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The chemical defensome: Environmental sensing and response genes in the *Strongylocentrotus purpuratus* genome

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Abstract

Metazoan genomes contain large numbers of genes that participate in responses to environmental stressors. We surveyed the sea urchin *Strongylocentrotus purpuratus* genome for homologs of gene families thought to protect against chemical stressors; these genes collectively comprise the ‘chemical defensome.’ Chemical defense genes include cytochromes P450 and other oxidases, various conjugating enzymes, ATP-dependent efflux transporters, oxidative detoxification proteins, and transcription factors that regulate these genes. Together such genes account for more than 400 genes in the sea urchin genome. The transcription factors include homologs of the aryl hydrocarbon receptor, hypoxia-inducible factor, nuclear factor erythroid-derived 2, heat shock factor, and nuclear hormone receptors, which regulate stress-response genes in vertebrates. Some defense gene families, including the ABCC, the UGT, and the CYP families, have undergone expansion in the urchin relative to other deuterostome genomes, whereas the stress sensor gene families do not show such expansion. More than half of the defense genes are expressed during embryonic or larval life stages, indicating their importance during development. This genome-wide survey of chemical defense genes in the sea urchin reveals evolutionary conservation of this network combined with lineage-specific diversification that together suggest the importance of these chemical stress sensing and response mechanisms in early deuterostomes. These results should facilitate future studies on the evolution of chemical defense gene networks and the role of these networks in protecting embryos from chemical stress during development.
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Introduction

A central question in biology is how cells and organisms maintain homeostasis in the face of an adverse environment. The need to deal with physical, chemical, and biological challenges has driven the evolution of an array of gene families and pathways affording protection from, and repair of, damage. Genes and proteins affording such protection for an organism collectively may be considered a “defensome.” A central part of this system is the “chemical defensome,” an integrated network of genes and pathways that allow an organism to mount an orchestrated defense against toxic chemicals. Chemical defense genes may be especially important for early embryos, which must cope with the environment during sensitive stages of differentiation and development. This study presents an overview of the genes potentially involved in toxicant sensing and protection in the sea urchin *S. purpuratus*.

Environmental chemicals handled by this defensome include microbial products, heavy metals, phytotoxins, and other natural compounds including biogenically derived polycyclic aromatic hydrocarbons and halogenated organic compounds.

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Although structurally diverse, many of these toxicants are hydrophobic, which facilitates their movement across membranes and entry into cells. Cellular homeostasis also requires inactivation and elimination of endogenous signaling molecules, such as steroids, and defense against endogenously generated toxicants such as reactive oxygen species (ROS), lipid peroxides, and heme degradation products.

As indicated in Fig. 1, the chemical defensome principally includes soluble receptors and other ligand-activated transcription factors that act as cellular sensors of toxicants or damage; oxidative, reductive, or conjugating biotransformation enzymes that transform chemicals to less toxic and easily excreted metabolites; efflux transporters that actively export substrates from the cell; and antioxidant enzymes protecting against externally and internally generated ROS or other radicals. While biotransformation generally results in detoxification, it is also a well-known double-edged sword; oxidation, *N*-acetylation, and sulfate or glutathione conjugation can lead to toxic and mutagenic metabolites in a cell and chemical specific manner (Gamage et al., 2006; Guengerich et al., 2003; Hein et al., 1993; Ioannides and Lewis, 2004; Surh, 1998). Bioactivation to toxic intermediates is therefore a consequence of chemical structural diversity and the occasional necessity for the creation of chemically reactive sites for subsequent conjugation reactions. Thus, there is a balance of protective and injurious processes, and between endogenous and exogenous functions (Nebert, 1991; Nebert and Russell, 2002).

The first line of cellular defense, against amphipathic or slightly lipophilic compounds in particular, is the active expulsion of these compounds by efflux proteins known as ATP Binding Cassette (ABC) or multidrug efflux transporters (Dean et al., 2001). Three well-known examples are the *p*-glycoproteins (PGP), members of the ABCB subfamily (Ambudkar et al., 1999); the multidrug resistance proteins (MRP) or ABCC proteins (Cole and Deeley, 1998); and multi-xenobiotic resistance (MXR) or ABCG proteins (Sarkadi et al., 2004).

Generally, once toxicants enter the cytoplasm, biotransformation is required to enhance elimination or inactivation. Oxidative (Phase I) modification of chemicals to form more hydrophilic metabolites is often the initial step leading to excretion. Oxidation is carried out by flavoprotein monooxygenase (FMO) and cytochrome P450 (CYP) enzymes, especially members of the CYP1, CYP2, CYP3, and CYP4 families. Oxidation often is followed by reductive or conjugative modification by glutathione-*S*-transferases (GSTs), sulfotransferases (SULTs), UDP-glucuronosyl transferases (UGTs), *N*-acetyl transferases (NATs), aldo-keto reductases (AKRs), epoxide hydrolases (EPHXs), and NAD(P)H-quinone oxidoreductases (NQOs). These enzymes perform primary or secondary conjugation reactions on toxic substrates (GST, SULT, UGT, NAT) or reduce or hydrolyze toxicants (NQO, AKR, EPHX).

Antioxidant defenses are critical components of defense systems in organisms living in aerobic environments. Reactive oxygen can be produced by toxicants, exposure to ultraviolet radiation, or during normal metabolism, and can damage DNA, lipids, and proteins, resulting in pathologies and toxicity. Antioxidant defensive proteins include superoxide dismutases (SODs), catalases (CATs), and peroxidases, including glutathione peroxidase (GPXs), and thioredoxins (TXNs).

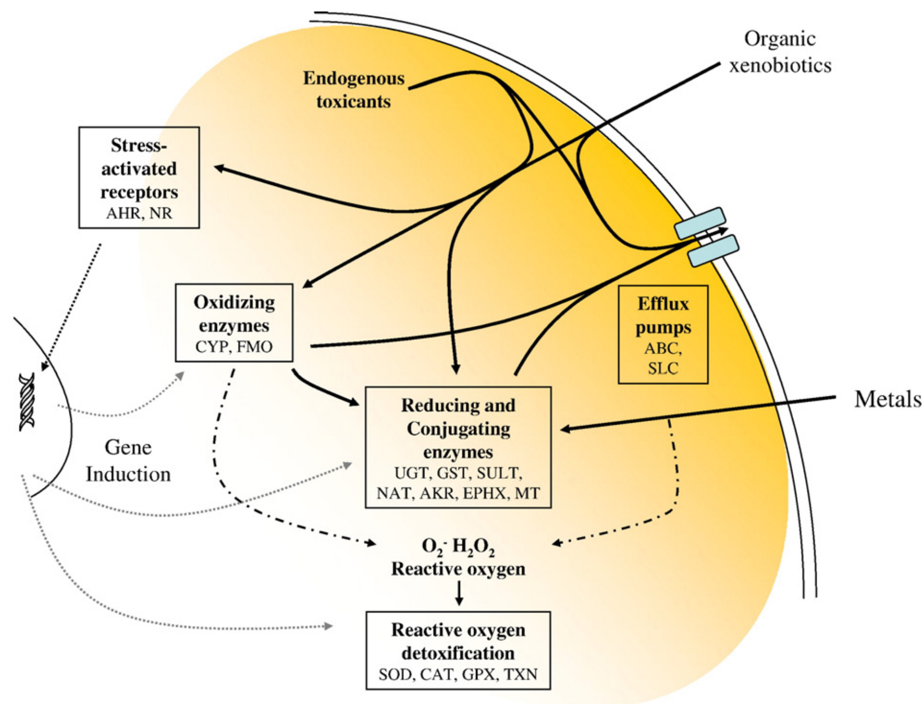

Fig. 1. Conceptual organization of the chemical defensome. Organic and inorganic toxicants are actively exported, and also subjected to a variety of biotransformative reactions including oxidation, reduction, conjugation, and hydrolysis. Solid lines indicate possible pathways for exogenous toxicants, dotted lines represent possible gene induction in response to stress-activated receptors, and dot-dashed lines indicate possible sources of toxicant-stimulated endogenous production of reactive oxygen. Gene families responsible for the some of the activities are indicated in appropriate boxes, and are abbreviated as found in the text.

Several families of vertebrate proteins are involved in sensing organic chemicals and governing expression of proteins that biotransform and/or transport chemicals and their metabolites. The PAS (Per-ARNT-SIM) protein family contains transcription factors involved in signaling during development, establishing and maintaining circadian rhythms, and sensing environmental variables such as oxygen and small molecules (e.g., hormones and xenobiotics) (Gu et al., 2000; Kewley et al., 2004). The primary member of this family that is a chemical sensor, but also has developmental roles, is the aryl hydrocarbon receptor (AHR), which is activated by planar aromatic hydrocarbons and a variety of other chemicals (Denison and Nagy, 2003). AHR subsequently regulates the expression of some *CYP* genes and genes encoding conjugating and reducing enzymes (Nebert et al., 2004; Whitlock, 1999).

The nuclear receptor (NR) superfamily comprises a second group of chemical sensors. The pregnane-X-receptor (PXR), constitutive androstane receptor (CAR), non-mammalian PXR homologs BXR and CXR, peroxisome proliferator-activated receptors (PPARs), liver-X-receptor (LXR), and farnesoid-X-receptor (FXR) are activated by a variety of xenobiotic and endobiotic compounds and regulate a coordinated response involving various CYP2s, CYP3s, and CYP4s, conjugating enzymes, and transporters (Bock and Kohle, 2004; Klaassen and Slitt, 2005; Xie and Evans, 2001). A third set of sensors includes nuclear factor erythroid-derived 2 (NFE2)-related factor 2 (NRF2, NFE2-like2) and related cap'n'collar (CNC)-basic-leucine zipper (bZIP) family proteins, which are activated by oxidants and electrophiles and form heterodimers with small Maf proteins to stimulate transcription of genes encoding antioxidant enzymes such as GST, NQO, glutamate-cysteine ligase (GCL), SOD, and others (Nguyen et al., 2003b).

The sea urchin is a well-established model for the study of development, and thus is a prime candidate for addressing mechanisms of chemical toxicity and defense during embryonic stages in early deuterostomes. A number of studies have described mechanisms by which chemicals affect embryonic development in sea urchins (e.g., Green et al., 1997; Hamdoun et al., 2004; Roepke et al., 2005; Smital et al., 2004; Vega and Epel, 2004), but the defense mechanisms are not well understood. This description of chemical defense genes in sea urchin will facilitate research to better understand the mechanisms of response to chemical challenge in embryos, and how enzymes and signaling pathways having both stress response and developmental roles accomplish these dual functions in concert.

