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Development of the GENIPOL European Flounder (*Platichthys flesus*) Microarray and Determination of Temporal Transcriptional Responses to Cadmium at Low Dose.

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We have constructed a high density, 13 270-clone cDNA array for the sentinel fish species European flounder (*Platichthys flesus*), combining clones from suppressive subtractive hybridization and a liver cDNA library; DNA sequences of 5211 clones were determined. Fish were treated by single intraperitoneal injection with 50 micrograms cadmium chloride per kilogram body weight, a dose relevant to environmental exposures, and hepatic gene expression changes were determined at 1, 2, 4, 8, and 16 days postinjection in comparison to saline-treated controls. Gene expression responses were confirmed by real-time reverse transcription polymerase chain reaction (RT−PCR). Blast2GO gene ontology analysis highlighted a general induction of the unfolded protein response, response to oxidative stress, protein synthesis, transport, and degradation pathways, while apoptosis, cell cycle, cytoskeleton, and cytokine genes were also affected. Transcript levels of cytochrome P450 1A (CYP1A) were repressed and vitellogenin altered, real-time PCR showed induction of metallothionein. We thus describe the establishment of a useful resource for ecotoxicogenomics and the determination of the temporal molecular responses to cadmium, a prototypical heavy metal pollutant.

Introduction

Resolving mechanisms of toxicity is a key requirement for understanding responses of organisms to environmental pollutants (*1*). Global assessments of molecular pathways affected by toxicants have been achieved using "omics" technologies such as microarrays, contributing to understanding the health status of tissues or organisms. Great progress has been made with genetic model organisms; however, there are advantages in using species of direct environmental relevance (*2*), where comparisons can be made between animals sampled from sites of environmental concern and those treated with a toxicant or mixture under laboratory conditions. Functional genomics does not have to be limited to a few selected model organisms (*3*). Therefore, we aimed to apply microarraying to aquatic ecotoxicology, both to improve our understanding of mechanisms of toxicity in fish (*4*, *5*) and to discover molecular biomarkers and gene expression fingerprints which are predictive of potential toxicity.

Our studies focused on the use of European flounder, *Platichthys flesus*, a teleost flatfish common in estuaries throughout Western Europe, many of which are impacted by sediment-associated toxicants including endocrine disruptors, heavy metals, polycyclic aromatic hydrocarbons, and dioxins. This species feeds mostly on benthic invertebrates and has been adopted by the OSPAR Joint Assessment and Monitoring Programme as the sentinel species for biological effects monitoring in inshore/estuarine waters of the OSPAR maritime area (*6*).

Cadmium is an environmental pollutant that is both toxic and carcinogenic. Although it has no intrinsic redox capacity, cadmium exerts many effects via the production of reactive oxygen species (*7*). While the kidney is considered the main target organ for cadmium toxicity, cadmium is also an acute hepatotoxicant (*8*). We treated flounder with a relatively low dose of $CdCl_2$ (0.05 mg/kg). Dry weight sediment cadmium concentration has been reported at up to 7 mg/kg in contaminated UK estuaries (*9*). Furthermore, hepatic cadmium concentrations up to 0.12, 2.8, and 0.38 mg/kg (wet weight) have been reported in European flounder (*10*, *11*) and other flatfish (*12*) from European waters. The dose level employed is similar to the maximum level of cadmium in fish for human consumption set by EU directive 2001/22/EC (0.05 mg/kg fish muscle tissue).

We report the construction of a high-density 13 270-clone glass-slide-based microarray incorporating previously isolated flounder clones (*13*, *14*, *15*) with clones picked from a normalized flounder liver cDNA library, the sequencing and functional annotation of a subset of these clones and the transcriptomic responses of flounder to cadmium treatment. We have investigated the utility of toxicogenomics in an environmentally relevant species to characterize the subtle temporal hepatic response to an environmentally relevant dose of the heavy metal so as to define the transient or delayed modification of major biological processes through gene ontology analyses. Through an understanding of the modes of action, dose−response relationships can be better understood, biomarkers can be optimized, and risk assessment improved.

Experimental

cDNA Library Construction. A normalized cDNA library containing transcripts of toxicant-responsive genes was constructed by combining the Carninci and Hayashizaki protocol (*16*) with a SMART library construction kit (Clontech-Takara Bio, Saint-Germain-en-Laye, France). A mixed total RNA sample was prepared from livers of three control immature fish, one gonadally mature female, one gonadally mature male, and groups of three animals sacrificed 48 h after intraperitoneal injection with prototypical toxicity-inducing agents (Sigma-Aldrich, Gillingham, UK); 3-methylcholanthrene (25 mg/kg), Aroclor 1254 (50 mg/kg), lindane

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(5 mg/kg), *t*-butylhydroperoxide (5 mg/kg), perfluorooctanoic acid (100 mg/kg), $CdCl_2$ (0.05 mg/kg), 3-methyltestosterone (10 mg/kg), pregnenolone 16-alpha-carbonitrile (10 mg/kg), 17-beta-estradiol (10 mg/kg), or carriers (1 mL/kg linoleic acid or 1% saline). This approach was taken in order to enrich for transcripts induced by a wide variety of model toxicants.

Poly(A^+) mRNA was prepared, one aliquot was used to prepare first strand full length cDNA as "tester" using a SMART cDNA library construction kit (Clontech-Takara Bio) following the manufacturer's instructions. A second aliquot, to act as "driver", was biotinylated by incubation at 37 °C for 1 h using a *Label*IT kit (Mirus, Madison, USA). Two hundred ng cDNA ("tester") was denatured at 80 °C for 1 min and then normalized by incubation at 42 °C for 96 h ($R_o t = 47$) with 300 ng biotinylated mRNA probe ("driver") and 10 mg dT_{16} oligonucleotide in 250 mM NaCl, 5 mM EDTA, 25 mM HEPES, 70% formamide so that abundant classes of mRNA hybridized more rapidly. cDNA/mRNA hybrids were removed using streptavidin-coated magnetic beads (PerSeptive Bio-Systems, Framlingham, USA), washed, then treated with RNase (Promega, Southampton, UK). The purified normalized cDNAs were used for second strand synthesis by long-distance PCR with Advantage2 *Taq* polymerase using SMART primers, after treatment with proteinase K, cDNA was digested with *Sfi*I and size-fractionated using CHROMA SPIN-400 columns (Clontech-Takara Bio). *Sfi*I-digested cDNAs were directionally cloned into dephosphorylated λTriplEx2 vector (Clontech-Takara Bio) using λ phage packaging system (MaxPlax, Epicentre, Cambio, Dry Drayton, UK). The recombinant library was converted to a pTriplEx2 cDNA library using the manufacturer's recommended protocol, amplified and titerd. This was designated the flounder "IL" cDNA library.

