Echinodermata: The Complex Immune System in Echinoderms

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Echinoderm Life History and Phylogeny

Echinoderms are benthic marine invertebrates living in communities ranging from shallow nearshore waters to the abyssal depths. Often members of this phylum are top predators or herbivores that shape and/or control the ecological characteristics

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of their habitats. Five classes are defined within echinoderms: Crinoidea (sea lilies and feather stars), Ophiuroidea (brittle stars), Asteroidea (sea stars and sea daisies), Holothuroidea (sea cucumbers), and Echinoidea (sea urchins and sand dollars) (Fig. 1a–e). As a consequence of rapid divergence that occurred shortly after the origin of the echinoderm phyla, which emerged an estimated 570 million years ago (Pisani et al. 2012), the phylogenetic relationships among the classes have been difficult to establish, and vary depending on the data set and phylogenetic methods (Fig. 1f) (Janies et al. 2011). Crinoids are unequivocally the basal group, whereas the monophyly of asteroids and ophiuroids remains in debate. The Echinodermata and Hemichordata phyla constitute the Ambulacraria, which is the basal group

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within the deuterostome lineage (Blair and Hedges 2005) and sister to the chordates (Fig. 1g). The nearly 8000 extant echinoderms (from the Greek, meaning "spiny skin") share several aspects of life cycle and body plan. Most echinoderm species develop indirectly through a dispersal-stage planktonic larva, which exhibits bilateral symmetry and swims and feeds through the activities of surface cilia and the ciliary band. Metamorphosis occurs when the larva descends to the benthos from the littoral zone and everts the adult rudiment into a juvenile with five spines and five tube feet. Most adults share the characteristics of a radial body plan with pentameral symmetry, a rigid calcite endoskeleton, and a water vascular system that functions as a hydraulic mechanism for tube foot extension and locomotion, sensory activity (including responses to light/dark (Ullrich-Lüter et al. 2011), and food capture (Hyman 1955).

Early Evidence for Echinoderm Immune Responses

Allograft Rejection Defines an Innate Immune System in Echinoderms

For centuries, the prevailing theory was that animals without backbones lacked immune systems. Invertebrates infected with pathogens either recovered or were deleted from the gene pool, and populations remained at steady state as a consequence of a large number of offspring. When this assumption was first challenged, the most straightforward strategy to identify immune responses in echinoderms was to use allograft rejection experiments designed to determine whether individuals could differentiate between self and nonself. When skin allografts and autografts were transplanted among individuals of the sea star *Dermasterias imbricata*, allografts were always rejected, whereas autografts were accepted and healed into the grafted site (Fig. 2a, b) (Hildemann and Dix 1972; Karp and Hildemann 1976).

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Similar experiments using body wall transplants demonstrated that nonself-recognition mechanisms are also present in the white sea urchin *Lytechinus pictus* (Coffaro and Hinegardner 1977). Although the kinetics of the second-set allograft rejections are significantly faster than the first-set rejections in this species, the kinetics of first-set versus third-party rejections are identical, suggesting a lack of specific immune memory (Fig. 2c) (Smith and Davidson 1992). This demonstrated that echinoderms have an immune system that is efficient for host protection, but that it functions with innate mechanisms in the absence of adaptive capabilities.

Foreign Particles Are Cleared Rapidly from the Coelomic Cavity

The use of allograft rejection to demonstrate immune capabilities in echinoderms, albeit effective, is an artificial experimental approach. Unlike the well-established systems in colonial tunicates and hydroids (Nydam and DeTomaso 2011; Rosengarten and Nicotra 2011; Taketa and DeTomaso 2015), natural allografts do not occur in echinoderms. As an alternative approach to assess nonself recognition capacity, echinoderms have been evaluated for their abilities to clear foreign particles and cells injected into their coelomic cavity. A wide range of particles and molecules have been employed in these studies, including foreign proteins, T4 bacteriophage, carbon particles, carmine, Sephadex, and latex beads, which are all cleared effectively (reviewed in Smith and Davidson (1994)). In response to injection with sheep red blood cells (RBCs), the sea cucumber *Holothuroidea polii* clears these cells within 8 days, which correlates with the appearance of dark brown bodies in the coelomic cavity (Canicatti and D'Ancona 1989). These bodies are consistent with aggregates of hemoglobin from the RBCs encapsulated by coelomocytes that have activated phenoloxidase activity and melanin production. In the sea urchin *Strongylocentrotus nudus*, intracoelomic injection of sheep RBCs is followed by the production of reactive oxygen intermediates (Ito et al. 1992). Furthermore, the rate of RBC phagocytosis is increased as a consequence of preincubation or opsonization with *Strongylocentrotus nudus* coelomic fluid (CF). Echinoderms also respond to foreign cells from other species within the phylum, and the injection of coelomocytes from the sea urchin *Arbacia punctulata* into the sea star *Asterias forbesi* results in the swift clearance of the sea urchin cells and corresponds to a transient decrease in the sea star coelomocytes (Reinisch and Bang 1971). A more

Fig. 1 (continued) *Patiria miniata*. (**e**) The Ophiuroidea are represented by the brittle star *Amphiura filiformis*. (Reprinted from Arnone et al. (2015).) (**f**) There are two possible relationships among the echinoderm classes. Two total evidence trees include nuclear sequences (18S RNA, 28S RNA, H3 histone genes), mitochondrial sequences (12S RNA, 16S RNA, tRNA cluster, cytochrome c oxidase 1), and morphological characters. The left tree is the result of direct optimization, whereas the tree on the right results from the 50% majority rule in MRBAYES. Both trees are modified from Janies et al. (2011) to show the differences in the relationships among the classes. (**g**) A simplified phylogenetic tree of the Deuterostomia shows the relationships between the Ambulacraria (which include the Echinodermata) and the Chordata. The Protostomia are indicated as the outgroup

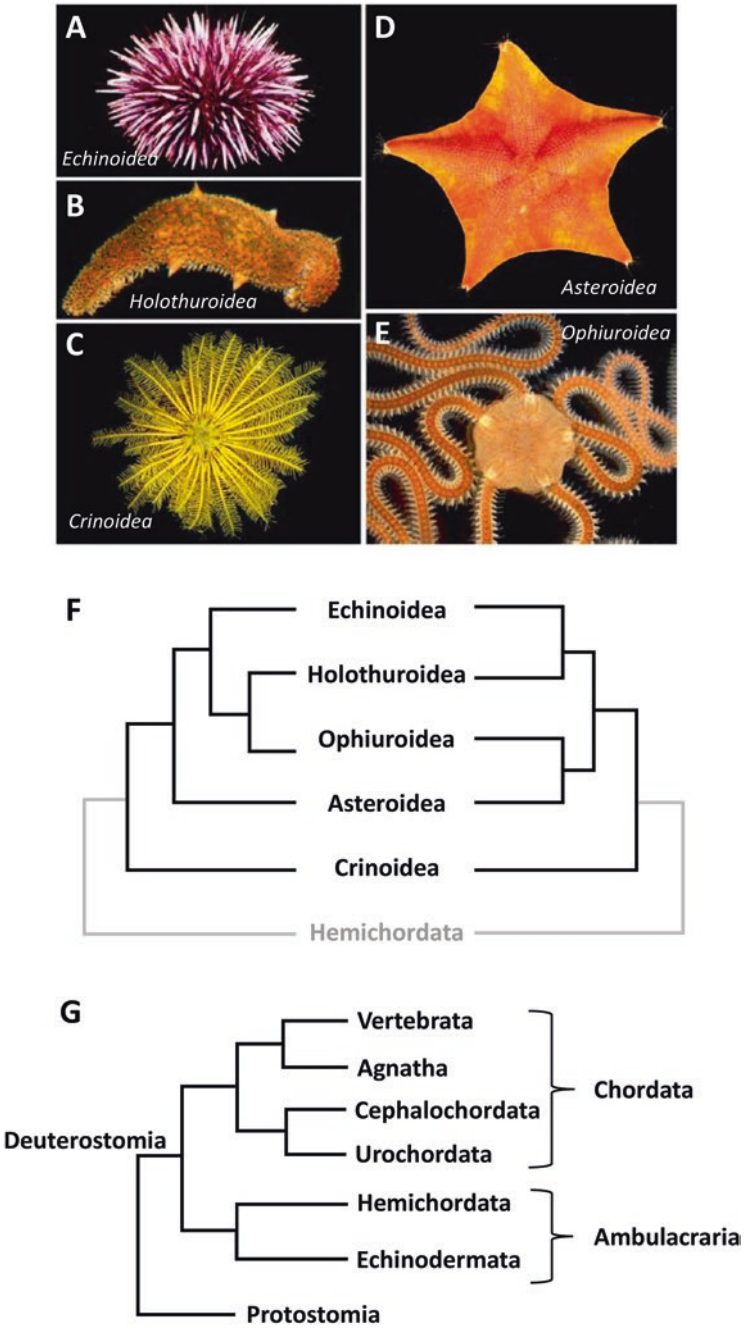

Fig. 1 *Five classes of the Echinodermata*. (**a**–**e**) Species from each of the five classes of echinoderms are shown. (**a**) The Echinoidea comprise sea urchins and sand dollars, and are represented by the purple sea urchin, *Strongylocentrotus purpuratus*. (**b**) The Holothuroidea are represented by the sea cucumber *Parastichopus parvimensis*. (**c**) The Crinoidea are represented by the sea lily *Oxycomanthus intermedius*. (**d**) The Asteroidea comprise sea stars and sea daisies, and are represented by the bat star,

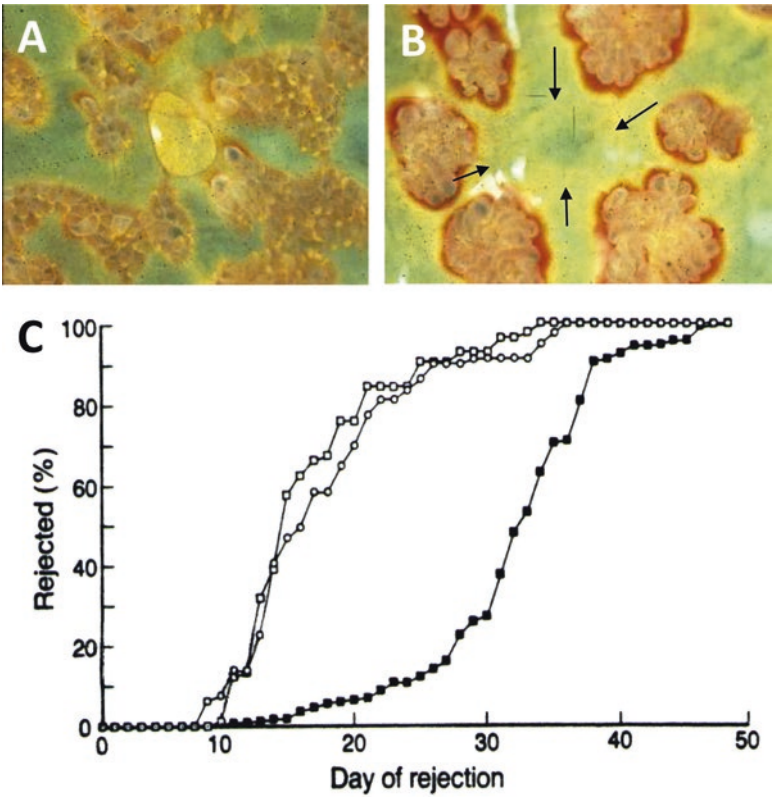

Fig. 2 *Tissue transplantation establishes echinoderm immune activity as innate in character*. (**a**) Allograft rejection in the sea star *Dermasterias imbricata*. Skin transplantation shows rejecting allograft tissue that is lighter in color than the normal green color of the sea star skin. (Modified from Karp and Hildemann (1976).) (**b**) Autograft fusion in *D. imbricata*. The control autograft shows fused tissue that is the same color as normal sea star skin. The edges of the accepted graft are indicated by arrows. The reddish areas on the surface of the sea star are normal dermal papillae. (Modified from Karp and Hildemann (1976).) (**c**) Allograft rejection of body wall transplants in the sea urchin *Lytechinus pictus* demonstrates innate immune function in echinoderms. About half of the first-set allografts (black boxes) are rejected by 35 days, whereas about half of the second-set allografts (open squares) are rejected by 15 days. However, the rejection kinetics for the third-party allografts (open circles) are the same as those for the second-set allografts, indicating a lack of specific immune memory. (Reprinted from Smith and Davidson (1992), with permission from Elsevier)

immunologically relevant approach to characterize the immune response, however, is to assess the ability of echinoderms to clear pathogenic bacteria from the coelomic cavity. Injections of unidentified marine bacteria or a freshwater fish pathogen into the coelomic cavity of the purple sea urchin, *Strongylocentrotus purpuratus*, results in clearance of 90–99% of the microbes within 3–6 h (Yui and Bayne 1983). Similarly, the congeneric *Strongylocentrotus droebachiensis* clears the echinoid

pathogen *Vibrio anguillarum* in 10 h (Plytyzc and Sejelid 1993). In both species, clearance of injected microbes is accompanied by a transient decrease in the numbers of coelomocytes in the coelomic cavity. Together, these results suggest that the transient decrease in the coelomocyte concentration in the CF in response to injections of foreign cells is a measurable parameter of phagocytosis and encapsulation, and illustrates that coelomocytes in echinoderms are the primary mediators of immune response.

Diseases of Adult Echinoderms

Mass Mortality Events in Echinoderms

The presence of an active immune system in echinoderms is evident from diseases that manifest as symptoms and death of large populations. However, the survival of the phylum in general (and the recovery from diseases by certain species in particular) is based on their immunological responses to those diseases. Although echinoderms do not generally succumb to bacteria that are injected experimentally, echinoderm diseases in nature have led to mass die-offs, which have been recorded for coastal species in shallow habitats and are often associated with sudden and dramatic ecological fluctuations. Overarching forces that tend to drive fluctuations in marine habitats stem from natural events such as hurricanes (which detach warm-core rings from the Gulf Stream and move warm water into the cold coastal waters of the northwestern Atlantic) and El Niño phases (which push warm water up and down the North American Pacific coast) (Scheibling and Hennigar 1997; Burge et al. 2014). Environmental fluctuations such as the quick changes in water temperature and salinity that are induced by these forces likely increase stress and reduce echinoderm resistance to waterborne pathogens, which leads to disease outbreaks and mass mortality events (reviewed in Burge et al. (2014); Jurgens et al. (2015)). Perhaps the greatest threat to the health of the coastal benthic community is the impact of increased human populations near coastlines, tourism, and commercial overfishing (Blois et al. 2013; Moritz and Agudo 2013; Norris et al. 2013; Stocker et al. 2013). Continued anthropogenic global climate change will result in extinctions, reduced species diversity, and drastic changes in ecosystems (Blois et al. 2013; Moritz and Agudo 2013; Norris et al. 2013), as well as an increased likelihood of disease outbreaks (Burge et al. 2014; Harvell et al. 1999).

Numerous disease-causing microorganisms have been isolated from echinoderms that maintain either pathogenic or symbiotic interactions with their hosts. These agents include bacteria, fungi and yeast, viruses, and parasites including amoebas, ciliates, cyanophytes, flagellates, apicomplexans, haplosporidians, algae, mesozoans, sponges, cnidarians, entoprocts, nematodes, turbellarians, trematodes, annelids, polychaetes, gastropods, bivalves, barnacles, crustaceans, amphipods, copepods, pycnogonids, tardigrades, bryozoans, and pearl fish (Turton and Wardlaw

1987; Jangoux 1990; Stein and Halvorsen 1998; Gudenkauf et al. 2014). Although these pathogens and parasites have been isolated from various echinoderms, few have been linked to a specific disease phenotype (e.g., spotted gonad disease in the sea urchin *Strongylocentrotus intermedius* (Shimizu 1994)) or have been verified experimentally as the pathogen of a mass mortality event. Thus, most of the reported mass mortalities are due to unknown or unobserved agents (reviewed in Lawrence (1996); Schultz (2016)).

The largest and most widespread report of a massive mortality event, and for which the pathogen remains unknown, occurred in populations of the long-spined black sea urchin, *Diadema antillarum*, in the Caribbean and Western Pacific near Central America. This species suffered a sudden massive die-off in 1982–1984 with repeated die-offs in 1985 and 1991–2, resulting in population declines ranging from 85% to 100% in localities across the entire Caribbean (Bak et al. 1984; Lessios et al. 1984; Lessios 1988; Moses and Bonem 2001; Hughes et al. 1985). The only populations of *D. antillarum* that were unaffected by this event were located outside the Caribbean, in the eastern Atlantic Ocean (e.g., in the Canary and Portuguese Islands (Lessios 1988)). No other Caribbean sea urchin species were disturbed during this time, suggesting that the causative agent infected *D. antillarum* specifically (Lessios et al. 1984). Although the involvement of pollution and global warming could not be excluded, early reports of these events speculated about a pathogenic cause (Liddell and Ohlhorst 1986) and a waterborne pathogenic causative agent was supported by the path of infection, which followed surface water currents (Lessios 1988). Both viral (Gudenkauf et al. 2014) and microbial (Bauer and Agerter 1987) pathogens were proposed; however, isolates from *D. antillarum* resulted in many microbial organisms, of which any might have been the lethal pathogen (Bauer and Agerter 1994). Mass die-offs of an apex herbivore have an immediate and significant impact on the ecology of coral reefs, which shift from predominantly coral to algal cover. This outcome followed predictions from experimental exclusion of *D. antillarum* from a reef patch, which released algal growth control from herbivory, so corals were outcompeted and smothered (Sammarco 1980). On the larger format of the Caribbean basin, removal of the top herbivore resulted in drastic ecological changes in the reef communities and fish populations (Lessios 1988; Carpenter 1988, 1990; Robertson 1991). As the populations of *D. antillarum* slowly recover, the species has become a key indicator for the recovery of reef communities because they promote the recruitment of new reef-building corals (Ogden et al. 1973; Edmunds and Carpenter 2001) and continued ecological changes in the reefs.

Bald Sea Urchin Disease

A few echinoderm disease outbreaks have been linked to specific pathogens (Becker et al. 2008). One example is the bald sea urchin disease, a bacterial infection that affects several species of sea urchins (e.g., *Mesocentrotus franciscanus*, *S. purpuratus*, and *Paracentrotus lividus)* (Pearse et al. 1977; Maes and Jangoux 1984; Becker et al. 2007; Johnson 1970). (For a revision to the strongylocentrotid family, see

Kober and Bernardi (2013)). A resurgence of bald sea urchin disease was noted in *S. purpuratus* near Santa Barbara, California, during the winter of 2016–2017, coincident with an El Niño event (Pierre and Smith, personal observations, 2016). The disease is characterized by lesions typically associated with injuries or abrasions on the lateral and oral surfaces of the animal. Subsequent surface infections show loss of spines and other appendages, including tube feet and pedicellariae, and appear as bare (or bald) patches of exposed test that may include partial destruction of the upper test layer (Fig. 3a). In some cases, the lesions are associated with a green discoloration of the affected area (Fig. 3b) (Johnson 1970; Maes and Jangoux 1984). Infiltration of immune cells (see section "Echinoderm Immunity Is Mediated by Coelomocytes") into the affected area followed by tissue regeneration and recovery may occur if the infection covers <30% of the animal surface (Jangoux 1990). Bald sea urchin disease is considered to be a consequence of infection with *Vibrio anguillarum* and/or *Aeromonas salmonicida*, because isolates from diseased animals can reproduce the disease in healthy purple sea urchins (*S. purpuratus*) (Gilles and Pearse 1986). Molecular analyses of bacteria isolated from disease lesions in the sea urchin *P. lividus* show an enrichment of α- and γ-proteobacteria (*Vibrio* and *Aeromonas*, respectively) as well as *Cytophagales*, *Flavobacteraceae*, and *Bacteriodetes* groups (Becker et al. 2008). Although the symptoms of bald sea urchin disease are similar in different echinoid species, the microbial basis of the disease is likely complex and infection with different microbes may manifest similar symptoms.

Echinoderm Paramoebiasis

The amoeba *Paramoeba invadens* has been isolated from the CF of sea urchins. This protozoan causes epizootic outbreaks of paramoebiasis disease in the green sea urchin *S. droebachiensis* resulting in mass mortality events along the Atlantic coast of Nova Scotia, which are associated with warm-core rings from hurricanes (Jones 1985; Scheibling and Hennigar 1997; Miller and Colodey 1983; Scheibling et al. 2010). The disease can be transferred to healthy sea urchins by immersion in seawater containing *P. invadens* (Jellett et al. 1988) or by injection of the amoeba into the coelomic cavity (Jones et al. 1985; Jones and Scheibling 1985). Symptoms of the disease appear 10–15 days after exposure as muscle necrosis leading to loss of tube foot function, substrate detachment, mouth gaping, spine loss, and tissue discoloration (Jellett et al. 1988). Infected animals exhibit a decreased coelomocyte concentration in the CF and an increased protein concentration, which corresponds to an impaired coelomocyte-mediated clotting response.

