epithelial abnormalities
apoptotic cells / 100 tubules
% of tubules containing diploid spermatids
11 8 4 3
8 3 6 4
9 6 3 3
Exposure A
Exposure B
Exposure C
EtOH cont
ZEA +
ZEA ++
ZEA +++


tubule diameter
% of tubules showing epithelial abnormalities
apoptotic cells / 100 tubules
% of tubules containing diploid spermatids
Exposure A
Exposure B
Exposure C
12 5 4 7
12 4 6 5
5 5 4 4
DMSO cont
MEHP +
MEHP ++
MEHP +++


tubule diameter
200
180
160
140
120
100
80
60
40
20
0
12 7 5 6
Exposure A
12 4 4 3
Exposure B
5 5 6 10
Exposure C
% of tubules showing epithelial abnormalities
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
apoptotic cells / 100 tubules
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
% of tubules containing diploid spermatids
12,0
10,0
8,0
6,0
4,0
2,0
0,0
DMSO cont
LIN +
LIN ++
LIN +++

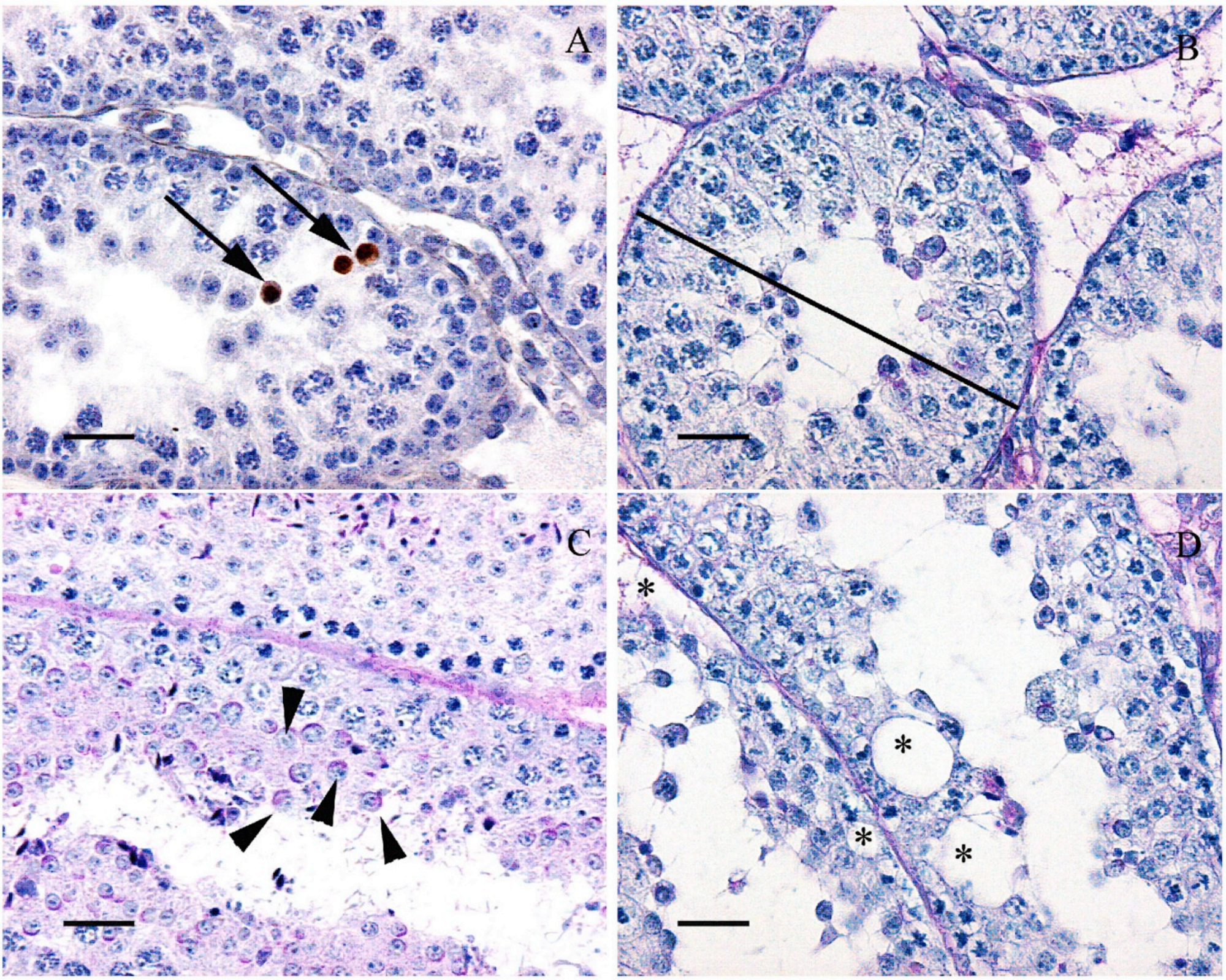

A
B
C
D

Table S1. Genes selected for quantitative real-time PCR validation purposes and their functions.

Gene Name	*Gene Symbol*	*Function*	*Process*
chromatin modifying protein 4B	Chmp4b	Transfer/carrier protein, Other transfer/carrier protein	Intracellular protein traffic
spermatogenesis associated 18	Spata18	Molecular function unclassified	Biological process unclassified
ect2 oncogene	Ect2	Select regulatory molecule, G-protein modulator	Oncogenesis
eukaryotic translation initiation factor 5A2	Eif5a2	Nucleic acid binding, Translation factor, Translation initiation factor	Protein metabolism and modification, Protein biosynthesis
prominin 1	Prom1	Membrane traffic protein	Intracellular protein traffic, Other intracellular protein traffic
Yip1 domain family, member 5	Yipf5	Molecular function unclassified	Biological process unclassified
DnaJ (Hsp40) homolog, subfamily B, member 8	Dnajb8	Chaperone, Other chaperones	Protein metabolism and modification, Protein folding
spermatid associated	Spert	Molecular function unclassified	Biological process unclassified
RAB5A, member RAS oncogene family	Rab5a	Select regulatory molecule, G-protein, Small GTPase	Signal transduction, Intracellular protein traffic, General vesicle transport, Exocytosis, Regulated exocytosis, Endocytosis, Receptor mediated endocytosis