Methods

Gene annotation

Defensome genes were identified by Hidden Markov Model searches (Hmmer v2.3.2; Eddy, 1998) of the GLEAN3 gene predictions with conserved domains of known defense genes using the PFAM models (for all genes except PAS, NR, and ABC genes). PAS, NR, and ABC genes were identified by BLAST searches of the GLEAN3 gene predictions derived from the first sea urchin genome assembly. Gene identities were confirmed by reciprocal BLAST of the predicted genes against Genbank or, where possible, by comparison to cloned cDNAs. Conserved ABC domains were also identified using Prosite. Other genes used in annotation were derived from the *Ciona intestinalis* assembly version 2, the *Danio rerio* assembly version 5, the *Takifugu rubripes* assembly version 4, the *Xenopus tropicalis* assembly version 4, and the *Gallus gallus* genome assembly version 1.

Alignments were constructed using ClustalX (Jeanmougin et al., 1998) or Muscle v3.6b (Edgar, 2004) and adjusted by hand as necessary. Phylogenetic trees were constructed by analyzing amino acid sequences using minimum evolution (PAUP* v4b10; Swofford, 2003), neighbor joining (PAUP*), maximum parsimony (PAUP*), and Bayesian (MrBayes v3.1.2; Ronquist and Huelsenbeck, 2003) techniques. The WAG model of amino acid substitution with a gamma distribution of substitution rates was used in all Bayesian analyses (WAG+I+G). In most cases, full-length predicted genes or complete domains were used in phylogenetic analyses. Partial gene sequences may be pseudogenes or may be due to incomplete genome assembly. However, pseudogene "number" is essentially impossible to determine except by BLAST searches of a complete genome with individual exons (e.g., Nelson et al., 2004).

Different types of evidence are available for the genes discussed in this paper. Predicted genes are derived from the GLEAN3 predictions of the whole genome shotgun assembly (The Sea Urchin Genome Sequencing Consortium, submitted for publication). Many of these predicted genes are supported by expression data from a whole genome tiling array (Samanta et al., 2006). EST or cDNA evidence confirms a number of the genes presented here, as noted in the Results. Whenever possible, we have tried to follow the nomenclature guidelines outlined by the HUGO gene nomenclature committee (http://www.gene.ucl.ac.uk/nomenclature/guidelines.html). One exception is that annotated urchin-specific gene names are prefaced with Sp-, and SPU numbers refer to the gene model numbers in the initial public release of the sea urchin genome (The Sea Urchin Genome Sequencing Consortium, submitted for publication).

The exact nomenclature of many genes presented in this paper is tentative because of the uncertainty in classifying genes to specific families or subfamilies within the major superfamilies represented in these analyses. We have attempted to follow the nomenclature guidelines for many of the defined gene superfamilies (Hyndman et al., 2003; Jez and Penning, 2001; Mackenzie et al., 2005; Nebert and Vasiliou, 2004; Nelson et al., 1993; Vasiliou and Nebert, 2005; Vasiliou et al., 2006) as well as the nomenclature accepted by the Human Genome Nomenclature Committee, but due to evolutionary distances many of the assignments are tentative. Thus, genes here are given names indicating our best understanding of the homologous relationships, but this should in most cases not be taken as formal assignments. Moreover, homologous genes do not necessarily possess identical functions, and thus inferred roles of sea urchin genes will require confirmation.

Expression

Embryonic gene expression was assessed with data from a whole genome tiling array using a mixed pool of poly-A RNA obtained from untreated eggs, early blastula, gastrula, and prism stages (Samanta et al., 2006). This tiling array consists of ~11 million 50mer oligonucleotides that covered the entire *S. purpuratus* genome with gaps of 10nt between consecutive probes, synthesized on glass slides. A hybridization signal 9-fold above the mean raw signal of 1.3 (arbitrary units) was interpreted as significant expression during embryonic development.

Results

Receptors and signal transduction

Aryl hydrocarbon receptor (AHR) and related bHLH-PAS proteins

PAS-family genes encode proteins involved in developmental signaling, including those responding to changes in internal and external environments. The sea urchin genome contains at least 14 predicted PAS genes representing 12 of the 14 known

metazoan PAS subfamilies (Supplemental Table S1). These include genes involved in circadian rhythms (*CLOCK*, *BMAL/ARNTL*), neurogenesis (*NXF/NPAS4*, *SIM*, *AHR*), and hypoxia and chemical signaling (*HIF-1α*, *ARNT*, *AHR*, *NCoA*). Of greatest relevance to chemical defense are *AHR* and *HIF1α* and their dimerization partner *ARNT* (Table 1). *AHR* and *HIF1α* appear to have been duplicated in sea urchin, because single forms occur in protostomes and *Ciona* (Supplemental Table S1). One of the *AHR* genes (*Sp-Ahr*) is most closely related to vertebrate AHRs, while the other (*Sp-Ahrl/Sp-AHR-like*) is a partial gene sequence that is more closely related to the *Drosophila* AHR homolog *spineless* (*ss*). The two HIF genes resemble vertebrate paralogs *HIF-1α* (*HIF1A*) and *HIF-2α* (*EPAS1*). Whether the apparent duplication of these urchin genes reflects a need for greater or more specialized adaptive responses to small organic molecules (AHR) or hypoxia (HIF) is unknown.

Oxidative stress-responsive transcription factors

The CNC-bZIP family in vertebrates includes four core members: nuclear factor erythroid-derived 2 (*NFE2*) and NFE2-related factors (*NFE2-like*, also known as *NRF*s) 1, 2, and 3 (Nguyen et al., 2003b), whereas *Drosophila* contains a single gene, *Cnc* (Mohler et al., 1991; Veraksa et al., 2000). The *S. purpuratus* genome includes a single predicted CNC-bZIP gene, designated *Sp-Cnc* (*SPU_011174*; Table 1). In phylogenetic analyses (Fig. 2), *Sp-Cnc* occurs basal to the four human CNC-bZIP genes, suggesting that CNC-bZIP genes underwent duplication and diversification in the chordates after the divergence of echinoderms from other deuterostomes.

Other proteins involved in regulation of the oxidative stress response in metazoans include the BTB-bZIP proteins BACH1 and BACH2 (Oyake et al., 1996), and the small Maf proteins (MafF, MafG, and MafK), which in vertebrates are heterodimeric partners of NF-E2, NRFs, and BACH proteins (Igarashi and Sun, 2006; Nguyen et al., 2003b). We have been unable to find a homolog of either human BACH gene in the current urchin assembly. One large Maf gene (*Sp-Maf, SPU_025888*) and one small Maf gene (*Sp-Mafs, SPU_030167*) have been reported in urchin (Coolen et al., 2005) (Table 1); both possess the conserved extended homology region (EHR) and bZIP domains that characterize vertebrate Maf proteins. In addition to these proteins, we also found an urchin homolog of the vertebrate gene *KEAP1* (Kelch-like-ECH-associated protein 1); in mammals, *KEAP1* encodes a protein which in the absence of oxidative stress retains NRF2 in the cytoplasm and enhances its proteasomal degradation (Nguyen et al., 2003a).

Nuclear receptors

Thirty-three predicted NR genes were identified in the *S. purpuratus* genome (Howard-Ashby et al., 2006). Of interest with regard to the defensome are four NRs (*SPU_015456*, *SPU_017404*, *SPU_004526*, and *SPU_027598*) that appear to be related to NRs in the 1H subfamily, which also contains vertebrate FXR and LXR and arthropod EcR (ecdysone receptor) (Table 1; Fig. 2). One of these genes (*SPU_027598*, *Sp-FXR*) is related to both LXR and FXR, and is of particular interest because the ligand-binding domain clusters with the LXR in phylogenetic analyses, whereas the DNA-binding domain clusters with the FXR (Supplemental Figs. S1 and S2). The other three *NR1H*-like genes form a monophyletic group that may represent a new subfamily within the *NR1* family (Fig. 3). Expression studies in untreated embryos show that the four *NR1H*-like genes are

Table 1
Xenobiotic receptors and conditional transcription factors in the sea urchin genome

Gene family or group	Gene name	Alternate names	Urchin model	Most closely related to
bHLH-PAS	*Sp-Ahr*		*SPU_013788*	Vertebrate *AHR*
	Sp-Ahrl [a]	Ahr-like	*SPU_012296*	*Drosophila ss*
	Sp-Arnt		*SPU_000129*	Vertebrate *ARNT*
	Sp-Hif-1α		*SPU_030140*	Vertebrate *HIF-1α*
	Sp-Hif-2α [a]	EPAS	*SPU_026758*	Vertebrate *HIF-2α*
CNC-bZIP and related	*Sp-Cnc*	NFE2	*SPU_011174*	Vertebrate *NFE2*, *NRF1* [b], *NRF2* [b], *NRF3* [b], and *Drosophila Cnc*
	Sp-Keap1		*SPU_011306*	Vertebrate *Keap1*
	Sp-Mafs	small Maf	*SPU_030167*	Vertebrate *MafK*
Nuclear receptors	*Sp-Ppar1*	NR1C	*SPU_019332*	Vertebrate *PPARα*
	Sp-Ppar2	NR1C	*SPU_021289*	Vertebrate *PPARγ*
	Sp-Fxr/Lxr	NR1H	*SPU_011348*	Vertebrate *LXR*
	Sp-NR1H6a	NR1L1?	*SPU_017404*	Vertebrate *RARβ*
	Sp-NR1H6b	NR1L2?	*SPU_015456*	Vertebrate *LXR*
	Sp-NR1H6c	NR1L3?	*SPU_004526*	Vertebrate *LXR*
	Sp-Hnf4	NR2A	*SPU_021192*	Vertebrate *HNF4*
	Sp-Rxr	NR2B1	*SPU_028422*	Vertebrate *RXRα*
	Sp-Err	NR3B2	*SPU_004723*	Vertebrate *ERRβ*
	Sp-Shr2	NR2C2	*SPU_008117*	Vertebrate *TR4*
Metal and heat response	*Sp-Mtf*		*SPU_016490*	Vertebrate *MTF1*
	Sp-Hsf		*SPU_013409*	Vertebrate *HSF1*

[a] Predicted genes represented by partial gene models.
[b] The approved gene symbols for these genes in human are NFE2L1 (NRF1), NFE2L2 (NRF2), and NFE2L3 (NRF3).