Viral and Bacterial Diseases of Sea Cucumbers

Diseases of edible, maricultured echinoderms result in significant losses to farmers and prompt investigations into causative agents of these diseases. The sea cucumber

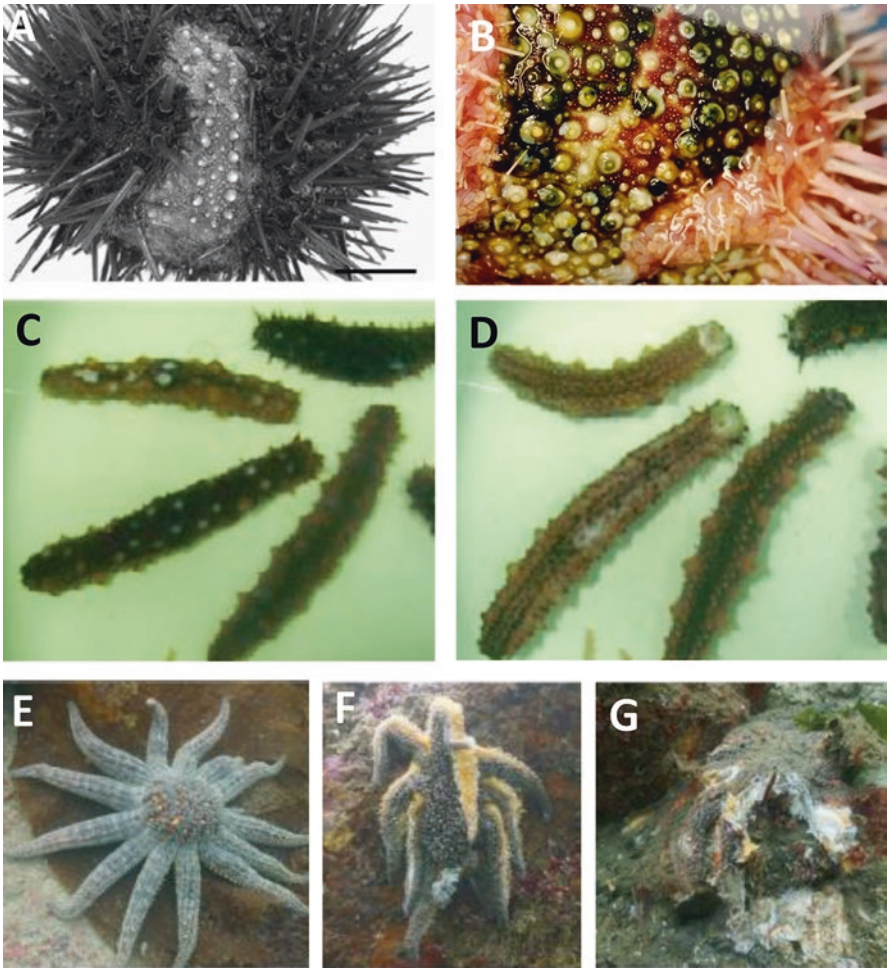

Fig. 3 *Diseases of echinoderms*. (**a**) Bald sea urchin disease in the sea urchin *Paracentrotus lividus* is characterized by spine loss and, in advanced cases, loss of ectoderm (*scale bar* = 1 cm). (Reprinted from Becker et al. (2008), with permission from Elsevier.) (**b**) Bald sea urchin disease in the sea urchin *Echinus esculentus* shows spine loss and discoloration of the exposed test. (Image courtesy of Dr. Anne Böttger.) (**c**, **d**) Skin ulceration and peristome tumescence syndrome virus (SUPTSV) infection in sea cucumbers. (Reprinted from Liu et al. (2010a), with permission from Elsevier.) (**c**) Skin ulcers are apparent on the dorsal side of the sea cucumber *Apostichopus japonicus* after immersion in seawater with SUPTSV. (**d**) Ulcers from SUPTSV infection on the ventral side surround the mouth. (**e**, **f**) Progression of sea star wasting disease (SSWD) in the sunflower star, *Pycnopodia helianthoides*. (Reprinted from Schultz et al. (2016), with minor modifications to image sizes following restrictions cited in https://creativecommons.org/licenses/by/4.0/legalcode.) (**e**) A healthy sunflower star. (**f**) An infected sunflower star shows loss of turgor, body wall disintegration, and exposure of internal organs. (**g**) A lethal infection of a sunflower star results in disintegration with the appearance of skeletal ossicles on the substrate

Apostichopus japonicus is subject to two diseases with distinct symptoms: a skin ulceration disease and a peristome tumescence disease. In mariculture facilities in China, these have triggered mass mortalities, which typically occur during the winter (Deng et al. 2009; Liu et al. 2010a). Skin ulceration disease is characterized by lesions around the mouth and/or cloacal openings, which can spread across the entire animal (Fig. 3c, d), and it is associated with cessation of feeding, head shaking, mouth sensitivity, and general atrophy. Peristome tumescence disease is characterized by ulceration of the integument muscle (Wang et al. 2005; Liu et al. 2010a). Although the cause for both diseases was believed to be bacterial (Wang et al. 2005; Zhang et al. 2006; Deng et al. 2009), repeated tissue sampling from intestine and tentacles of diseased animals identified the pathogen as a spherical enveloped virus of 100–250 nm, which causes both diseases and was subsequently called skin ulceration and peristome tumescence syndrome virus (SUPTSV) (Liu et al. 2010a). Two bacterial species, *Pseudoalteromonas tetraodonis* and *Pseudoalteromonas* sp., were also isolated from infected tissue samples that produced similar but different disease symptoms. Early symptoms of the bacterial infection in *A. japonicus* show loss of tentacle activity plus ulcers on the dorsal skin and abdominal parapodia, which increase in size and number and eventually merge. The differences between the diseases indicate that the bacterial pathogens primarily cause ulceration of the skin, whereas SUPTSV initially targets cellular function in the tentacles and peristome. Although each of the pathogens induces symptoms with overlapping characteristics, the likelihood of mixed infection under natural or aquaculture conditions is high, in which both types of early symptoms could develop rapidly from multiple pathogens (Liu et al. 2010a).

Sea Star Wasting Disease

The first observed outbreak of sea star wasting disease (SSWD) was in 1977–1978 in the Gulf of California and affected the predatory sun star *Heliaster kubiniji* (Dungan et al. 1982). Between 1978 and 1998, repeated outbreaks of SSWD caused mass mortalities of multiple species of asteroids in the California Channel Islands (Engle et al. 1994; Eckert et al. 1999; Blanchette et al. 2005). The ochre sea star, *Pisaster ochraceus*, at two different sites on Vancouver Island, British Columbia, also showed evidence of SSWD (Bates et al. 2009). Although SSWD primarily affects asteroids, symptoms can also be displayed by sea urchins (*S. purpuratus*, *M. franciscanus*, and *Lytechinus pictus*) and brittle stars (*Ophioplocus esmarki* and *Ophiopteris papillosa*) (Eckert et al. 1999). SSWD outbreaks tend to coincide with warmer waters that occur during El Niño events (Engle et al. 1994; Eckert et al. 1999; Blanchette et al. 2005). The presence and severity of SSWD and its association with temperature can be replicated experimentally by maintaining sea stars at different temperatures (Bates et al. 2009).

An unprecedented SSWD epidemic in early 2013 killed millions of asteroids of up to 20 species along the eastern Pacific coastline of North America (Hewson et al. 2014; Stokstad 2014). Symptoms include necrotic lesions, body wall edema and dermal inflammation, loss of body turgor and lethargy, behavior changes, limb curling and autotomy, and death, which appears as a rapid degradation or "melting" of the animal, leaving behind the skeletal elements on the substrate (Fig. 3e–g) (Hewson et al. 2014). The absence of bacterial and eukaryote pathogens in lesions from infected animals suggested that the causative agent for SSWD might be a virus. Consistent with this prediction, filtered seawater (0.22 μm filters) collected from tanks containing infected sea stars or similarly filtered tissue supernatants from infected animals cause SSWD in healthy animals (Hewson et al. 2014; Fuess et al. 2015). Massively parallel sequencing of tissues isolated from infected versus healthy sea stars revealed that the most common sequence in infected sea stars is that of a densovirus (Parvoviridae), subsequently termed sea star–associated densovirus (SSaDV). Densoviruses bind to a transferrin receptor that is expressed on coelomocytes in many echinoderm species, which may be the basis for the multispecies epidemic. Analysis of museum specimens also identified SSaDV in tissues collected in 1942 (prior to any reports of wasting disease), suggesting that the virus has been present in the environment for a long time, that it may have a nonasteroid reservoir, and that environmental changes or other stressors either to asteroids or the virus may have initiated the epidemic (Hewson et al. 2014). This suggestion is in accordance with the 2013 summary report from the Intergovernmental Panel on Climate Change, reporting new prediction models for continued emissions of greenhouse gases that will lead to global climate change throughout the twenty-first century and likely result in an increasing incidence of disease outbreaks and mass mortalities with less time for recovery (Burge et al. 2014). Thus, understanding the functions of the innate immune responses to pathogens in echinoderms continues to be important because many serve as keystone species for the coastlines of our world.

Immunogenomics: Immune Genes Encoded in Echinoderm Genomes

Genomes from species within most echinoderm classes (Fig. 1) have been sequenced, including sea urchins (Echinoidea; *S. purpuratus* and *Lytechinus variegatus*), brittle stars (Ophiuroidea; *Ophiothrix spiculata* and *Ophionereis fasciata*), sea stars (Asteroidea; *Patiria miniata* and *Patiriella regularis*), and sea cucumbers (Holothuroidea; *Parastichopus parvimensis* and *Australostichopus mollis*) (Sodergren et al. 2006; Cameron et al. 2009; Long et al. 2016) (see also www.echinobase.org). Of these, the purple sea urchin, *S. purpuratus*, was the first large marine invertebrate organism to have its genome sequenced (Sodergren et al. 2006). Through a community-wide collaboration, the annotation of many of the approximately 23,000 gene models encoded in this 814-Mb sequence was accomplished with an emphasis on molecules involved in several aspects of the life history (see *Developmental Biology*, volume 1, 2006). Some of the most striking results to

emerge from the analysis were homologues of genes that function in either immune cell development or immune response (Hibino et al. 2006). Comprehensive surveys for these homologues relied on both primary sequence similarity (e.g., BLAST-based searches) and a domain-based strategy to identify more distantly related molecules (see Buckley and Rast (2011)). In total, over 1000 immune genes fall into the following categories: pattern recognition receptors (PRRs) and other immune receptors, intracellular signaling, transcription factors, cytokines and growth factors, and immune effector genes, as well as genes involved in coagulation and the complement system, and homologues of genes that function in the vertebrate adaptive immune system (Table 1) (Hibino et al. 2006; Rast et al. 2006). Many of these genes (e.g., the complement genes, the *SpRAG*-like gene cluster, and the *SpTransformer* [formerly *Sp185/333*] effector gene family) are described elsewhere in this chapter. This analysis complemented previous studies of the echinoderm immune response and unexpectedly revealed that the system is complex, sophisticated, robust, and likely highly flexible for detecting and responding to a wide range of potential pathogens in the marine environment.

Pattern Recognition Receptors

An exceptional example of the complexity of the echinoderm immune system is the significant expansion of gene families encoding PRRs, specifically the Toll-like receptors (*SpTLR*s; 253 genes), NOD-like receptors (*SpNLR*s; >200 genes), and scavenger receptors containing multiple scavenger receptor cysteine-rich domains (*SpSRCR*; 1095 domains in 218 genes) (Table 1) (Rast et al. 2006; Hibino et al. 2006; Rast and Messier-Solek 2008; Messier-Solek et al. 2010; Buckley and Rast 2015). This set of sea urchin immune genes contrasts with homologous repertoires in the well-characterized vertebrate (e.g., mammalian) and protostome (e.g., *Drosophila* and *C. elegans*) systems, which typically harbor 5–20 genes in each of these families. Genes within the expanded *SpTLR* multigene family encode transmembrane proteins with a cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain that mediates signaling, and a ligand-binding ectodomain consisting of a series of leucine-rich repeats (LRRs) capped by specialized N-terminal and C-terminal LRRs (Rast et al. 2006; Hibino et al. 2006; Buckley and Rast 2012, 2015; Messier-Solek et al. 2010). The *SpTLR* genes form 11 subfamilies that exhibit differential expression patterns in larval and adult tissues, although some are primarily expressed at sites of immune interaction (e.g., coelomocytes and the gut). Genes within several of the subfamilies are subject to significant levels of diversifying selection. In some subfamilies, specific residues under positive selection are predicted to cluster spatially within the ectodomain, which is consistent with the speculation that these regions may be important for binding the pathogen or PAMP and for dimerization, as in other systems (Choe et al. 2005; Sackton et al. 2007). The *SpTLR* genes are intronless, clustered within the genome, and characterized by numerous insertion/deletion events. Of the 194 complete *SpTLR* genes, 127 encode a full-length SpTLR protein, while the remaining 59 (30.1%) are predicted to be pseudogenes based on

Table 1 The immune gene repertoire encoded in the *Strongylocentrotus purpuratus* genome sequence[a]

Gene	Function in other systems	Copy number
A. Immune genes		
Pattern recognition receptors		
TLRs	Extracellular pathogen recognition	253
NLRs	Intracellular pathogen recognition	203
SRCR domain–containing proteins	Extracellular or secreted pathogen recognition	1095 domains; 218 gene models
The complement system		
C3/4/5	Central components of the complement pathways	2
Thioester-containing protein		5
Mannose-binding lectin	Activates the lectin pathway	1
C1q	Activates the classical pathway	4
Ficolin	Activates the lectin pathway	46[b]
C2/Factor B	Involved in alternative activation pathway	3
CD59	Inhibitor of the membrane attack complex	4
Immune effector genes		
Macpf	Similar to proteins in terminal complement pathway; kills target cells by creating pores in the membranes	21
SpTrf	Not present in other systems, antipathogen activity	15–50
Cytokines		
IL-17	Induces inflammation and cell migration; maintains barrier integrity	35
MIF	Regulates inflammatory responses	9
B. Transcription factors		
Hematopoietic factors		*Vertebrate orthologues*
bHLH factors	SpScl	Scl, TAL-2, Lyl-1
	SpId	Id1, Id2, Id3, Id5
	SpE-protein	HEB, E2A, Id-2
GATA factors	SpGata1/2/3	Gata-1, Gata-2, Gata-3
	SpGata4/5/6	Gata-4, Gata-5, Gata-6
Homeobox factor	SpNot	None
Other	SpGcm	None; homologous to gcm in *Drosophila melanogaster*

IL interleukin, *MIF* macrophage migration inhibitory factor, *NLRs* Nod-like receptors, *SRCR* scavenger receptor, cysteine-rich, *TLRs* Toll-like receptors

[a]Gene numbers in this table are compiled from Hibino et al. (2006), Buckley et al. (2017), Buckley and Rast (2012, 2015), Buckley et al. (2008a), and Oren et al. (2016a)

[b]The ficolin genes include all gene models that encode fibrinogen domains, some with coiled coil domains but none with N-terminal collagen domains (Hibino et al. 2006)

frameshifts or premature stop codons. The high levels of diversity within the *SpTLR* genes, their structural similarities to TLR proteins in other organisms, and their expression in immune-related tissues suggest important pathogen detection functions within the immune system.

The evolutionary strategy of gene family expansion to generate PRR diversity is not limited to the purple sea urchin. The estimated sizes of the *TLR* gene family in two other strongylocentrotid species are similar to that of *S. purpuratus*: there are 276 *TLR* genes in *Mesocentrotus franciscanus*, 238 in *S. fragilis*, and 68 in *L. variegatus* (Buckley and Rast 2012). Outside the echinoderms, similarly large *TLR* gene families are present in in other invertebrate deuterostomes (e.g., *Branchiostoma floridae* (Holland et al. 2008)) and several lophotrochozoan species (Davidson et al. 2008; Zhang et al. 2015). The expansions may provide invaluable benefits to these animals by increasing the potential microbial recognition capacity. This is relevant for host defense and also to shape a beneficial microbiota, particularly given observations that normal commensal microbes can become opportunistic pathogens under stress conditions (e.g., bald sea urchin disease).

The Transcriptional Response to Sea Star Wasting Disease

In addition to genome sequences, transcriptomic data are increasingly available from echinoderms in various states of immune challenge. These strategies have been used not only to identify echinoderm pathogens (e.g., SSDaV) but also to characterize the host response to the pathogen. When the coelomocyte transcriptome from the sunflower star, *Pycnopodia helianthoides*, infected with SSWD (Fig. 3f, g) is compared with that from the bat star, *Patiria miniata*, and the purple sea urchin, *S. purpuratus*, 52% and 26% (respectively) of the encoded proteins can be predicted (Fuess et al. 2015). Sunflower star coelomocyte gene expression is particularly high for genes involved in complement pathways (see section "The Complement System"), including homologues of C3, factor B, ficolins, and properdin, which are likely important for opsonization to augment phagocytosis. Sunflower stars also upregulate members of TLR signaling pathways (e.g., MyD88 and NFκB), cytokines (e.g., the IL-6 receptor and IL-17), and genes that encode proteins involved in melanin/prophenyloxidase activation, arachidonic acid metabolism that may mediate phagocytosis, inflammation, pain, and chemotaxis. Concurrently, increases in expression of genes encoding proteins involved in extracellular matrix remodeling such as proteases and collagenases is consistent with significant changes in the connective tissues and animal morphology exemplified by "melting" (Fig. 3g). These transcriptomic data highlight aspects of the echinoderm immune response that are complex and are both novel within this phyla and more broadly conserved among animals.

SpRAG1L and *SpRAG2L* Expression and Function in Sea Urchins

The complex array of pattern recognition receptors in the *S. purpuratus* genome suggests that sea urchins have a sophisticated system of pathogen detection that differs from that in jawed vertebrates (Hibino et al. 2006). The jawed vertebrates rely heavily on the protection provided by the adaptive immune system, a central hallmark of which is the antigen receptor genes immunoglobulin (Ig) and T-cell receptor (TCR), which exist as gene segments in a nonfunctional configuration in the germline. Their assembly into functional Ig and TCR genes in developing lymphocytes is mediated by an enzyme complex consisting of the products of the recombination activating genes 1 and 2 (*RAG1* and *RAG2*) (reviewed in Gellert (2002)). These genes were originally identified exclusively in the genomes of jawed vertebrates and thus were intimately linked to the presence of diversifying Ig and TCR genes and the presence of an adaptive arm of the immune system (reviewed in Schatz (2004)). However, the *S. purpuratus* genome includes a pair of genes with striking similarities to vertebrate *RAG* genes on the basis of the locus structure, as well as the deduced amino acid sequence and domain structure of the encoded proteins (Fig. 4a) (Fugmann et al. 2006). Consequently, these genes are called *SpRAG1L* (*SpRAG1-Like*) and *SpRAG2L*, respectively. Their transcripts are predominantly expressed in coelomocytes in the adult sea urchin and, just like their vertebrate counterparts, these genes are always coexpressed (Fugmann et al. 2006). Furthermore, the encoded SpRAG1L and SpRAG2L proteins form complexes with each other and with selected vertebrate RAG1 and RAG2 proteins. SpRAG1L has low but clearly detectable recombinase activity on an artificial vertebrate recombination substrate when ectopically expressed in murine 3T3 cells (Carmona et al. 2016). Given that the DNA binding domain of SpRAG1L is one of the least conserved regions of the protein, it is likely that higher activity levels would be detected on the cognate but yet unidentified DNA target of the SpRAG1L/SpRAG2L complex in the sea urchin genome. In addition, SpRAG2L binds specifically to histones that are methylated on the fourth lysine in histone H3 (H3K4me2/3), which mirrors interactions observed for mammalian RAG2 (Wilson et al. 2008). Finally, homologues of the *SpRAG1L* and *SpRAG2L* genes are present in the genomes of other echinoderms, including the sea urchin *L. variegatus*, the bat star, *P. miniata*, and the brittle star *Ophiothrix spiculata* (Fig. 4b) (Kapitonov and Koonin 2015; KM Buckley, JP Rast, and SD Fugmann unpublished data, 2015). Together, with the recent discovery of a *RAG1/RAG2-like* gene pair (likely a transposable element) in the amphioxus *Branchiostoma belcheri* (Huang et al. 2016), these observations suggest that an ancestral *RAG1/RAG2* gene pair was present in the genome of the last common deuterostome ancestor prior to the emergence of the adaptive immune system in jawed vertebrates. While the possibility exists that the encoded proteins are involved in gene rearrangements, their functions in echinoderm immunity remain to be elucidated.