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13, testis-specific serine kinase 6	Ndufa13,Tssk6	Molecular function unclassified, Kinase, Protein kinase, Non-receptor serine/threonine protein kinase	Biological process unclassified, Protein metabolism and modification, Protein modification, Protein phosphorylation
A kinase (PRKA) anchor protein 3	Akap3	Select regulatory molecule, Kinase modulator	Protein targeting and localization, Protein targeting
ubiquitin specific peptidase 2	Usp2	Protease, Cysteine protease	Protein metabolism and modification, Proteolysis
small nuclear RNA activating complex, polypeptide 2	Snapc2	Transcription factor, Basal transcription factor	Nucleoside, nucleotide and nucleic acid metabolism, mRNA transcription, mRNA transcription regulation
ring finger protein (C3HC4 type) 19	Rnf19	Molecular function unclassified	Protein metabolism and modification, Protein modification, Cell structure and motility, Cell structure
retinoblastoma-like 2	Rbl2	Transcription factor, Other transcription factor, Nucleic acid binding	Nucleoside, nucleotide and nucleic acid metabolism, mRNA transcription, mRNA transcription regulation, Oncogenesis, Tumor suppressor, Cell cycle, Cell cycle control
cell adhesion molecule with homology to L1CAM	Chl1	Cell adhesion molecule, CAM family adhesion molecule	Signal transduction, Cell communication, Cell adhesion-mediated signaling, Developmental processes, Ectoderm development, Neurogenesis, Cell adhesion
defensin beta 19	Defb19	Molecular function unclassified	Biological process unclassified
ring finger protein 10	Rnf10	Molecular function unclassified	Biological process unclassified
glycoprotein, synaptic 2	Gpsn2	Oxidoreductase, Dehydrogenase	Biological process unclassified
zinc finger, FYVE domain containing 9	Zfyve9		
testis derived transcript	Tes	Nucleic acid binding	Electron transport, Other metabolism
phospholipid scramblase 2	Plscr2		
cullin 3	Cul3	Miscellaneous function, Other miscellaneous function protein	Protein metabolism and modification, Proteolysis, Oncogenesis, Cell cycle, Cell cycle control, Apoptosis, Induction of apoptosis