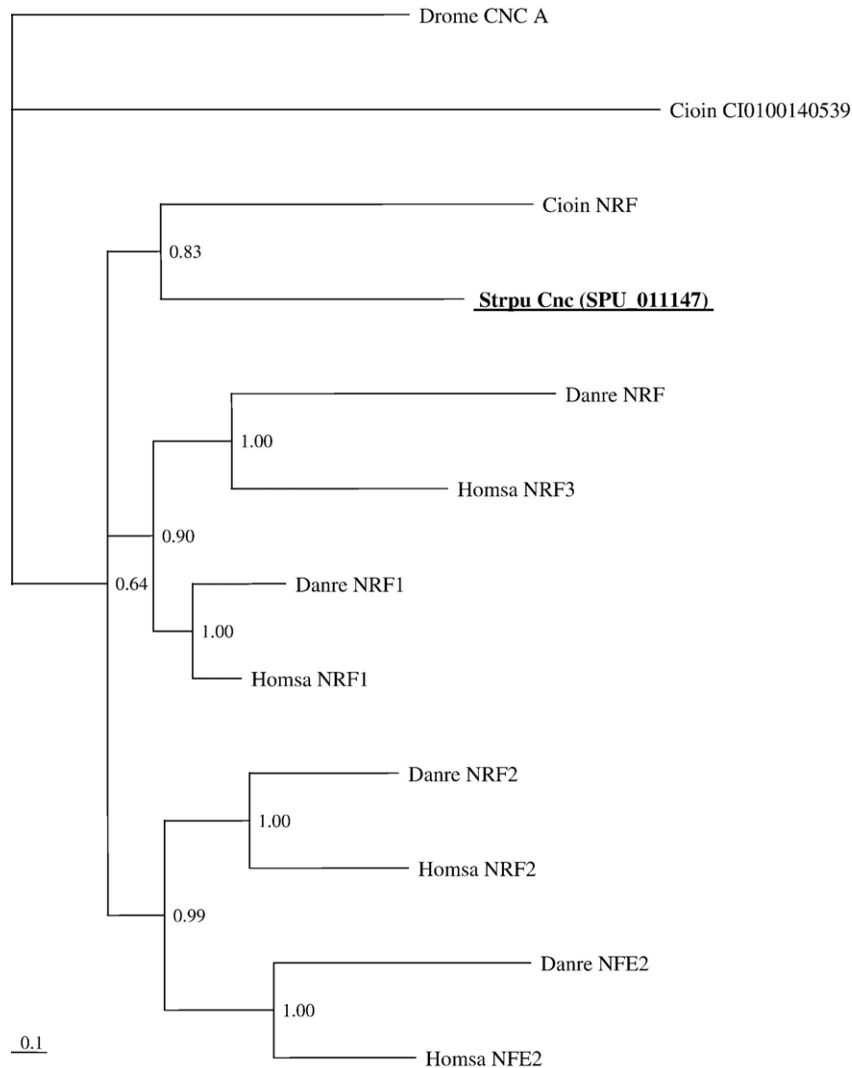

Fig. 2. Unrooted Bayesian phylogenetic tree of *NFE2*-related *CNC* genes, including vertebrate, tunicate, and sea urchin homologs. *Drosophila CNCA* serves as the outgroup. Numbers at branch points are the posterior probabilities calculated from 5 million generations of Bayesian MCMC. The approved gene symbols for the human genes designated here by the common names *NRF1*, *NRF2*, and *NRF3* are *NFE2L1*, *NFE2L2*, and *NFE2L3*, respectively. Species abbreviations are Drome, *Drosophila melanogaster*, Cioin *Ciona intestinalis*, Danre *Danio rerio*, Homsa *Homo sapiens*, and Strpu *Strongylocentrotus purpuratus*. The distance bar represents 0.1 amino acid substitutions per site.

expressed at higher levels in early development (0–6 hpf), decrease at 6–18 hpf, and then exhibit increasing transcript abundance during later stages (24–48 hpf) (Howard-Ashby et al., 2006). By contrast, orthologs of genes in the *NR1I* family (*PXR*, *CAR*, *VDR*) have not been detected in the current genome assembly, nor in EST libraries.

S. purpuratus also possesses homologs of *RXR* (*NR2B*) and *HNF4* (*NR2A*) (Table 1). RXRs in vertebrates, and their invertebrate homolog USP, act as dimerization partners of NR1H and NR1I proteins to regulate transcription. HNF4 is a ligand-independent NR with an important role as a co-regulator of FXR function (Eloranta and Kullak-Ublick, 2005).

Other NRs involved in xenobiotic response in vertebrates include PPARs (NR1C), which have target genes involved in lipid metabolism, energy homeostasis, and cell differentiation, and estrogen receptor (*ER*; *NR3A*). *S. purpuratus* possesses two *PPAR* paralogs (*SPU_021289* and *SPU_019332*; Table 1), but no ERs. However, an estrogen receptor-related receptor (*ERR*; *NR3B*) homolog (*SPU_004723*) was identified. ERRs are targets for endocrine disrupting compounds (Yang and Chen, 1999) and are closely related to estrogen receptors (ERs), which regulate many aspects of hormone responses in vertebrates. In addition, a previously cloned NR, *Sp-Shr2* (*NR2B/C*-like, *SPU_00817*; Kontrogianni-Konstantopoulos et al., 1996), has recently been shown to bind estradiol (Roepke et al., 2006). *Sp-Shr2* exhibits ubiquitous expression throughout development (Howard-Ashby et al., 2006) but also appears to be upregulated in eggs in response to maternal estradiol treatment (Roepke et al., 2006).

Other stress-sensing genes present in the urchin genome include single homologs of the metal-responsive transcription factor *MTF1* (*Sp-Mtf*, *SPU_016490*), and the heat shock factor *HSF1* (*Sp-Hsf*, *SPU_013409*).

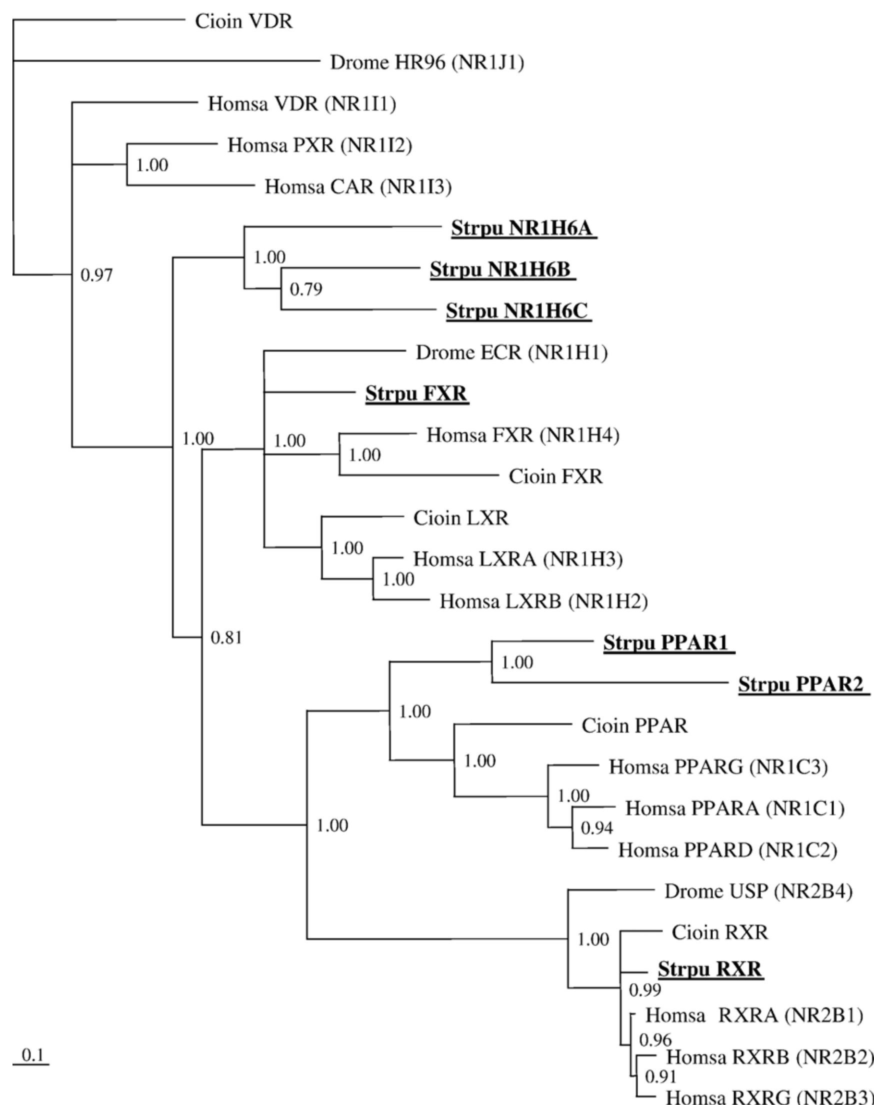

Fig. 3. Unrooted Bayesian phylogenetic tree of *NR* genes involved in toxicant responses in vertebrates. Sequences include vertebrate, tunicate, and sea urchin genes in the NR1J, *NR1I*, *NR1H*, *NR1C*, and *NR2B* subfamilies. Numbers at branch points are the posterior probabilities calculated from 5 million generations of Bayesian MCMC. Species abbreviations are as in Fig. 2. The distance bar represents 0.1 amino acid substitutions per site.

Efflux transporter proteins

Many toxic compounds are pumped across membranes, against concentration gradients. This energy-dependent efflux of toxicants is mediated by members of the ATP-binding cassette (ABC) superfamily. In humans the 48 confirmed ABC transporters are organized into 7 subfamilies designated ABC A through G (Dean and Annilo, 2005). A subset of these families, ABCB, C and G, includes proteins known to export toxicants. These proteins are commonly called the multidrug transporters due to their ability to pump out multiple therapeutic drugs, a major obstacle to the efficacy of chemotherapy in cancer and the treatment of several pathogens (Dean et al., 2005).

Sea urchins have 65 *ABC* genes organized into 8 subfamilies (Supplemental Figs. S3–S9, Table S2) including the three multidrug transporter subfamilies (*ABC B*, *C* and *G*). Although there is considerable variation in the total number of ABC genes within eukaryotic genomes (Annilo et al., 2006), the relative proportion of each family has tended to stay constant (Fig. 4). In the case of sea urchins, the *ABCC* family is about 25% larger than in other deuterostome genomes, with at least 30 genes, accounting for almost half of all the *ABC* transporters (Fig. 5). Much of the expansion is in the *Sp-ABCC5* and *Sp-ABCC9* families, whereas orthologs of the vertebrate gene *ABCC2* (also called *MRP2*) are absent. As one indication of their functional importance, 25 of these 30 genes show significant levels of expression at some stage of development (Samanta et al., 2006).