Microarray Construction. Approximately 12 700 colonies were picked from the IL cDNA library, PCR was carried out upon bacterial cultures using a Mastercycler 96-well machine (Eppendorf, Cambridge, UK). PCR products were electrophoresed on 1% agarose gels to identify single-banded products. Additional clones consisted of identified flounder genes derived from earlier projects (*13*, *14*, *15*), plaice (*Pleuronectes platessa*) detoxification enzyme and nuclear transcription factor genes, cloned at Stirling, and a dab (*Limanda limanda*) Rb clone (from Dr. J. Rotchell, University of Sussex, UK). Controls consisted of empty wells, water, spotting buffer, plasmid DNA; pRL-TK (Promega), pBluescriptIISK+ (Stratagene, La Jolla, U.S.), pTripleEx2 (Clontech-Takara Bio) and pCR2.1 (Invitrogen, Paisley, UK), PCR products; polylinker regions of pBluescriptIISK+, pTriplEx2 and pCR2.1, beta-lactamase, *Renilla* luciferase genes, and Lucidea controls (Amersham, Amersham, UK).

PCR products were purified, then resuspended in sterile-filtered spotting buffer (50% DMSO plus 0.3× SSC). Microarrays were printed using a Biorobotics MGII arrayer with a 48-split-pin head. Each clone was spotted in duplicate, producing 48 subarrays, each containing 576 spots, for a total of 27 648 spots per slide. The microarray slides used were GAPSII, Ultra-GAPS (Corning, Schiphol-Rijk, Netherlands) or Nexterion A (Schott, Jena, Germany).

Sequencing and Analysis of Clones. Pollutant-responsive clones from the "IL" library were sequenced from the 5′ end using the TriplEx 5LD primer (Clontech-Takara Bio). Sequences were base-called using Phred (*17*), trimmed to remove low quality and vector regions using Lucy2 (*18*), clustered by CAP3 (*19*) and batch BLAST-ed using Soap-HT-BLAST (*20*) or Blast2GO (*21*). A *P*-value cutoff of 1e-6 was used for sequence annotation. Sequences which clustered by CAP3 or were contigs of the same gene were treated as replicate spots during microarray analyses. Blast2GO uses NCBI's QBlast or local WWW-Blast installation to search and find similar sequences to input sequences. Sequences were annotated with Gene Ontology terms associated with putative homologous genes using Blast2GO. Blast2GO extracted the GO terms associated with each of the obtained hits and returned an evaluated GO annotation for the query sequences. All sequences were submitted to dbEST at NCBI.

Treatments. *P. flesus* were artificially reared from gametes obtained from three females and three males and maintained on a commercial pelleted trout diet in a flowing seawater aquarium system (8 °C, salinity 32 ± 1ppt, 60 light/40 dark) at Port Erin Marine Laboratory, Isle of Man, UK. Larvae were fed poly unsaturated fatty acid supplemented *Artemia* and weaned onto an artificial diet at day 20 which was then substituted for a commercial trout diet (Trout no. 3) after metamorphosis (31 ± 3 days). At 2−3 months post-hatch, juveniles were transferred to a recirculating seawater system at Stirling University, Scotland and were on-grown at 11 °C, salinity 32ppt. Sexually immature fish of 75−100 g were treated by intraperitoneal injection with cadmium chloride (50 micrograms/kg) in 1% saline or saline alone (1 mL/kg). All exposures were carried out under UK Home Office licenses PPL 60/2360 and 60/3279 in aerated static seawater tanks sited in a constant temperature containment aquarium. Water was replaced every 2 days. After 1, 2, 4, 8, and 16 days, animals were killed by a blow to the head. Body, liver, and kidney were weighed and samples of liver tissue were immediately homogenized in TriReagent (Sigma-Aldrich) and stored at −80 °C before extraction of total RNA using the manufacturer's recommended protocol. Liver somatic indices (LSI) were calculated by (liver weight/body weight) × 100. Any fish with detectable gonad development were eliminated from the experiment.

Microarray Experiments. Twenty micrograms total RNA was treated to remove DNA contamination using a DNA-free kit (Ambion, Huntingdon, UK), then reverse transcribed using 200U Superscript II (Invitrogen) with oligo-dT (*12−18*) primers before re-purification (Qiagen, Crawley, UK). cDNA (500ng) was labeled with Cy5-dCTP or Cy3-dCTP (Amersham) using 40U Klenow polymerase (Invitrogen) with random primers. Labeled cDNA was purified (Qiagen) and fluorophore incorporation measured by spectrophotometry. A Bioanalyser 2100 Biosizing total RNA nano assay was used to assess the integrity of selected total RNA samples, while a Bioanalyser 2100 Biosizing mRNA nano assay was used to quantify the cDNA concentrations of selected samples prior to Cy-dye labeling (Agilent, Stockport, UK).

All array experiments employed the reference cDNA technique (*22*). This reference cDNA hybridized to each probe on the array. Each of the probes corresponded to PCR amplicons of a gene fragment cloned into either pTriplEx2, pCR2.1 or pBluescriptIISK+ amplified using vector primers. The reference cDNA was made by combining equimolar quantities of PCR amplicons from recircularised pTriplEx2, pCR2.1 and pBluescript plasmids amplified with these vector primers. The reference cDNA was labeled as above but purified with Amicon YM30 filters (Millipore, Watford, UK).

