Toxicogenomic investigation of *Tetrahymena thermophila* exposed to dichlorodiphenyltrichloroethane (DDT), tributyltin (TBT), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

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Dichlorodiphenyltrichloroethane (DDT), tributyltin (TBT), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are persistent in the environment and cause continuous toxic effects in humans and aquatic life. *Tetrahymena thermophila* has the potential for use as a model for research regarding toxicants. In this study, this organism was used to analyze a genome-wide microarray generated from cells exposed to DDT, TBT and TCDD. To accomplish this, genes differentially expressed when treated with each toxicant were identified, after which their functions were categorized using GO enrichment analysis. The results suggested that the responses of *T. thermophila* were similar to those of multicellular organisms. Additionally, the context likelihood of relatedness method (CLR) was applied to construct a TCDD-relevant network. The T-shaped network obtained could be functionally divided into two subnetworks. The general functions of both subnetworks were related to the epigenetic mechanism of TCDD. Based on analysis of the networks, a model of the TCDD effect on *T. thermophila* was inferred. Thus, *Tetrahymena* has the potential to be a good unicellular eukaryotic model for toxic mechanism research at the genome level.

microarray, enrichment analysis, gene interaction network, pollutant

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Some pesticides and industrial additives that are byproducts of industrial development can be deleterious to human health and aquatic ecosystems when released into the environment. Substances that interfere with normal biological processes by mimicking natural hormones are known as environmental hormones. Within this class of chemicals, dichlorodiphenyltrichloroethane (DDT), tributyltin (TBT), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are well-known for being hazardous. The first intentionally released chemical found to be estrogenic was DDT [1]. Although banned from use many years ago, DDT is still widely used in some developing countries. DDT also poses a great threat

to both ecosystems and human health because it persists in the environment, bioaccumulates in organisms, and exhibits chronic toxicity. The widely used antifouling agent TBT is another common contaminant that has been shown to be capable of embryotoxicity [2], genotoxicity [3] and endocrine disruption [4]. Overall, TBT induces imposex and causes androgenic effects in some aquatic organisms [5]. The compound TCDD is a widely distributed environmental contaminant that originates from industrial processes and has numerous associations with estrogenic action at the molecular level. TCDD has been linked to a wide variety of biological responses, ranging from induction of cytochrome P450 1A (CYP1A) to tumorigenesis [6]. Owing to their potential to cause harm, these three toxicants have been the

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focus of a great deal of research over the last few decades. A few genes have been found to be closely related to these chemicals (e.g., *CYP1a1* and TCDD) [7]; however, research at the genome- and proteome-levels in a suitable eukaryotic model is required to study the complicated molecular mechanisms responsible for their toxic effects and environmental hormone actions.

Tetrahymena thermophila, a free-living ciliated protist, has become well-established as a unicellular eukaryotic model organism during the past several decades. To date, research using *T. thermophila* has made great contributions to fundamental biological discoveries such as dynein [8], catalytic RNA [9], telomeric repeats [10,11], telomerase [12], the function of histone acetylation [13], and RNAi mechanisms in macronucleus reprogramming [14]. Analysis of expressed sequence tags in the genome of *Tetrahymena* has demonstrated that it has a high degree of functional homology with human and mammalian genomes [15]. The organism also shares many core processes with a wide variety of eukaryotes (including humans) [16] that are not found in other single-celled model systems (e.g., yeast). *Tetrahymena* is convenient as a model organism because of its small size and ease of culture. These characteristics and its advantages for toxicological studies have led to its use as a model to detect aquatic toxicity and ecotoxicological effects for many years [17]. Recently, differentially expressed genes in *T. thermophila* exposed to DDT or TBT have been detected using genome-wide screening conducted by suppression subtractive hybridization [18,19]. Moreover, completion of the macronuclear sequencing project [16] and establishment of the first genome-wide microarray platform in *T. thermophila* [20] with the *Tetrahymena* Gene Expression Database (TGED, <http://tged.ihb.ac.cn>) [21] offers extensive resources for further functional genomic studies, including toxigenomics. Thus, there is an opportunity to make use of this unicellular model to study the toxicological mechanism of environmental hormones at the genomic level.

In the present study, genes differentially expressed in response to exposure to DDT, TBT and TCDD were screened using a microarray technique and then categorized by function using GO annotation information and enrichment analysis. The results showed that typical functions were affected by different types of toxic environmental hormones in *T. thermophila*, as in some metazoans. Furthermore, analysis of the gene-interaction network revealed a molecular mechanism coincident with the characteristics of TCDD, which suggested that *T. thermophila* had the potential to be a unicellular eukaryotic model for investigation of toxic molecular mechanisms at the genome level.