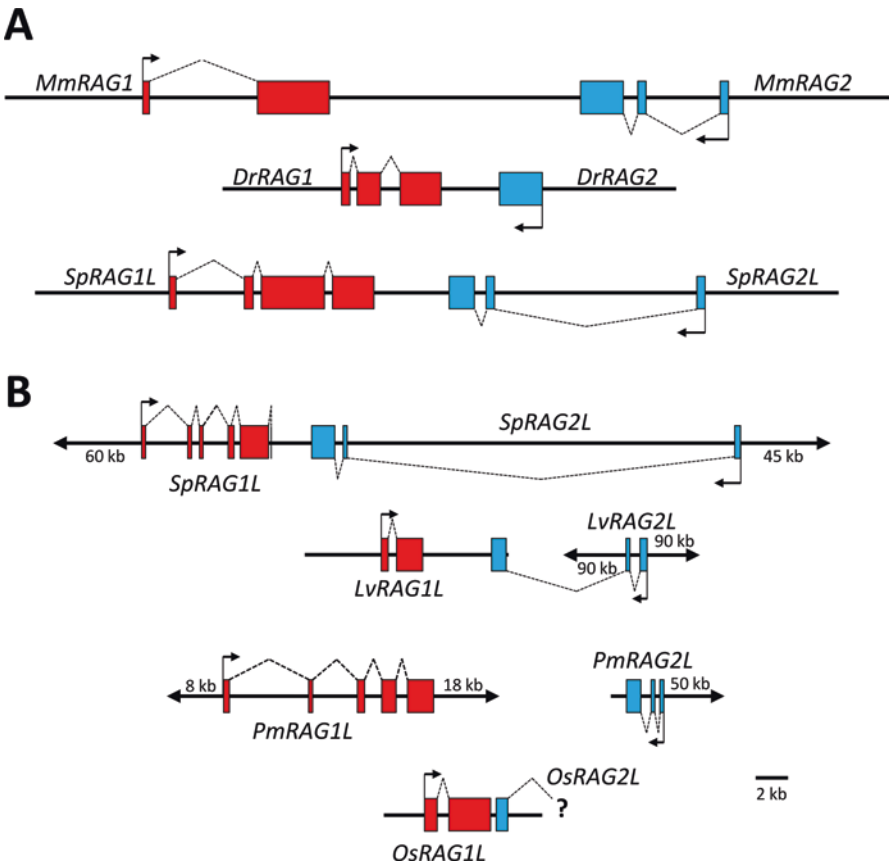

Fig. 4 *SpRAGL genes in echinoderms*. (**a**) *SpRAG-like* (*SpRAGL*) genes in the purple sea urchin, *Strongylocentrotus purpuratus*, are clustered and in the same tail-to-tail orientation as *RAG* genes in the mouse *Mus musculus* (*MmRAG*) and the zebrafish, *Danio rerio* (*DrRAG*). The exons of the *RAG1* and *RAG2* homologues are shown in red and blue, respectively. (Modified from Fugmann et al. (2006) and reprinted with permission, copyright 2006, National Academy of Sciences, USA.) (**b**) Genomic loci of *RAG1L* and *RAG2L* genes in echinoderms. Scaffolds containing the genomic loci of *SpRAG1L* and *SpRAG2L* from the sea urchin *Strongylocentrotus purpuratus* and *LvRAG1L* and *LvRAG2L* from *Lytechinus variegatus* are shown to scale with those from the sea star *Patiria miniata* (*PmRAG1L* and *PmRAG2L*) and the brittle star *Ophiothrix spiculata* (*OsRAG1L* and *OsRAG2L*). Arrowheads indicate the parts of the genomic scaffolds that have been omitted for clarity, and the sizes of these omitted regions are indicated. The putative transcription start site for each gene is marked with a bent arrow. Note that the *OsRAG2L* gene is incomplete in the publicly available draft version of the *Ophiothrix spiculata* genome (9/2016). Information to generate this figure was derived from the respective genome sequences at www.echinobase.org

Echinoderm Immunity Is Mediated by Coelomocytes

The ability of echinoderms to reject allografts, clear foreign cells and particles from the CF, and survive infection from a variety of pathogen agents all indicate the importance of the collection of cells within the CF that are known as coelomocytes. Echinoderm coelomocytes are highly heterogeneous, with a wide range of cell types and sizes that have been identified (Kindred 1924; Boolootian and Giese 1958; Hetzel 1963; Endean 1966; Smith 1981; Smith and Davidson 1992; Chia and Xing 1996; Gross et al. 1999; Ramírez-Gómez and García-Arrarás 2010; Smith et al. 2010). The description of coelomocytes presented here adopts a simplified classification scheme based on selected previous reviews (Ramírez-Gómez and García-Arrarás 2010; Pinsino and Matranga 2015). Cell categories are based on morphological criteria and classified into at least six cell types, although not all six have been identified in all classes or species. Coelomocytes are defined as phagocytes (also called leukocytes), spherule cells (also called spherulocytes, amoebocytes, morula cells, or granulocytes), vibratile cells, hemocytes, progenitor cells, and crystal cells, of which some are more restricted in their phylogenetic distribution (Smith 1981). In general, coelomocyte types are relatively conserved among sea urchin (Echinoidea) and sea star (Asteroidea) species and less conserved among different species of sea cucumbers (Holothuroidea). Although the original work to identify and characterize cell types was done using transmitted bright field light microscopy with multiple histological staining techniques, more recent reports have employed fluorescence microscopy to differentiate coelomocyte subpopulations by cellular cytoskeletal structure and protein localization, as well as electron microscopy to identify ultrastructural characteristics. Some functions of coelomocyte subsets are becoming better defined, while the functions and activities of other types remain poorly understood. In the section on "Immune Cells in Adult Echinoderms", the six coelomocyte types are discussed as they relate to their presence and function in sea urchins, sea cucumbers, and sea stars. Note that relatively little is known about coelomocytes in the brittle stars (Ophiuroidea) and sea lilies (Crinoidea).

Immune Cells in Adult Echinoderms

Phagocytes

Phagocytes are often the most abundant coelomocytes in echinoderms, particularly in sea urchins and sea stars. Their functional characteristics are associated with phagocytosis and/or encapsulation of foreign invaders, allograft rejection, and cytolytic and cytotoxic responses (Gross et al. 1999), in addition to the expression and secretion of antimicrobial peptides (AMPs) (Li et al. 2010a, b; 2014a) (see section "Echinoderm AMPs"). They can undergo shape changes from a petaloid/bladder form to a filopodial form and can self-aggregate, which is involved with CF clotting and syncytia formation (Kindred 1924; Boolootian and Giese 1959; Edds 1977; Majeske et al. 2013b). Sea urchin phagocytes range in size from 20 to 50 μm; they are typically the most abundant coelomocyte type and make up approximately 40–80% of the total cells found in the CF of sea urchins, depending on the species (Fig. 5a–d) (Bertheussen

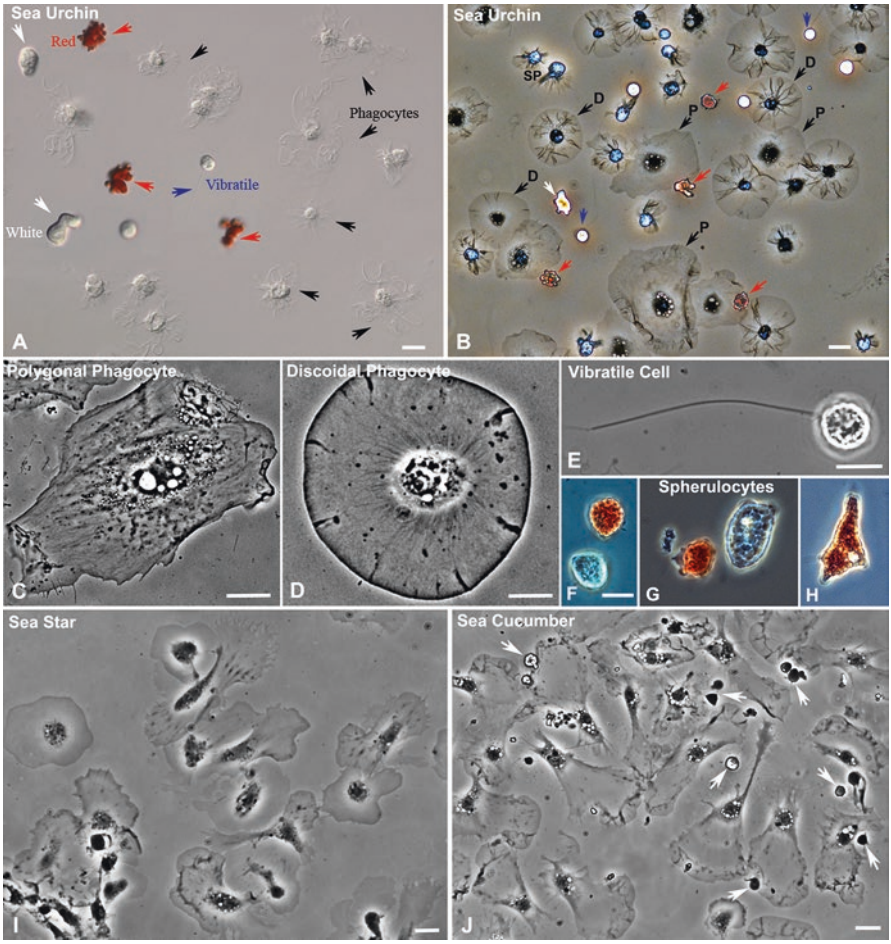

Fig. 5 *Echinoderm coelomocytes*. Live coelomocytes collected from sea urchins (**a**–**h**), a sea star (**i**), and a sea cucumber (**j**). Cells from the sea urchins *Paracentrotus lividus* (**a**: DIC imaging) and *Strongylocentrotus droebachiensis* (**b**–**h**: phase contrast imaging) include two types of large phagocytes. (**a**, **b**) Black arrows indicate phagocytes, red arrows indicate red spherule cells, white arrows indicate white or colorless spherule cells, and blue arrows indicate vibratile cells with single flagellae. (**b**) Phagocytes are indicated as discoidal phagocytes (D), polygonal phagocytes (P), and small phagocytes (SP). The red and colorless spherule cells and the vibratile cells are indicated as in panel (**a**). (**c**–**e**) High-resolution phase contrast imaging of large phagocytes (**c**, **d**) emphasizes the difference in the cytoskeletal and organellar organization in the polygonal cells (**c**) compared with the discoidal cells (**d**) and demonstrates the presence of the cytoplasmic granules and flagellum of a vibratile cell (**e**). (**f**–**h**) Red and colorless spherule cells in the process of spreading on glass and progressing from a spherical shape (**f**) to a more amoeboid shape (**g**, **h**). (**i**) Coelomocytes from the sea star *Asterias forbesi* are only phagocytes, which often group together into aggregates. (**j**) Coelomocytes from the sea cucumber *Sclerodactyla briareus* include numerous phagocytes with broad lamellipodial edges, as well as a variety of other cell types, which may include spherule cells, hemocytes, and/or progenitor cells (white arrows). The scale bars are 5 μm in panel (**a**) and 10 μm in all other panels. The magnifications in panels (**f**–**h**) are equivalent

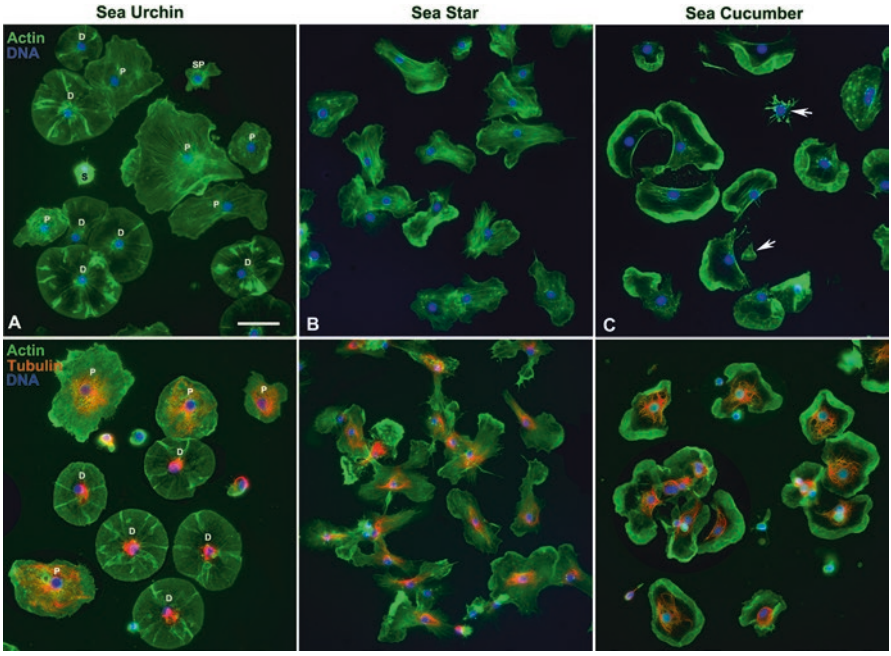

Fig. 6 *Phagocyte morphology varies among species from different echinoderm classes*. All species have phagocytes that stain for actin filaments (green), microtubules (red), and DNA (blue). (**a**, **d**) Large phagocytes from the sea urchin *Strongylocentrotus droebachiensis* are discriminated as polygonal (P) and discoidal (D) by actin staining and cytoskeletal morphology. A small phagocyte (SP) and a white or red spherule cell (S) are also shown. (**b**, **e**) Phagocytes from the sea star *Asterias forbesi* are primarily cells with an elaborate actin cytoskeleton, which is most similar to sea urchin polygonal phagocytes. Other distinct morphological subtypes of phagocytes are not obvious. (**c**, **f**) Phagocytes from the sea cucumber *Sclerodactyla briareus* have phagocytes with strong labeling of a dense actin network in the lamellipodial region at the cellular edge, and many have similar organization of the actin cytoskeleton. Some nonphagocyte cells are present (white arrows). The scale bars are 20 μm. The magnifications in panels (**a–c)** and panels (**d–f)** are equivalent

and Seljelid 1978; Jellett et al. 1988; Smith et al. 2010). Large phagocytes in suspension include a subset of cells that appear with veils of cytoplasm and a central nucleus, which are often referred to as bladder or petaloid cells (Figs. 5b, d and 6a, d) (Edds 1977; Henson et al. 1999). When spread on glass, they become discoidal in shape, which is the result of uniform spreading of the actin-rich lamellipodial cytoskeleton associated with a radial array of actin bundles (Figs. 5b, d and 6a, d) (Henson et al. 1999). A second subset of large phagocytes in sea urchins appear polygonal in shape when spread on a substrate, where they display a cytoskeleton with elongated actin bundles oriented parallel to the long axis of the cell that are reminiscent of actin stress fibers in mammalian tissue culture cells (Figs. 5a, c and 6a). These two cell types also differ in nuclear morphology, cytoplasmic organelle distribution, microtubule cytoskeleton and motor proteins, the extent of actin-mediated centripetal flow, and associated phagocytosis (Figs. 5b–d and 6a, d) (Edds 1993; Henson et al. 1992, 1999).

However, both types of large phagocytes undergo a transformation to a filopodial morphology in vivo as part of the clotting process in response to wounding of the animal, or as an in vitro response to osmotic shock or elevation in intracellular calcium (Smith 1981; Henson and Schatten 1983; Edds 1993; Henson et al. 1992, 1999; Chia and Xing 1996). In addition to the large phagocytes, there is a population of small phagocytes, the abundance of which appears dependent on the immunological activation status of a given individual sea urchin (Figs. 5b and 6a) (Gross et al. 2000; Brockton et al. 2008). A subset of these cells stain intensely for the sea urchin immune response proteins of the SpTransformer family (see section "The *SpTransformer* Gene Family in Euechinoids") (Brockton et al. 2008).

Phagocytes in sea stars (Figs. 5i and 6b, e) constitute up to 95% of the coelomocytes, which also undergo petaloid to filopodial shape changes (Coteur et al. 2002a; Pinsino et al. 2007) and show differences in size and granularity (Coteur et al. 2002a). Four subsets have been tentatively defined on the basis of morphology and size: small (10–15%), large (5%), and vesiculate (70–75%) phagocytes, and cells with short pseudopodia (10–15%) (Jangoux and Vanden Bossche 1975). There may be subpopulations of sea star phagocytes that differ in their level of phagocytic activity, their ability to undergo immunomodulation, and their relative aggregation as part of a CF clotting process (Kanungo 1982; Coteur et al. 2002a, b). Live cell imaging (Fig. 5i) for actin and microtubule staining (Fig. 6b, e) indicates that sea star phagocytes have variable morphology and are often found as aggregates, and that sea stars do not appear to have an equivalent of the distinct phagocyte subtypes that are present in sea urchins.

In sea cucumbers, phagocytes make up about one third of the coelomocytes in the CF in some species and can appear in either petaloid or filopodial morphologies similar to phagocytes in sea urchins, although clear distinctions among phagocyte types are not evident (Figs. 5j and 6c, f) (Hetzel 1963; Chia and Xing 1996; Eliseikina and Magarlamov 2002; Xing et al. 2008; Ramírez-Gómez and García-Arrarás 2010). Cells in the petaloid or bladder morphology exhibit extensive motility of their cytoplasmic veils and are highly phagocytic, and the process of phagocytosis has been linked to the transformation from the petaloid to the filopodial morphology (Chia and Xing 1996). Cytoskeletal staining of settled sea cucumber phagocytes shows broad lamellipodial regions with the characteristic dendritic actin network consistent with extensive centripetal motility, as well as centralized microtubule arrays (Fig. 6c, f). Phagocytes in sea cucumbers contribute to cell aggregation and clotting, and make up about two thirds of the cells found in early stages of aggregation (Taguchi et al. 2016).

Spherule Cells

Spherule cells (also called amoebocytes) are rounded or ovoid shaped (8–20 μm in diameter) in suspension with a small nucleus that has condensed chromatin, and large cytoplasmic granules containing mucopolysaccharides and protein (Fontaine and Lambert 1977; Canicatti and D'Ancona 1989; Smith 1981; Chia and Xing 1996; Pinsino and Matranga 2015). Functions assigned to this coelomocyte type include antibacterial activity, inflammation, wound healing, encapsulation, graft

rejection, and cytotoxic activity (reviewed in Gross et al. (1999)). Sea urchins typically contain red and colorless (also called white) spherule cells (Fig. 5a, b, f–h) that make up 5–40% of the total coelomocyte population (Ramírez-Gómez and García-Arrarás 2010; Smith et al. 2010), depending on the species as well as the pathophysiological condition, which varies among animals. The spherule cells are round in suspension but once in contact with a substrate they exhibit actin-based amoeboid-like motility and can extend elongate processes (Figs. 5a, b, f–h and 6a). The red spherule cells have a distinct pigmentation due to the chemical echinochrome A within the cytoplasmic granules, which is a naphthaquinone double-ring structure that forms peroxide in the presence of extracellular concentrations of calcium (Perry and Epel 1981) and exhibits antibacterial activity against a variety of marine and nonmarine microbes (Service and Wardlaw 1984). It is produced from the activities of a distinctive set of enzymes that are expressed in larval pigment cells (see section "Pigment Cells") and red spherule cells in adults (Calestani et al. 2003). Colorless spherule cells (also called white spherule cells) in sea urchins display cytotoxic activity against mammalian cells in vitro, an activity that is increased by the presence of phagocytes (Arizza et al. 2007). The spherule cell type in sea cucumbers (Fig. 5j) appears to be particularly variable with multiple subtypes, defined in the literature on the basis of color (colorless, green, or yellow), presence of granules, reaction to histological and histochemical stains, and/or appearance in transmission electron micrographs (reviewed by Chia and Xing (1996)), although there is no general agreement on a standardized nomenclature for these subtypes. Like sea urchin spherule cells, these cells in sea cucumbers exhibit pseudopodia-based amoeboid-like motility (Hetzel 1963; Fontaine and Lambert 1977). In sea stars, red and colorless spherule cells have been reported only as a relatively rare coelomocyte type (Pinsino et al. 2007).

Vibratile Cells

Vibratile cells are spherical and 5–10 μm in diameter, with an irregular nucleus and large cytoplasmic granules, and they are distinctive because of their single long flagellum. They are highly motile, which has been suggested to assist in circulation of the CF (Smith 1981; Chia and Xing 1996). Vibratile cells degranulate during the CF clotting process, may be a source of some clotting proteins, and may also function in hemostasis (Chia and Xing 1996). In sea urchins, vibratile cells are relatively abundant, making up 8–20% of the total population in the CF (Fig. 5a, b, e) (Vethamany and Fung 1972; Arizza et al. 2007; Jellett et al. 1988; Matranga et al. 2006). In live preparations of freshly withdrawn CF, these cells have also been observed in sea cucumbers (Eliseikina and Magarlamov 2002), although they are rarely reported for sea stars (Pinsino et al. 2007). In general, vibratile cells are not consistently present in all echinoderm classes and their functions are not understood.

Hemocytes

Hemocytes are relatively large (10–23 μm) coelomocytes that are biconcave or spherical with a round nucleus and hemoglobin in their cytoplasm (Hetzel 1963; Smith 1981; Chia and Xing 1996). They are prominent in some species of sea cucumbers, have been preliminarily reported in some sea stars, and are thought to

function in oxygen transport (Hetzel 1963; Eliseikina and Magarlamov 2002; Pinsino et al. 2007).

Progenitor Cells

Progenitor cells, also called lymphocytes, are small, spherical cells of 2–8 μm in diameter, with large round nuclei, prominent nucleoli, and a thin rim of minimal cytoplasm. These cells show morphological similarities to small lymphocytes in vertebrates (Smith 1981) and have been hypothesized to be stem cells from which other coelomocytes are derived, although this hypothesis has not been tested. They are the predominant coelomocyte type in several sea cucumber species (Eliseikina and Magarlamov 2002; Taguchi et al. 2016), although their presence in sea urchins and sea stars has not been well documented and, if present, they may not be found in the CF.

Crystal Cells

Crystal cells are rhomboid shaped and 2–24 μm in length, with a crescent-shaped heterochromatic nucleus and a rectangular vacuole (Chia and Xing 1996; Eliseikina and Magarlamov 2002). These cells appear to be restricted to sea cucumbers, in which they are a rare coelomocyte type, making up fewer than 0.5% of the total coelomocyte population. Structurally similar cells have been noted within the CF that have a central vacuole containing small crystal-like structures, which appear to become crystal cells in live preparations under conditions of increasing osmotic pressure (Eliseikina and Magarlamov 2002). The function of crystal cells is unknown.

Immune Cells in Larval Sea Urchins

Most echinoderm species develop through an intermediate larval stage, which lives for several weeks to months prior to metamorphosis into a juvenile form. That these free-swimming, feeding larvae survive in microbe-rich seawater for this length of time suggests that even in this morphologically simple life stage, echinoderms rely on robust immune systems. The immune cells of echinoderm larvae were originally discovered by Metchnikoff on the basis of his experiments inserting rose prickles into the blastocoel of a sea star larva (Metchnikoff 1893). He observed migrations of mesenchymal cells toward the foreign surface and its subsequent encapsulation. Although this was the first demonstration of phagocytosis and encapsulation in an echinoderm (or in any animal), in the century since Metchnikoff's landmark work, which earned him the 1908 Nobel Prize in Physiology or Medicine, mesodermal immune cells have been classified for many different species of echinoderm larvae (Furukawa et al. 2009; Hibino et al. 2006; Ho et al. 2016; Kominami et al. 2001; Silva 2000). Collectively, larval immunocytes share several similarities with adult coelomocytes, yet they also exhibit some key differences.