Dye-labeled cDNA (60 pmoles incorporated) was mixed with dye-labeled reference control DNA (12 pmoles incorporated). These were hybridized to the array in 60 μL under a 25 × 60I Lifterslip (VWR, Lutterworth, UK) at 42 °C for 18 h (*13*). Five cadmium-treated and five control fish from each of the five timepoints were arrayed individually. After stringent washing, slides were scanned in an Axon 4000B scanner (Molecular Devices, Wokingham, UK). Data were captured using Genepix software (Molecular Devices), and each slide was checked in detail for spots with poor morphology. These spots were designated as "not found" and were filtered out in later analyses. Arrays showing poor hybridization, or experimental artifacts such as areas of high background, were discarded. The data used in analyses consisted of local background-subtracted median intensities.

Analyses. The Genespring software package (Agilent) was used to analyze microarray data. To normalize, median expression over control was brought into concordance using median polishing (Agilent). Data were then Lowess transformed. Each microarray in a treatment group was then normalized to the mean of the relevant control group. Only data from spots designated as "present" were used. Data for spots from clones which had showed multiple banding during QC of the PCRs, spots with low raw data intensity, standard deviation of >1.4 between replicate spots or control channel intensity less than the calculated base/proportional score (Agilent) in 50% of samples were removed. Lists of differentially expressed genes were generated by finding genes that differed by more than 2-fold in normalized data and were statistically significantly different by parametric Welch *t*-test between test and control groups. The *P*-value cutoff was 0.05 and the Benjamini and Hochberg multiple testing correction was used (*23*).

Blast2GO integrates the Gossip package (*24*) for statistical assessment of annotation differences between two sets of sequences. For each timepoint we compared lists of statistically significantly induced or repressed genes with a reference list of all detectable genes. Fisher's Exact test *P*-values for representation of each GO term were generated.

Validation. To assess the reproducibility of the array data, dye reverse experiments and technical replications were carried out. Quantitative reverse transcription polymerase chain reaction (RT−PCR) was used for validation of microarray data, using an aliquot of the cDNA made for arraying or cDNA generated by random priming from an independent preparation of total RNA. We used a Quantica real-time nucleic acid detection system (Techne, Stone, UK) with ABsoulte QPCR SYBR Green master Mix (ABgene, Epsom, UK) and primers for the following *P. flesus* genes: metallothionein; MT-F 5′-CTGCGAATGCTCCAAGACTG-3′, MT-R 5′-GTGGTGTCGCATGTCTTCC-3′ product 159bp. CYP1A; CYP1A-F 5′-GTTTCGATACCGTCTCTACTGC-3′, CYP1A-R 5′-AGGAAGCGATCTGGGTTGAAG-3′, product 345 bp. Glutathione reductase; GR-F 5′-GGCTTCTTGCGTGAATGTTGG-3′, GR-R 5′-CGAGGTTGCTACGATAAATGCG-3′, product 190bp. Peroxiredoxin; PRX-F 5′-CCTCTTGTAGCAGATCTCACC-3′, PRX-R 5′-GGCGAAGTCAGTGTGCTGGA

A-3′, product 180bp. Cu/Zn SOD; SOD-F 5′- TGGAGACAACACAAACGGG-3′, SOD-R 5-CATTGAGGGTGAGCATCTTG-3′, product 172 bp. HSP30B; H30-F 5′-GCTCCAGGAGTTCAGACAGG-3′, H30-R 5′-ACACACTCTGCTGTGGTTGC-3′, product 182 bp. 18S rRNA; 18S-F 5′-GCATCCCAAGATCCAACTAC-3′, 18S-R 5-ACAGCAGCCGCGGAAATT-3′, product 100bp. Quansoft software was used for fluorescence detection (Barloworld Scientific, Stone, UK). For direct correlation with the array data absolute quantification was obtained from calibration curves of known concentrations of the PCR amplicons cloned in pCR2.1 (Invitrogen). For independent comparison using cDNA generated by random priming, relative quantification was performed using a comparative CT method where expression of 18S rRNA gene was used as a reference value (*25*).

Results and Discussion

Library and Sequences. The titer of the recombinant phage library was 3.28×10^6 pfu/mL. On conversion to the corresponding pTriplEx2 cDNA library and amplification, the titer was 7.5×10^8 colonies/mL. Of 12 738 colonies picked, 10 606 showed a visible single band upon agarose gel electrophoresis. Of 5353 clones sequenced, 5211 (97%) were successful. The redundancy of sequenced clones was 59%. Of 2232 unique sequences, 338 matched with ESTs, 797 gave no significant match, and 1097 matched to named proteins, of these 813 possessed GO annotation. Genbank accession numbers are DV565292 to DV570502. Details of sequenced clones are shown in Supporting Information Table 1, representing a considerable addition of sequence data for this species. Thirteen highly redundant genes were individually amplified, labeled, and hybridized to the microarray, allowing the annotation and elimination of these clones from further sequencing runs.

Validation. The standard correlation (Pearson correlation around zero) of gene expression between technical replicates was >0.95 in all cases. The standard correlation between data was >0.96 for comparisons between three forward and three reverse-labeled arrays. Validation of microarray data was carried out using quantitative PCR for glutathione reductase, Cu/Zn superoxide dismutase, and metallothionein. Comparison of microarray and qPCR data showed R^2 correlation values of 0.98, 0.79, and 0.89, respectively (Figure 1 A−C). In a previous experiment using a 500 clone array, we assessed the responses of flounder to 2 mg/kg $CdCl_2$, sampling fish at day 3 after injection (*15*). Supporting Information Table 3 shows a comparison of data from Sheader et al. (*15*), and from this experiment. Despite treating with 40-fold less $CdCl_2$, all genes found to be induced with 2 mg/kg at day 3 were also apparently induced with 0.05 mg/kg at day 1, however, these changes were not sustained to day 4. Conversely, those genes found to be repressed with 2 mg/kg $CdCl_2$ at day 3 were also apparently repressed with 0.05 mg/kg at day 4 but not necessarily at day 1. A higher dose of toxicant may be expected to elicit a more sustained response; therefore, we consider the data comparable at these early timepoints.