1 Materials and methods

1.1 Cell culture, toxicant exposure and RNA isolation

T. thermophila strain Cu428 was grown axenically at 30°C

in SPP medium [22], which consists of 2% proteose peptone (Becton, Dickinson and Company, BD), 0.1% yeast extract (Oxoid), 0.2% glucose (Sinopharm Chemical Reagent Co., Ltd.) and 0.003% ferric citrate (Sigma), until the mid log phase (approximately 3×10^5 cells mL⁻¹). The cultures were then exposed to different toxicants for 24 h or DMSO (Sigma) as a negative control. To obtain results that were as clear as possible, the highest concentrations that would not affect the growth rate of *T. thermophila* (DDT 4×10^{-6} mol L⁻¹, TBT 5×10^{-9} mol L⁻¹, and TCDD (SUPELCO) 1×10^{-9} mol L⁻¹) were selected based on the results of a preliminary experiment (data not shown). After homogenization of cells using QiaShredder spin columns, the total RNA was extracted using an RNeasy Protect Cell Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration of the samples was then quantified by UV spectrophotometry at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE) and the RNA integrity was verified using a Bioanalyzer 1000 (Agilent, Palo Alto, CA).

1.2 Microarray processing

High-density *T. thermophila* genome-wide oligonucleotide DNA microarrays were designed according to the predicted genome 2006 [16]. Details regarding the chip design and microarray operation are available elsewhere [20]. Raw data were deposited in the Gene Expression Omnibus Database under data set accession numbers GSE26515 including three samples: GSM651904 for TCDD, GSM651903 for DDT, GSM651902 for DMSO and GSM651901 for TBT.

1.3 Quantitative real-time PCR analysis

Real time fluorescent quantitative PCR (Q-PCR) tests for microarray assay were conducted as follows. RNA was extracted from *T. thermophila* and treated in the same method as for the microarray experiment, after which it was digested with Dnase (Promega), then reverse-transcribed into double-stranded cDNA using M-MLV reverse transcriptase RNase H⁺ (TOYOBO). A FastStart Universal SYBR Green Master mix (Roche) was used as the reagent mix for Q-PCR, which was conducted using an Opticon2 96-well reactor (Bio-rad). The thermal cycling program was as follows: 5 min of denaturation at 94°C followed by 40 cycles of 20 s at 94°C, 20 s at 54–57°C, 20 s at 72°C, and 1 s at 70°C for plate reading. This was followed by a 10 min extension at 72°C, after which a melting curve was generated by heating the samples from 70°C to 90°C at a rate of 0.5°C s⁻¹. Real-time RT-PCR reactions were conducted in triplicate for each gene. Data were collected using the Opticon2 software (MJ Research) and the fold change was calculated using the comparative quantification model [23] assuming 100% efficiency. The 18S ribosomal RNA gene unaltered by exposure to toxicants was selected as a reference. Target

gene IDs and primer sequences are listed in Appendix Tables S1 and S2 in the electronic version.

1.4 Microarray data analysis

To identify the genes that responded to the chemicals, we identified those with expression levels that were up- or down-regulated by more than 2-fold when compared to DMSO treated samples using the Arraystar software.

1.5 Gene ontology annotation and enrichment analysis

Functional annotations and descriptions of each gene were required for GO classification. We annotated the entire genome using Blast2GO [24]. The P -value was set as 10^{-6} , while the default values were used for the other parameters. Enrichment analysis was then conducted using the Gossip package [25] integrated with Blast2GO. Genes responding to different toxicants were used separately as an independent test dataset, and the entire genome was used as a reference dataset. The hypothesis is briefly presented below. Null hypothesis (H_0): Compared with the reference, a GO term in the test group is not enriched. Alternative hypothesis (H_1): The GO term is enriched in the test set when compared with the reference. The null hypothesis was tested using Fisher's exact test. A GO term with a P -value < 0.01 was taken to indicate significance [25].

1.6 TCDD-relevant network construction and function analysis

We applied the context likelihood of relatedness (CLR) algorithm [26] with a Z-score threshold of 3.49 to construct a *Tetrahymena* gene-interaction network using data from 67 NimbleGen single channel microarrays, including 52 microarrays from Gorovsky's lab and Miao's lab, 11 from the Pearlman's lab and four from Liu's lab [21]. Three stages of the life cycle were involved in these microarrays, growth, starvation and conjugation. For growth, cells were harvested when grown to 1×10^5 , 3.5×10^5 and 1×10^6 cells mL^{-1} . For starvation, cells at a density of 2×10^5 cells mL^{-1} were collected at 0, 3, 6, 9, 12, 15, and 24 h. For conjugation, equal amounts of B2086 and CU428 cells were mixed, and samples were harvested at 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 h after mixing. A combined filter criterion was used [27] to remove genes with low information content. Based on between-sample variability and the gene minimal signal, the filter removed the following genes: (i) those that have an expression difference or variation between samples ($\text{Exp}_{\text{highest-lowest}}$) lower than the median of all the expression differences calculated for each gene ($\text{Exp}_{\text{highest-lowest}} < \text{median } \text{Exp}_{\text{highest-lowest}}$) and (ii) those that have a mean expression signal between samples lower than the median of all the expression signals calculated for each gene. After filtering, 12973 genes were excluded and 15091 genes were retained

for use in constructing the gene-interaction network [21].