Pigment Cells

Pigment cells in the larval sea urchin *S. purpuratus* (Fig. 7a1) are granular, stellate cells, which migrate through the blastocoel and embed within or are closely apposed to the larval ectoderm (Krupke et al. 2016), and by 10 days post fertilization (dpf),

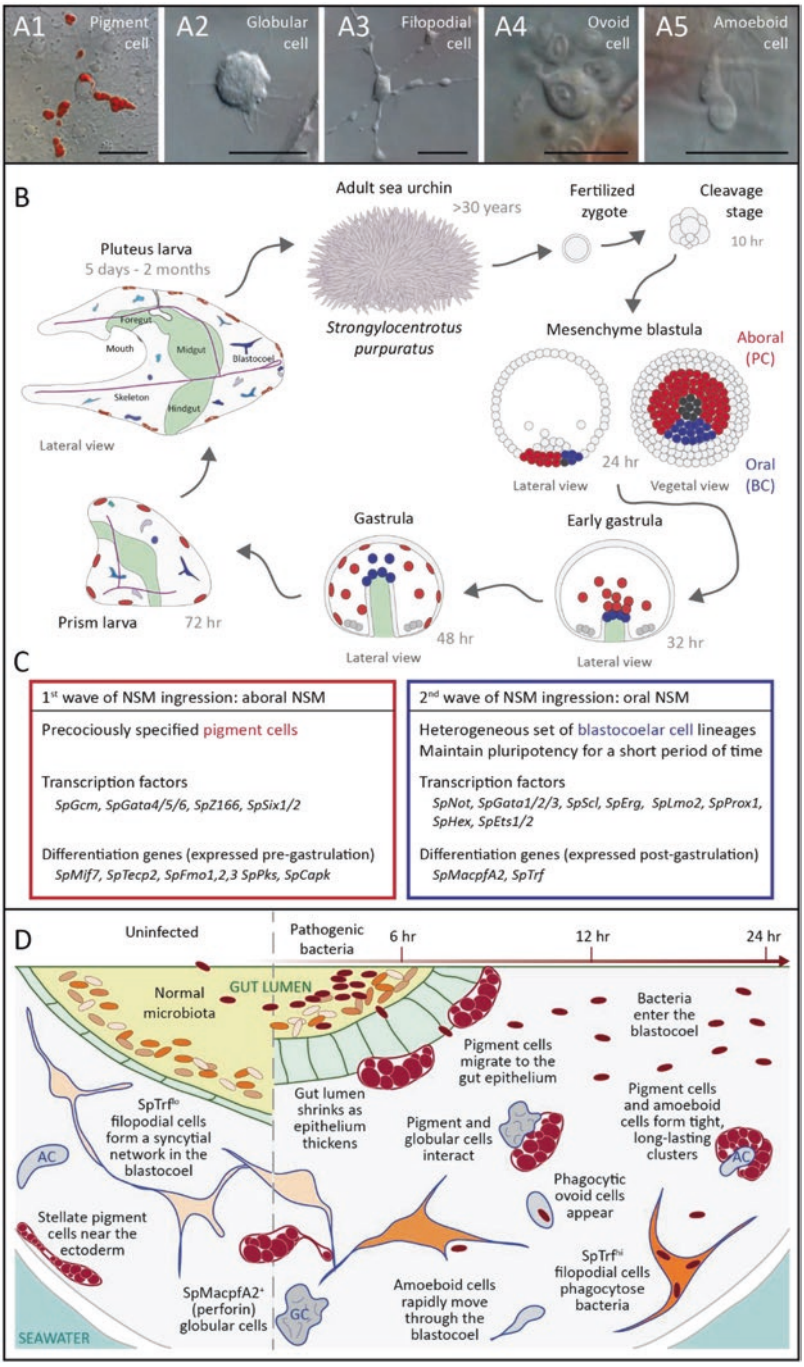

Fig. 7 *Purple sea urchin larvae have mesodermally derived immune cell types that respond rapidly to gut infection*. (**a**) The purple sea urchin larva contains five types of immune cells. Pigment cells (a1) are present in the ectoderm and have stellate morphology in their resting state.

the larvae have approximately 80 pigment cells. These cells have two to four pseudopodia, which constantly move within the plane of the ectoderm (Gibson and Burke 1987; Ho et al. 2016) and are filled with ~40 small, red granules (1–2 μm in diameter). The red pigment is due to echinochrome A, which is also present in the adult red spherule cells (see section "Spherule Cells") that mediate wound healing and antibacterial responses, and these two cell types are likely homologous. Larval pigment cells express a suite of genes that are involved in several aspects of the immune response (Table 1). These include genes encoding the transcription factor c-rel (Ransick and Davidson 2012), the cytokine macrophage migration inhibitory-like factor 7 (SpMif7; also known as dopachrome tautomerase (SpDopT) (Rast et al. 2002; Ho et al. 2016)), a thioester-containing protein (SpTecp2), the scavenger receptor SpSRCR142 (Ho et al. 2016), and polyketide synthase (SpPKS), an enzyme involved in echinochrome biosynthesis (Calestani et al. 2003). In vertebrates, SRCRs are involved in pathogen recognition and clearance, and adult coelomocytes also express a complex and dynamic array of *SpSRCR* genes (Pancer 2000). Whole-mount in situ hybridization (WMISH) shows that while both *SpSRCR142* and *SpTecp2* are expressed in cells that also express *SpPKS*, conversely cells that are *SpPKS* negative do not express *SpSRCR142* or *SpTecp2*, suggesting that pigment

Fig. 7 (continued) Four types of blastocoelar cells are classified on the basis of morphology and behavior: globular cells (a2), a subset of filopodial cells that form a network within the blastocoel (a3), ovoid cells (a4), and amoeboid cells (a5). The scale bars are 20 μm. (**b**) The purple sea urchin has a biphasic life history. The life cycle times shown apply to *S. purpuratus*. Fertilized zygotes undergo rapid cleavage to form a mesenchyme blastula by 24 h post fertilization (hpf). The mesoderm is partitioned along the oral (blue)–aboral (red) axis and contains precursors of pigment cells (PC) and blastocoelar cells (BC). Pigment cell precursors in the aboral mesoderm ingress into the blastocoel at the onset of gastrulation at ~30 hpf. Expression of *SpGata1/2/3* and *SpScl* homologues mark the oral mesoderm, which gives rise to several blastocoelar cell types that ingress late in gastrulation (>42 hpf). Larvae initiate feeding and contain a tripartite gut (foregut, midgut, and hindgut), as well as a calcite skeleton. Pigment cells are often found apposed to the ectoderm. The blastocoel contains several morphologically distinct types of immunocytes illustrated in panel (**a**). The sizes of the adult sea urchin and the cleavage stages are not drawn to scale. (**c**) Larval immunocytes are patterned from two fields of mesoderm. Pigment cell (red box) and blastocoelar cell (blue box) precursors reside in the aboral and oral region of the mesoderm, respectively (a schema of the vegetal view of blastula-stage mesoderm is shown in panel (**b**)). The relevant gene expression for each cell type is listed within each box. For a more detailed gene regulatory network of immunocyte patterning, see Fig. 8. (**d**) An organism-wide larval response to gut-associated bacterial perturbation occurs among different cells and tissues. Larvae fed high concentrations of the marine bacteria *Vibrio diazotrophicus* and imaged with time-lapse microscopy over the course of the infection exhibit a reproducible and reversible cellular response across the entire animal. A timeline of the larval response is shown above the graphic summary of the changes that occur in the gut morphology, cell behavior, and gene expression levels. In uninfected conditions, dendritic pigment cells are apposed to the ectoderm and low levels of *SpTrf* (formerly known as *Sp185/333*) gene expression are evident in select filopodial cells. *Vibrio diazotrophicus* exposure induces a robust thickening of the gut epithelium and elicits cell migrations. Pigment cells and amoeboid cells migrate to the gut within 24 h of exposure to *Vibrio*, and upregulation of *SpTrf* occurs in subsets of filopodial cells. As bacterial cells escape from the gut into the blastocoel, they are phagocytosed by SpTrf-positive ($SpTrf^+$) cells. AC amoeboid cell, GC globular cell. (Modified from Ho et al. (2016) and reprinted with permission from the Nature Publishing Group)

cells may be more heterogeneous than previously appreciated. Mature pigment cells express a battery of genes that imply various cellular functions. These include the single copy gene encoding the ephrin homologue (*Eph*), which facilitates cellular trafficking to the ectoderm (Krupke et al. 2016), the gene encoding the multidrug resistance transporter ABCC5a (Shipp et al. 2015), and genes involved in metabolism, including *cyclin-dependent AMP kinase* (*SpCAPK*; Rast et al. (2002)), sulfotransferase (*SpSult*), and several members of a flavin-monoxygenase family (*SpFmo1*, *−2*, *and −3*, and *−a*).

Blastocoelar Cells

Blastocoelar cells in the larval sea urchin are a mixed population of cells that are present in the blastocoel and exhibit several distinct morphologies, behaviors, and functions (Fig. 7a2–5), particularly with regard to immune function (Ho et al. 2016). Blastocoelar cells are phagocytic (Silva 2000), exhibit immune surveillance–like behavior, and participate in cell–cell interactions during the course of an immune response. Four distinct types of blastocoelar cells have been described and are termed globular, filopodial, ovoid, and amoeboid cells (Ho et al. 2016).

Globular Cells

Globular cells are large (10–15 μm), vessicular, and relatively slow moving (2 μm/min) (Fig. 7a2). Several of these cells wander in the blastocoel while others cluster in the tips of larval arms and at the aboral apex of the body. Globular cells exhibit surveillance-like behavior by extending short pseudopodial projections. They express *SpMacpfA2*, a perforin-like gene, which is a member of the multigene family that encodes proteins characterized by conserved membrane attack complex/perforin (MACPF) domains (Table 1). MACPF domains are also present in vertebrate perforins (lytic molecules secreted onto target cells by NK cells and T killer cells), in *Mpeg1* (a macrophage-specific gene conserved in fish and tetrapods), and in the complement proteins C6–C9 (which are members of the terminal pathway (Anderluh et al. 2014)), leading to the speculation that these cells may have killer activity.

Filopodial Cells

Filopodial cells are small cells (5–7 μm in diameter) that form a syncytial network within the blastocoel (Fig. 7a3) (Tamboline and Burke 1992). These cells extend two to five long, branching filopodia (10–50 μm) that span the blastocoel and connect the filopodial cells with the gut, epidermis, and skeletal rods. Although filopodial cells show little net movement within larvae, the nucleus and cytoplasm move extensively along the cellular processes. At the late gastrula stage (50 h post fertilization (hpf)) there are 10–15 filopodial cells per embryo in *S. purpuratus*, and by 72 hpf there are 20 in the early (prism) larval stage (Tamboline and Burke 1992). In response to immune challenge, subsets of filopodial cells express the immune effector *SpTransformer* gene family (see section "The *SpTransformer* Gene Family in Euechinoids") and are able to phagocytose bacteria in the blastocoel (Ho et al. 2016).

Ovoid Cells

Ovoid cells are oval-shaped cells measuring approximately 10–15 μm along their long axis (Fig. 7a4). They are motile, granular cells, which likely emerge rapidly from the filopodial networks, and appear at the sites of microbes within the blastocoel (Ho et al. 2016). When present, they are very efficient phagocytes.

Amoeboid Cells

Larvae (10 dpf) have two to five motile amoeboid cells (Fig. 7a5) within the blastocoel, which are medium-sized (5–10 μm), comma shaped, rapidly motile (5 μm/min), and morphologically similar to colorless spherule cells in the adult. Amoeboid cells traffic rapidly between the gut epithelium and the larval ectoderm, and maintain interactions with several other cell types. This activity is attenuated during immune responses in infected larvae, such that their migration rate slows (< 2 μm/min) as they interact with other cell types. Their most frequent and longest interactions are with pigment cells, which are dynamic and can last several hours.

Immune Cells in Larval Sea Stars

Immune cell morphology and behavior in asteroid larvae are similar to those in echinoid larvae. In the sea star *Patiria pectinifera*, most mesenchyme cells in bipinnaria larvae (4 dpf), which account for ~1% of the total larval cell number, are distributed beneath the body wall and move randomly, forming a dynamic network structure (Furukawa et al. 2009). This distributed pattern is effective for phagocytosis of both cellular constituents released from the ectodermal cells and foreign substances that access the blastocoel through the body wall. On the basis of their strong phagocytic activity and the characteristics of the larval immune cells, the mesenchyme cells are functionally equivalent to blastocoelar cells in larval echinoids. However, differences in size and morphology are not observed for the sea star cells, and no functional subgroups of cells have been defined.

Larval Immune Cell Development

The development of larval immune cells is best characterized in the purple sea urchin, *S. purpuratus* (Gibson and Burke 1985, 1987; Hibino et al. 2006; Ho et al. 2016; Krupke et al. 2016; Materna and Davidson 2012; Materna et al. 2013; Ransick and Davidson 2006, 2012; Schrankel et al. 2016; Solek et al. 2013; Tamboline and Burke 1992) and in the Japanese sea urchin *Hemicentrotus pulcherrimus* (Kominami 2000; Kominami et al. 2001; Shoguchi et al. 2002; Tokuoka et al. 2002; Kominami and Takata 2003; Katow 2004; Ohguro et al. 2011). Fate-mapping experiments show that two lineages of larval immune cells develop from the asymmetric allocation of a one-cell-thick ring of nonskeletal mesodermal (NSM) cells, which are patterned in early embryogenesis (Fig. 7b) (Kominami and Takata 2003; Ruffins

and Ettensohn 1996). The first is the precociously specified pigment cell lineage, which arises early in gastrulation (Fig. 7b, c). The second lineage develops into the heterogeneous blastocoelar cell population (Gibson and Burke 1985; Tamboline and Burke 1992; Hibino et al. 2006; Ho et al. 2016). Pigment cells express lineage-specific markers by 20 hpf, whereas the blastocoelar cell precursors remain undifferentiated for several hours (Fig. 7b, c). They do not express terminal differentiation markers until 48 hpf or later, when they differentiate into globular, filopodial, amoeboid, or ovoid cells (see section "Immune Cells in Larval Sea Urchins") (Solek et al. 2013; Schrankel et al. 2016).

Immune cell development in echinoderm larvae has illuminated a regulatory heritage that is shared across phyla (Schrankel et al. 2016; Solek et al. 2013). These processes are largely controlled by a suite of transcription factors that regulate hematopoiesis on the basis of their homology to proteins in vertebrates and *Drosophila*. Analysis of the *S. purpuratus* genome identified representatives of each major transcription factor family. As echinoderms diverged prior to the several rounds of whole-genome duplication within the vertebrate lineage, most of these factors are encoded by single gene orthologues (Table 1). Interactions among these proteins are described using well-characterized gene regulatory network (GRN) models (Fig. 8).

Gene Regulatory Networks that Control Larval Immune Cell Differentiation

The first step in larval immune cell differentiation is specification of a ring of NSM cells in the mesenchyme blastula, which are positioned at the point at which invagination will occur during gastrulation (Fig. 7b). NSM cell specification is initiated when Delta/Notch (D/N) signaling (Materna and Davidson 2012; Sherwood and McClay 1999; Sweet et al. 2002) activates expression of the gene encoding the transcription factor glial cells missing (*SpGcm*; Ransick and Davidson 2006). The NSM ring is segregated along the oral–aboral axis (OA; also termed the dorsal–ventral axis) in a process that involves the reciprocal activities of Nodal and BMP2/4 signaling (Duboc et al. 2010; Lapraz et al. 2015). Cells within the oral side of the NSM ring develop into the blastocoelar cell lineages, whereas pigment cell precursors form in the aboral two thirds of the ring (Fig. 7b). Following the early activation of *SpGcm* throughout the NSM, its expression resolves to the aboral side, where it specifies the pigment cell lineage in concert with the transcription factor SpGata4/5/6 (also known as Gatae (Lee and Davidson 2004; Materna et al. 2013)). Together, SpGcm and SpGata4/5/6 activate downstream differentiation gene batteries (Calestani et al. 2003; Calestani and Rogers 2010), and perturbation of either factor results in "albino" larvae that lack pigment cells.

The regulatory state of the oral NSM is initiated by the homeobox factor SpNot, which works synergistically with D/N signaling to activate genes involved in blastocoelar cell specification (Fig. 8) (Materna et al. 2013; Materna and Davidson 2012). Upon development to the mesenchyme blastula stage, blastocoelar cells

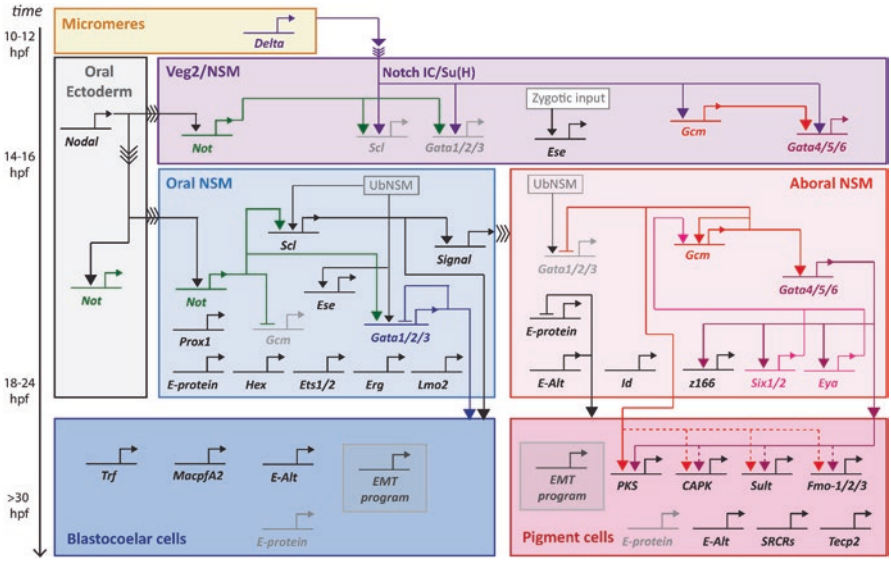

Fig. 8 *A gene regulatory network model describing larval immune cell differentiation in the purple sea urchin (S. purpuratus) embryo*. Larval immune cells develop from a ring of mesodermal precursor cells over the course of about 24 h. An approximate time scale is shown on the right. Colored boxes indicate regulatory states in tissues or cell types. Regulatory interactions are indicated by arrows (indicating gene activation) or crossbars (indicating gene repression). Signal transduction events among regulatory states are shown as chevrons. Genes shown in colors are transcriptionally active; genes in gray are not expressed. Interactions between regulatory genes and downstream terminal target genes are indicated by connecting lines. The nonskeletal mesoderm (NSM) territory is initially specified following Delta/Notch (D/N) signaling from the micromeres, which activates *SpGcm* and *SpGata4/5/6* expression in a coherent feed-forward loop. Nodal signaling from the oral ectoderm subsequently activates *SpNot* expression in the NSM by 16 hpf. After 18 hpf, parallel inputs from D/N and SpNot activate *SpGata1/2/3* and *SpScl* expression throughout the NSM, which is spatially variable and low. SpNot represses *SpGcm* in the oral NSM after 18 hpf and enriches *SpGata1/2/3* and *SpScl* expression in these cells. A ubiquitous NSM activator (UbNSM) and an unknown SpScl-dependent mechanism maintain expression of the oral NSM transcription factors. The aboral NSM regulatory state is specified when SpGcm and SpGata4/5/6 restrict *SpGata1/2/3* expression and also induce directly a terminal differentiation battery encoding pigment synthesis genes (dotted lines indicate predicted inputs that have not been verified experimentally). Proper expression of *SpGata1/2/3* and *SpScl* in the NSM are required for immunocyte epithelial-mesenchymal transition (EMT) and potential activation of downstream markers in blastocoelar cells, such as the expression of an alternative isoform of the *SpE-protein* gene (*SpE-Alt*). SpE-Alt activity negatively regulates expression of the canonical *SpE-protein* isoform within developing immunocytes. (This GRN model integrates data from Ransick and Davidson (2006), Duboc et al. (2010), Materna et al. (2010), Materna and Davidson (2012), Ransick and Davidson (2012), Materna et al. (2013), Solek et al. (2013), and Schrankel et al. (2016). A complete GRN describing endomesoderm development can be found at http://sugp.caltech.edu/endomes/)

express a suite of genes encoding transcription factors including *SpErg* (Rizzo et al. 2006), *SpGata1/2/3* (Davidson et al. 2002; Duboc et al. 2010; Solek et al. 2013), *SpLmo2* (Duboc et al. 2010; Solek et al. 2013), and *SpScl* (Duboc et al. 2010; Solek et al. 2013). The expression levels of *SpGata1/2/3*, *SpScl*, and *SpErg* peak shortly

after the mesenchyme blastula stage, after which they are sharply downregulated (Solek et al. 2013). Notably, these transcription factors are also expressed in adult coelomocytes (Pancer et al. 1999). The usage of SpGata, SpScl, and SpErg transcription factors in the sea urchin reflects deeply conserved regulation of deuterostome immune cell development. In zebrafish and tetrapods, gata1/2/3 and scl factors are also activated by Notch and BMP during hematopoietic stem cell emergence (Davidson and Zon 2004; Kim et al. 2014; Oren et al. 2005; Walmsley et al. 2002). In vertebrate hematopoietic stem cells, *gata-2*, *scl*, and *fli-1* genes are molecularly connected in a bistable positive feedback loop that maintains a self-renewing, pluripotent state, and immune cell differentiation occurs only upon the downregulation of *gata-2* (Narula et al. 2010, 2013; Pimanda et al. 2007). Similarly, in sea urchin larvae, immune gene markers are not expressed until several hours after *SpGata1/2/3* and *SpScl* are downregulated (Fig. 8).

In vertebrates, gata1/2/3, scl, and lmo2 factors form multifactor transcriptional complexes that regulate various stages of hematopoiesis (Wilson et al. 2010), and their activities are modulated by the E-protein basic helix-loop-helix (bHLH) factors (E2A, E2–2, and HEB) (reviewed in De Pooter (2010) and Kee (2009)). Similar interactions are believed to occur in the development of sea urchin larval immune cells. The *S. purpuratus* genome sequence contains a single orthologue encoding these bHLH factors, known as *SpE-protein*, which is expressed as two isoforms: a longer, canonical form (SpE-Can) and a truncated, alternative form (SpE-Alt), which is generated from a secondary transcriptional start site and is homologous with alternative transcripts in vertebrate *HEB* and *E2-2* (Skerjanc et al. 1996; Wang et al. 2006). *SpE-Can* is expressed ubiquitously in the early embryo (Howard-Ashby et al. 2006; Schrankel et al. 2016; Solek et al. 2013). *SpE-Alt* expression initiates in the aboral NSM and expands to blastocoelar cells once they initiate ingression from the vegetal plate and immune marker expression (Fig. 8). Perturbation of *SpE-Alt* expression leads to diminished migratory activity of both pigment and blastocoelar cells, and abrogates terminal marker expression in blastocoelar cells. Furthermore, the two isoforms exhibit mutually exclusive expression patterns; as *SpE-Alt* expression increases in immunocytes after gastrulation, *SpE-Can* transcription is diminished in these cells. This affect requires SpE-Alt activity, which highlights a novel regulatory connection in E-protein biology.