Gross Pathology. There was no evidence of gross liver pathology. Liver somatic indices showed no significant differences between cadmium- and saline-treated fish at the same time-points after IP injection. These findings are in accordance with the use of a sub-hepatotoxic dose of cadmium.

Gene Expression Responses. Cadmium treatment resulted in differential expression of 899 sequenced genes at one or more of the five timepoints during the 16 day experiment, in comparison with time-matched saline-injected controls. Of these, 382 were identifiable with known proteins, shown in Supporting Information Table 2. We discuss only these identifiable genes, the remainder provide opportunities for discovery of novel responses. The acute response to cadmium treatment at day 1 showed gene expression changes of greatest magnitude. Gene ontology analysis using Blast2GO was carried out to compare representation of GO terms between lists of changing genes and a list of all detectable GO annotated genes. The results of these comparisons are shown in Table 2. The majority of changes in GO groups were at day 1, fewer at intermediate timepoints, then an increase at day 16. We postulate that this reflects inter-individual variability in the transition from damage to recovery, stabilizing at day 16, where terms such as cellular proliferation are more prevalent. Functionally related groups of genes differentially regulated at day 1 are illustrated by a Blast2GO-graph showing terms statistically significantly ($P < 0.025$ in single *t*-test) over-represented in a list of up-regulated genes at day 1 (Figure 2). Broadly, the GO biological process classes show differences in unfolded protein response, oxidative stress response, ion homeostasis, protein synthesis, transport and degradation, apoptosis, cytoskeleton, G1 to S phase transition of the cell cycle, and immune function.

Unfolded Protein Response. Genes related to the unfolded protein response, heat shock genes (Hsp), and molecular chaperones, were induced transiently at day 1 postinjection. These included Hsp30B, an alpha-Crystallin family member (*26*), also found by real-time PCR to be highly induced (Table 1), Hsps 40, 90-alpha and -beta, and chaperonins (Figure 3A). Cadmium is known to bind to proteins

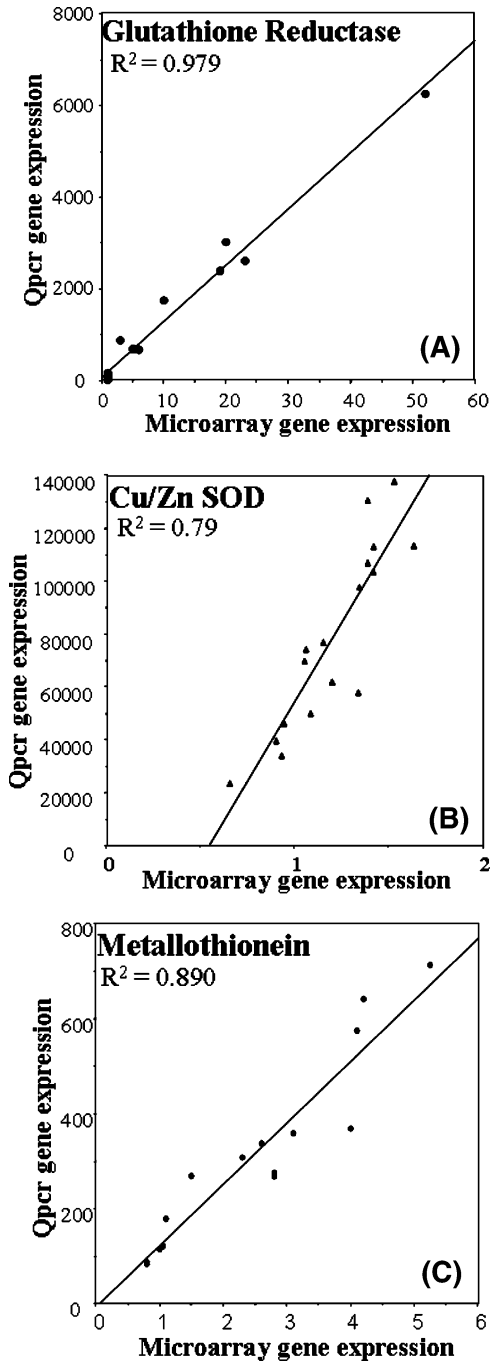


FIGURE 1. (A) Expression of glutathione reductase mRNA determined by microarray (fold change) and q-RTPCR (arbitrary units relative to absolute expression. (B) Expression of Cu/Zn superoxide dismutase mRNA determined by microarrays (fold change) and q-RTPCR (arbitrary units relative to absolute expression). (C) Expression of metallothionein mRNA determined by microarrays (fold change) and q-RTPCR (arbitrary units relative to absolute expression).

at cysteine residues and cause thiol oxidation, which can lead to protein unfolding. The induction of heat shock genes is a well-known response to cadmium treatment (*27*). This may be seen as both a protective response and an indication of macromolecular damage. Hsp70 appeared highly induced but showed high variability.