Table S2. Genes preferentially expressed in spermiogenesis (EI-EIII) and round spermatids (Rtds I-V) that appear co-clustered in Figure 3 (EI-III, orange bars, and Rtds I-V, grey bars).

E-I

Gene	Ref seq DNA	Description
Tax1bp1	NM_025816.1	Tax1 (human T-cell leukemia virus type I) binding protein 1
Col4a3bp	NM_023420.1	Procollagen, type IV, alpha 3 (Goodpasture antigen) binding protein
Clpx	NM_011802.1	caseinolytic peptidase X (E.coli)
Iqwd1	AK161893	IQ motif and WD repeats 1
1700007B14Rik	AK077102	Adult male testis cDNA, IQ calmodulin-binding motif containing protei
Anubl1	AK089032	AN1, ubiquitin-like, homolog (Xenopus laevis)
Psma6	NM_011968.1	proteasome (prosome, macropain) subunit, alpha type 6
Mjd	NM_029705.1	Machado-Joseph disease (ataxin 3) homolog (human)
Vashl	NM_144879.1	Vasohibin-like protein
Otud4	AK122429	OTU domain containing 4
Cpeb4	NM_026252.2	cytoplasmic polyadenylation element binding protein 4
Cast	NM_009817.1	Calpastatin, mRNA (cDNA clone MGC:12116 IMAGE:3710078)
Trim36	NM_178872.3	Tripartite motif-containing 36, mRNA
Dgkh	XM_484397	similar to diacylglycerol kinase, eta isoform 1
Tnp1	NM_009407.1	transition protein 1
Hils1	NM_018792.1	histone H1-like protein in spermatids 1
1700010M22Rik	NM_025490.1	SIMILARITY: Contains 1 SCP2 domain
Psme4	NM_134013.2	Proteasome (prosome, macropain) activator subunit 4, mRNA

E-II

Gene	Ref seq DNA	Description
Prkcd	NM_011103.1	Protein kinase C, delta (Prkcd)
Sh3bp5	NM_011894.1	SH3-domain binding protein 5 (BTK-associated)
Nt5c1b	NM_027588.2	5'-nucleotidase, cytosolic IB
Cdrt4	NM_025496.1	CMT1A duplicated region transcript 4
Isg20l2	NM_177663.2	interferon stimulated exonuclease gene 20-like 2
Q9DC38	AK004585	Adult male lung cDNA hypothetical protein
4931440F15Rik	NM_176829.1	hypothetical protein LOC216622
Sstk	NM_032004.1	Serine/threonine protein kinase SSTK (Sstk)
Tssk2	NM_009436.1	Testis-specific serine kinase 2 (Tssk2)
1700003N22Rik	NM_145538.1	similar to Ubiquitin-conjugating enzyme variant Kua
2810453I06Rik	NM_026050.1	hypothetical protein LOC67238
Aif1	NM_019467.2	Allograft inflammatory factor 1
Nup35	NM_027091.1	nucleoporin 35
D2Ertd750e	NM_026412.1	TRAF4 associated factor 1 homolog
1700012A03Rik	NM_029587.1	hypothetical protein LOC76382
Dnajb8	NM_019964.1	DnaJ (Hsp40) homolog, subfamily B, member 8 (Dnajb8)
Csnk1g2	NM_134002.1	casein kinase 1, gamma 2
Spata18	NM_178387.2	Spermatogenesis associated 18 homolog (rat)
Fscn3	NM_019569.2	Fascin homolog 3, actin-bundling protein, testicular (S. purpuratus)
Otub2	NM_026580.1	OTU domain, ubiquitin aldehyde binding 2
Dp1l1	NM_139292.1	Deleted in polyposis 1-like 1 (Dp1l1), mRNA
Gsg1	NM_010352.1	Germ cell-specific gene 1
4931407G18Rik	NM_027631.1	testis development protein NYD-SP26
Rnf138	NM_019706.2	Ring finger protein 138, transcript variant 2
Iqcf3	NM_026645	IQ motif containing F3
5730596K20Rik	NM_146091.2	hypothetical protein LOC109168
1700029H14Rik	NM_025601.1	hypothetical protein LOC66501
Ppm1a	NM_008910.2	protein phosphatase 1A, magnesium dependent, alpha isoform
Psmf1	NM_144889.2	proteasome (prosome, macropain) inhibitor subunit 1