Subsequently, genes with enriched functions in TCDD set were selected as cores and genes that might interact with them were extracted, after which the interactions among the extracted genes were identified according to the network. This enabled construction of a network of TCDD-relevant genes. The network graph was exported by cytoscape 2.7 [28] using the spring embedded layout.

The relevant genes assembled in clusters that were consistent with the branches of the ontology tree; therefore, we named each cluster after the corresponding enriched term. Next, we conducted GO enrichment analysis of each group separately, setting FDR (false discovery rate) 0.05 as the cut-off.

2 Results and discussion

2.1 Identification of differentially expressed genes and categorization of functions

Twenty-two genes were selected at random for real time fluorescent quantitative PCR (Q-PCR) confirmation (Appendix Table S1 in the electronic version). The Q-PCR data generally confirmed the microarray results. Linear regression analysis demonstrated a strong correlation between the two methods, with $R=0.8154$ (Figure 1).

Consistent with the chronic toxicity of environmental hormones, our former study showed that exposure to high concentrations of DDT, TCDD and TBT would not significantly affect the growth rate of *T. thermophila* (data not shown). A genome level screening using microarray would be more suitable for molecular mechanism research of the chronic toxicants. The microarray analysis conducted using Arraystar showed that 148, 254 and 608 genes were up- or down-regulated by more than 2-fold in response to exposure to DDT, TBT, and TCDD, respectively. The overlapping of these genes was summarized using a Venn diagram in Figure 2, and the details are provided in Appendix Table S3 in

Figure 1 Correlation between log-transformed levels of gene expression measured by microarray and Q-PCR.

Figure 2 Venn diagram of genes up- or down-regulated when exposed to the three toxicants. The number in the bracket refers to the GO annotated genes.

the electronic version. We conducted gene ontology (GO) annotation of the entire genome to predict the biological roles of these screened genes and categorize them by function. Gene ontology is structured into three domains, molecular functions showing the elemental activities of a gene product, biological processes associating a gene product with the functioning of integrated living units, and cellular components describing where the gene product is active. GO annotation for the entire genome of *T. thermophila* was

obtained using Blast2GO [24], and 7745 of 27769 genes received functional annotations. For each toxicant-treated gene set, 43 genes in the DDT set, 117 genes in the TCDD set and 66 genes in the TBT set were annotated (Appendix Table S3 in the electronic version).

Enrichment analysis is a common method of biologically interpreting microarray data [29] that has been used widely for automatic functional categorization of gene lists. We conducted enrichment analysis of differentially expressed genes in each toxicant-treated set using the Gossip package [25] integrated in Blast2GO to determine what gene functions were mainly involved in toxicant exposure. The output was divided into three independent domains (biological processes, molecular functions, and cellular components); however, we chose to summarize the results using only the molecular function ontology. This method was selected because it can reflect the basic activation of cellular processes, making it possible to transfer information about function among different organisms and easier to unify existing expression information from genome-wide toxicological studies of different organisms [30].

There were five branches in the DDT ontology tree (Figure 3), but most of the genes that were involved were assigned to only two of them (Appendix Table S4 in the electronic version). One branch reflected peptidase activity, which is common in oxidatively stressed cells [31]. The other branch reflected anion transmembrane-transporting ATPase activity, which was a combination of two types of function, transmembrane-transporter activity and ATPase

Figure 3 Ontology tree of enriched molecular functions in the DDT set. The area framed in green is the deepest branch, representing ABC transporter genes. The color of the boxes indicates the *P*-value.

activity. Overall, seven genes were represented in this branch, and five of these were ABC (ATP-binding cassette) transporters (Appendix Table S4 in the electronic version). ABC transporters comprise a large family of membrane-bound proteins that can actively transport a wide range of compounds through membranes against concentration gradients via the consumption of ATP. Many ABC transporters have been identified in *T. thermophila* [16], and these proteins are probably responsible for transporting material to maintain cellular homeostasis, which is consistent with the environmental sensitivity needed for the organisms to adapt to a free-living mode of life. Furthermore, several ABC transporters have been reported to protect organisms against xenobiotics [32–35], some of which were reported to be directly related to DDT [34,35]. As in some multicellular organisms, ABC transporters in *T. thermophila* may play an important role in the protective response to counteract exposure to DDT.