One *ABCC* gene, *Sp-ABCC9A*, has been cloned from sea urchin eggs and encodes a protein of 1519 amino acids (*SPU_025903*; 33 exons spanning 24 kb of genomic DNA). Although there are high levels of efflux activity in sea urchin embryos, the transcript is present at trace quantities, <1 copy/cell, suggesting that this mRNA is translated and degraded

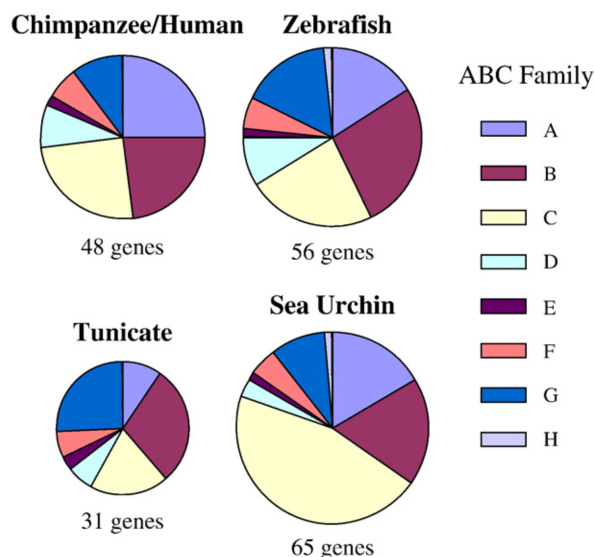

Fig. 4. Distribution of *ABC* transporters between *ABC* families A–H in primate, zebrafish, tunicate, and urchin.

during oogenesis (Hamdoun et al., 2004). The main xenobiotic efflux in early embryos is an MK-571 sensitive MRP-like efflux activity (Hamdoun et al., 2004), and may arise from this Sp-ABCC9A gene product.

The drug-binding pocket of Sp-ABCC9A is unlike mammalian SUR and MRP ABCC drug-binding pockets; it appears to be a hybrid, having both a tyrosine residue, characteristic of mammalian MRP, and a leucine residue found only in SUR-type mammalian ABCCs. Consistent with the MRP-like efflux activity, rather than SUR-like activity, associated with the presence of this gene in early embryos, we have observed no change in phenotype in sea urchin eggs or embryos treated with a sulfonylurea or a potassium channel opener (100 μM tolbutamide or 100 μM diazoxide). However, ligands of mammalian LXR and FXR that have been shown to upregulate mammalian *ABCC* (*MRP*) genes (Kast et al., 2002; Kullak-Ublick and Becker, 2003) also induce *ABCC9A* in sea urchin (Hamdoun et al., unpublished data).

A second *ABC* gene cloned from eggs is *Sp-ABCB1* (*SPU_001752*, 27 exons spanning 32 kb of genomic DNA). This gene is one of 12 *ABCB* genes present in the sea urchin and is orthologous to mammalian *ABCB1*/*PGP*. *Sp-ABCB1* codes for a 1329-amino-acid protein and is detected in embryos shortly after fertilization. Based on inhibition characteristics, Sp-ABCB1 accounts for less of the embryo's observed calcein efflux activity than Sp-ABCC9A (Hamdoun et al., 2004).

The *ABCG2* gene is the primary protein in the vertebrate G family involved in toxicant protection, conferring resistance to several toxic and chemotherapeutic agents (Allikmets et al., 1998; Miyake et al., 1999). There are four *ABCG2*-like genes in the urchin genome (*SPU_021184*, *SPU_014013*, *SPU_024785*, and *SPU_014007*), the same number as in tunicates and zebrafish. Substrates of ABCG proteins in mammals include chlorophyll breakdown products such as pheophorbide A (Robey et al., 2004).

Ion transporters

Other toxicant efflux proteins include members of the organic anion transporters (OAT), the organic anion transporting polypeptides (OATP), and the organic cation transporter (OCT) families (Sweet, 2005; Sweet et al., 2001). The OAT and OCT form a subfamily of the solute transporter branch (SLC22A) of the major facilitator superfamily (Sweet, 2005; Sweet et al., 2001), whereas the closely related OATP are members of the SLC21 subfamily. OAT substrates studied in vertebrates include the herbicide 2,4-dichlorophenoxyacetic acid, heavy metals such as mercury and cadmium, and neurotransmitter metabolites (Sweet, 2005). The sea urchin genome contains 30 *OATP* genes, mostly (60%) members of the *OATP4* subfamily. We also found 46 members of the *SLC22* branch, which includes both *OAT* and *OCT* genes. Most of the urchin genes in the *SLC22* family show greater similarity to insect *SLC22* genes than to vertebrate homologs. These gene counts represent significant expansions of the *SLC22* family, which consists of only 5 genes in humans and mice (Sweet, 2005).

Oxidative biotransformation

Cytochromes P450

The sea urchin contains 120 *CYP* genes, and those related to *CYP* gene families 1–4 constitute a large proportion (80%) of the total, suggesting pressure to expand functionality in these gene families. Multiple gene duplications appear to have taken place in the echinoderm lineage, leading to urchin-specific gene clades that are phylogenetically basal to known vertebrate *CYP* families. This complicates definitive assignment of names, based on current *CYP* nomenclature guidelines. However, we are able to provisionally classify these genes at the family level, based on similarities to vertebrate *CYP* genes.

In vertebrates, CYP1s are critical xenobiotic-metabolizing enzymes that catalyze the activation of numerous polycyclic aromatic hydrocarbon carcinogens. Many *CYP1* genes are induced by polycyclic and planar halogenated aromatic hydrocarbons and various natural compounds via the AHR (Whitlock, 1999). Eleven *CYP1*-like genes are present in the urchin genome, more than twice the five *CYP1*s in *C. intestinalis* or the 3–4 *CYP1*s in most vertebrates. Two primary clades – *CYP1As* and *CYP1B/1Cs* – resulted from an early divergence in an ancestral 'protochordate' prior to the divergence of tunicates from chordates (Goldstone et al. unpublished data), but phylogenetic analyses show that the sea urchin *CYP1*-like genes do not fall into these clades (data not shown). Nine of the urchin genes are single coding exon genes, and three of these are adjacent in the genome assembly, suggesting tandem duplication following reinsertion of a processed transcript. These three adjacent genes exhibit very different embryonic and larval expression during normal development (*SPU_010719* is weakly expressed, *SPU_010720* is strongly expressed, and *SPU_010721* does not show significant expression) suggesting different enzymatic roles in the organism (Supplemental Table S3).

In mammals, the highly diverse CYP2 enzymes are responsible for a tremendous variety of substrate oxidation

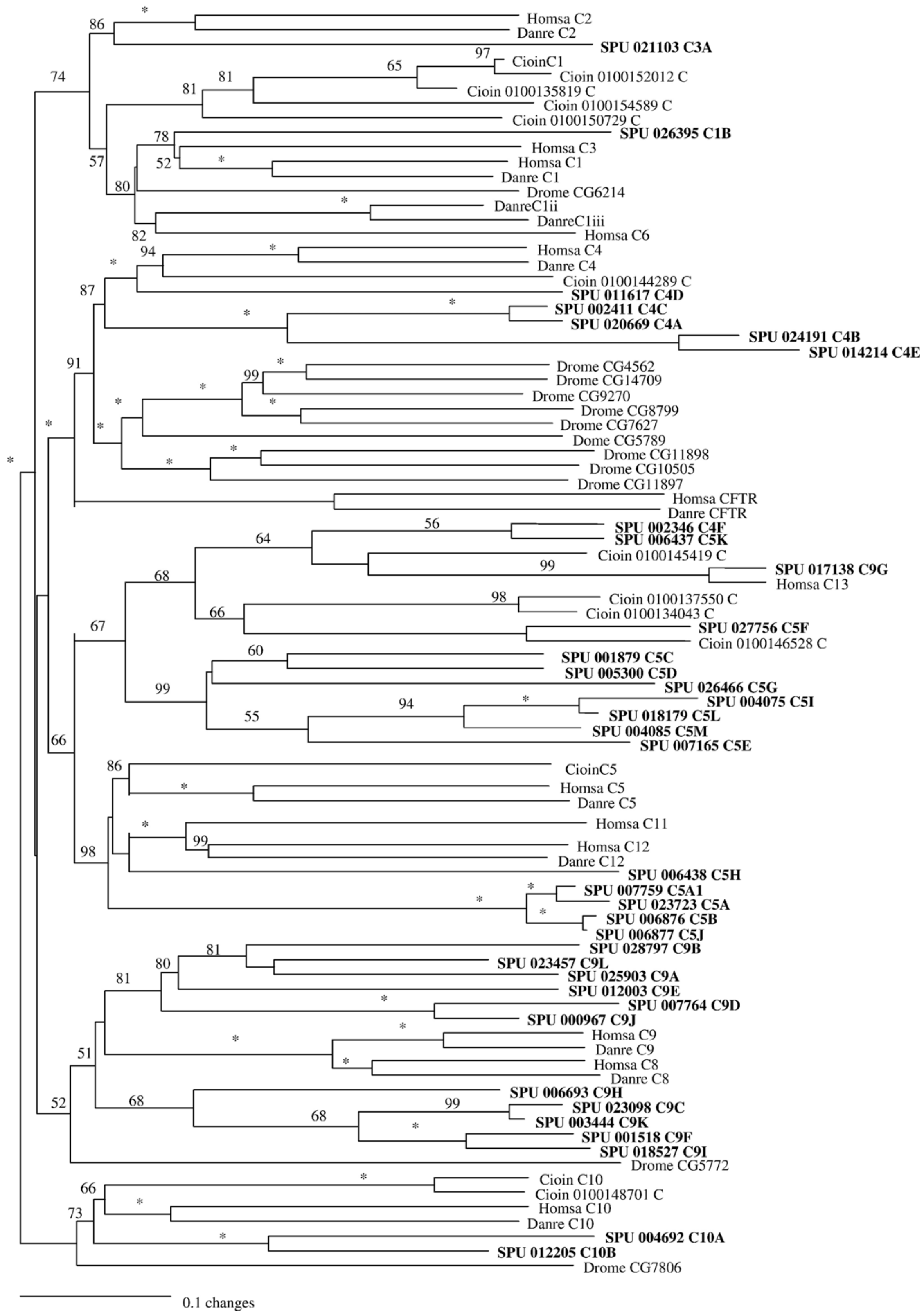

Fig. 5. Unrooted minimum evolution phylogenetic tree of *ABCC* genes, including vertebrate, tunicate, sea urchin, and *Drosophila* sequences. Numbers at branch points are bootstrap values resulting from 250 bootstrap replicates. Branches marked with an asterisk exhibit 100% support. Species abbreviations are as in Fig. 2.