Among echinoderm species, some aspects of larval immune cell development are highly conserved whereas others exhibit considerable variation. The best example of this is the absence of pigment cells outside the echinoid lineage. At the molecular level, however, in the asteroid *P. miniata*, orthologues of *SpEts1/2* and *SpGata1/2/3* are coexpressed in mesodermal progenitor cells during the blastula stage (McCauley et al. 2010). Subsets of these cells migrate and behave similarly to sea urchin larval blastocoelar cells.

The Immune Response in Echinoderm Larvae

The immune cells of echinoderm larvae display a variety of immune behaviors, which were originally discovered by Metchnikoff on the basis of his experiment of inserting rose prickles into the blastocoel of a larval sea star (Metchnikoff 1893).

Since Metchnikoff's landmark work that earned him the Nobel Prize in 1908, mesoderm-derived immune cells have been classified across many different species of echinoderm larvae (Furukawa et al. 2009; Hibino et al. 2006; Ho et al. 2016; Kominami et al. 2001; Silva 2000). More recent descriptions that duplicated the Metchnikoff experiment, but at an earlier point in embryonic development of the sea star *Patiria pectinifera* and the sea urchin *Lytechinus variegatus*, have defined the stage at which the embryos become competent for phagocytosis (Silva 2000; Furukawa, 2009). When foreign particles are injected into the blastocoel at the mid-gastrula stage of the sea star, they are quickly phagocytosed by the mesenchyme cells that are released from the tip of the archenteron (R. Furukawa, unpublished observation, 2009). This is in agreement with the description by Metchnikoff a century ago. When the yeast *Saccharomyces cerevisiae* is injected into the blastocoel at the hatched blastula stage, it is not phagocytosed by the mesenchyme cells until mid-gastrula, which indicates the onset of abilities not only for phagocytosis but also for detection of nonself. These results indicate that phagoytosis begins at the same developmental stage for both sea stars and sea urchins.

The Immune Response in Sea Star Larvae

In the bipinnaria larvae of the sea star *Patiria pectinifera*, the distribution pattern of the mesenchyme cells within the blastocoel is effective for phagocytosis of both cellular constituents released from the ectodermal cells and foreign substances that access the blastocoel through the body wall (Furukawa et al. 2009). Although larval immune cells phagocytose foreign particles injected into the blastocoel within 2 h, when small amounts of small foreign particles (≤~10 μm in diameter) are injected into the blastocoel, individual cells in the blastocoel readily phagocytose the particles. When larger amounts of foreign materials are injected, or when particle sizes are large relative to the mesenchyme cell sizes, multiple cells converge on the site from within the blastocoel and undergo cell–cell fusion to form a syncytial multinucleated giant cell that encapsulates the aggregated particles. Similar syncytial formations have also been noted for adult phagocytes in vitro (Majeske et al. 2013b). The number of recruited immune cells is dependent on the amount and size of the foreign substance (Furukawa et al. 2009), indicating that the process of clearing the blastocoel is strictly regulated by the larval immune system.

The immunoreactive migration and encapsulation of foreign particles by mesenchyme cells in the sea star *P. pectinifera* are regulated by two macrophage migration inhibitory factors (MIFs): *Ap*MIF2 and *Ap*MIF1 (Furukawa et al. 2016). (The names of these proteins and those described below have the prefix *Ap*, reflecting their description relative to the genus name of *Asterina* rather than *Patiria*; this change was made on the basis of the revision of the Asterinidae (O'Laughlin and Waters 2004).) *Ap*MIF1 and *Ap*MIF2 act sequentially first to stimulate and then to inhibit chemotactic activity, respectively, and thereby coordinate the regulated recruitment of the appropriate numbers of mesenchyme cells during the immune response. During this chemotactic migration, a member of the dedicator of cytokinesis 1 (DOCK180; ~180 kDa) superfamily in the sea star *P. pectinifera*, *Ap*DOCK,

regulates F-actin organization at the leading edge of lamellipodia in mesenchyme cells (Furukawa et al. 2012a). Actin organization under control of *Ap*DOCK is also essential for the persistence of encapsulation. Perturbation of *Ap*DOCK results in imperfect lamellipodial formation and in deficient membrane ruffling at the leading edge of mesenchyme cells. These immune effectors are evolutionally conserved, and homologues are present in the sea urchin genome sequence. Therefore, the immune cell behaviors of chemotaxis, cytoskeletal modifications, and syncytia formation in the sea urchin may also be regulated by the orthologues of MIF and DOCK.

The Immune Response in Sea Urchin Larvae

After exposure to the marine bacteria *Vibrio diazotrophicus*, which are ingested as a potential food source, sea urchin larvae initiate a system-wide suite of stereotypic cellular and transcriptional responses (Ho et al. 2016). Within 6 h of bacterial exposure, the gut epithelium thickens, reducing significantly the luminal volume of the midgut (Fig. 7d) (Buckley et al. 2017). By 12 h post exposure (hpe), pigment cells in both the ectoderm and blastocoel migrate more rapidly, whereas amoeboid cell velocity decreases. After 24 h, pigment cells accumulate at the gut epithelium, although some of this cellular activity may be in response to *Vibrio* bacteria that penetrate the gut epithelium and enter the blastocoel.

Coincident with this cellular response, larvae exhibit a series of transcriptional changes in both the gut and the peripheral immune cells. Surveys of gene activity indicate that of the >1000 genes annotated with immune function (Table 1) (Hibino et al. 2006), 200 exhibit a greater than threefold change in expression in the course of the larval response to contact with *Vibrio*. These genes encode immune receptors, intercellular signaling molecules, signal mediators, transcription factors, and effector molecules (K. M. Buckley and J. P. Rast, unpublished data, 2015). Embryos express the complement component *SpC3* and transcript levels from this gene increase in response to bacterial contact (Shah et al. 2003), which may suggest an opsonic function for the SpC3 protein similar to that shown in adult sea urchins (Clow et al. 2004). There are several genes in the sea urchin genome sequence encoding thioester-containing proteins that may have complement function in opsonizing pathogens. Thioester-containing protein 2 (*SpTecp2*) is expressed in larval pigment cells, where it is slightly upregulated in response to *Vibrio* infection (Ho et al. 2016). Conversely, macrophage inhibitory factor 7 (*SpMif7*) is quickly downregulated in pigment cells in response to *Vibrio*. Homologues of Mif factors act as cytokines in other systems and they may have similar functions in infected larvae. Finally, the immune effector gene family *SpTransformer* (reviewed in Ghosh et al. (2010) and Smith and Lun (2017); see also section "The *SpTransformer* Gene Family in Euechinoids") is upregulated in filopodial blastocoelar immune cells by 24 h after *Vibrio* exposure (Ho et al. 2016).

In the course of the larval immune response to *Vibrio diazotrophicus*, the most strongly activated genes belong to two families of interleukin-17 (IL-17) homologues (Buckley et al. 2017). IL-17 cytokines are of central importance in the vertebrate immune response, where they are expressed in lymphocytes as well as in

epithelial barrier cells (Korn et al. 2009; Song et al. 2011). The *S. purpuratus* genome encodes 35 *IL-17* genes (Table 1), which form ten subfamilies based on sequence similarity. Genes within two of these families (*SpIL-1-1* and *SpIL17-4*) are upregulated in larvae within 2–4 hpe to *Vibrio* and expression is largely attenuated within 24 h. Analyses using whole-mount in situ hybridization and BAC-based fluorescent reporter protein constructs indicate that expression of these transcripts is restricted to gut epithelial cells in infected larvae. To assess the functions of these cytokines in the larval immune response, SpIL-17 signaling was perturbed by interference with correct splicing of its receptor *SpIL-17R1* (Buckley et al. 2017). Larvae subject to this perturbation exhibit decreased transcription of several genes associated with IL-17 in vertebrates (*tnfaip3*, *nfkbiz*, *cebpα*, and *cebpγ*) in response to immune challenge. Notably, expression of the *SpIL17-4* genes is also reduced, which hints at potential feedback mechanisms among these factors. Finally, homologues of the *IL-17* genes are present in the genomes of four additional sea urchin species (including the cidaroid *Eucidaris tribuloides*), as well as the asteroid *Patiria miniata* (Buckley et al. 2017), suggesting an ancient and conserved function for these cytokines within echinoderm immunity. Together, these data point to a complex larval immune response including both features that are novel to the echinoderm lineage and those that were present in the last common deuterostome ancestor.

Immune Response Genes and Proteins in Echinoderms

SRCR Genes and Proteins

The *S. purpuratus* genome sequence has nearly 1100 regions that encode SRCR domains. Similarly expanded *SRCR* repertoires are present in the genome sequences of the sea urchin *L. variegatus* and the sea star *Patiria miniata*, suggesting that this feature is common in echinoderms (Buckley and Rast 2015). Proteins containing multiple SRCR domains (termed SRCR proteins; see section "Pattern Recognition Receptors") are involved in the innate immune system of metazoan animals (Sarrias et al. 2004). The diversity of the sea urchin *SpSRCR* repertoire was predicted from a transcriptional analysis of coelomocytes prior to the genome sequencing project (Pancer 2000, 2001). Analysis of coelomocyte transcripts by northern blot shows that individual sea urchins express unique patterns of *SpSRCR* transcripts, and genome blots confirmed that this intraspecific variation was consistent with variations in the *SpSRCR* gene family (Pancer 2000). Furthermore, expression profiles exhibit dynamic shifts after immune challenge. This level of polymorphism in the population of *S. purpuratus* and the variability in expression of these genes in coelomocytes suggest not only complex functions of the encoded proteins but a complex system to control expression of this multigene family.

Although the function of SpSRCR proteins in the immune system is unknown, ApSRCR1 acts as a bacterial opsonin in both the larval and adult stages of the sea star *Patiria pectinifera* (Furukawa et al. 2012b). The *Ap*SRCR1 protein has nine SRCR domains, one short consensus repeat (SCR), one transmembrane region, and a very

short cytoplasmic tail. It is localized to cytoplasmic vesicles in larval mesenchyme cells and adult coelomocytes. When bacteria invade the larval blastocoel or the coelomic cavity of the adult, *ApSRCR1* gene expression is upregulated and the extracellular region of the *Ap*SRCR1 protein is secreted into the larval blastocoel or the adult coelomic cavity. This fragment binds to and aggregates bacteria and promotes phagocytosis by the larval and adult immune cells. The SpSRCR7.2 protein in the sea urchin *S. purpuratus* has a similar structure (with seven SRCR domains, an SCR, and a transmembrane region (Pancer 2000)) and may have similar activities to *Ap*SRCR1. The diverse structure of the *SRCR* gene family within and among species from different echinoderm classes and the dynamic expression patterns among individuals are consistent with important innate immune response functions for host protection.

The *SpTransformer* Gene Family in Euechinoids

The purple sea urchin, *Strongylocentrotus purpuratus*, responds to a variety of immune challenges, such as marine bacteria and PAMPs, with a rapid upregulation of the *Sp185/333* gene family (Rast et al. 2000; Nair et al. 2005; Terwilliger et al. 2006, 2007). Because no sequence similarity is apparent within species from other phyla, other echinoderm classes, or the cidaroid family of echinoids, the transcripts were named on the basis of matches to one full-length cDNA sequence (DD185) (Rast et al. 2000) and one partial cDNA sequence (EST333) (Smith et al. 1996) from sea urchin coelomocytes. The original name was meant to avoid implications for protein function, which were unknown and could not be predicted on the basis of the sequence. However, analysis of one recombinant protein has elucidated its structure and function (see section "SpTrf Protein Functions"), which has provided the opportunity for a name change to *SpTransformer* (*SpTrf*) (Lun et al. 2016, 2017a, b) (for reviews, see Ghosh et al. (2010), Smith (2012), and Smith and Lun (2017)). The name *SpTrf* will be used hereafter in this chapter.

The *SpTrf* cDNA sequences are remarkably diverse and characterized by large blocks of shared sequences called *elements*, which are present in mosaics and result in a variety of *element patterns* (Fig. 9) (Terwilliger et al. 2006, 2007). *SpTrf* genes are composed of two exons, of which the second exon is characterized by six types of repeats: a series of two to four imperfect tandem repeats near the 5′ end of the second exon, as well as five types of interspersed repeats located toward the 3′ end (Buckley and Smith 2007). In addition to the variability among the element patterns, genes of identical sequence are not shared among animals, indicating the level of diversity within the population (Buckley and Smith 2007). The sequence diversity of the genes and transcripts, as well as their upregulation in response to challenge (Rast et al. 2000; Nair et al. 2005; Terwilliger et al. 2007), predict that these genes are involved in the sea urchin immune response.

SpTrf Genes Are Clustered in the Genome

The *S. purpuratus* genome sequence (v4.2) contains six tandem *SpTrf* genes that are clustered in a single locus. However, the sequence diversity of genes isolated from

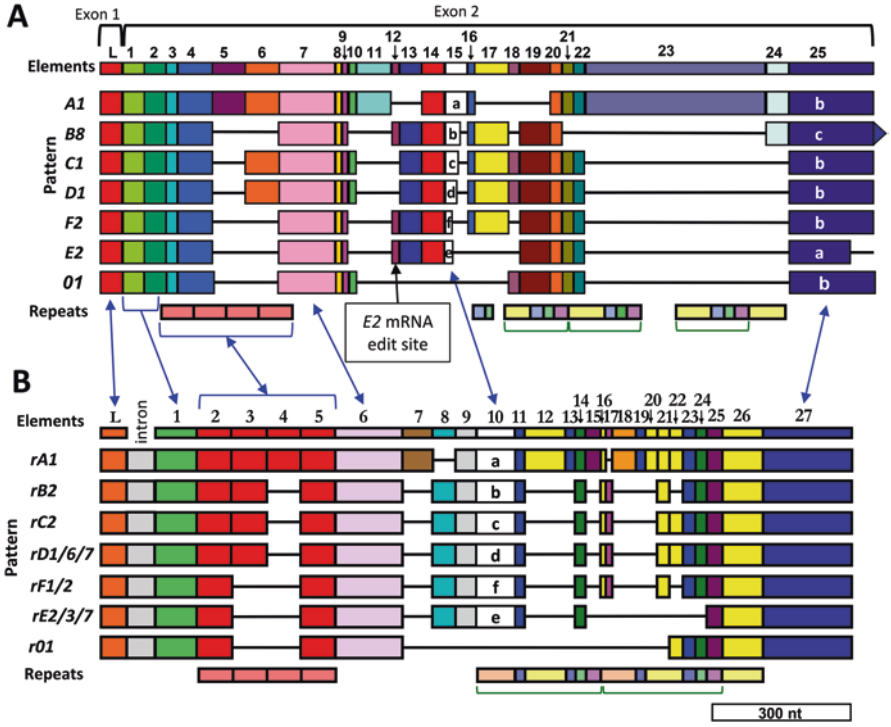

Fig. 9 *The SpTrf transcript sequences are diverse*. (**a**) The cDNA-based alignment of message sequences is based on Terwilliger et al. (2006, 2007). Blocks of sequences, called elements, are shown as colored rectangles, and large gaps are indicated by horizontal black lines. Gene exons are indicated at the top. The leader (L) is encoded by exon 1, and the elements that are present in the mature protein are encoded by exon 2. The names of element patterns (listed to the left) are based on the sequence of element 15, which is highly diverse for both sequence and length. Different versions of element 15 are associated with different sets of elements that appear as element patterns. A common edit site in the *E2* transcripts (indicated) encodes a truncated protein lacking the histidine-rich region. Element 25a, b, and c correlate with 1, 2, and 3 stop codons. (**b**) The repeat-based alignment is based on gene sequences reported by Buckley and Smith (2007), in which the edges of the elements correspond, where possible, with the edges of the repeats. The intron (~400 nt) is indicated as gray boxes and is not shown to scale. For both alignments, all possible elements are shown at the top, and repeats within the coding regions are shown in different colors at the bottom. Green brackets surround subsets of interspersed repeats that are duplicated. Blue arrows between the two alignments indicate corresponding regions. (Reprinted from Smith and Lun (2017))

the genomic DNA from individual animals (Buckley and Smith 2007) plus estimates from bacterial artificial chromosome (BAC) library screens suggest that the gene copy number in single genomes may be much higher (see Buckley et al. (2008a), reviewed in Smith (2012) and Smith and Lun (2017)). To clarify this discrepancy, inserts of several BAC clones (from the library that was the basis for the sea urchin genome assembly) were sequenced and analyzed (Miller et al. 2010; Oren et al. 2016a). The BACs contain 15 *SpTrf* genes with a typical structure of two exons; they are flanked by short tandem repeats (STRs) and are distributed in three

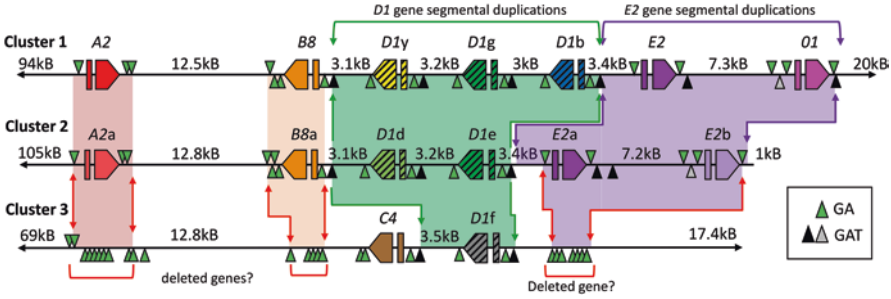

Fig. 10 *The SpTrf genes are arranged in three genomic clusters at two loci*. The genes within clusters 1, 2, and 3 range in size from 1170 to 1894 nt and are spaced apart by 3.0–12.8 kB. All genes have two exons, as indicated by the rectangle (first exon) and pentagon (second exon), which also indicates gene orientation. Element patterns (see Fig. 9) are listed above each gene; however, those with the same element pattern do not necessarily have identical sequences. All genes are surrounded by short tandem repeats (STRs; green triangles) of GA dinucleotides. Long stretches of GA STRs of up to 3 kB may be the remnants of deleted regions (red arrows), including deleted genes in cluster 3 (red brackets). Segmental duplications that include *D1* genes (green shading *and* green arrows) and *E2* genes (plus the *01* gene) (purple shading and purple arrows) are flanked by GAT STRs (black triangles indicate >35 repeats, gray triangles indicate 4–17 repeats). Clusters 1 and 2 are likely allelic on the basis of matches in the flanking regions outside the gene clusters, even though the numbers of genes do not match within locus I. The flanking regions outside cluster 3 indicate that it is positioned separately at locus II. (Reprinted from Smith and Lun (2017))

clusters of seven, six, and two genes (Fig. 10). Clusters 1 and 2 are likely allelic, indicating that the sequenced genome may contain only two *SpTrf* loci. The scaffold in the assembled genome harboring *SpTrf* genes is a hybrid sequence of allelic clusters 1 and 2, which is likely a consequence of the repetitive nature of the *SpTrf* region (Oren et al. 2016a).

Among the three clusters, the genes have similar orientations, intergenic spacing, and relative positioning (Fig. 10) (Miller et al. 2010; Oren et al. 2016a). Furthermore, all of the *SpTrf* genes are associated with GA STRs and some with GAT STRs located in intergenic regions at strategic positions on both sides of each gene and at the edges of segmental duplications (Miller et al. 2010; Oren et al. 2016a). This repetitive structure may contribute to gene diversification by promoting genomic instability, as has been noted in other systems (Pearson et al. 2005; Thys et al. 2014), perhaps through *SpTrf* gene deletion and by blocking the progression of sequence homogenization of clustered genes from gene conversion (Miller et al. 2010; Oren et al. 2016b). In addition to the short STRs that flank all genes, cluster 3 has two long stretches of GA STRs that correlate with the relative positions of genes in clusters 1 and 2 (Fig. 10). These long STRs may be remnant signatures of genes deleted from cluster 2 as a consequence of repeat-mediated genomic instability (Oren et al. 2016a). On the other hand, shorter GAT STRs flank five segmental duplications that appear in tandem in the three gene clusters and include nearly identical genes. The structure of the *SpTrf* family—including the modular element patterns, the repeats within and near the genes, and the gene

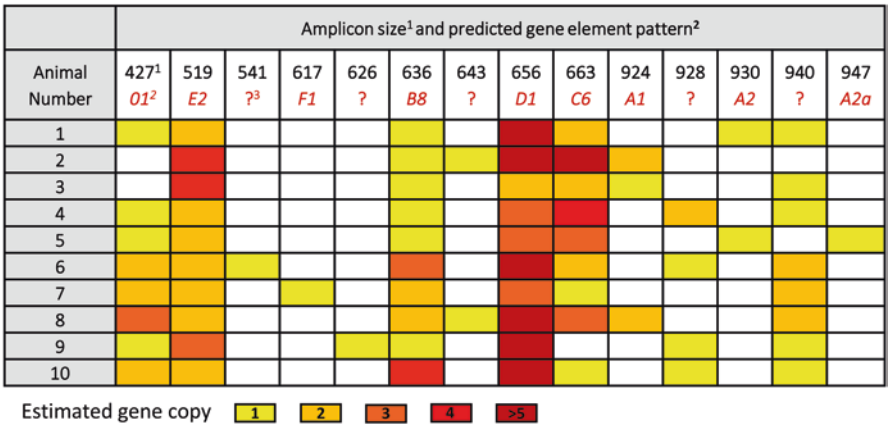

Fig. 11 *The SpTrf gene repertoire is different among individual sea urchins. SpTrf* gene profiles of ten sea urchins are shown. Element patterns from alleles are predicted from the amplicon length for the second exon based on fragment analysis on an ABI 3130 capillary sequencer (Oren et al. 2016a). Each sea urchin shows a unique gene repertoire. Genes with the *D1* element pattern (see Fig. 9 for element patterns) are present in all ten animals, in agreement with gene sequencing (Buckley and Smith 2007). Estimates of copy numbers for each allele are based on the allele frequencies in each animal under the assumption that the least abundant amplicon per animal represents a single copy gene. [1]Amplicon size is based on primers that amplify the second exon (Buckley and Smith 2007). [2]Element pattern prediction is based on amplicon sizes that match to known gene sequences (Buckley and Smith 2007). [3]Question marks indicate amplicon sizes that do not correlate with a known element pattern length. (Modified from Oren et al. (2016a))

clustering—likely contribute to genomic instability, which may underpin the exceptional diversity of this family.