With respect to TBT, all five genes assigned to the single enriched branch were annotated as serine protease inhibitors (Appendix Table S5 in the electronic version). TBT has been reported to cause apoptosis [36,37], in which activation of protease is known to play an important role [38]. Some protease inhibitors have been shown to inhibit apoptosis in the early stages of apoptosis [39]. Among these inhibitors, serine protease inhibitor has been used to block TBT-induced apoptosis at an intermediate stage for experiments involving morphological observation [39]. According to our microarray results, the five inhibitor genes of *T. thermophila* were all up-regulated after exposure to TBT. Thus, the over-expression of serine protease inhibitors is likely a mechanism to protect *T. thermophila* against apoptosis caused by TBT.

Although TCDD is known to affect a wide range of cellular processes, its metabolic functions and roles in protective mechanisms in cells are still unclear. Additionally, the enriched functions and the relevant genes that TCDD influences are more complex than those affected by the other two chemicals we investigated (Appendix Table S6 in the electronic version). The enriched functions of TCDD were categorized into the following four types: (a) protein tyrosine/serine/threonine phosphatase activity, (b) NAD-dependent deacetylase activity, (c) acid-amino acid ligase activity, and (d) cysteine-type peptidase activity.

Coincident with the epigenetic mechanism of TCDD [6], functions a, b and c are involved in signal transmission, regulation of gene expression, and post-translational modification, respectively. Function d was common among organisms exposed to oxidative stress [31], which is one of the effects believed to be caused by TCDD [40]. Furthermore, function a is likely related to kinase activity, which is affected by exposure to TCDD [41] and plays an important role in signal regulatory pathways. Additionally, three genes in function c encoded ubiquitin-conjugating enzymes (Appendix Table S6 in the electronic version), which correlates

with the ubiquitination reported in response to exposure to TCDD [42,43]. Taken together, these findings indicated that these enriched functions all corresponded well with the characteristics of TCDD.

It is well known that the toxicity mechanisms of these three compounds are quite different. DDT has been found to be estrogenic [1], TBT is believed to cause androgenic effects [5], and TCDD affects a wide range of complicated biological processes and is thought to be nongenotoxic [44]. Accordingly, microarray analysis of exposure of *Tetrahymena* to the toxicants suggested that the functions of the differentially expressed genes varied among toxicant-treated sets. These functions coincided with the characteristics of the toxicants, and some of them have been reported in multicellular organisms [31–35,41–43]. Thus, *T. thermophila* has the potential to be a unicellular model for toxic research because it can reflect general patterns of biological responses to these toxicants that match those of other eukaryotic organisms.

2.2 Network analysis of TCDD in *T. thermophila*

Organisms have an immense and flexible program of gene expression controlled by the dynamic interactions of hundreds of transcriptional regulators and are thus able to adjust themselves to the irregular and often unpredictable stresses of their environment. Explaining the mechanisms that determine the expression of genes will help elucidate the effects of various stressors; thus, identification of gene interaction is especially important. To unravel the complexity of interactions and regulatory mechanisms involved in expression, several methods of calculating networks have been calculated to clarify the relationships of genes in pathways. Although high-throughput methods have been used widely for years, they have primarily been used to screen a few responding genes rather than for network analysis at the genome level. Taking the three toxicants we investigated as examples, recent studies on network analysis of environmental pollutants have primarily focused on classical genes and pathways [45–51]. Thus, a suitable model and a corresponding method for network analysis at the genome level are required.

During exposure to TCDD, the number of differentially expressed genes is much larger than that observed in response to exposure to DDT and TBT, as is the number of independent enriched functions. Moreover, the enriched molecular functions in TCDD concern macromolecular modification and signal transmission; therefore, we inferred that their interaction with other genes might reflect the biological responses to TCDD. Consequently, we constructed a network to investigate the mechanism of response to TCDD exposure that included the function-enriched genes and genes interacting with them calculated by context likelihood of relatedness (CLR) algorithm [26]. The network graph generated using a spring-embedded layout turned out to be T-shaped (Figure 4, Table 1) and could be divided into four

Figure 4 The T-shaped network of TCDD-relevant genes, with genes clustered into four modules. Module 1 includes PTP1, TTL, and Ubi1, module 2 includes PTP2, Ubi2, and NDD, module 3 includes PTP3, and module 4 includes CTP. The subnetwork division is shown on the graph.

Table 1 Gene distribution in Figure 4

Description	Number
Ubiquitin	194
Ubi1	146
Ubi2	48
PTP	264
PTP1	167
PTP2	51
PTP3	46
CTP	69
NDD	71
TTL	40
PTP×CTP×Ubi	1
CTP×Ubi	2
NDD×PTP×CTP	3
NDD×PTP×CTP×Ubi	1
PTP×Ubi	31
NDD×CTP×Ubi	3
NDD×Ubi	16
PTP×CTP	5
NDD×PTP×Ubi	19
NDD×PTP	44
PTP×TTL×Ubi	2
TTL×Ubi	1

modules. Most genes were distributed between modules 1 and 2, with many edges linking them. Most genes in modules 3 and 4 interacted only with those in module 2 and only

rarely with those in module 1.