Skd3	NM_009191.2	Suppressor of K+ transport defect 3 (Skd3), mRNA
Spata19	NM_029299.1	spermatogenesis associated 19
R3hcc1	BC059860	R3H domain and coiled-coil containing 1
Pdzk1	NM_021517.1	PDZ domain containing 1
Nurit	NM_026457.1	Testis-specific leucine zipper protein nurit mRNA,
Adam1a	NM_172126.2	Fertilin alpha precursor (ADAM 1)
4930431B09Rik	BC046309	PREDICTED
Tisp78	NM_144827.3	Spermatogenesis associated 20
Mfap3l	NM_027756.2	Microfibrillar-associated protein 3-like
Chn2	NM_023543.1	chimerin (chimaerin) 2
Tmco5	NM_026104.1	transmembrane and coiled-coil domains 5
5031400M07Rik	NM_020586.1	hypothetical protein LOC80517
Fts	NM_010241.1	Fused toes, mRNA
1700009N14Rik	XM_131323	hypothetical protein LOC75471
Ppp2r2b	NM_027531.1	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52)
Akap4	NM_009651.1	A kinase (PRKA) anchor protein 4 (Akap4), mRNA
4921517D22Rik	NM_183290.1	hypothetical protein LOC70900
Tmem56	NM_178936.2	Transmembrane protein 56 (Tmem56)
Mgat4a	NM_173870.1	mannoside acetylglucosaminyltransferase 4, isoenzyme A
1700029J11Rik	AK006503	Adult male testis cDNA, product:hypothetical protein
Ube2v2	NM_023585.2	Ubiquitin-conjugating enzyme E2 variant 2 (Ube2v2)
4933417A18Rik	NM_025750.1	hypothetical protein LOC66761
Odf2	NM_013615.1	outer dense fiber of sperm tails 2
Orc3l	NM_001007589.1	origin recognition complex, subunit 3-like (S. cerevisiae)
4921530L21Rik	NM_025733.1	hypothetical protein LOC66732
4921530D09Rik	AK014983	Adult male testis cDNA, hypothetical protein
1700011H14Rik	NM_025956.2	hypothetical protein LOC67082
Ccdc50	NM_001025615.1	Coiled-coil domain-containing protein 50 (Protein Ymer)
4921528O07Rik	AK019544	Adult male testis cDNA, hypothetical ARM repeat containing protein
Plcz1	NM_054066.2	Phospholipase C, zeta 1 (Plcz1), mRNA
Hip2	NM_016786.2	Huntingtin interacting protein 2
Spag4l	NM_029599.1	Sperm associated antigen 4-like (Spag4l), mRNA
Tes	NM_011570.2	Testin, testis derived transcript (Tes), transcript variant 1
Gpd2	NM_010274.2	glycerol phosphate dehydrogenase 2, mitochondrial
Lnp	NM_027133.1	limb and neural patterns
Zp3r	NM_009581.1	Zona pellucida 3 receptor (Zp3r), mRNA
Sec24b	NM_207209.1	SEC24 related gene family, member B (S. cerevisiae)
Pdia3	NM_007952.1	protein disulfide isomerase associated 3