The structure and gene content of the *SpTrf* family may be resolved for the individual sea urchin for which the genome was sequenced; however, the diversity of the family in terms of gene sequence and presence among different animals is extremely high (Buckley and Smith 2007). Analysis of the *SpTrf* gene repertoire in ten different individuals shows significant differences, and the family in each genome may be unique, with some genes common in the population and others more rare (Fig. 11) (Oren et al. 2016a). While the diversification mechanisms promoting this characteristic have not been determined, bioinformatic predictions of the *SpTrf* gene sequences suggest the involvement of gene deletion, duplication, recombination, and conversion (Buckley and Smith 2007; Miller et al. 2010; Oren et al. 2016a). In other families of clustered genes, these processes often result in pseudogenes for rapidly diversifying immune gene families (Oren et al. 2016b), as is exemplified by the clustered sea urchin *SpTLR* family (see section "Immunogenomics: Immune Genes Encoded in Echinoderm Genomes") (Buckley and Rast 2012). Consequently, it is unusual that no *SpTrf* gene fragments and only one pseudogene (likely a retroposon) have been identified from 198 gene sequences, suggesting that the diversification process may be highly regulated (Oren et al. 2016a).

SpTrf gene expression is rapidly upregulated in adult coelomocytes in response to immune challenge. Notably, however, analysis of transcript prevalence reveals that individual coelomocytes contain transcripts of a single *SpTrf* sequence and likely express a single *SpTrf* gene (Majeske et al. 2014). This restricted expression may be regulated on the basis of detection of specific pathogens, which may reflect the usage of a complex pathogen detection mechanism such as the large *SpTLR* gene family (Buckley and Rast 2012) (see also section "Immunogenomics: Immune Genes Encoded in Echinoderm Genomes"). It remains unclear whether a single *SpTrf* gene is expressed per cell or if all but one of the genes are actively repressed. The structure and diversity of the *SpTrf* gene family, variability of the family among individual sea urchins, and restricted expression patterns in individual cells indicate that this system is dynamic, flexible, highly sophisticated, and functions to maintain the survival of *S. purpuratus* in the microbe-rich marine environment.

SpTrf Protein Diversity and Expression

The predicted structure of the SpTrf proteins has an N-terminal hydrophobic signal sequence, which is likely cleaved during protein processing. The mature, full-length protein, which is encoded by the second exon, has a glycine-rich region with an arginine, glycine, aspartic acid (RGD) motif; a histidine-rich region; and a C-terminal region (Fig. 12a) (Terwilliger et al. 2006). All full-length proteins characterized to date lack cysteines. The sequence diversity of SpTrf proteins is a consequence of the mosaic element patterns, as well as mRNA editing, which may result in single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) leading to missense sequence and early stop codons that expand the predicted size range of proteins (4–55 kDa) (Buckley et al. 2008b; Terwilliger et al. 2006, 2007). An individual sea urchin may express up to 260 different SpTrf protein variants according to analysis by two-dimensional (2D) western blots (Dheilly et al. 2009), which is significantly more than the ~50 estimated genes. Native SpTrf (natSpTrf) proteins isolated from individual sea urchins are unexpectedly larger than the protein size prediction, implying that the natSpTrf protein variants multimerize to form nondenaturable, high molecular weight protein complexes (Brockton et al. 2008; Dheilly et al. 2009). Repertoires of full-length natSpTrf proteins differ among individual sea urchins after multiple immune challenges with the same or different bacterial species (Sherman et al. 2015).

Fig. 12 (continued) and a C-terminal region (gray). (**b**) A small phagocyte has SpTrf proteins on the surface and associated with small vesicles within the cell. (**c**) A polygonal phagocyte has SpTrf proteins within vesicles in the cytoplasm. (**d**) A minority of discoidal phagocytes have SpTrf proteins in very small perinuclear vesicles. (**e**) Red spherule cells are negative for SpTrf proteins. (**f**) Vibratile cells are negative for SpTrf proteins. (**g**) A cross-section of gut from *S. purpuratus* shows SpTrf-positive cells within the columnar epithelium. The gut lumen is at the top of the image and the coelomic space is at the bottom. (**h**) The axial organ has many SpTrf-positive cells. Images (**b**) and (**d**–**g**) are captured from fluorescence microscopy, and images (**c**) and (**h**) are from confocal microscopy. The scale bars are 10 μm in images (**b**–**f**) and 100 μm in images (**g**) and (**h**). (Reprinted from Smith and Lun (2017))

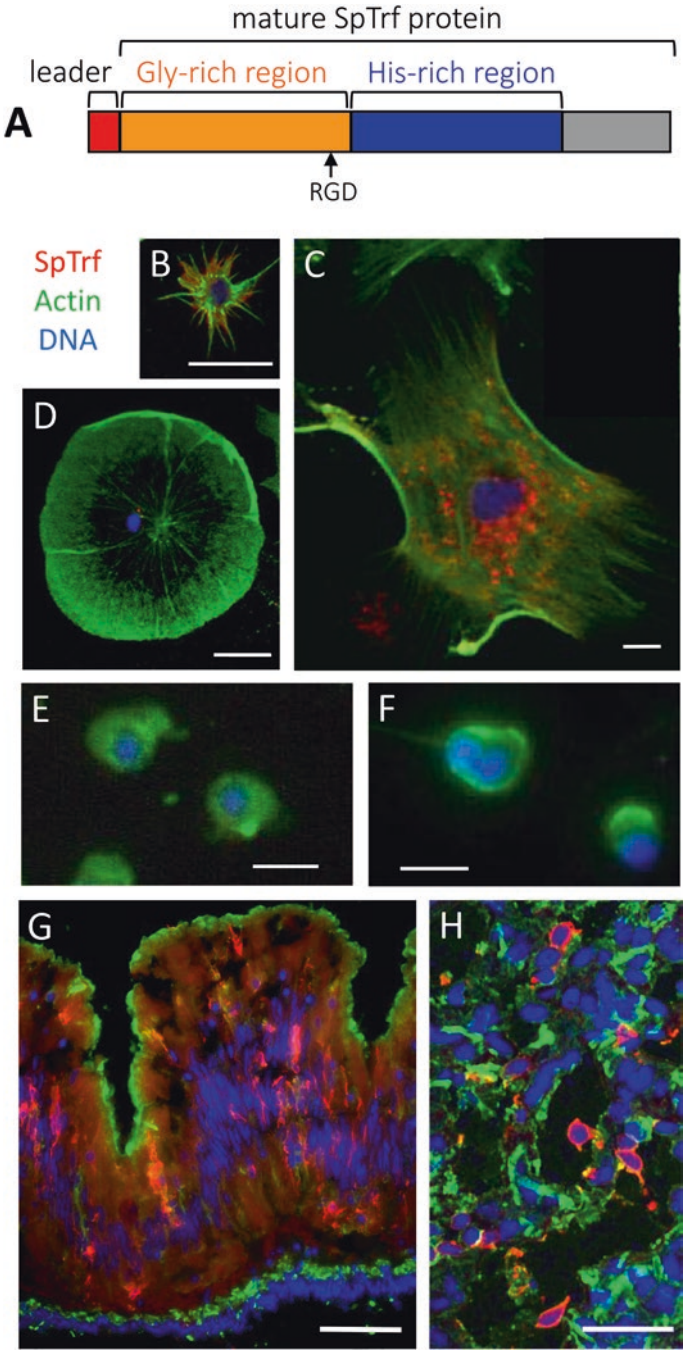

Fig. 12 *SpTrf proteins are expressed in phagocytes.* (**a**) The standard SpTrf protein structure is composed of a leader (red), which is likely cleaved during processing, and a mature protein with a glycine-rich region (orange) with an arginine, glycine, aspartic acid (RGD) motif, a histidine-rich region (blue),

The amino acid sequences of the SpTrf proteins do not predict any obvious structural or functional features. Yet the nucleotide diversity of the genes and transcripts, the swift expression in response to immune challenge (Nair et al. 2005; Terwilliger et al. 2006, 2007; Buckley and Smith 2007), mRNA editing (Buckley et al. 2008b), and variable protein repertoires among animals (Dheilly et al. 2009; Sherman et al. 2015) suggest important immune activities. Most immunoquiescent (IQ) sea urchins (those maintained in recirculating, closed aquaria with downregulated immune activity) show decreased or no expression of the *SpTrf* genes (Nair et al. 2005; Terwilliger et al. 2007). Transcripts isolated from IQ animals tend to encode truncated SpTrf proteins that lack the histidine-rich region as a consequence of mRNA editing (Figs. 9a and 12a) (see Buckley et al. (2008b) and Sherman et al. (2015), reviewed in Smith and Lun (2017)). Edited transcripts encoding truncated proteins missing the histidine-rich region are elevated in IQ sea urchins, whereas after an immune challenge, transcripts encoding full-length SpTrf proteins increase. This suggests not only regulation for at least some of the transcript edit sites but also that the truncated proteins may have surveillance functions based on their presence prior to an immune response, and that after challenge, the histidine-rich region may have pathogen-binding activity.

The SpTrf proteins are expressed by the phagocytes where they are associated with membranes of perinuclear vesicles in discoidal, polygonal, and small phagocytes, and are present on the surface of the plasma membrane of small phagocytes (Fig. 12b–f) (see Brockton et al. (2008), Dheilly et al. (2011a), and Majeske et al. (2014), reviewed in Smith and Lun (2017)). The SpTrf proteins are also expressed in all major organs in adult sea urchins, including the axial organ, pharynx, esophagus, intestine, and gonad (Fig. 12g, h) (Majeske et al. 2013a). SpTrf protein expression increases in most of these organs after an immune challenge; however, gene expression, SpTrf protein content, and numbers of SpTrf-positive cells increase significantly only in the axial organ (Table 2), suggesting that it may be an organ with immune functions. Although it is not known whether SpTrf expression in the adult organs is specific to phagocytes that have infiltrated the adult tissues, the implication from *SpTrf* gene expression in larvae, which is restricted to the blastocoelar cells (Fig. 7d) (Ho et al. 2016), suggests that expression and production of SpTrf proteins in the adult is also restricted to the phagocytes.

Table 2 SpTrf responses to challenge with LPS

Tissue	mRNA	SpTrf protein	SpTrf$^+$ cells
Pharynx	–[a]	+	–
Esophagus	–	+	–
Intestine	+[b]	+	–
Gonad	–	–	–
Axial organ	+	+	+

This table is modified from Majeske et al. (2013a)

[a]–, decreased expression of genes or proteins, or decreases in SpTrf-positive (SpTrf$^+$) cells

[b]+, increased expression of genes or proteins, or increases in SpTrf$^+$ cells

SpTrf Protein Functions

The diversity and expression patterns of the SpTrf proteins suggested antipathogen activities. Binding activities of the natSpTrf proteins from different sea urchins exhibit variable and diverse binding to Gram-positive and Gram-negative bacteria, sheep red blood cells (SRBCs), and insect cells from the lepidopteran *Spodoptera frugiperda* (Sherman et al. 2015; Lun et al. 2016). Detailed functional analysis of these proteins is complicated by their sequence diversity, complex multimerization patterns, and potential interactions with other proteins. Thus, a recombinant protein called rSpTrf-E1 (formerly rSp0032) was generated for functional analyses (see Fig. 9a for the E1 element pattern), in addition to recombinant fragments of rSpTrf-E1 spanning the complete glycine-rich region (rGly-rich fragment), the C-terminal portion of the glycine region (rC-Gly), and the histidine-rich region (rHis-rich fragment) (Lun et al. 2016). rSpTrf-E1 binds specifically and tightly to *Vibrio diazotrophicus* and baker's yeast (*Saccharomyces cerevisiae*) but does not bind to two Gram-positive *Bacillus* species (Fig. 13a). The rGly-rich and rHis-rich fragments bind to *Vibrio* and yeast, and also to *Bacillus*, suggesting that interactions among these two regions in the full-length protein may underlie the binding specificities in the full-length protein. The rC-Gly fragment multimerizes upon isolation, indicating that this region may mediate multimerization for all SpTrf proteins (Lun et al. 2016). rSpTrf-E1 binds to flagellin from *Vibrio diazotrophicus* (Table 3) and from *Salmonella typhimurium*, LPS from *Escherichia coli*, and β-1,3-glucan from *Saccharomyces* but does not bind to peptidoglycan (PGN) from *Bacillus* subtilis (Fig. 13a). Competition assays indicate that rSpTrf-E1 binding to LPS, flagellin, and β-1,3-glucan is specific and irreversible (Fig. 13b).

The outcome of rSpTrf-E1 binding to several different PAMPs suggests the possibility of structural changes in the protein that allow interactions with different binding targets (Lun et al. 2016). Bioinformatic prediction from amino acid sequences indicates that most if not all SpTrf proteins, including rSpTrf-E1 and HeTrf sequences (from *Heliocidaris erythrogramma*; see section "*HeTransformer* Genes in *Heliocidaris erythrogramma*"), have a hydrophobic α helical leader and that the mature proteins are entirely hydrophilic and intrinsically disordered proteins (IDPs) (Fig. 13c). The flexibility of IDPs allows conformational plasticity of proteins to adopt different conformations upon encountering a range of binding targets (Uversky 2010). Circular dichroism (CD) confirms that rSpTrf-E1 is intrinsically disordered and is capable of undergoing structural transformations to mostly α helical in the presence of sodium dodecyl sulfate (SDS) (de Latour et al. 2010) and 2,2,2-trifluorethanol (TFE) (Table 4) (Lun et al. 2017a). LPS also induces a conformational transformation of rSpTrf-E1 from disorder to mostly α helical. Although the rGly-rich and rHis-rich fragments are also predicted to be IDPs, CD analysis shows that they are partially α helical, they intensify their α helical content in SDS, and they unexpectedly transform to β strands in TFE (Table 4). These fragments demonstrate opposite structural transformation in the presence of LPS, in which the rGly-rich fragment transforms from α helical to β strand and the rHis-rich fragment intensifies its α helical content. These results predict unusual

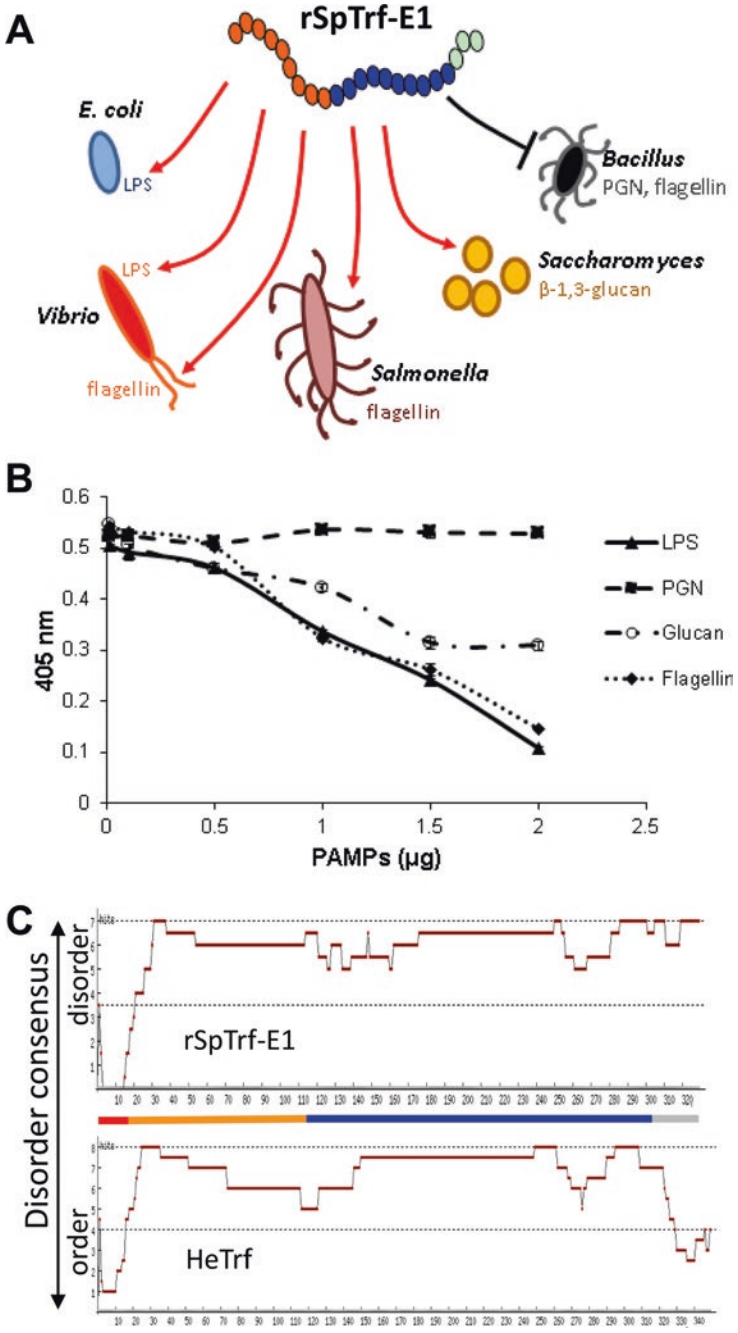

Fig. 13 *rSpTrf-E1 has multitasking binding activity, and Trf proteins are intrinsically disordered.* (**a**) rSpTrf-E1 is shown as an unfolded disordered protein. (Republished from Smith and Lun (2016), with minor revisions.) The colors correlate with protein regions in Fig. 12a. *Red arrows* indicate binding targets of rSpTrf-E1. The black line indicates that rSpTrf-E1 does not bind to *Bacillus*

Table 3 rSpTrf-E1 binds to *Vibrio* flagellin

Sample	MW	Peptide sequences identified[b]	Significant matches	Match; GenBank accession number
rSpTrf-E1 and *Vibrio*[a]	60 kDa	IAETTSFGGNK DDAAGLQISNR	Flagellin B OS; *Vibrio anguillarum*	Q56702
		SQILSQASSSILAQAK IAETTSFGGNK DDAAGLQISNR	Polar flagellin B/D OS; *Vibrio parahaemolyticus*	Q56572
		GDGEEETDAAQQIGDGLGGR RGDGEEETDAAQQIGDGLGGR	SpTrf protein	NP_001073016.1

This table is modified from Lun et al. (2016)

[a]*Vibrio diazotrophicus* was incubated with rSpTrf-E1 and washed, and proteins were separated by SDS-PAGE. Bands that correlated in size to SpTrf-positive bands on a parallel western blot were evaluated by MS

[b]rSpTrf-E1 is ~37 kDa and flagellin is 45–50 kDa; however, the SpTrf-positive band of 60 kDa includes sequences of both rSpTrf-E1 and flagellin

conformational plasticity for the sea urchin SpTrf protein family, perhaps leading to flexible binding activity with a wide range of foreign targets.

The Trf proteins associate with membranes of perinuclear vesicles and the plasma membranes of phagocytes (Brockton et al. 2008; Majeske et al. 2014; Dheilly et al. 2011a), and this association is unexpected because they lack transmembrane domains and glycophosphatidylinositol linkages (Terwilliger et al. 2006, 2007). However, rSpTrf-E1 and the rGly-rich and rHis-rich fragments all bind to phosphatidic acid (PA), which is a small cone-shaped phospholipid with a single phosphate as the head group (Lun et al. 2017b). In the presence of PA, rSpTrf-E1 transforms from an IDP to mostly α helical (Table 4). When rSpTrf-E1 binds to synthetic liposomes composed of 10% PA and 90% phosphatidylcholine (PC), it induces membrane curvature in liposomes, which is associated with PA clustering and changes in liposome shape in the form of budding, invagination, and fusion (Fig. 14a, b). These morphological changes may result from the positively charged amino acids in rSpTrf-E1 binding to the negatively charged phosphate head group of PA, followed by rSpTrf-E1 multimerization; this causes PA clustering (Fig. 14c–h), which is known to induce membrane curvature (Zimmerberg and Kozlov 2006). rSpTrf-E1 and the rHis-rich fragment cause leakage of luminal

Fig. 13 (continued) species or to peptidoglycan (PGN). No binding to flagellin from *Bacillus* is assumed, but this has not been tested. (**b**) rSpTrf binds tightly and specifically to multiple PAMPs. (Reprinted from Lun et al. 2016.) When LPS, β-1,3-glucan (glucan), flagellin, or PGN are preincubated with rSpTrf-E1, LPS reduces rSpTrf-E1 binding to LPS bound to an ELISA well. Glucan and flagellin also complete and reduce rSpTrf-E1 binding to LPS. Preincubation with PGN does not interfere with rSpTrf-E1 binding to LPS. (**c**) rSpTrf-E1 (GenBank accession number DQ183168, from Terwilliger et al. (2006)) and an HeTrf protein (GenBank accession number AFK91970, from Roth et al. (2014)) of similar sizes are predicted to be disordered with an N-terminal α helical leader on the basis of the Disorder Prediction Meta-Server (DisMeta, http://www-nmr.cabm.rutgers.edu/bioinformatics/disorder/). The y axis indicates the confidence level of the disorder consensus based on the outcome of the seven or eight predictor programs for each protein. A represenation of rSpTrf-E1 is shown between the graphs, and colors indicate regions of the protein, as in Fig. 12a

Table 4 rSpTrf-E1 and the recombinant fragments change secondary structure in response to buffer additives and binding targets

	Secondary structure in different reagents[b]				
Protein structure	PO_4	SDS	TFE	LPS	PA
rSpTrf-E1	Disordered	α helical	α helical	α helical	α helical
% α helical	1–2%[c]	79%	95.1%	78.5%	71.8%
Helix tightness[a]		0.59	1	0.66	0.7
rGly-rich fragment	α helical	α helical	β strand	β strand	ND
% α helical	15–17%	75.1%	NA[d]	NA	
Helix tightness		0.78	NA	NA	
rHis-rich fragment	α helical	α helical	β strand	α helical	ND
% α helical	19–30%	70.7%	46.2%	72.8%	
Helix tightness		0.76	NA	0.78	

Reprinted from Smith and Lun (2017)

[a]Helix tightness is estimated from the *R* value obtained from circular dichroism spectra and is used to infer the width of an α helical twist. A standard helix has an *R* value of 1. A 3_{10} helix has an *R* value of 0.4, which has a smaller diameter and is longer for a similar number of amino acids (Vieira-Pires and Morais-Cabral 2010; Lun et al. 2017a).