After conducting the network analysis, we investigated the components of each module and the distribution of function-enriched genes. We found that, although the genes clustered in the graph, those that interacted with the same core could be distributed in different modules. For example, the genes interacting with the core genes annotated to protein tyrosine/serine/threonine phosphatase activity (PTP) were distributed in modules 1, 2 and 3. Similarly, the ubiquitin-related genes (Ubi) were distributed between modules 1 and 2. Thus, we named the gene clusters composed of function-enriched genes and those that interacted with them according to their location in the modules. Module 1 includes PTP1, Ubi1 and tubulin-tyrosine ligase (TTL), module 2 includes PTP2, Ubi2 and NAD-dependent protein deacetylase activity (NDD), module 3 contains only PTP3, and module 4 contains cysteine-type peptidase activity (CTP). To determine if the clusters functioned as different modules, we conducted GO enrichment analysis of each cluster separately. The results are shown in Table 2.

In module 1, the enriched functions were generally related to DNA or chromatin structure and the process and machinery of protein synthesis, all of which are likely related to regulation of gene expression on the level of transcription or protein synthesis. In modules 2 and 4, proteolysis and protein amino acid phosphorylation were common. It should be noted that most genes annotated as carbohy-

Table 2 GO enrichment analysis of each group in the relevant network

Module	Cluster	Biological process		Molecular function		Cellular component	
Module 1	PTP1	GO: 0007389	pattern specification process	GO: 0003682	chromatin binding	GO: 0000785	chromatin
		GO: 0006333	chromatin assembly or disassembly	GO: 0003676	nucleic acid binding	GO: 0005634	nucleus
		GO: 0006323	DNA packaging				
	Ubi1	GO: 0006333	chromatin assembly or disassembly	GO: 0019843	rRNA binding	GO: 0005730	nucleolus
		GO: 0051231	spindle elongation	GO: 0003682	chromatin binding	GO: 0000785	chromatin
		GO: 0022613	ribonucleoprotein complex biogenesis	GO: 0003735	structural constituent of ribosome	GO: 0022627	cytosolic small ribosomal subunit
		GO: 0006412	translation			GO: 0022625	cytosolic large ribosomal subunit
		GO: 0009790	embryonic development			GO: 0009507	chloroplast
						GO: 0005773	vacuole
						GO: 0005886	plasma membrane
	TTL	none		none		none	
Module 2	NDD	GO: 0005975	carbohydrate metabolic process	GO: 0016798	hydrolase activity, acting on glycosyl bonds	GO: 0000159	protein phosphatase type 2A complex
		GO: 0040029	regulation of gene expression, epigenetic	GO: 0017136	NAD-dependent histone deacetylase activity	GO: 0005874	microtubule
		GO: 0016458	gene silencing	GO: 0003924	GTPase activity	GO: 0005773	vacuole
		GO: 0051258	protein polymerization	GO: 0005525	GTP binding		
		GO: 0030705	cytoskeleton-dependent intracellular transport	GO: 0019888	protein phosphatase regulator activity		
		GO: 0009855	determination of bilateral symmetry				
	PTP2	GO: 0006468	protein amino acid phosphorylation	GO: 0004672	protein kinase activity	none	
		GO: 0005975	carbohydrate metabolic process	GO: 0016798	hydrolase activity, acting on glycosyl bonds		
		GO: 0006508	proteolysis	GO: 0008233	peptidase activity		
		GO: 0051241	negative regulation of multicellular organismal process				
	Ubi2	none		GO: 0016798	hydrolase activity, acting on glycosyl bonds	none	
Module 3	PTP3	none		none		none	
Module 4	CTP	GO: 0006508	proteolysis	GO: 0008233	peptidase activity	none	
		GO: 0006468	protein amino acid phosphorylation	GO: 0004672	protein kinase activity		
		GO: 0005975	carbohydrate metabolic process	GO: 0016798	hydrolase activity, acting on glycosyl bonds		
				GO: 0001882	nucleoside binding		
				GO: 0019239	deaminase activity		
				GO: 0008810	cellulase activity		

drate metabolic process, which was enriched in clusters of both modules, were protein kinases (Appendix Table S7 in the electronic version). This was likely because the phosphorylation of amide was included in carbohydrate metabolism. In addition, the distribution of ubiquitin-related genes should be noted. Ubiquitination is commonly observed in stressed cells, and polyubiquitin and monoubiquitin produce different results on modified proteins. Polyubiquitination is usually a sign of proteasome proteolysis [52]. Ubiquitin-related genes were found in both modules 1 and 2 (Table 3), but polyubiquitin genes were found only in module 2, which was closely related to the process of proteolysis. The

case of polyubiquitin supported at least the partial reflection of the true biological processes occurring in *T. thermophila* based on our categorization of proteins into modules.