Oxidative Stress. In accord with the role of oxidative stress in the toxicity of cadmium (*7*), gene inductions indicative of a response to oxidative stress were seen at day 1 and were generally more sustained than the heat shock protein response (Figure 3B). These included Cu/Zn superoxide dismutase, catalase, peroxiredoxin, glutaredoxin, and a glutathione peroxidase, while induction of glutathione reductase was not statistically significant. Inductions of Cu/Zn superoxide dismutase, peroxiredoxin and glutathione reductase were independently confirmed by real time PCR (Table 1). A transcript for selenium water dikinase (selenophosphate synthetase 2), was induced in a majority of fish from days 1 to 8 after injection. This is a key component of the selenoamino-acid metabolic pathway, producing the selenocysteine necessary for some antioxidant proteins. In addition, transcripts for selenoprotein M and selenium binding protein 1 (Hsp56) were also induced by cadmium treatment, although the potential for protection by these proteins against oxidative stress has not been established. A transcript similar to MAP kinase interacting serine-threonine kinase 2 (Mnk2) was also induced, implying perturbation of the MAP kinase signal transduction pathways. Transcripts coding for enzymes involved in metabolism and detoxification of lipid peroxidation products showed early induction following cadmium treatment, including microsomal glutathione *S*-transferase (usGST), carbonyl reductase which converts 4-oxonon-2-enal to 4-hydroxynon-2enal (4-HNE), a theta class GST which metabolizes hydroperoxides (*28*) and GST-A which conjugates these hydroxynon-enals in flatfish (*29*) (Figure 3J). A transcript of an aldose reductase-like gene, coding for an enzyme which further detoxifies these conjugates (*30*), was induced at days 2−16 posttreatment. Paraoxonase 2 was induced, which has been implicated in defense against oxidative-stress-induced lipid peroxidation (*31*). Although cadmium has no intrinsic redox capacity, cadmium-induced lipid peroxidation has been postulated as being mediated by release of iron from molecules such as ferritin (*32*), here we observe the induction of ferritin H and M chain transcripts, maybe representing a compensatory mechanism.

Protein Synthesis, Transport, and Degradation. Overall protein synthesis has been shown to be inhibited by cadmium treatment in cell culture (*33*), though at concentrations below 10 micromolar, synthesis recovers within 6 h and can subsequently exceed control levels. We show induction of protein synthesis-related genes such as those encoding ribosomal proteins, ribosomal biogenesis factors, and translation initiation factors (Figure 3C). Of 36 such genes found to be differentially regulated at day 1, 35 were induced. A similar induction of such genes was seen in liver during carbon-tetrachloride treatment of rats (*34*) and we echo the hypotheses that this could represent a mechanism to restore damaged protein biosynthetic machinery or reflect an increased requirement for synthesis of protective proteins such as chaperones and antioxidant enzymes. Additionally we show induction of transcripts linked with protein transport (Figure 3D). The proteasome is the major route for degradation of endogenous damaged and misfolded ubiquitinated proteins. In yeast, proteasomal components have been shown to be inducible by cadmium (*35*). In cadmium-treated flounder, we found that 11 of 13 differentially regulated proteasomal and ubiquitin-conjugating gene transcripts were induced at day 1 (Figure 3E), with continued induction to day 4 in most individuals. The induction of genes for these protein degrading components suggests that protein damage and turnover are increased in response to cadmium.

Cytoskeleton. Of a group of cytoskeletal genes, some are induced and others repressed (Figure 3F). Those induced include cysteine- and glycine-rich protein 2, syndecan 2, dynein light chain 2, ARP3, and alpha tubulin, those repressed include thymosin beta 4, adducin 3, ANKT, and beta-actin. Previous studies have shown that cadmium treatment can lead to disruption of the cytoskeleton (*36*), which is known to be susceptible to damage by reactive oxygen species and