E-III

Gene	Ref seq DNA	Description
Fabp3	NM_010174.1	fatty acid binding protein 3, muscle and heart
1700023L04Rik	AK076883	hypothetical protein LOC76419
Thrap3	NM_025856.1	thyroid hormone receptor associated protein 3
Dnajb13	NM_153527.2	DnaJ (Hsp40) related, subfamily B, member 13
4930579J09Rik	NM_133689.1	IIIG9 protein, a novel mRNA expressed along brain ventricles
Stard10	NM_019990.1	START domain containing 10 (Stard10), mRNA
Osbp2	NM_152818.2	Oxysterol binding protein 2 (Osbp2), mRNA
Arpm2	NM_028513.2	Actin related protein M2 (Arpm2), mRNA
4930579G22Rik	NM_026916.1	hypothetical protein LOC69034
1700056E22Rik	AK006811	hypothetical protein LOC73363
1700054O13Rik	NM_026096.1	Huntingtin interacting protein M
Nr1d1	NM_145434.1	Nuclear receptor subfamily 1, group D, member 1 (Nr1d1)
BC051628	NM_199312.1	hypothetical protein LOC332713

Rtids-l

Gene	Ref seq DNA	Description
Ddx20	NM_017397.2	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20 (Ddx20), mRNA
Col4a3bp	NM_023420.1	Procollagen, type IV, alpha 3 (Goodpasture antigen) binding protein
4921521K07Rik	NM_027599.1	hypothetical protein LOC70902

Ngly1	NM_021504.2	N-glycanase 1 (Ngly1), mRNA
1700007B14Rik	AK077102	IQ Calmodulin binding motif containing protein
Anubl1	AK089032	AN1, ubiquitin-like, homolog (X. laevis)
Psma6	NM_011968.1	proteasome (prosome, macropain) subunit, alpha type 6
Tsnaxip1	NM_024445.2	translin-associated factor X (Tsnax) interacting protein 1
Mjd	NM_029705.1	Machado-Joseph disease (ataxin 3) homolog (human)
4631422O05Rik	BC062166	4631422O05 gene
B130052G07Rik	NM_144879.1	Vasohibin-like protein
Otud4	AK122429	OTU domain containing 4/HIV-1 induced protein HIN-1
Cpeb4	NM_026252.2	cytoplasmic polyadenylation element binding protein 4
Trim36	NM_178872.3	Tripartite motif-containing 36, mRNA
Dgkh	XM_484397	M. musculus diacylglycerol kinase, eta, transcript variant 1
4932412H11Rik	NM_172879.1	hypothetical protein LOC242838
Tnp1	NM_009407.1	transition protein 1
Hils1	NM_018792.1	histone H1-like protein in spermatids 1
1700010M22Rik	NM_025490.1	SIMILARITY: Contains 1 SCP2 domain