[b]PO_4, phosphate buffer (10 mM, pH 7.4); SDS, sodium dodecyl sulfate; TFE, 2,2,2-trifluoroethanol; LPS, lipopolysaccharide from *Escherichia coli*; PA, phosphatidic acid in the form of small vesicles.

[c]The percentage of the secondary structure for either the α helix or β strand is deconvoluted from the CD spectra using the DichroWeb online server (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml; Whitmore and Wallace 2004, 2008).

[d]N/A, not applicable. The deconvolution to calculate the β strand percentage is not feasible for this sample (Lun et al. 2017a).

ND, not done.

material from 10% PA liposomes, whereas dimerized rSpTrf-E1 and the rGly-rich fragment do not, suggesting that the histidine-rich region of rSpTrf-E1 is required for this activity (Fig. 15). The lack of activity by dimerized rSpTrf-E1 suggests that multimerization of natSpTrf proteins may be an intrinsic control mechanism to limit their activities in vivo.

Association with negatively charged lipids may be a possible mechanism for binding of natSpTrf proteins to membranes (Brockton et al. 2008; Dheilly et al. 2011a), and content leakage may indicate cytotoxic activities upon binding to pathogens. Although the underlying binding mechanisms remain elusive, the multitasking binding ability and intrinsically disordered nature of rSpTrf-E1 provide an initial insight into how this novel immune protein family may bind to a wide range of pathogens and other foreign targets. The natSpTrf proteins may opsonize a broad array of pathogens and, given the many isoforms that are likely present in individual sea urchins, each may have slightly different but overlapping binding activities and serve as a very effective immune response system in euechinoids.

HeTransformer Genes in *Heliocidaris erythrogramma*

Structure of the *HeTrf* Transcripts and Genes

Outside of investigations of the *SpTrf* family in *S. purpuratus*, the most extensive characterization of the *Trf* family has been reported for the sea urchin *Heliocidaris*

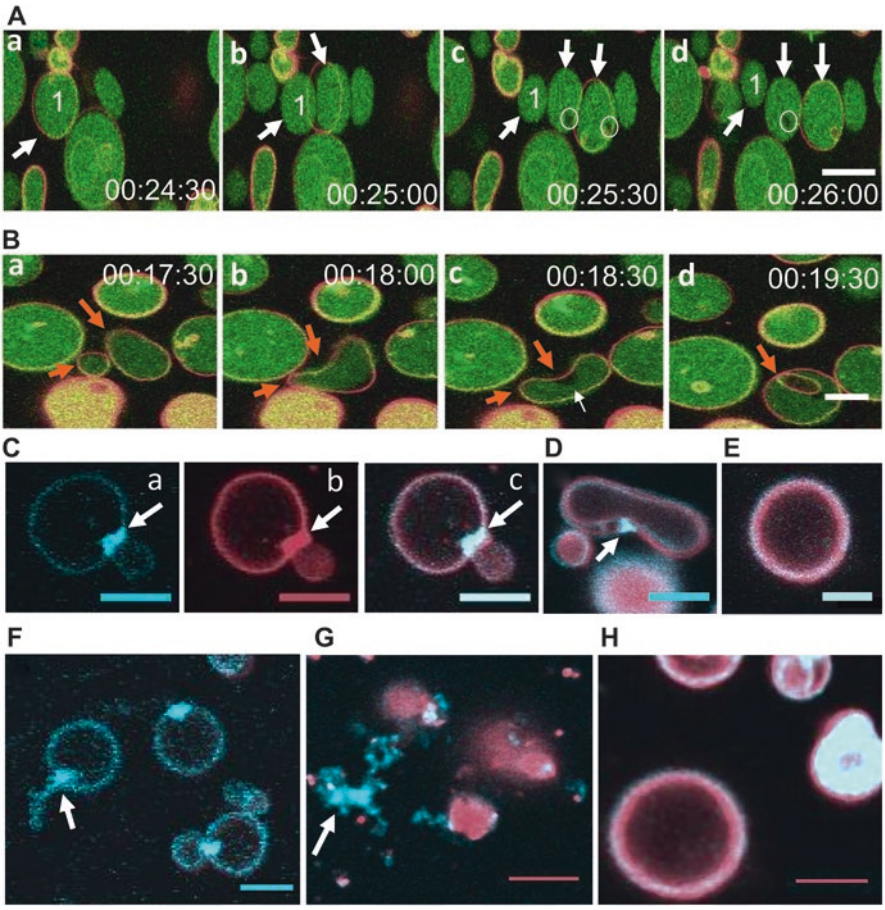

Fig. 14 *rSpTrf-E1 alters liposome membranes*. Liposomes (10% PA; 90% PC) are visualized by dextran labeled with AlexaFluor® 488 (dextran-488; green) in the lumen and the lipophilic dye DiD (red) in the membrane. (**a**) Incubation of liposomes with rSpTrf-E1 results in budding. Liposome 1 buds into three liposomes (white arrows) over 30 s. Dextran-488 leakage is suggested by black spaces within some of the liposomes (white circles in a**c** and a**d**). (**b**) Two liposomes of different sizes fuse in the presence of rSpTrf-E1 (b**a**–**b**, orange arrows). The fused liposome has a dark region in the lumen (**c**, white arrow) near the concave region of the membrane. This liposome invaginates at the site of the concave membrane and generates an internal vesicle that does not contain dextran-488 (b**d**, orange arrow). The confocal image capture was set for 30-s intervals as indicated for (**a**) and (**b**). (**c**) Clusters of blue-labeled phosphatidic acid (blue-PA; arrow) are present at sites of contacting membranes for two liposomes after incubation for 20 min with rSpTrf-E1. The membrane is labeled with the lipophilic dye DiD (red). (c**a**) blue-PA; (c**b**) red DiD; (c**c**) merge. (**d**) A cluster of blue-PA (arrow) is present at the convex curve of a liposome membrane after incubation with rSpTrf-E1 for 20 min. This image is a merge of red and blue channels. (**e**) Blue-PA is evenly distributed in a control liposome after 20 min in the absence of rSpTrf-E1. The image is a merge of the blue-PA and DiD (red). (**f**) Clusters of blue-PA (blue channel only) are present in liposome membranes incubated with rSpTrf-E1 for 20 min. In one liposome, the blue-PA cluster appears to be partially extracted from the membrane (arrow). (**g**) After 2 h of incubation with rSpTrf-E1, blue-PA appears as disordered clusters that are separated from the liposomes (arrow). (**h**) In the absence of rSpTrf-E1, liposomes show an even distribution of blue-PA that remains in the membrane after 2 h. (All images are captured by confocal microscopy. The scale bars are 10 μm.) (Modified from Smith and Lun (2017))

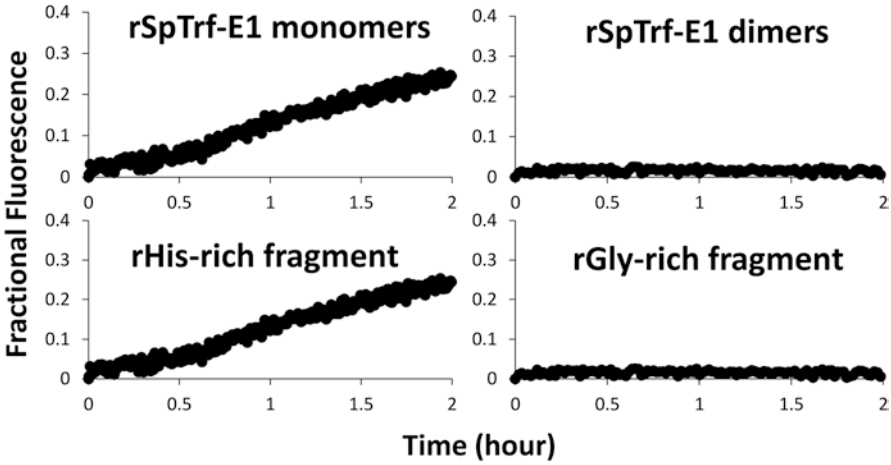

Fig. 15 *rSpTrf-E1 and the rHis-rich fragment induce leakage of luminal contents from liposomes*. Liposomes composed of 10% PA and loaded with 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS; fluorescent dye) plus p-xylene-Bis-pyridinium bromide (DPX; quencher) are incubated for 2 h with recombinant proteins. Luminal leakage separates ANTS from DPX by dilution into the buffer, and fluorescence is recorded relative to control liposomes that are lysed to measure leakage equivalent to 100% (fractional fluorescence). Dye leakage from liposomes is detected only with monomeric rSpTrf-E1 or the rHis-rich fragment, whereas neither the dimerized rSpTrf-E1 nor the rGly-rich fragment induce leakage. (Modified from Smith and Lun (2017))

erythrogramma, which lives in the southern hemisphere (Dheilly et al. 2011a; Roth et al. 2014). The *HeTrf* genes are closely related to those in *S. purpuratus*, with a conserved overall structure of two exons with substantial levels of sequence homology (Roth et al. 2014). Both *Trf* families have mosaic patterns of elements in transcripts and genes although the introns are different (Fig. 16a, b). The sequences and numbers of elements differ slightly among the *HeTrf* and *SpTrf* sequences (compare Figs. 16a and 9), but the structure of the transcripts and genes are generally similar (Terwilliger et al. 2006, 2007; Buckley and Smith 2007; Roth et al. 2014). Sequence diversity in the *HeTrf* sequences is largely based on element pattern differences, but SNPs, indels, and a variety of repeats also contribute (Roth et al. 2014). Sequence alignments indicate that negative selection against codon diversification is common in both families, with significant negative selection apparent in 4.7% of *HeTrf* codons and 11% of *SpTrf* codons. In contrast, codons subject to positive selection are more rare (2.5% in *HeTrf* and 2.6% in *SpTrf*). These differences likely result from differential selection pressures from variable versus stable binding targets in the relative habitats of these two sea urchin species.

HeTrf Proteins: Diversity and Cellular Localization

The Trf protein repertoires of both species show significant differences among individual animals and significant changes in response to immunological challenges (Dheilly et al. 2009; 2011a; Sherman et al. 2015). The HeTrf and SpTrf proteins are

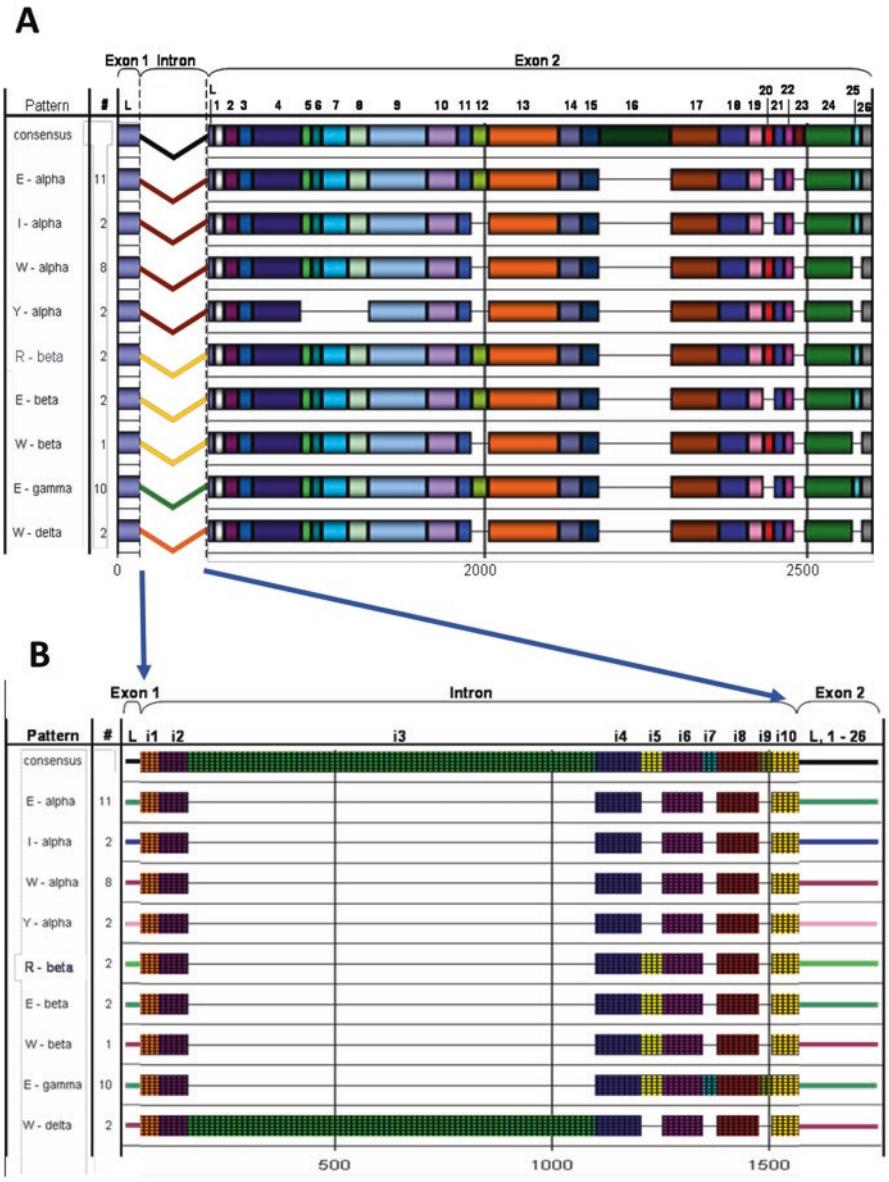

Fig. 16 *An alignment of HeTrf gene sequences identifies 39 unique element patterns.* (**a**) Exon element patterns. The two exons are separated by an intron that is positioned within the region encoding the leader (L). Nine combinations of exon and intron elements of 29 element patterns are shown. All possible elements are shown at the top as a consensus pattern. The numbers (#) of genes with unique nucleotide sequences that comprise a certain element pattern are shown in the second column. (**b**) An expanded area in (**a**) shows the regions that encode the leader and the intron. The introns align optimally after the insertion of large gaps that define ten intron elements and four intron element patterns that are designated as alpha, beta, gamma, and delta. Individual intron elements are termed i1 to i10 and are shown as blocks of different colors with hatched patterns. Exons are shown as horizontal lines that flank the intron elements, with different colors representing different exon element patterns. (Reprinted from Roth et al. (2014))

similar, with a hydrophobic leader, glycine-rich and histidine-rich regions, and several potential N- and O-linked glycosylation sites. However, most HeTrf sequences lack the RGD motif near the intersection of the glycine-rich and histidine-rich regions (Fig. 12a) (Roth et al. 2014). Despite differences in element patterns (compare Figs. 9 and 16a), the protein families have some well-conserved blocks of predicted amino acid sequence with identities of up to 100%. The HeTrf proteins exhibit a diverse range of molecular weights and isoelectric points, which is similar to that of SpTrf proteins (Brockton et al. 2008; Dheilly et al. 2009; Roth et al. 2014; Sherman et al. 2015). Under strong reducing conditions, both HeTrf and SpTrf proteins have far larger apparent molecular weights than predicted from transcripts, which may reflect a combination of posttranslational modifications and multimerization.

The HeTrf proteins are predominantly associated with the cell surface and perinuclear vesicles of coelomocytes (Roth et al. 2014), and associate directly with the membranes of vesicles originating from the trans face of the Golgi apparatus, later fusing with the plasma membrane (Dheilly et al. 2011a). HeTrf proteins are also expressed by the rare fusiform cells of *H. erythrogramma*, a cell type that is absent in *S. purpuratus* (Dheilly et al. 2011a). Most significantly, the HeTrf proteins associate with what appear to be phagosomes containing ingested bacteria within cells found in the gut epithelium (Dheilly et al. 2011a). The location of the HeTrf-positive cells in the gut is in agreement with gut-associated SpTrf-positive cells (Fig. 12g) (Majeske et al. 2013a). These observations suggest that HeTrf proteins may be involved in the opsonization of microbes that have invaded the gut epithelium (see section "The Immune Response in Sea Urchin Larvae" for larval responses to *Vibrio* infection in the gut).

Phylogenetic Differences in the Two Families of *Trf* Genes

Phylogenetic analysis of *HeTrf* and *SpTrf* transcripts indicates that the sequences cluster into two separate, species-specific clades (Fig. 17), suggesting that the two Trf families evolved independently after the genera diverged about 35 million years ago (Palumbi and Lessios 2005; Roth et al. 2014). This divergence may be a consequence of the different life history traits in these two sea urchins, in which *S. purpuratus* develops via a relatively long-lived larval stage that feeds in the plankton, whereas *H. erythrogramma* produces relatively few large eggs that develop directly into juveniles (Laegdsgaard et al. 1991; Palumbi and Lessios 2005). However, it is also likely that the divergence between the two *Trf* gene families is the result of pathogen pressure not only for the larval *S. purpuratus* in the water column but also for the adults living in different habitats and feeding on different macroalgae and substrate biofilms. This is expected for gene families encoding proteins that interact with the environment and are under pressure from pathogens. Swift diversification of the *SpTrf* gene family has been predicted not only from the gene clusters and the associated repeats but also as an advantage in the arms race with pathogens (Smith and Coscia 2016).

The Complement System

The vertebrate complement system is composed of more than 50 proteins (Volanakis 1998) functioning in three proteolytic activation cascades that converge to cleave

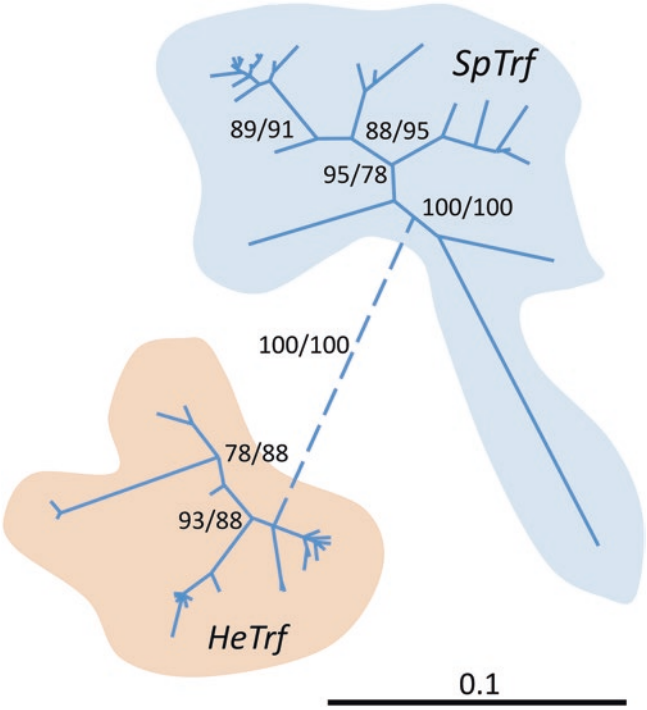

Fig. 17 *The Trf transcript sequences from Strongylocentrotus purpuratus and Heliocidaris erythrogramma cluster separately*. The phylogenetic dendrogram shows that *SpTrf* (blue) and *HeTrf* (orange) sequences form two distinct clades. Each branch represents a single distinct *SpTrf* or *HeTrf* sequence. The central branch (dashed line) separating the two clades is shortened for display purposes and reflects a corrected genetic distance of 0.27949 that is supported by bootstrap values of 100/100. A subset of bootstrap values are shown that indicate values for neighbor joining and maximum parsimony analyses (NJ/MP). All nodes with bootstrap values of <50 are collapsed. The scale bar indicates the corrected genetic distance. (Revised from Roth et al. (2014))

and activate the central component of complement, C3, which has an important opsonization function and is required to activate the terminal or lytic pathway. The classical and lectin activation pathways are triggered by the recognition of nonself antigens or PAMPs, which initiate a cascade of serine proteases that activate the effector complement proteins. The alternative activation pathway is constitutively activated by the C3 "tick-over" mechanism in which the thioester site is spontaneously activated and exposed (Tomlinson 1993). The central component, C3, functions in all pathways, and once it is cleaved, the C3a fragment exerts proinflammatory functions by interacting with receptors on self cells and the larger C3b fragment with the activated thioester site binds to foreign cell surfaces. Furthermore, cleavage of C3 to C3b initiates the positive feedback loop of the alternative pathway that amplifies the production of cleaved and activated C3 fragments to augment opsonization of a binding target, which increases immune responsiveness and effectiveness (Hugli 1990). Many complement components are secreted as zymogens

(inactive precursors), which are activated by cleavage to fragments with effector functions. In addition, there are numerous proteins with regulatory and inhibitory activity to control the system and block or protect self cells from attack. The inhibitory regulators ensure that the cascades are activated in a controlled manner such that effector activities occur at the right time and place and that host cells are protected from complement-mediated lysis (Zipfel and Skerka 2009).