Overall, the network graph and the functional enrichment supported the functional division of genes potentially related to TCDD-response into two subnetworks. Subnetwork 1, which was composed of module 1 (PTP1 and Ubi1), was related to regulation of gene expression on both the transcriptional and translational levels. Subnetwork 2, which was composed of module 2 (PTP2 and NDD) and part of module 4 (CTP), was related to signal transmission and protein degradation. Furthermore, we inferred a TCDD-

Table 3 Ubiquitin-related genes in each group^{a)}

	Group	ID	Name	Link	Total
Module 1	Ubi1	TTHERM_00686150	ribomal-ubiquitin fusion protein ubi5	96	164
		TTHERM_00339620	ubiquitin	90	164
		TTHERM_01082950	ubiquitin-conjugating enzyme family protein	164	164
	PTP1	TTHERM_00550720	ubiquitin-conjugating enzyme e2-17 kD	48	184
		TTHERM_00048860	ubiquitin specific peptidase 20	85	184
		TTHERM_00290870	ubiquitin carboxyl-terminal hydrolase family protein	102	184
	TTL	TTHERM_01205390	ubiquitin carboxyl-terminal hydrolase family protein	13	43
		TTHERM_01326800	ubiquitin carboxyl-terminal hydrolase family protein	10	43
Module 2	NDD	<u>TTHERM_00633030</u>	<u>polyubiquitin</u>	90	157
		TTHERM_00469360	ubiquitin- hect domain	40	157
		TTHERM_00011330	ubiquitin-conjugating enzyme family protein	43	157
	Ubi2	<u>TTHERM_00633030</u>	<u>polyubiquitin</u>	49	102
		TTHERM_00456960	ubiquitin-conjugating enzyme	102	102
	PTP2	<u>TTHERM_00633030</u>	<u>polyubiquitin</u>	48	140
		TTHERM_00633020	ubiquitin family protein	51	140
		TTHERM_00011330	ubiquitin-conjugating enzyme family protein	15	140
		TTHERM_01341620	ubiquitin-conjugating enzyme family protein	94	140

a) Total indicates the number of genes in the cluster and link indicates the genes in the cluster linking to them. Polyubiquitins are underlined.

effect model (Figure 5) based on the function analysis. Normal transcription regulation might be impacted by TCDD (PTP1, NDD), leading to abnormal signal transmission (PTP2, NDD) that would then affect downstream cellular proteolysis (PTP2, CTP). Conversely, both the process and machinery of protein synthesis were disturbed (Ubi1). As a result, abnormal protein accumulated, and cellular homeostasis and the biological processes of *T. thermophila* were then damaged. Based on Figure 3, subnetwork 1, which was composed of PTP1, Ubi1 and TTL, seemed to be consistent with the early effects in the model, while subnetwork 2, which was composed of PTP2, Ubi2 and NDD, seemed more likely to reflect the consequent effects. The two subnetworks may be intermediated by NDD because it enriched both gene-expression-regulation related functions linked to the early effects and phosphatase regulator activity linked to the late effects. Based on these findings, this model warrants further investigation.

The results presented here suggest that *Tetrahymena thermophila* responds differently when exposed to different

types of environmental hormones, and that these responses are similar to those observed in some multicellular organisms [31–35,41–43]. Furthermore, interaction network analysis of the exposure to TCDD provided rich information suggesting that *T. thermophila* would be a good unicellular, eukaryotic model for investigation of molecular mechanisms triggered by exposure to toxicants at the whole-genome level.

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Figure 5 The hypothesized model of TCDD effects on *T. thermophila*.