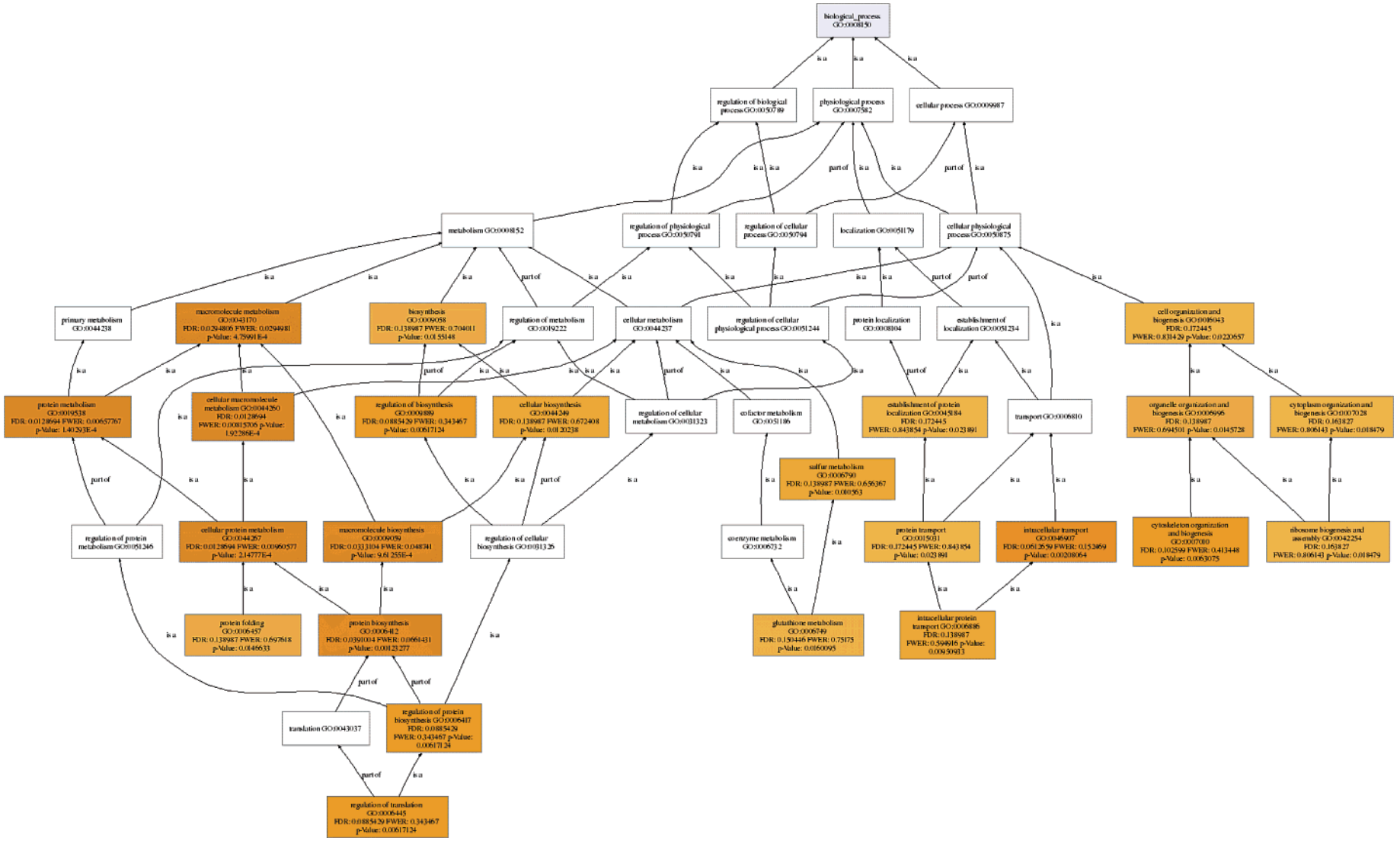


FIGURE 2. Blast2GO directed acyclic graph showing "biological process" GO terms over-represented ($P < 0.025$) among clones induced at day 1 after cadmium treatment in comparison with all detectable clones (colored orange).

TABLE 1. Comparison of Log_2 Gene Expression of Flounder Treated with 0.05 mg/kg $CdCl_2$ Versus Saline Treated Controls on days 1 and 16 after Intraperitoneal Injection[a]

	day 1		day 16	
gene	qPCR	array	qPCR	array
metallothionein	1.92*	0.51	−0.68	−0.52
CYP1A	−1.53*	−2.39*	−1.77*	−1.27*
glutathione reductase	2.31*	3.59	−0.24	−0.97
peroxiredoxin	2.90*	1.42*	0.22	0.40
Cu/Zn SOD	2.85*	1.00*	−0.25	0.73
HSP30B	11.22*	4.24*	−0.19	0.85

[a] Measured by quantitative PCR (qPCR) and microarray (array). * designates P value < 0.05 by t-test, with Benjamini and Hochberg multiple testing correction for microarray data.

consequent raised intracellular free calcium (*37*). Our data suggest that modulation of cytoskeletal gene expression may contribute to cytoskeleton disruption with cadmium treatment. Such disruption may also affect protein transport and cell cycle.

Apoptosis. GO analyses highlighted apoptosis as one of the major pathways affected on the first day after cadmium treatment. Of 13 differentially expressed apoptosis-related transcripts, 10 were induced and three repressed at day 1 (Figure 3G). Cytochrome c was induced more than 4-fold on day 1. It is well-known for its involvement in the intrinsic pathway of apoptosis by release from the mitochondria to the cytosol where, together with apoptosome partners Apaf1 and pro-caspase 9, active caspase 9 is produced to activate pro-caspase 3. It has also been shown that cytochrome c transcription can be induced by pro-apoptotic compounds in mammalian cell culture and that overexpression of cytochrome c enhances caspase activation and promotes cell death in response to apoptotic stimulation (*38*). Programmed cell death 6 (apoptosis-linked gene 2/ALG-2) (*39*) has been implicated as part of an alternative intrinsic, cytochrome c independent, pathway for apoptosis, closely linked to endoplasmic reticulum (ER) stress (*40*). The neuroendocrine factor reticulon 1 has also been identified as a component of ER-related apoptosis (*41*). ER stress is thought to arise from alterations in calcium homeostasis and accumulation of unfolded proteins. The observations above may imply a link between cadmium-induced protein damage and ER-stress mediated apoptosis. Integral membrane protein 2B, thioredoxin, and thymidine phosphorylase have been linked with apoptosis (*42*, *43*, *44*), while anticoagulant protein C (*45*) and P8 (COM-1) (*46*) have been observed to inhibit apoptosis. Ethylmalonic encephalopathy 1 (HSCO) and COMM domain containing 3 have both been shown to inhibit NF-κB activity, but NF-κB can show pro- or anti-apoptotic activity depending on context (*47*, *48*). APG16L is a component of the autophagosome, which carries out bulk degradation of cytoplasmic components during cell death (*49*).

Diablo (Smac/direct IAP binding protein with low pI) and survivin (bacterial IAP repeat containing 5A) both appear transcriptionally repressed after cadmium treatment. Survivin inhibits caspase activity and thus apoptosis, while diablo, on release from mitochondria, inhibits survivin activity, promoting apoptosis (*50*). Diablo transcript is repressed throughout the timecourse, while survivin expression is restored and indeed increased at later timepoints. Pro-apoptotic changes, including the induction of cytochrome c, programmed cell death 6, APG16L and reticulon 1, tend to be more pronounced at the early timepoints (Figure 3G), whereas anti-apoptotic changes such as induction of anticoagulant protein C, P8/COM1 and thymidine phosphorylase and repression of ITM2B and diablo appear to occur at all timepoints. This may represent a pro-apoptotic situation upon acute cadmium treatment which is later reversed thus altering the balance between apoptosis and survival across the time course of cadmium treatment and recovery.

Cell Cycle. Transcripts associated with the G1 to S phase transition of the cell cycle were induced, these included G1 to S phase transition 1 (*51*) and GTP-binding protein 1 (Rhou/Wrch), chaperonin subunit 7, involved in maturation of cyclin E, Ran GTPase and NHP2, involved in the cell cycle (Figure 3H). Transcripts repressed included those for Zw10, a mitotic checkpoint gene, cyclin H, part of the cdk-activating kinase complex and septin 5 (Cdcrel-1). Previous studies have shown that cadmium can disrupt the cell cycle (*52*). Our data would support cell cycle disruption as a consequence of low dose cadmium administration. Cell-cycle gene expression changes could be linked to a post-apoptotic compensatory hyperplasia. Such an increase in cellular proliferation has been noted in intestine and liver of cadmium exposed fish (*53*, *54*).