reactions (reviewed in Lewis et al., 2004). In the sea urchin, the number of *CYP2*-like genes is expanded relative to most other known genomes, consisting of 73 predicted genes, more than 60% of the total CYP complement, in contrast to the 30–49% in vertebrate genomes (Fig. 6). Only one *CYP2*-like gene can be confidently assigned to a subfamily, *CYP2U* (*SPU_008632*),

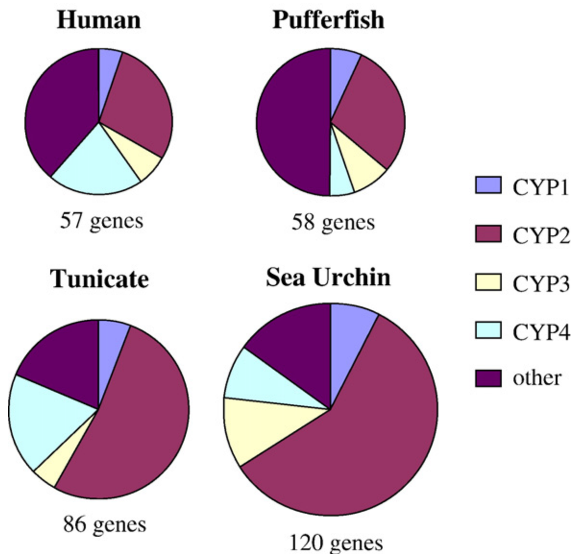

Fig. 6. Distribution of genes in families *CYP1–CYP4* in human, pufferfish, tunicate, and urchin.

suggesting functional conservation in that subfamily. The majority of *CYP2*-like genes (51/73) exhibit embryonic or larval expression, with five genes (*SPU_010576*, *SPU_016816*, *SPU_020753*, *SPU_020756*, *SPU_021087*) exhibiting very high embryonic expression levels (Supplemental Table S3).

In humans, CYP3A enzymes catalyze the metabolism of 40% to 60% of all clinically used drugs (Guengerich, 1999), and also metabolize endogenous hormones, bile acids, fungal and plant products, and environmental pollutants (reviewed by Guengerich, 1999; Maurel, 1996; Thummel and Wilkinson, 1998). The tunicate *C. intestinalis* has four *CYP3*-like genes (Verslyke et al., 2006) that are phylogenetically similar to vertebrate *CYP3* genes and not closely related to the insect *CYP6s* and *CYP9s* in gene structure. Ten complete and seven incompletely assembled *CYP3*-like genes were identified in the sea urchin genome. Phylogenetic analysis indicates that these genes cluster with other "clan 3" genes including vertebrate and tunicate *CYP3* genes, the insect *CYP6* and *CYP9* genes, and the vertebrate CYP5 genes. All of the complete *CYP3*-like sea urchin genes contain 12–13 exons, remarkably similar to the 12–13 exons in tunicate and vertebrate *CYP3* genes, and distinctly different from the 1–6 exons in insect CYP6 and CYP9 genes.

The *CYP4* family is one of the more ancient animal *CYP* families, with members found in both protostomes and deuterostomes. Many CYP4 substrates are peroxisome proliferators, and *CYP4s* in mammals are regulated by PPAR (Dogra et al., 1998; Waxman, 1999). The sea urchin genome contains 10 *CYP4*-like genes; Bayesian phylogenetic analysis shows that 3 of these genes cluster with the vertebrate and tunicate *CYP4F* genes, whereas 6 form a cluster with the vertebrate *CYP4V* genes (data not shown). Two partial *CYP4C* genes, *CYP4C19* and *CYP4C20*, have been isolated from the white sea urchin, *Lytechinus anamesis* (Snyder, 1998), and orthologs of these exist in the *S. purpuratus* genome (*SPU_002380* and *SPU_012081*, respectively). Based on Bayesian analysis, the *Lytechinus* sequences cluster with the vertebrate *CYP4Vs*, suggesting a reconsideration of the *Lytechinus* sequence classification. Nine of the urchin *CYP4*-like genes have 11–12 exons, similar to the numbers of exons in vertebrate *CYP4* genes. The urchin *CYP4* family, similar to the *CYP1*, *CYP2*, and *CYP3* families, exhibits apparent tandem duplications, based on the clustering of several genes on the same scaffold. Half of the *CYP4* genes exhibit embryonic or laval expression, with *SPU_005931* exhibiting high expression and a subset expressed at lower levels (*SPU_002380*, *SPU_002382*, *SPU_02835*, *SPU_007335*; see Supplemental Table S3).

Flavoprotein monooxygenases

The flavin-containing monooxygenase (FMO) enzymes catalyze the NADPH-dependent N- or S-oxidation of heteroatom-containing compounds similar to some of the activities of CYP enzymes (Cashman, 2002, 2005; Ziegler, 2002). However, FMO substrates are generally restricted to soft nucleophiles, although less is known about mammalian FMO activity than CYP activity due to the instability of FMO enzymes (Cashman, 2005). FMO activity is not restricted to xenobiotic metabolism: for example, an FMO was found to be essential for the echinochrome pigment formation in *S. purpuratus* mesenchyme pigment cells (Calestani et al., 2003). The human genome contains 6 functional *FMO*s and 5 pseudogenes, arranged in two main clusters on separate chromosomes (Hernandez et al., 2004). The sea urchin genome contains 16 *FMO* genes, divided among 4 of the 5 families of *FMO*s, *FMO1*, *2*, *3*, and *5*, with the majority most closely related to *FMO5*. One of the urchin *FMO* genes (*SPU_017639*) is more closely related to insect *FMO*s (disulfide oxidoreductases) than to vertebrate *FMO*s. At least two of the *FMO5* genes are located on the same scaffold, suggesting that urchin *FMO*s may be clustered, similar to the mammalian genomic *FMO* arrangement.

Aldehyde dehydrogenases

Highly reactive electrophilic aldehydes are oxidized by aldehyde dehydrogenase (ALDH) enzymes; these compounds include endogenous aldehydes formed by the metabolism of amino acids, carbohydrates, lipids, amines, steroids, and a wide range of environmental chemicals. While this activity may be protective, with some substrates, ALDH oxidation can also lead to substrate bioactivation rather than detoxification. For example, one of the most important ALDH reactions in vertebrate development is the irreversible oxidation of retinal to retinoic acid (Lee et al., 1991). ALDH enzymes may also act to maintain cellular homeostasis by maintaining the cellular redox balance via ROS scavenging and the production of reducing equivalents as NADPH or NADH. Of the 19 ALDH proteins in humans, the 7 members of the ALDH1 family (including ALDH2) and the two members of the AHR-regulated ALDH3A subfamily appear to play the most significant roles in detoxification (Vasiliou and Nebert, 2005). Sea urchins posses 20 *ALDH* genes (Table 2; Supplemental Table S4), of which 5 are *ALDH1*-like (including three *ALDH2* mitochondrial precursor-like genes) and 2 are *ALDH3A*-like. All but one of the *ALDH1* and *ALDH3*-like genes are expressed during development, the exception being one *ALDH2*-like gene (*SPU_007284*).

Table 2
Gene counts of biotransformative genes in humans and sea urchins

Classification	Gene	Urchin	Human
Oxidative	*CYP1*-like	11	3
	CYP2-like	73	16
	CYP3-like	10	4
	CYP4-like	10	12
	FMO	16	6
	ALDH (total)	20	19
	ALDH1 and 3	7	9
Conjugative	*GST*	38	17
	MGST	12	3
	SULT	36	13
	UGT	49	13 [a]
	NAT	1	2 (10) [b]
Reductive	*AKR-like* (total)	10	8
	AKR1-like	6	4
	EPHX	5	2
	NQO	0	2

[a] Not including multiple first exon expression in UGT1.
[b] Eight non-arylamine NAT genes are in the human genome.

Other oxidases

Homologs of another important dioxygenase/peroxidase gene, prostaglandin-endoperoxide synthase (*PTGS*; also known as cycloxygenase, *COX*), appear to be missing from the current genome assembly. If this result holds, it would suggest that *PTGS* genes have been lost on the echinoderm lineage, because at least two *PTGS* genes are present in vertebrates, and two PTGS genes have been cloned from coral (Jarving et al., 2004).

Reductive and conjugative biotransformation

Sea urchins posses a large number of xenobiotic-conjugating enzymes, including genes for 36 cytosolic sulfotransferase (*SULT*) genes, 49 distinct *UGT* genes, and 38 *GST* genes (see Table 2). This represents a very large diversification in these gene families relative to those in mammals: the human genome contains 13 *SULT*, 13 *UGT* and 17 *GST* genes (Gamage et al., 2006; Mackenzie et al., 2005; Nebert and Vasiliou, 2004; Pearson, 2005). However, insect genomes may contain more members of these gene families; for example, the *Drosophila melanogaster* genome contains 33 UGT genes (Luque and O'Reilly, 2002). The urchin UGT enzymes are principally divided among the *UGT1*-like and *UGT2*-like families (13 *UGT1*-like and 29 *UGT2*-like). Assigning *UGT* homology is complicated by the fact that *UGT1* genes in mammals consist of one gene with as many as 14 different first exons, a trait also shared by members of the *UGT2A* subfamily (Mackenzie et al., 2005). Based on the large number of distinct genes in the sea urchin, exon duplication similar to that observed in the mammalian *UGT* families is not the only method of *UGT* diversification. However, further analysis is required to determine whether alternative first exons are expressed in sea urchins.