- 1 Sonnenschein C, Soto A M. An updated review of environmental estrogen and androgen mimics and antagonists. J Steroid Biochem, 1998, 65: 143–150
- 2 Marin M G, Moschino V, Cima F, et al. Embryotoxicity of butyltin compounds to the sea urchin paracentrotus lividus. Mar Environ Res, 2000, 50: 231–235
- 3 Jha A N, Hagger J A, Hill S J, et al. Genotoxic, cytotoxic and developmental effects of tributyltin oxide (tbto): An integrated approach to the evaluation of the relative sensitivities of two marine species. Mar Environ Res, 2000, 50: 565–573
- 4 Crisp T M, Clegg E D, Cooper R L, et al. Environmental endocrine disruption: An effects assessment and analysis. Environ Health Persp, 1998, 106: 11–56
- 5 Antizar-Ladislao B. Environmental levels, toxicity and human exposure to tributyltin (tbt)-contaminated marine environment. A review. Environ Int, 2008, 34: 292–308
- 6 Crews D, McLachlan J A. Epigenetics, evolution, endocrine disruption, health, and disease. Endocrinology, 2006, 147: S4–S10
- 7 Santostefano M J, Ross D G, Savas U, et al. Differential time-course and dose-response relationships of tcdd-induced cyp1b1, cyp1a1, and cyp1a2 proteins in rats. Biochem Bioph Res Co, 1997, 233: 20–24
- 8 Gibbons I R. Studies on protein components of cilia from tetrahymena pyriformis. Proc Natl Acad Sci USA, 1963, 50: 1002–1010
- 9 Kruger K, Grabowski P J, Zaug A J, et al. Self-splicing RNA: Auto-excision and autocyclization of the ribosomal RNA intervening sequence of tetrahymena. Cell, 1982, 31: 147–157
- 10 Blackburn E H, Gall J G. Tandemly repeated sequence at termini of extrachromosomal ribosomal-RNA genes in tetrahymena. J Mol Biol,