Immune and Inflammation. GO terms related to chemokines were more prevalent in the list of transcripts downregulated at day 1 after cadmium treatment (Table 2). In addition, a number of other immune-function related genes were seen to be downregulated with acute cadmium exposure (Figure 3I). Of this group, including both cytokines and immunoglobulins, 18 of 21 differentially expressed transcripts were repressed at early timepoints though by day 16 the expression of a subset had increased. Cadmium has long been known to exert immunotoxicological effects (*55*) and has been shown to decrease expression of cytokines in blood mononuclear cells (*56*) but conversely induce expression of cytokines in hepatoma cells, Kupffer cells and primary hepatocyte cell cultures (*57*, *58*). The expression of inflammation-related genes in liver tissue is likely to be complex, due to the variety of cell types and the possibility of macrophage and neutrophil infiltration.

Traditional Biomarkers and Crosstalk. A number of genes are used, or have been proposed, as molecular biomarkers for early detection of pollutant responses in fish. In addition to the molecular chaperones, oxidative stress responsive genes and glutathione-*S*-transferases discussed above, these include genes encoding phase I metabolic enzymes, such as cytochrome P450s (CYP), egg proteins such as choriogenins and vitellogenins, and metallothionein, a metal ion sequestering protein (Figure 3J). Metallothionein has been most commonly used as a marker for heavy metal contamination. Both real-time PCR and the microarray showed an induction of metallothionein, though this was not statistically significant for the microarray data. CYP2 family members were induced, while CYP1A was repressed, which was confirmed by real time PCR (Table 1). We have previously shown that cadmium can inhibit transcription from the flounder CYP1A promoter (*59*). The confounding effects of cadmium on use of CYP1A activity as a biomarker in environmental monitoring, where both heavy metals and organic compounds are often found in complex mixtures, has been highlighted previously (*60*). Vitellogenin A transcripts were transiently repressed at day 1 after cadmium treatment but were induced by 2−3-fold at later timepoints. These data would tend to support a weak endocrine disrupting effect of cadmium, which has recently been the subject of much attention (*61*, *62*), though this appears likely to be mediated indirectly as no acute induction was seen. Although cadmium may affect the expression of biomarkers of endocrine disruption, this is unlikely to affect monitoring of estrogen mimics as these can alter vitellogenin and choriogenin gene expression in male flatfish by orders of magnitude (*63*).

We have found that cadmium treatment affects transcription of many genes including those related to oxidative