Cytosolic GSTs are soluble proteins that catalyze the transfer of glutathione to electrophilic substrates. The 38 urchin *GST*s represent 6 of the major *GST* classes (alpha, pi, sigma, theta, zeta, omega; Supplemental Fig. S10, Table S5). Nine urchin *GSTs* cluster phylogenetically with vertebrate alpha *GST*s. Alpha, pi, and mu GSTs in mammals are responsible for the conjugation of polycyclic aromatic hydrocarbon metabolites (Sundberg et al., 1997). Four omega-class *GST*s are present in the urchin genome, closely related to vertebrate and insect omega *GST*s with dehydroascorbate reductase, thioltransferase, and monomethylarsenate reductase activities (Whitbread et al., 2003). Omega-class *GST*s also are related to the onset of Alzheimer and Parkinson diseases (Schmuck et al., 2005). Seventeen urchin *GST* genes cluster with sigma-class *GST*s; in mammals, sigma GSTs are prostaglandin D synthases and are not primarily involved in detoxification mechanisms. The six urchin theta-class *GST* genes are most closely related to vertebrate theta-class *GST*s, rather than to the insect theta-class *GST*s, aiding the phylogenetic resolution of this widespread GST-family (Nebert and Vasiliou, 2004; Pearson, 2005). We found only one *GST-pi* (*SPU_024294*) and one *GST-zeta* (*SPU_025025*); GST-pi has been related to carcinogenesis in humans and mice (Henderson et al., 1998), whereas GST-zeta deficiency causes oxidative stress in mice (Blackburn et al., 2006). Embryonic and larval expression in untreated sea urchins varies among the toxicologically relevant *GST*s, with 5 alpha class, 3 theta class, all 4 omega class, and both the zeta and pi *GST*s expressed (Supplemental Table S5).

Microsomal GSTs (MGST, also known as MAPEG) are membrane-bound glutathione transferases that display wide substrate specificities similar to the cytosolic GSTs while being evolutionarily unrelated. Several MGSTs have specific roles in inflammatory responses in vertebrates (Trebino et al., 2003) and are involved in prostaglandin synthesis (Pettersson et al., 2005). Eleven full and one partially assembled *MGST*-like genes were found in the current urchin genome assembly (Table 2), representing all three classes of *MGSTs* (*MGST1*, *MGST2*, and *MGST3*). Nine are *MGST1*-like, only one is *MGST2*-like (*SPU_008286*), and the remaining two full and partially assembled genes are *MGST3*-like (*SPU_016492* and *SPU_023582*). In vertebrates, MGST2 may play an important role in *tert*-butylhydroperoxide resistance (Ma et al., 2004). Only 3 *MGSTs* are present in the human genome (Pearson, 2005). The expansion in *MGST1*-like genes in the urchin may be due to tandem duplication, because at least two gene clusters of *MGST1*-like genes (one of 4 genes and one of 2 genes) are present.

Sulfotransferase enzymes are broadly classified into cytosolic (soluble) and membrane bound SULTs. Cytosolic SULTs are responsible for the metabolism of xenobiotic and small endogenous substrates (SULT1 and SULT2), whereas membrane-bound SULTs are involved in endogenous peptide, lipid, and glycosaminoglycan sulfonation. SULT3 and SULT4 substrates have not yet been identified (Gamage et al., 2006). The 36 cytosolic *SULT* genes in the urchin genome are divided into two major divisions: 17 are *SULT1*-like and 19 are *SULT3*/*SULT4*-like (Supplemental Fig. S11). Phylogenetic analyses indicate that *SUTL1* diversification may have occurred independently in urchins and in vertebrates.

One arylamine *N*-acyltransferase (*NAT*)-like gene most similar to the two *NAT* genes in *C. intestinalis* appears in the urchin genome (SPU_012976), indicating that this family has not become diversified in sea urchins. In vertebrates, arylamine NAT enzymes catalyze the addition of an acetyl group to aromatic amines and hydrazines, a reaction generally resulting in detoxification (Boukouvala and Fakis, 2005). Multiple *NAT* homologs are present in bacterial and vertebrate genomes, including two arylamine NATs in humans mapped to the same chromosome locus (Boukouvala and Fakis, 2005), one of which has roles in neuronal development (Sugiura et al., 2003). Humans have eight other *N*-acetyltransferase genes, most of which do not have known functions.

The reducing and hydrolyzing Phase II enzymes are also represented in the sea urchin genome, and are slightly expanded relative to mammals (Table 2, Supplemental Fig. S12). There appear to be two membrane epoxide hydrolase (*EPHX*) genes similar to xenobiotic-inducible mammalian *EPHX1*, and three *EPHX* genes related to the vertebrate *EPHX2* (soluble EPHX) genes (mammals have two *EPHX* genes (Barth et al., 2004a,b; fatty acid epoxides are the major physiological substrates of EPHX2). Three sea urchin genes similar to mammalian epoxide hydrolase-related genes are also present; these genes do not have a known function.

Sea urchins possess 10 *AKR*-like genes but no *NQO*-like genes. This latter finding is in line with the observed lack of *NQO* genes in the worm, fly, sea squirt, or plants (Vasiliou et al., 2006). Six of the urchin *AKR*-like genes are most closely related to the vertebrate *AKR1* family genes (Supplemental Fig. S13), the products of which in mammals function as aldehyde and aldose reductases and dihydrodiol dehydrogenases, acting on PAH-diols formed by CYP and EPHX (Hyndman et al., 2003; Jez and Penning, 2001). The four additional *AKR*-like genes in urchin are more similar to invertebrate and fungal AKR genes than to vertebrate genes.

Antioxidant proteins

Reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radicals, can be derived from a variety of cellular processes and can contribute to diseases and pathologies generally due to altered gene expression or damage to biomolecules, including proteins, lipids, and DNA (Halliwell and Gutteridge, 1999; Lesser, 2006). General antioxidant defense genes include superoxide dismutase (SOD), catalase (CAT), and peroxidases, including glutathione peroxidases (GPXs), peroxiredoxin (PRDXs), and thioredoxins (TXNs). The sea urchin genome possesses one vertebrate-like SOD2 gene (mitochondrial manganese SOD; *SPU_024657*; see Table 3) and one manganese *SOD* gene that is more similar to bacterial Mn/Fe *SodM* (*SPU_021817*). Similar to chordates, there is one soluble Cu/Zn *SOD1* (*SPU_006214*). Urchins also posses a heme peroxidase important during fertilization, ovoperoxidase, which has superoxide dismutase activity (Heinecke and Shapiro, 1990; LaFleur et al., 1998) and is similar to human myeloperoxidase (Nomura et al., 1999). One CAT gene related to both insect and vertebrate CAT genes is present (*SPU_000281*). A variety of peroxidases are present, including 4 glutathione peroxidase genes, one of which is a single exon gene (*SPU_014373*), and one that is expressed during development (*SPU_014276*). At least three thioredoxin peroxidase genes are present (*SPU_006211*, *SPU_014869*, and *SPU_022529*), and expressed at high levels during normal development.

The glutathione pathway acts in both detoxification of xenobiotics via conjugating GSTs (see above) and in the detoxification of ROS via the glutathione–glutathione disulfide (GSH–GSSG) redox cycle. GSH must be supplied by reduction of GSSG by glutathione reductase (GSR), or by *de novo* synthesis via glutamate cysteine ligase (GCL) and glutathione synthase (GSS). GCL is a heterodimeric oxidative-stress responsive enzyme consisting of two subunits, a catalytic (GCLC) and a modifier (GCLM) subunit (Dickinson et al., 2004). In mammals, expression of both GCLC and GCLM is dependent on NRF2 (Dickinson et al., 2004; Gong and Cederbaum, 2006; Yang et al., 2005). The importance of GSH pathways in sea urchin development has been recognized for many years (Sakai and Dan, 1959), although there is no significant change in the overall GSH and GSSG concentrations during fertilization and early development (Fahey et al., 1976), possibly due to the importance of ovothiol as an antioxidant (Shapiro and Hopkins, 1991).

The urchin genome contains one homolog of both *GCLC* (*SPU_026291*) and *GCLM* (*SPU_002909*) which, along with *GSS* (*SPU_000830*), are expressed at high levels during developmental and larval stages (Table 3). *GSR* (*SPU_025989*) is also expressed at high levels during development, as has been known for some time (Ichio, 1977; Ii and Sakai, 1974a,b).

Metal detoxification

Three of the metal complexing genes found in the genome have previously been identified in sea urchins, the metallothioneins MTA, MTB1, and MTB2 (Nemer et al., 1991), which are active in response to excess metal stress but may also play roles in nutrient metal homeostasis and in apoptosis (reviewed in Vasak and Hasler, 2000). These three MT genes are all expressed at high levels in embryonic and larval sea urchins (Table 3). Interestingly, sea urchins possess an *SOD2*-like gene (*SPU_014006*) that may be related to the molluscan metal-binding proteins cavortin and pernin, which are hemolymph proteins with binding affinity for Fe but also Cu, Ni, Co, and Zn (Huvet et al., 2004; Scotti et al., 2001).

Other metal-binding genes in the urchin genome include a homolog of the Cu and Fe-binding protein ceruloplasmin (*SPU_027885*, also know as ferroxidase), a homolog of the Fe-binding protein transferrin (*SPU_026949*), and both heavy and light chains of the Fe storage protein ferritin (*SPU_004876* and *SPU_024366*). However, we have not been able to find in the current genome assembly homologs of the important heme-detoxification enzyme heme oxygenase 1 (*HMOX1*) or

Table 3
Gene families responsible for protection from oxidative damage and metal toxicity

Classification	Predicted *S. purpuratus* homolog	*S. purpuratus* gene model	Gene similarity or identity	Embryonic/larval expression [a]
Oxidative damage	*Sp-Sod*	*SPU_006214*	SOD1 (Cu/Zn)	+++
	Sp-Sod2	*SPU_021817*	SOD2 (Fe/Mn)	+
	Sp-Sod2b	*SPU_024657*	SOD2 (Fe/Mn)	−
	Sp-Opo	*SPU_016914*	Ovoperoxidase	
	Sp-Cat	*SPU_000281*	Catalase	+++
	Sp-Gpx1	*SPU_004397*	Glutathione peroxidase 7	−
	Sp-Gpx2	*SPU_014276*	Glutathione peroxidase 4	+
	Sp-Gpx3	*SPU_014373*	Glutathione peroxidase 1	−
	Sp Gpx4	*SPU_028532*	Glutathione peroxidase 2	++
	Sp Prx4	*SPU_006211*	Peroxiredoxin 4	+++
	Sp Prx2	*SPU_014869*	Peroxiredoxin 2	+++
	Sp Prx3	*SPU_022529*	Peroxiredoxin 3	++
Metal complexing	*Sp Mta*	*SPU_017989*	Metallothionein A	+++
	Sp Mtb1	*SPU_001866*	Metallothionein B1	+++
	Sp Mtb2	*SPU_017134*	Metallothionein B2	++
	Sp Cavortin	*SPU_014006*	Cavortin/pernin	+++
	Sp Cp	*SPU_027885*	Ferroxidase (ceruloplasmin)	++
	Sp Tf	*SPU_026949*	Transferrin	+++
	Sp Fth1	*SPU_004876*	Ferretin–heavy subunit	+++
	Sp Ftl	*SPU_024366*	Ferretin–light subunit	+++
	Sp Pcs1	*SPU_004431*	Phytochelatin synthase	+
	Sp Pcs2	*SPU_022493*		−
	Sp-Pcs3	*SPU_023132*		−
Glutathione cycling	*Sp-Gclc*	*SPU_026291*	Glutathione cyclase	+++
	Sp-Gclm	*SPU_002909*	Glutathione cyclase	+++
	Sp-Gss	*SPU_000830*	Gluathione synthase	++
	Sp-Gsr	*SPU_025989*	Glutathione reductase	+++

[a] Pooled embryonic and larval expression is derived from the whole genome tiling array of (Samanta et al., 2006). Relative expression is presented as the highest signal measured at an annotated exon: "−" below mean noise (1.3 units); "+", 1.3–10 units; "++", 11–20 units; "+++", greater than 21 units.

biliverdin reductase (*BLVR*), the second enzyme in heme degradation, despite the presence of a heme biosynthetic pathway as indicated by the gene encoding 5-aminolevulinate synthase, the rate-limiting enzyme in this pathway (Duncan et al., 1999) and other genes involved in heme biosynthesis, as well as heme proteins (e.g., CYP). *S. purpuratus* may possess alternative mechanisms of heme turnover and detoxification.