- 1978, 120: 33–53
- 11 Yao M C, Yao C H. Repeated hexanucleotide C-C-C-C-A-A is present near free ends of macronuclear DNA of tetrahymena. *Proc Natl Acad Sci-Biol*, 1981, 78: 7436–7439
- 12 Greider C W, Blackburn E H. Identification of a specific telomere terminal transferase-activity in tetrahymena extracts. *Cell*, 1985, 43: 405–413
- 13 Brownell J E, Zhou J X, Ranalli T, et al. Tetrahymena histone acetyltransferase A: A homolog to yeast gcn5p linking histone acetylation to gene activation. *Cell*, 1996, 84: 843–851
- 14 Mochizuki K, Fine N A, Fujisawa T, et al. Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in tetrahymena. *Cell*, 2002, 110: 689–699
- 15 Fillingham J S, Chilcoat N D, Turkewitz A P, et al. Analysis of expressed sequence tags (ESTs) in the ciliated protozoan tetrahymena thermophila. *J Eukaryot Microbiol*, 2002, 49: 99–107
- 16 Eisen J A, Coyne R S, Wu M, et al. Macronuclear genome sequence of the ciliate tetrahymena thermophila, a model eukaryote. *PLoS Biol*, 2006, 4: 1620–1642
- 17 Sauvant M P, Pepin D, Piccinni E. Tetrahymena pyriformis: A tool for toxicological studies. A review. *Chemosphere*, 1999, 38: 1631–1669
- 18 Feng L, Miao W, Wu Y. Differentially expressed genes of tetrahymena thermophila in response to tributyltin (TBT) identified by suppression subtractive hybridization and real time quantitative PCR. *Aquat Toxicol*, 2007, 81: 99–105
- 19 Miao W, Yu T, Orias E, et al. Identification of differentially expressed genes in tetrahymena thermophila in response to dichlorodiphenyltrichloroethane (DDT) by suppression subtractive hybridization. *Environ Microbiol*, 2006, 8: 1122–1129
- 20 Miao W, Xiong J, Bowen J, et al. Microarray analyses of gene expression during the tetrahymena thermophila life cycle. *PLoS ONE*, 2009, 4: e4429
- 21 Xiong J, Yuan D, Fillingham J S, et al. Gene network landscape of the ciliate tetrahymena thermophila. *PLoS ONE*, 2011, 6: e20124
- 22 Gorovsky M A, Yao M C, Keevert J B, et al. Isolation of micro- and macronuclei of tetrahymena pyriformis. *Methods Cell Biol*, 1975, 9: 311–327
- 23 Pfaffl M W, Horgan G W, Dempfle L. Relative expression software tool (REST[®]) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*, 2002, 30: e36
- 24 Conesa A, Gotz S, Garcia-Gomez J M, et al. Blast2go: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, 2005, 21: 3674–3676
- 25 Bluthgen N, Brand K, Cajavec B, et al. Biological profiling of gene groups utilizing gene ontology. *Genome Inform*, 2005, 16: 106–115
- 26 Faith J J, Hayete B, Thaden J T, et al. Large-scale mapping and validation of *Escherichia coli* transcriptional regulation from a compendium of expression profiles. *PLoS Biol*, 2007, 5: 54–66
- 27 Prieto C, Risueno A, Fontanillo C, et al. Human gene coexpression landscape: Confident network derived from tissue transcriptomic profiles. *PLoS ONE*, 2008, 3: e3911
- 28 Cline M S, Smoot M, Cerami E, et al. Integration of biological networks and gene expression data using cytoscape. *Nat Protoc*, 2007, 2: 2366–2382
- 29 Nam D, Kim S Y. Gene-set approach for expression pattern analysis. *Brief Bioinform*, 2008, 9: 189–197
- 30 Pedra J H F, McIntyre L M, Scharf M E, et al. Genom-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant drosophila. *Proc Natl Acad Sci USA*, 2004, 101: 7034–7039
- 31 Friguet B. Oxidized protein degradation and repair in ageing and oxidative stress. *FEBS J*, 2006, 280: 2910–2916
- 32 Abu-Qare A W, Elmasry E, Abou-Donia M B. A role for p-glycoprotein in environmental toxicology. *J Toxicol Env Heal B*, 2003, 6: 279–288
- 33 Buss D S, McCaffery A R, Callaghan A. Evidence for P-glycoprotein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. *Med Vet Entomol*, 2002, 16: 218–222
- 34 Leslie E M, Deeley R G, Cole S P C. Multidrug resistance proteins: Role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol Appl Pharm*, 2005, 204: 216–237
- 35 Shabbir A, DiStasio S, Zhao J B, et al. Differential effects of the organochlorine pesticide DDT and its metabolite P,P'-DDE on P-glycoprotein activity and expression. *Toxicol Appl Pharm*, 2005, 203: 91–98
- 36 Gennari A, Viviani B, Galli C L, et al. Organotins induce apoptosis by disturbance of $[Ca^{2+}]_i$ and mitochondrial activity, causing oxidative stress and activation of caspases in rat thymocytes. *Toxicol Appl Pharm*, 2000, 169: 185–190
- 37 Raffray M, Cohen G M. Thymocyte apoptosis as a mechanism for tributyltin-induced thymic atrophy *in vivo*. *Arch Toxicol*, 1993, 67: 231–236
- 38 Patel T, Gores G J, Kaufmann S H. The role of proteases during apoptosis. *FASEB J*, 1996, 10: 587–597
- 39 Ghibelli L, Maresca V, Coppola S, et al. Protease inhibitors block apoptosis at intermediate stages: A compared analysis of DNA fragmentation and apoptotic nuclear morphology. *FEBS Lett*, 1995, 377: 9–14
- 40 Stohs S J. Oxidative stress-induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Free Radic Biol Med*, 1990, 9: 79–90
- 41 Blankenship A, Matsumura F. 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced activation of a protein tyrosine kinase, pp60(src), in murine hepatic cytosol using a cell-free system. *Mol Pharmacol*, 1997, 52: 667–675
- 42 Ma Q, Baldwin K T. 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway-role of the transcription activation and DNA binding of AhR. *J Biol Chem*, 2000, 275: 8432–8438
- 43 Roberts B J, Whitelaw M L. Degradation of the basic helix-loop-helix/PER-ARNT-SIM homology domain dioxin receptor via the ubiquitin/proteasome pathway. *J Biol Chem*, 1999, 274: 36351–36356
- 44 McGregor D B, Partensky C, Wilbourn J, et al. An IARC evaluation of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans as risk factors in human carcinogenesis. *Environ Health Persp*, 1998, 106: 755–760
- 45 Alexeyenko A, Wassenberg D M, Lobenhofer E K, et al. Dynamic zebrafish interactome reveals transcriptional mechanisms of dioxin toxicity. *PLoS ONE*, 2010, 5: e10465
- 46 Boverhof D R, Burgoon L D, Tashiro C, et al. Temporal and dose-dependent hepatic gene expression patterns in mice provide new insights into TCDD-mediated hepatotoxicity. *Toxicol Sci*, 2005, 85: 1048–1063
- 47 Carney S A, Chen J, Burns C G, et al. Aryl hydrocarbon receptor activation produces heart-specific transcriptional and toxic responses in developing zebrafish. *Mol Pharmacol*, 2006, 70: 549–561
- 48 Fletcher N, Wahlstrom D, Lundberg R, et al. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters the mRNA expression of critical genes associated with cholesterol metabolism, bile acid biosynthesis, and bile transport in rat liver: A microarray study. *Toxicol Appl Pharm*, 2005, 207: 1–24
- 49 Kiyosawa N, Kwekel J C, Burgoon L D, et al. Species-specific regulation of PXR/CAR/ER-target genes in the mouse and rat liver elicited by O,P'-DDT. *BMC Genomics*, 2008, 9: 487
- 50 Menzel R, Swain S C, Hoess S, et al. Gene expression profiling to characterize sediment toxicity—a pilot study using *Caenorhabditis elegans* whole genome microarrays. *BMC Genomics*, 2009, 10: 160
- 51 Toyoshiba H, Yamanaka T, Sone H, et al. Gene interaction network suggests dioxin induces a significant linkage between aryl hydrocarbon receptor and retinoic acid receptor β . *Environ Health Persp*, 2004, 112: 1217–1224
- 52 Pickart C M, Fushman D. Polyubiquitin chains: Polymeric protein signals. *Curr Opin Chem Biol*, 2004, 8: 610–616