Rtds-II

Gene	Ref seq DNA	Description
Prkcd	NM_011103.1	Protein kinase C, delta (Prkcd), mRNA
Nt5c1b	NM_027588.2	5'-nucleotidase, cytosolic IB
Cdrt4	NM_025496.1	CMT1A duplicated region transcript 4
Isg20l2	NM_177663.2	interferon stimulated exonuclease gene 20-like 2
Q9DC38	AK004585	Adult male lung cDNA hypothetical protein
Sstk	NM_032004.1	Serine/threonine protein kinase SSTK (Sstk)
Tssk2	NM_009436.1	Testis-specific serine kinase 2 (Tssk2), mRNA
2810453I06Rik	NM_026050.1	hypothetical protein LOC67238
Aif1	NM_019467.2	Allograft inflammatory factor 1, mRNA)
1700012A03Rik	NM_029587.1	hypothetical protein LOC76382
Dnajb8	NM_019964.1	DnaJ (Hsp40) homolog, subfamily B, member 8 (Dnajb8)
Csnk1g2	NM_134002.1	casein kinase 1, gamma 2
Spata18	NM_178387.2	Spermatogenesis associated 18 homolog (rat)
Fscn3	NM_019569.2	Fascin homolog 3, actin-bundling protein, testicular (S. purpuratus)
Otub2	NM_026580.1	OTU domain, ubiquitin aldehyde binding 2
Gsg1	NM_010352.1	Germ cell-specific gene 1, mRNA 798)
4931407G18Rik	NM_027631.1	testis development protein NYD-SP26
Rnf138	NM_019706.2	Ring finger protein 138, transcript variant 2, mRNA
1700023E05Rik	NM_027970.1	hypothetical protein LOC71868
Iqcf3	NM_026645	IQ motif containing F3, mRNA
5730596K20Rik	NM_146091.2	hypothetical protein LOC109168
1700029H14Rik	NM_025601.1	hypothetical protein LOC66501
Psmf1	NM_144889.2	proteasome (prosome, macropain) inhibitor subunit 1
Skd3	NM_009191.2	Suppressor of K+ transport defect 3 (Skd3), mRNA
Spata19	NM_029299.1	spermatogenesis associated 19
Agpat3	NM_053014.2	1-acylglycerol-3-phosphate O-acyltransferase 3 (Agpat3)
R3hcc1	BC059860	R3H domain and coiled-coil containing 1
Pdzk1	NM_021517.1	PDZ domain containing 1
Rnf38	NM_175201.2	Ring finger protein 38, mRNA
Nurit	NM_026457.1	Testis-specific leucine zipper protein nurit), alternatively spliced
Adam1a	NM_172126.2	Fertilin alpha precursor (ADAM 1)
4930431B09Rik	BC046309	PREDICTED
Tisp78	NM_144827.3	Spermatogenesis associated 20, mRNA)
Wdr51b	NM_027740.3	WD repeat domain 51B
Mfap3l	NM_027756.2	Microfibrillar-associated protein 3-like, mRNA:5025281)
4930435E12Rik	AK077164	PREDICTED: similar to hypothetical protein FLJ32859
	NM_001013783.1	hypothetical protein LOC432552
Chn2	NM_023543.1	chimerin (chimaerin) 2
Tmco5	NM_026104.1	transmembrane and coiled-coil domains 5
5031400M07Rik	NM_020586.1	hypothetical protein LOC80517
1700009N14Rik	AK005799	hypothetical protein LOC75471

Akap4	NM_009651.1	A kinase (PRKA) anchor protein 4 (Akap4), mRNA
4921517D22Rik	NM_183290.1	hypothetical protein LOC70900

Rtds-III

Gene	Ref seq DNA	Description
4921530L21Rik	NM_025733.1	hypothetical protein LOC66732
4921530D09Rik	AK014983	adult male testis cDNA, hypothetical protein
1700011H14Rik	NM_025956.2	hypothetical protein LOC67082
Ccdc67	NM_181816.1	coiled-coil domain containing 67
4921528O07Rik	AK019544	Adult male testis cDNA, hypothetical ARM repeat structure
Plcz1	NM_054066.2	Phospholipase C, zeta 1 (Plcz1), mRNA
Chl1	NM_007697.1	Cell adhesion molecule with homology to L1CAM

Rtds-IV

Gene	Ref seq DNA	Description
Tesp1	NM_009355.1	Testicular serine protease 1 (Tesp1), mRNA
Spag4l	NM_029599.1	Sperm associated antigen 4-like (Spag4l), mRNA
Tekt2	NM_011902.1	Tektin 2, mRNA (cDNA clone MGC:5692 IMAGE:3582971)
1700025E21Rik	NM_029373.1	hypothetical protein LOC75647
Tes	NM_011570.2	Testin, testis derived transcript (Tes), transcript variant 1
Gpd2	NM_010274.2	glycerol phosphate dehydrogenase 2, mitochondrial
Rnf139	NM_175226.2	Ring finger protein 139, mRNA
Lnp	NM_027133.1	limb and neural patterns
Zp3r	NM_009581.1	Zona pellucida 3 receptor (Zp3r), mRNA