Phytochelatins are metal-binding peptides composed primarily of GSH groups that are important metal detoxifying genes in plants and fungi. It was believed that phytochelatins were present only in plants and fungi until phytochelatin synthase (PCS) was discovered in the nematode *C. elegans* (Clemens et al., 2001), and now it is clear that many other lineages contain PCS homologs (Clemens, 2006). Three *PCS* genes are present in the urchin genome (*SPU_004431*, *SPU_022493*, and *SPU_023132*), four *PCS* homologs are present in the genome of the sea anemone *Nematostella vectensis*, and one *PCS* gene is present in the genome of the tunicate *C. intestinalis*. Vertebrate genomes do not contain a gene homologous to *PCS*, suggesting that phytochelatin synthesis ability was lost some time after the divergence of tunicates from the lineage leading to the vertebrates.

Active efflux of toxic metals is another route to detoxification. Both SLC22 and ABC efflux proteins (see above) export metals directly or as glutathione (GSH) conjugates (Leslie et al., 2001; Sweet, 2005), and the urchin genome contains multiple genes in the subfamilies known to be involved in resistance to heavy metal toxicity.

Heat shock proteins

Heat shock proteins (HSP) have been implicated in the response to various toxicants including heavy metals, such as cadmium and arsenic, and free radicals (Feder and Hofmann, 1999). The induction of HSP mRNA and protein appears part of generalized cellular stress responses via heat shock factor (HSF). Sea urchins have several families of heat shock genes including *HSP 100*, *90*, *70*, *40*, and small α-crystalline *HSP*s (*HSP20*s). The major family of heat shock proteins is the *HSP70* family, containing at least 17 genes with at least 10 *HSP40/DNAJ* co-chaperones. Sea urchins also have at least 4 *HSP90* genes and 2 small α-crystalline *HSP20*s. *HSP* genes appear to be highly expressed during development, based on the tiling array data (Samanta et al., 2006); roughly 80% of the *HSP*s showed significant signals on the array. Notably, the array suggests that all four *HSP90* genes (*SPU_001586*, *SPU_020322*, *SPU_024082*, and *SPU_027527*) show very high levels of expression during development. This developmental expression may be related to a role for HSP90 in masking genetic variability in eukaryotic embryos (Queitsch et al., 2002). More recently, HSP90 has been shown to stabilize client proteins that are targets for toxicants, suggesting that HSP90 mediates the acquisition of drug

resistance by stabilization of other proteins (Cowen and Lindquist, 2005).

Discussion

The chemical defensome is an integrated network of genes and pathways that allow an organism to mount an orchestrated defense against toxic chemicals, including exogenous compounds (xenobiotics) and endogenously produced toxicants, such as free radicals. The term "defensome" has been used previously in the plant infection literature (de Torres et al., 2003), and it has been narrowly defined as a Rac GTPase-mediated dynamic plant immunity complex. Although we consider only the chemical defense genes in this paper, the usage in the plant infection field is contained within the broader overall defensome introduced here.

Almost all of the gene families or superfamilies contributing to chemical defense in vertebrates are also represented in the sea urchin. Similarly, many of the gene families or superfamilies that participate in the defensome in deuterostomes are found in protostomes, suggesting the origin of an orchestrated chemical defense in a bilaterian ancestor.

While there is general conformity in the presence of higher order gene groups across taxa, in most cases gene orthology is more difficult to determine. Orthologous relationships are discernable among the PAS proteins and among the ABC transporters. Such distinct relationships are less evident among the *CYP* genes, or amongst the *GST*, *SULT*, and *UGT* genes. Further examination comparing gene structure and synteny could reveal other orthologous relationships. Furthermore, gene orthologies may or may not be predictive of function, as seen with Sp-ABCC9A. The observed expansion of gene families compounds the difficulty in reliably classifying genes within these families or inferring function.

Gene family diversification

Many defense gene families have undergone diversification and expansion in the sea urchin. For example, in urchins there are 73 *CYP* genes related to the vertebrate *CYP2* family, larger than the 16 *CYP2s* known in humans or most other vertebrates (*M. musculus*: 51, *R. rattus*: 50, *B. taurus*: 23, *T. rubripes*: 17). However, it is possible to assign but a single urchin *CYP2* gene, *Sp-CYP2U*, to a subfamily represented in vertebrates. This result is not unexpected, as there are few *CYP2* subfamilies that occur in common even among some vertebrate groups, for example between fish and mammals (Nelson, 2003). Although far fewer in absolute numbers, both the *CYP3*- and the *CYP1*-related genes in urchin show a similar, or even greater degree of expansion relative to the number of *CYP3* or *CYP1* genes in vertebrates. As with the *CYP2*s, the current analyses do not indicate the subfamily relationships. Such diversity and lack of clear orthology confound the consideration of catalytic function of the CYP enzymes, i.e., their specific roles in the biotransformation of endobiotic and xenobiotic chemicals.

The total number of *ABCB* genes is similar across deuterostomes, with most species containing 1 or 2 *ABCB1*-like genes. Urchins have two *ABCB1* genes, the same as in fish and rodents. In contrast, in tunicates there are 5 *ABCB1* (*PGP*) subfamily genes (Annilo et al., 2006). Interestingly, there are no *ABCC2* genes in either urchins or tunicates; this gene subfamily may be specific to vertebrates. Given the expansion of the *ABCC8* and *9* subfamilies in urchins, we were surprised to find that these families appear to be absent in tunicates (Annilo et al., 2006). Whether this expansion in urchins is specific to *S. purpuratus* or is a general feature of all echinoderms is unknown.

Whether the greater number of genes in some gene families in the urchin (Fig. 7) is due to gene loss in other (e.g., chordate) lineages or to specific expansion in echinoderms is unclear. If the latter, it could suggest a more diverse array of chemicals in the environment of the organisms, or a more diverse set of endogenous substrates, perhaps including substrates that have some role in development. In vertebrates, CYPs in gene families other than 1–4 play roles in development, e.g., CYP26 and the oxidation of retinoids. However, vertebrate CYP1, 2, and 3 family members also oxidize prostaglandins, eicosanoids, and retinoids (Nebert, 1991; Puga et al., 2005; Stoilov et al., 2001) and could contribute to developmental control in urchins as well, as evinced by their expression during normal urchin development. The efflux during development of cyclic nucleotides or lipid-derived signaling molecules, such as platelet-activating factor or leukotrienes, may be involved in the expansion of *ABCC9* and *ABCC5* gene families.

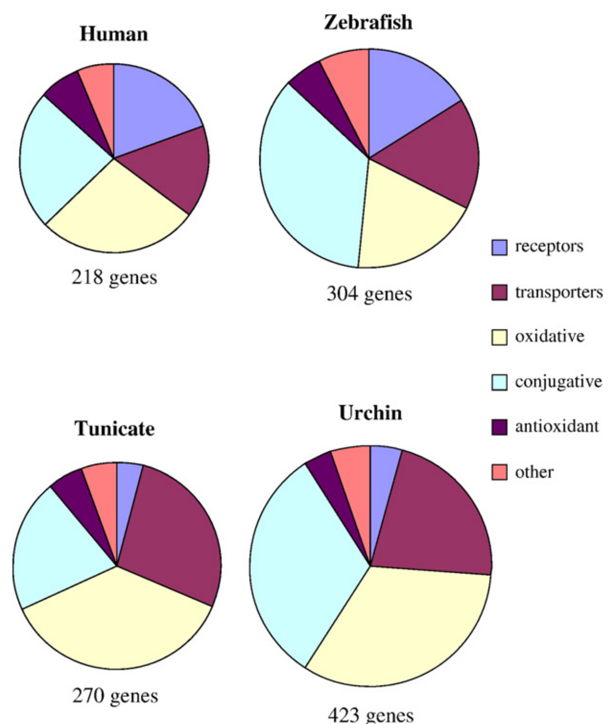

Fig. 7. Gene count comparisons for various classes of defensome genes. The area of each circle is proportional to the total number of genes classified into the defensome. Receptors include *bHLH-ZIP*, *NR*, and *CNC* receptors. Transporters are *ABC* and *OAT* transporters; oxidative modification genes include *CYP*, *FMO*, and *ALDH*; conjugative genes are *GST*, *MGST*, *UGT*, *SULT*, and *NAT*; antioxidant genes are *SOD*, *CAT*, *PXR*, and *GPX*. Other genes include *MT*, *AKR*, *EPHX*, and *NQO*.

Antioxidant gene families are also somewhat expanded in urchins relative to vertebrates. The presence of three *SOD* genes and an unusual peroxidase, ovoperoxidase, with SOD-like activity (Heinecke and Shapiro, 1990), provides further evidence that ROS may be an important stressor to sea urchins. ROS have been implicated in the mechanisms of chemically induced disease, often resulting from direct redox cycling of toxic compounds but also produced as a result of 'leakage' from oxidative metabolism (Halliwell and Gutteridge, 1999).