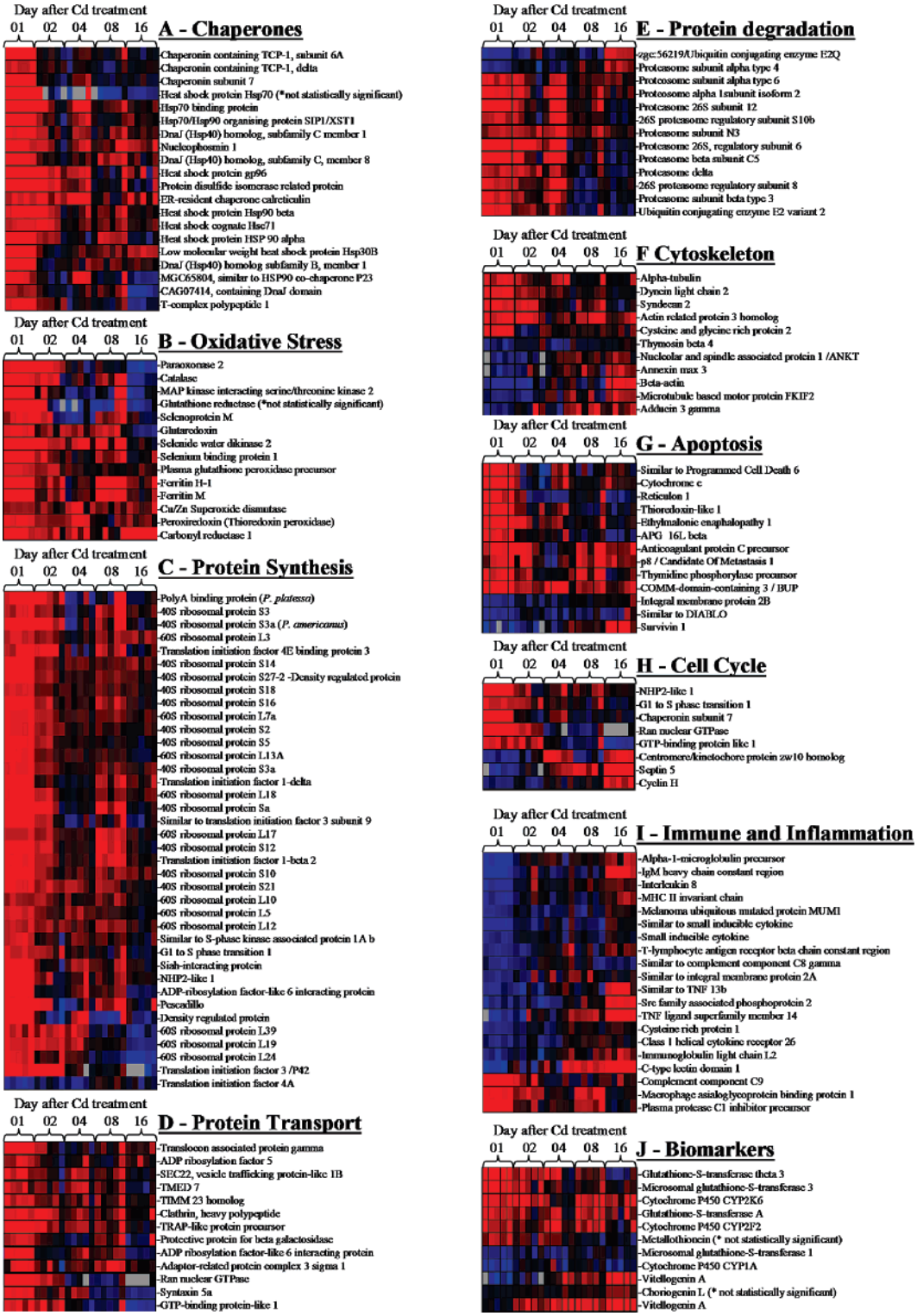
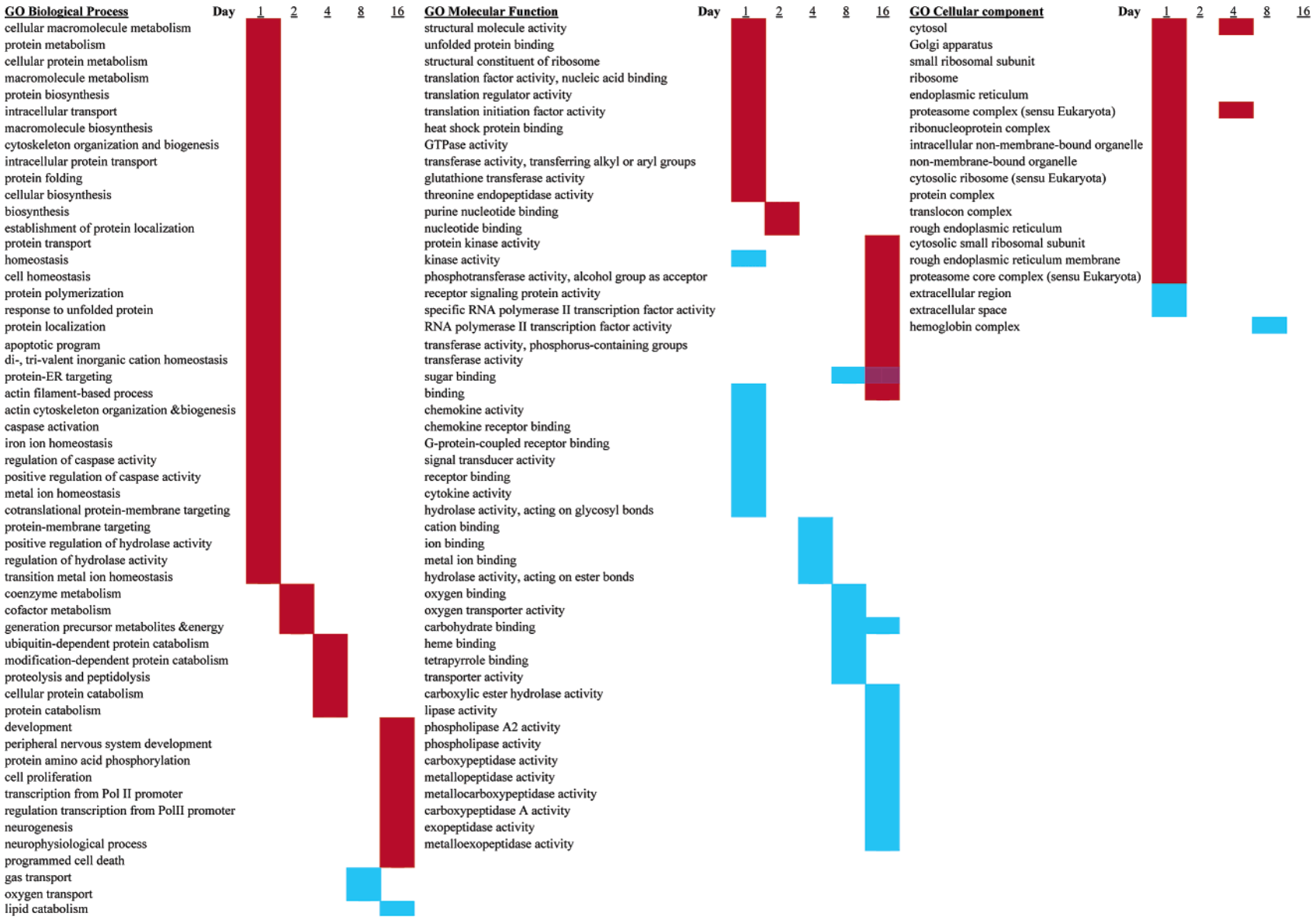


FIGURE 3. (A–J) Heatmap diagrams illustrating gene expression in response to cadmium at days 1–16 after intraperitoneal injection. Red denotes increased expression and blue denotes decreased expression in comparison with saline-injected control fish, black indicates unchanging expression, gray indicates no data. Highest color intensity was set to 3-fold change. Each column represents an individual fish.

stress response, unfolded protein response, protein synthesis, transport and degradation, apoptosis, cell cycle, and immune function. These changes may result from the combination of direct protein damage and oxidative stress, and are likely to be inter-related. For example, protein damage may be a factor in inducing ER-stress and the observed effects on cytoskeletal gene transcription may also contribute to potential cell cycle disruption. Many gene products play a dual role in the cell cycle and apoptotic axis. Our data support current biomarkers for heavy metal intoxication, such as metallothionein and heat shock protein 70, but show the wide range of additional biomarkers related to disturbance of different biological processes which will provide more comprehensive and conclusive evidence of stress response when used in concert. Detection of adverse effects, such as apoptosis and protein damage (inferred by induction of

TABLE 2. Gene Ontology Terms Over-Represented (Fisher's Exact Test $P < 0.05$) in Lists of Statistically Significantly Up- (Red) or Down-Regulated (Blue), Transcripts, or Both (Purple) with Cadmium Treatment in Comparison with All Detectable GO-Annotated Transcripts, Those Represented by a Single Transcript Were Removed



proteasomal genes) enable elucidation of modes of toxicant action. We are continuing to profile gene expression responses of flounder to additional toxicants and to determine diagnostic gene expression fingerprints for main classes of environmental contaminants. Moreover, changes in gene expression will inform further studies on adaptation and subpopulation selection based on genetic polymorphisms.

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Supporting Information Available

Three tables showing comparisons of sequenced clones. This material is available free of charge via the Internet at http://pubs.acs.org.

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