Rtds-V

Gene	Ref seq DNA	Description
1700023L04Rik	AK076883	Adult male testis cDNA, hypothetical protein
1700110M21Rik	AK007164	Adult male testis cDNA, hypothetical protein
Thrap3	NM_025856.1	thyroid hormone receptor associated protein 3
Dnajb13	NM_153527.2	DnaJ (Hsp40) related, subfamily B, member 13
4930579J09Rik	NM_133689.1	IIIG9 protein, a novel mRNA expressed along brain ventricles
Arpm2	NM_028513.2	Actin related protein M2 (Arpm2), mRNA
4930579G22Rik	NM_026916.1	hypothetical protein LOC69034
1700054O13Rik	NM_026096.1	Huntingtin interacting protein M
BC051628	NM_199312.1	hypothetical protein LOC332713

Table S3. Genes included in the most representative network from the hierarchical clustering analysis considering all experimental conditions of exposure

Symbol	*Entrez Gene Name*	*Location*	*Family*
BCAR3	breast cancer anti-estrogen resistance 3	Cytoplasm	other
C9ORF9	chromosome 9 open reading frame 9	Unknown	other
CD48	CD48 molecule	Plasma Membrane	other
CNOT1	CCR4-NOT transcription complex, subunit 1	Unknown	other
CNOT6	CCR4-NOT transcription complex, subunit 6	Nucleus	other
CNOT7	CCR4-NOT transcription complex, subunit 7	Nucleus	transcription regulator
DDX24	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24	Nucleus	enzyme
EEF1D	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	Cytoplasm	translation regulator
EGLN2	egl nine homolog 2 (*C. elegans*)	Cytoplasm	enzyme
EIF3B	eukaryotic translation initiation factor 3, subunit B	Cytoplasm	translation regulator
EIF3E	eukaryotic translation initiation factor 3, subunit E	Cytoplasm	translation regulator
EIF3H	eukaryotic translation initiation factor 3, subunit H	Cytoplasm	translation regulator
EIF4A1	eukaryotic translation initiation factor 4A, isoform 1	Cytoplasm	translation regulator
EIF4A2	eukaryotic translation initiation factor 4A, isoform 2	Cytoplasm	translation regulator
EIF4E	eukaryotic translation initiation factor 4E	Cytoplasm	translation regulator
EIF4G1	eukaryotic translation initiation factor 4 gamma, 1	Cytoplasm	translation regulator
EIF4G2 (includes EG:1982)	eukaryotic translation initiation factor 4 gamma, 2	Cytoplasm	translation regulator
FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	Cytoplasm	transporter
FABP4	fatty acid binding protein 4, adipocyte	Cytoplasm	transporter
FOXE1	forkhead box E1 (thyroid transcription factor 2)	Nucleus	transcription regulator
GUCY2C	guanylate cyclase 2C (heat stable enterotoxin receptor)	Plasma Membrane	kinase
HK1	hexokinase 1	Cytoplasm	kinase
HMOX2	heme oxygenase (decycling) 2	Cytoplasm	enzyme
Insulin		Unknown	group
MDH2	malate dehydrogenase 2, NAD (mitochondrial)	Cytoplasm	enzyme
PABPC1	poly(A) binding protein, cytoplasmic 1	Cytoplasm	translation regulator
PAX4	paired box 4	Nucleus	transcription regulator
PGK1	phosphoglycerate kinase 1	Cytoplasm	kinase
PLA2G16	phospholipase A2, group XVI	Nucleus	other
RGS2	regulator of G-protein signaling 2, 24kDa	Nucleus	other
RPS29	ribosomal protein S29	Cytoplasm	other
SRXN1	sulfiredoxin 1 homolog (*S. cerevisiae*)	Cytoplasm	enzyme
TMEM9B	TMEM9 domain family, member B	Plasma Membrane	other
TPI1	triosephosphate isomerase 1	Cytoplasm	enzyme
UPF3B	UPF3 regulator of nonsense transcripts homolog B (yeast)	Nucleus	transporter