The sea urchin embryo produces hydrogen peroxide at fertilization to facilitate the hardening of the fertilization envelope (Wong et al., 2004; Wong and Wessel, 2005), and their antioxidant mechanisms have been well studied (see Epel, 2003; Shapiro, 1991). Sea urchins posses antioxidants such as glutathione (Sakai and Dan, 1959), carotenoids (Griffiths, 1966; Griffiths and Perrott, 1976), tocopherols, ascorbic acid (Backstrom, 1957), and a unique embryo-specific thiol named ovothiol (Shapiro and Hopkins, 1991; Turner et al., 1986, 1988). These small molecule antioxidants are present in eggs at high concentrations (GSH and ovothiol total ~10 mM; Turner et al., 1986, 1988) and remain high throughout development (Schomer and Epel, 1998). ROS production can also be an important consequence of UV exposure (Lesser, 2006). Urchin embryos exposed to environmental levels of UV significantly upregulate SOD and undergo apoptosis (Lesser et al., 2003).

Diversity and conservation in signaling

Metazoan proteins containing a PAS domain are transcription factors involved in signaling during development, establishing and maintaining circadian rhythms, and sensing environmental variables such as oxygen and small molecules (e.g., hormones and xenobiotics) (Gu et al., 2000; Kewley et al., 2004). Most, but not all, of the PAS genes also possess a bHLH motif, consistent with their activity as heterodimeric DNA-binding proteins. In contrast to the apparent diversification in several active defense gene groups, PAS gene diversity in *S. purpuratus* is similar to that found in other invertebrate genomes, including the chordate *C. intestinalis*, but lower than that of vertebrates (Supplemental Table S1). The PAS gene family has undergone expansion in vertebrates, with two or three paralogs for each of the genes found in the genomes of *D. melanogaster*, *C. elegans*, and *C. intestinalis* (Hahn, 2002; Hahn et al., 2006).

Two predicted *AHR*-like genes were identified in the *S. purpuratus* genome; efforts to confirm these predicted sequences, and to complete the partial model for *Sp-Ahrl,* are underway. One of these *AHR* homologs (*Sp-Ahr; SPU_013788*) is more closely related to the vertebrate AHR, a ligand-activated transcription factor that regulates the expression of xenobiotic-metabolizing enzymes such as CYPs in response to dioxins, polycyclic aromatic hydrocarbons, and a variety of other compounds (Nebert et al., 2004). The other putative *AHR* homolog (*Sp-Ahrl; SPU_012296*) shares the greatest sequence identity with *Drosophila spineless* (*ss*). The latter was originally identified because of its role in the specification of distal antennal and leg segments (Duncan et al., 1998) and is involved in the specification of photoreceptor identity in the fly retina (Wernet et al., 2006). The developmental roles of ss, of the *C. elegans* AHR homolog AHR-1 (Huang et al., 2004; Qin and Powell-Coffman, 2004), and of the mammalian AHR (Walisser et al., 2005) are consistent with suggestions that AHR functions include ligand-dependent developmental signaling (Nebert, 1991; Puga et al., 2005). However, unlike the vertebrate AHRs, the known invertebrate AHR homologs do not bind typical ligands of the vertebrate AHR (Butler et al., 2001; Powell-Coffman et al., 1998), suggesting an important distinction in AHR functions that is not well understood. The possible existence of duplicated *AHR* genes in *S. purpuratus* is especially intriguing, and could clarify the evolutionary origin of the distinct roles of this protein in metazoans (Hahn, 2002).

Two predicted sea urchin *HIF* genes share sequence similarity with vertebrate *HIF1*α and *HIF2*α (*EPAS1*) genes, but whether each of the urchin genes is orthologous to its most closely related vertebrate homolog is not yet clear. HIF proteins mediate the response to low oxygen through a signaling mechanism that is evolutionarily conserved and required for development in most metazoans (Lavista-Llanos et al., 2002; Shen et al., 2005). HIF signaling may involve reactive oxygen, and there is some overlap between the target genes of the HIF and CNC proteins (see below) (Hu et al., 2003; Scortegagna et al., 2003). Conceivably, one or both Sp-HIFα proteins could contribute to the oxidative stress response.

An oxidative stress response involving CNC-related proteins appears to be conserved in animals, although there are differences in the specific roles and mechanisms involved (An and Blackwell, 2003; Veraksa et al., 2000; Walker et al., 2000). The four mammalian CNC-bZIP proteins possess distinct but overlapping functions. NFE2 is a hematopoietic-specific transcription factor that regulates globin gene expression (Andrews et al., 1993; Ney et al., 1993) but may also regulate an oxidative stress response in mature erythrocytes (Chan et al., 2001). *NRF1*, *NRF2*, and *NRF3* (*NFE2L1*, *NFE2L2*, and *NFE2L3*) are more widely expressed (Chan et al., 1993) and function both in the oxidative stress response (Kwong et al., 1999; McMahon et al., 2001) and during development (Kobayashi et al., 1999; Leung et al., 2003). Thus, the single *CNC* and small *Maf* genes in *S. purpuratus* are likely to resemble their homologs in other metazoans in having dual functions during development and in protection against oxidative stress.

S. purpuratus contains a number of nuclear receptor genes, including several with potential roles in defense. In vertebrate animals, genes in the *NR1* family (*PXR*, *CAR*, *LXR*, *FXR*), regulate xenobiotic- and endobiotic-inducible adaptive responses involving *CYP*, *ABC*, and other genes encoding biotransformation enzymes and transporters (Bock and Kohle, 2004; Klaassen and Slitt, 2005; Krasowski et al., 2005; Xie and Evans, 2001). Similar functions as xenobiotic sensors are not known to be prominent for invertebrate NRs, but arthropod ecdysone receptors (EcR, NR1H1) and the *C. elegans* nuclear receptor DAF-12 (NR1J) mediate endobiotic (steroidal) signaling important to reproduction and development and involving CYPs (Motola et al., 2006). *Sp-Fxr* is most closely related to

vertebrate *FXR* and *LXR*, which are activated by oxysterols and bile acids, respectively (Eloranta and Kullak-Ublick, 2005; Handschin and Meyer, 2005), suggesting a role in endobiotic signaling. The apparent absence of urchin genes related to *NR1I* genes (*PXR*, *CAR*), which are key xenobiotic sensors in vertebrates, suggests an important difference in sensor repertoire between these deuterostome groups. However, the existence of a set of novel *NR1H*-related genes, possibly forming a new subfamily, raises the possibility that these genes may be involved in xenobiotic sensing—an important question for future research.

Embryos lack specialized tissues and organs that are integral to adult stress and toxicant response. At the earliest stages of development, each cell must carry out all of the protective functions typically carried out by multiple cells or tissues in an adult. Therefore, chemical defense genes may be especially critical for resilience and survival of these early stages. Some defense gene products may be packaged into eggs during oogenesis to provide adequate defense during early cleavage stages when divisions are rapid and nucleus:cytoplasm ratios are low (Epel, 2003; Giudice et al., 1999; Hamdoun et al., 2004), a period in which gene expression is unlikely to result in efficient upregulation of protein concentration. Later in development the defense genes are synthesized both constitutively and in response to environmental stress.

The defense genes operate in a network regulated by the stress-sensing transcription factors. Recent work has shown that, in yeast, environmental responses such as stress responses have shorter regulatory cascades than endogenous functions such as cell cycle control, implying faster signal propagation (Lunscombe et al., 2004). Those results indicate that transcription factors governing a stress response have greater regulatory influence, and that they regulate in simpler combinations. The delay between activation and response in transcriptional cascades is also shorter (fewer cell cycles) for sensory responses than for developmental patterning, perhaps reflecting the irreversibility of patterning (Rosenfeld and Alon, 2003). The connectivity, path-length, and interconnectivity of this stress-sensing and response network are greater in humans than in yeast (Lee et al., 2002), an evolutionary distance bridged, in part, by sea urchins.

Two important factors may be at work in defensome gene regulation: coregulation and redundancy. Coordinate regulation of defensome genes, such as AHR regulation of CYPs and UGTs or the coregulation of CYP3A4, UGT1As, and ABCC2 by CAR/PXR in mammals (Bock and Kohle, 2004; Xu et al., 2005), may be a method of accelerating the response to toxicant exposure. Redundancy of the signal transduction may also be an important mechanism for rapid responses, allowing many different types of stress signals to lead to generalized cellular stress response. For example, in humans, ABCB1 is induced by a large variety of cues, including protein denaturing stressors, ensuring virtually ubiquitous inducibility (Shtil and Azare, 2005). The expansion of gene families such as ABCC is not reflected by a corresponding expansion in the numbers of nuclear hormone receptors (Fig. 7). The regulatory proteins governing the expression of many defensome genes are not known even in vertebrates, and thus there could be transcription factors yet to be described that are involved in regulation of the active stress defense genes in sea urchin.

A special challenge in embryonic development may be the integration of the regulation of defensive genes with the gene programs that are active during differentiation. Receptor activation by xenobiotic ligands may interfere with the endogenous ligand-dependent developmental signaling, particularly via AHR, bZIP-CNC, and NR proteins. Thus, whereas an organism may be adapted to an “anticipated” environment, exposure to high concentrations of xenobiotic compounds may alter developmental cascades enough to cause teratogenesis and lethality.

Many of the gene products presented here are also involved in protection from environmental factors, such as oxidative stress, that lead to senescence. Understanding the action of these genes in sea urchins should provide insights into the mechanisms that result in the lowered senescence and increased longevity (>100 years) observed in adult sea urchins (Ebert and Southon, 2003). A similar relationship between defense genes and longevity genes has been seen in the *C. elegans* genes that control dauer formation, a whole organism stress response during development (Antebi et al., 2000; Yabe et al., 2005).

The sea urchin is an excellent model for the study of the problem of cellular defense during embryonic development, due to the technologies established for genetic manipulation (e.g., control of transcription factor expression; Bogarad et al., 1998) and to the extensive work done to determine gene regulatory networks (Longabaugh et al., 2005). Development inherently can be a robust process; which parts of the process are more susceptible to disruption, and from which stressors, is not clear. The description of this defense gene set will allow us to test when and how embryos can mount generalized cellular stress responses and to understand how these stress responses are coordinated by the action of conserved stress sensing transcription factors. Despite the fact that the individual genes within the network may vary across organisms, this network may be comprised of evolutionarily conserved modules, “kernels”, which are retained across evolution (Davidson and Erwin, 2006; von Dassow et al., 2000). If these modules prove to be highly conserved, xenobiotics that disrupt them could pose a particular challenge to cellular homeostasis in a broad range of animals. Comparing the susceptibility of sea urchin embryos with that of vertebrate embryos, for a range of chemicals, could lead to fundamental insights into how these defensome kernels function to protect deuterostome embryos from the myriad chemical challenges that could derail development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.066.

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