SpC3, the Sea Urchin Homologue of the C3 Complement Component

The first evidence of a complement system in an echinoderm was suggested for the green sea urchin, *S. droebachiensis*, in which phagocytosis of RBCs by adult phagocytes could be augmented when opsonized by human C3 (Kaplan and Bertheussen 1977; Bertheussen 1981a, b, 1982; Bertheussen and Seljelid 1982), and could be decreased or blocked by inhibitors of mammalian complement (Bertheussen 1983). The first molecular evidence of an echinoderm complement component was based on transcript sequences from *S. purpuratus* phagocytes with homology to vertebrate C3 and called *SpC3* (see Smith et al. (1996), Al-Sharif et al. (1998), and Gross et al. (2000), reviewed in Smith et al. (2001)). *SpC3* transcripts are present in unfertilized eggs and persist throughout embryogenesis; however, *SpC3* gene expression increases in gastrulae when the embryos are cultured with *Vibrio diazotrophicus* (Shah et al. 2003). In adults, the preprocessed SpC3 form is stored in vesicles (Fig. 18a) as a single protein of 210 kDa, which is slightly larger than human C3 (190 kDa) (Al-Sharif et al. 1998; Gross et al. 2000). Upon secretion, SpC3 is cleaved at the βα junction during final processing to the α chain (130 kDa) and β chain (80 kDa), which are linked by disulfide bonds between cysteines in conserved positions (Al-Sharif et al. 1998). SpC3 protein levels are very low in the CF of IQ sea urchins but are readily induced upon injection with LPS (Clow et al. 2000, 2004). Homologues of C3 and complement receptor type 2 (CR2) are among the most abundant proteins in the CF of *S. purpuratus* and *H. erythrogramma*, which underscores the importance of these proteins in the echinoderm immune system (Smith 2002; Dheilly et al. 2013).

The SpC3 opsonization function in sea urchins is likely similar to that in vertebrates and is enhanced by challenge with LPS (Clow et al. 2004). Preincubation of

Fig. 18 (continued) an irrelevant antibody (Smith et al. 1992). [2]Yeast opsonized with LPS-activated CF was incubated with α-SpC3 antibody and subsequently washed with 1 mM glycine, pH 2, to remove the α-SpC3 but not the covalently bound complement protein. [3]Yeast opsonized with LPS-activated CF and incubated with α-SpC3. [4]Yeast opsonized with LPS-activated CF. [5]Yeast opsonized with ASW. [6]The phagocytic stimulation index (PSI) is the number of yeast cells phagocytosed per 100 coelomocytes. PSI are shown as means ±SEM from six phagocytosis experiments. *Significance ($p < 0.05$) is based on a Student's two-tailed *t* test. (Republished from Clow et al. (2004) in the Journal of Experimental Biology.) (**c**) SpC3 undergoes autolysis, indicating a functional thioester site. The western blot shows autolysis of SpC3 in CF under denaturing conditions plus heating at pH 10 to generate the 50 kDa fragment of the α chain that is recognized by the α-SpC3 antibody (lane 1). The full-length α chain in SpC3 is 130 kDa. The 80 kDa fragment of the α chain after autolysis is not recognized by the antibody and is not visible. Incubation of SpC3 with methylamine ($MeNH_2$) or yeast to engage the thioester site blocks autolysis (lanes 3–5). The autoradiograph demonstrates that the SpC3 α chain is labeled after incubation with ^{14}C-labeled methylamine (^{14}C-$MeNH_2$). M protein standard. (Modified from Smith (2002) and reprinted with permission from Elsevier)

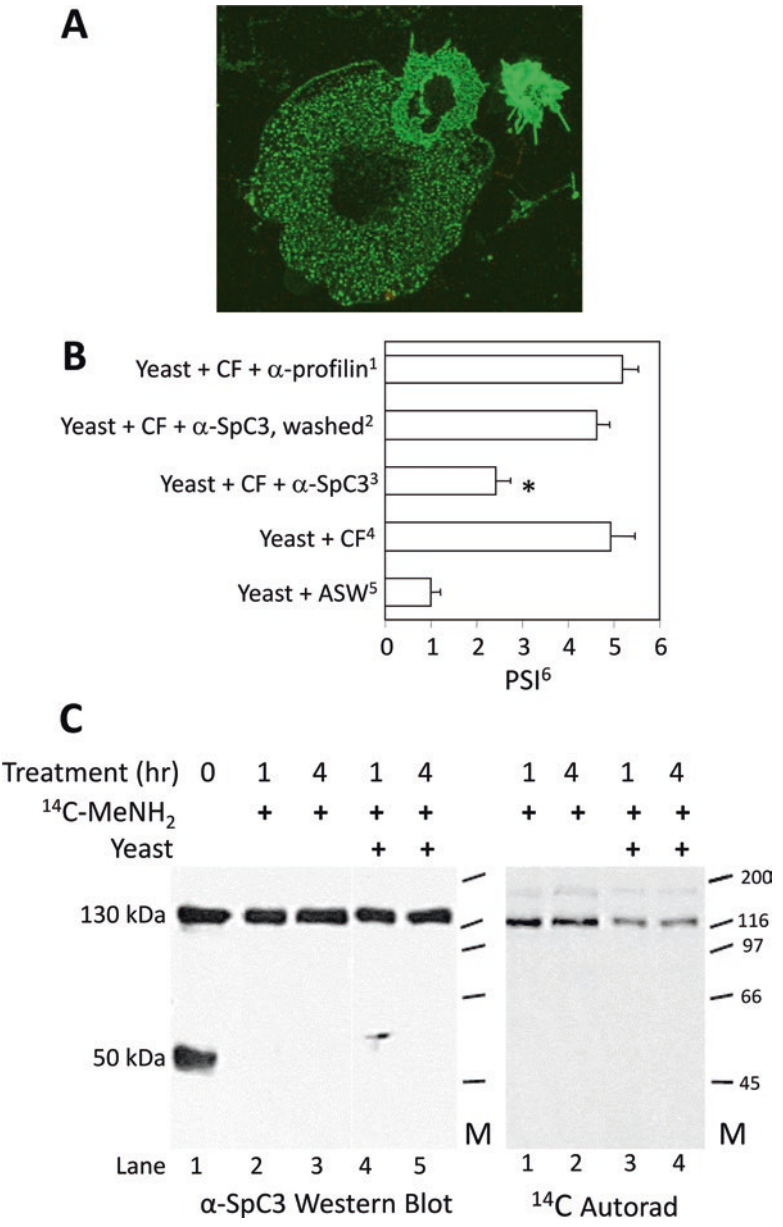

Fig. 18 *The complement system in echinoderms*. (**a**) Three coelomocytes from the purple sea urchin, *Strongylocentrotus purpuratus*, show SpC3 (green) within small cytoplasmic vesicles. The antibody to SpC3 (Al-Sharif et al. 1998) binds to the N-terminal end of the α chain. The central dark area within the cells is the unstained nucleus. The image was captured by confocal microscopy (republished from Gross et al. (2000), with permission from Springer Publishing). (**b**) SpC3 opsonizes yeast and increases phagocytosis by sea urchin coelomocytes. Cell-free coelomic fluid (CF) was obtained on day 3 from sea urchins after injections of LPS on days 1 and 2. Yeast (*S. cerevisiae*) was mixed with either CF or artificial seawater (ASW; Instant Ocean) and evaluated for phagocytosis by sea urchin coelomocytes. [1]Yeast opsonized with LPS-activated CF followed by incubation with α-profilin,

yeast with CF that includes SpC3 significantly increases phagocytosis by sea urchin phagocytes in comparison with nonopsonized controls, demonstrating the opsonization function of SpC3 (Fig. 18b) (Clow et al. 2004). Mammalian C3 and C4 undergo autolysis under heat and high pH, which requires an active thioester site and results in α chain cleavage into an N-terminal fragment of 50 kDa (Sim and Sim 1981). A similar autolytic reaction is observed for sea urchin SpC3, which also generates an α chain fragment and can be blocked by deactivation of the thioester (Fig. 18c) (Smith 2002). These data support homology between echinoderm SpC3 and vertebrate C3, which is based on both sequence and function.

SpBf, the Sea Urchin Factor B Homologue

In vertebrates, the second component in the alternative pathway is Factor B (Bf), which is a serine protease that interacts with cleaved and activated C3b. A transcript sequence with homology to Factor B (*SpBf*) is also expressed by coelomocytes (Smith et al. 1998). *SpBf* is constitutively expressed by phagocytes, and the deduced protein has a typical mosaic domain structure of five short consensus repeats (SCRs) at the N terminus, a conserved Factor D cleavage site, a von Willebrand Factor domain, and a serine protease domain (Smith et al. 1998). Although Factor D has not been identified in echinoderms, the positions of its cleavage sites are conserved in all Bf/C2 orthologues identified to date. Alternative splicing varies the number of SCRs in SpBf, which may result in different binding affinities for SpC3 (Terwilliger et al. 2004) or may interact with the several thioester-containing proteins encoded in the genome (see section "Additional Complement Homologues in the Sea Urchin Genome Sequence" and Table 1). If the sea urchin complement system functions as in other animals, SpBf may interact with activated SpC3, forming an SpC3–convertase complex similar to that in the alternative pathway of vertebrates, including a positive feedback loop and effective opsonization of foreign targets (Fig. 19). Variable expression of SpC3 may be a simple mechanism for regulating complement activity that is based on SpC3 protein concentration in the CF in response to immune challenge (Terwilliger et al. 2004).

Additional Complement Homologues in the Sea Urchin Genome Sequence

Annotation of genes in the *S. purpuratus* genome sequence indicated that initial reports of transcript sequences had identified only a subset of the genes homologous to vertebrate complement components. There are two *SpC3* genes, of which the second, *SpC3-2*, is expressed in embryos, and four additional genes that encode thioester-containing proteins—of which three are embryonically expressed—have not been investigated for function (Table 1) (see supplementary material in Hibino et al. (2006)). There are three paralogous *SpBf* genes that all encode SpBf proteins with five SCR domains (also called sushi domains or complement control protein (CCP) modules). It is noteworthy that a BAC clone insert identified a genomic linkage between *SpC3* and *SpBf*, which are also linked in the class III region of the major histocompatibility complex in vertebrates (Rast et al. 2000). A single mannose-binding lectin (*SpMBL*) homologue and four paralogues of *SpC1q* may

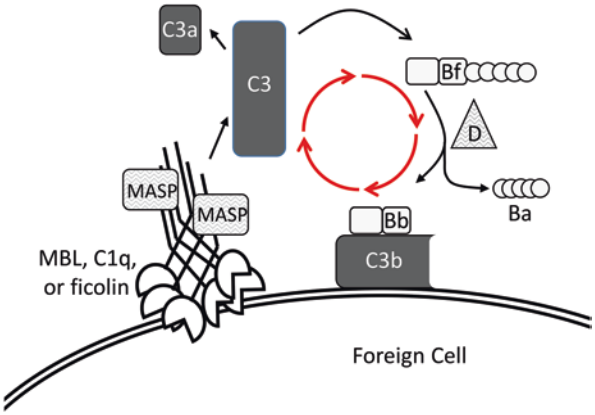

Fig. 19 *The echinoderm complement system* The echinoderm complement system may be activated by a lectin-like pathway and augmented by an alternative pathway feedback loop. Pathogens or other foreign surfaces may be detected by homologues of mannose-binding lectin (MBL), C1q, or ficolins that lead to cleavage and activation of the C3 homologue by mannose-binding-lectin-associated serine protease (MASP) homologues. Cleaved C3b binds to the foreign surface and associates with a Factor B (Bf) homologue, which is cleaved (Bb) and activated by a Factor D (D) homologue. This forms a C3-convertase-like complex (C3b and Bb) that cleaves and activates more C3 through an activation feedback loop (red arrows) to coat the pathogen with opsonizing C3b. The genes encoding Factor D and the MASPs have not been identified in the sea urchin genome sequence, although they have been identified in other echinoderms (see Table 5), and are indicated by patterned shapes. Properdin function is not shown. (Modified from Smith et al. (1999))

encode proteins that may activate a lectin-like complement pathway (Fig. 19) (Smith et al. 1999). Orthologues of members of the terminal or lytic pathway have not been identified in echinoderm genome sequences. Although there are 21 gene models that encode perforin-related proteins with MACPF domains (Table 1), the proteins are unlikely to function in the sea urchin terminal complement pathway, because they do not have the expected domain architecture. However, it is noteworthy that in the *S. purpuratus* genome there are two genes encoding CD59 homologues that, in vertebrates, block assembly of the membrane attack complex (MAC).

Because complement proteins with thioester sites can form covalent bonds with exposed amines or hydroxyls on any molecule, they can bind any cell surface, including self, and can result in inappropriate cell lysis, opsonization, and inflammatory reactions. Given that covalent binding between C3 or C4 and nonself is an important mechanism for identifying and clearing pathogens, self cells in vertebrates are protected from autologous complement attack by the complement regulatory system. Initial evidence for a complement regulatory system in the sea urchin is predicted from conserved cleavage sites in the SpC3 sequence (Al-Sharif et al. 1998). Two possible regulatory proteins identified from *S. purpuratus* coelomocyte transcripts encode the mosaic proteins: Sp Complement Related protein Long form (SpCRL) and Short form (SpCRS). The deduced protein sequences have 4–18 SCR domains with diverse sequences but which show sequence similarities to SCR

sequences in Factor I, Factor H, and a Factor I membrane attack complex (FIMAC) domain shared with members of the terminal or lytic pathway in vertebrates (Multerer and Smith 2004). Both genes are expressed in coelomocytes, gut, gonad, pharynx, esophagus, and axial organs, and are not induced by immune challenge with LPS, suggesting a constitutive protective function against complement attack for all self cells. Many complement regulatory proteins and several of the complement receptors are characterized by domain compositions that often include multiple tandem SCRs. Domain-based searches of the *S. purpuratus* genome (v4.2) indicate that 252 additional gene models contain SCRs. Although SCRs are present in a wide variety of complement proteins, many with SCRs are also likely to have nonimmune functions. Hence, functional analysis of these candidate complement regulatory proteins and receptors will be required to identify which of these proteins are additional components of the sea urchin complement system.

Complement Genes in Other Classes of Echinoderms

Since the original identification of complement homologues in the purple sea urchin, complement homologues have been identified in many other invertebrates (Table 5). This conservation underscores the importance of the antipathogen activities of a complement system that is likely based on opsonization. Contrary to initial reports (Ramírez-Gómez et al. 2008), complement homologues are present in the sea cucumber *Apostichopus japonicus*, including *MBL* (Vasilenko et al. 2012), two gene copies of *C3* (*AjC3* and *AjC3-2* (Zhou et al. 2011)) and one homologue of *Bf* (*AjBf* (Zhong et al. 2012)). AjC3-2 has all of the expected domains for a C3 protein; however, the sequence shows poor conservation of binding sites for CR1, CR2, C3aR, Bf, and Factor H, as well as cleavage sites for Factor I (Zhou et al. 2011). Maternal transcripts for both *AjC3* isoforms are present in eggs, and gene expression increases gradually during embryogenesis and peaks during larval stages. In adults, *AjC3* homologues are expressed in coelomocytes and upregulated in response to immune challenge. The *Bf* gene (*AjBf-2*) in *A. japonicus* is highly expressed in the tentacles, body wall, and coelomocytes (Zhong et al. 2012). Unlike *SpBf* in *S. purpuratus*, *AjBf-2* is expressed in the coelomocytes and body wall, and responds to LPS challenge. Only a few homologues of complement proteins have been identified in the Asteroidea. In the sea star *Asterias rubens*, the deduced peptide sequence from a partial sequence shows similarities to C3. Expression increases in both coelomocytes and the hepatopancreas in response to LPS (Mogilenko et al. 2010). Expression of complement homologues is also noted in the antiviral response in the sunflower star, *Pycnopodia helianthoides*, with a strong complement response to SSaDV (see section "Sea Star Wasting Disease") (Fuess et al. 2015). A complement system is likely present in all echinoderms that relies on lectin-based and C3 tick-over activation to opsonize and augment phagocytosis of foreign targets.

Complement System Activation in Echinoderms

The activities of the echinoderm complement system, based on the factors described above, is likely amplified by the positive feedback loop of the alternative pathway

Table 5 Complement components are present in a wide range of invertebrates

Phylum	Species	Common name	Component	References
Echinodermata	*Strongylocentrotus purpuratus*	California purple sea urchin	C3, Bf	Smith et al. (1996), Al-Sharif et al. (1998), Smith et al. (1998), Terwilliger et al. (2004)
	Apostichopus japonicus	Sea cucumber	MBL, C3, Bf	Vasilenko et al. (2012), Zhou et al. (2011), Zhong et al. (2012)
	Asterias rubens	Sea star	C3	Mogilenko et al. (2010), Leclerc et al. (2013)
	Pycnopodia helianthoides	Sunflower star	C3, Bf/C2, ficolin, properdin	Feuss et al. (2015)
Chordata	*Halocynthia roretzi*	Tunicate	C3	Nonaka and Azumi (1999)
	Botryllus schlosseri	Colonial tunicate	C3, Bf, MASP1, MBL, ficolin	Franchi and Ballarin (2014, 2017)
	Branchiostoma japonicum	Amphioxus	Properdin	Gao et al. (2017)
			C3, Bf	Suzuki et al. (2002), He et al. (2008)
			C1q	Gao et al. (2014)
			Ficolin	Huang et al. (2011)
	Ciona intestinalis	Tunicate	MASP, MBL	Skjoedt et al. (2010)
			C3	Marino et al. (2002)
Arthropoda	*Tachypleus tridentatus*	Chinese horseshoe crab	C3	Ariki et al. (2008)
	Hasarius adansoni	Adanson's house jumper	C3	Sekiguchi et al. (2012)
Mollusca	*Euprymna scolopes*	Hawaiian bobtail squid	C3	Castillo et al. (2009)
	Sinonovacula constricta	Razor clam	C3	Peng et al. (2017)
	Ruditapes decussatus	Carpet-shell clam	C3, Bf	Prado-Alvarez et al. (2009)
	Mytilus galloprovincialis	Mussel	C3	Gerdol and Venier (2015)
Cnidaria	*Swiftia exerta*	Gorgonian coral	C3	Dishaw et al. (2005)
	Nematostella vectensis	Starlet sea anemone	C3, Bf, MASP	Kimura et al. (2009)
	Diadumene lineata	Orange-striped green sea anemone	C3	Fujito et al. (2010)
	Acropora millepora	Branching stony coral	C3	Miller et al. (2007)

(Fig. 19). This activity would be mediated by the formation of a C3–convertase–like complex consisting of a C3b homologue bound to a target surface through the thioester bond and associated with a Bf homologue. Echinoderm genomes likely encode a Factor D homologue (currently unidentified) that would cleave Bf and activate the serine protease function of Bb. Bb plus C3b would associate to form the C3–convertase–like complex to cleave and activate additional C3 in the feedback loop of the alternative pathway. Although homologues of MASP genes have not been identified in the sea urchin genome sequence, they have been reported in other invertebrates (Table 5) and their protease activity associated with target binding by homologues of MBL, C1q, and ficolins in echinoderms is assumed. On the basis of the inability to identify members of the terminal or lytic pathway in echinoderms or the equivalent of the MAC through either biochemistry, transcriptomics, or genomic analysis, it is likely that the ancestral function of the complement system in deuterostomes is opsonization of targets with covalently bound tags to augment phagocytosis, which results in effective host protection.

Antimicrobial Peptides in Echinoderms

Antimicrobial Peptide Characteristics

The survival and fitness of echinoderms in a marine environment near coastlines or estuaries that are heavily populated by microorganisms suggest that these invertebrates have an effective innate immune system (Tincu and Taylor 2004). In many invertebrate lineages, a central component of immunity is mediated by the activities of humoral components such as antimicrobial peptides (AMPs). AMPs are typically low molecular weight proteins (2–50 kDa; 50–100 amino acids) (Ganz 2003; Maroti et al. 2011) with significantly different structures, conformations, and functions, with antimicrobial activity against a broad spectrum of bacteria, viruses, and fungi. Many are evolutionarily conserved and are widely distributed in a wide range of organisms (Leippe 1999; Pag and Sahl 2002; Garcia-Olmedo et al. 1998). Many AMPs have a net positive charge, many form amphipathic structures that are stable in aqueous and hydrophobic solutions, and some are posttranslationally modified, including proteolytic release from larger precursors (Zasloff 2002; Hancock and Sahl 2006; Li et al. 2015). AMPs are typically classified as α helical, β pleated sheet, or a mix of both, in addition to extended regions of disordered loops (Melo et al. 2009). Some AMPs have no stable structure in solution and are considered to be IDPs that fold to their final active conformation upon binding to a target (Zhang et al. 2014).

The recruitment and interaction of AMPs with bacterial membranes is initially based on an attraction between the cationic portion of the AMPs and the negatively charged microbial membrane containing phosphatidylglycerol, cardiolipin, or phosphatidylserine (Scott et al. 1999; Zhao et al. 2001; Yeaman and Yount 2003). Some AMPs act as multifunctional microbicides that interfere with bacterial membranes through electrostatic attraction (Scott et al. 1999; Zhao et al. 2001), followed by

attachment (Heller et al. 2000; Huang, 2000), insertion (Yang et al. 2001; Lee et al. 2004), and formation of pores with hydrophilic channels in the lipid bilayer (Lee et al. 2004; Wang et al. 2015), in which the pore lumen is partly lined by peptides and phospholipid head groups (Matsuzaki et al. 1996). Other AMPs interact with intercellular molecules (Wang et al. 2015; Yonezawa and Sugiura 1992; Park et al. 1998) and inhibit protein and/or DNA synthesis (Boman et al. 1993; Subbalakshmi and Sitaram 1998) or cell wall synthesis (Brotz et al. 1998; Le et al. 2016). AMPs may neutralize endotoxins and cellular chemotaxis, and may modulate immune responses by altering cytokine production, angiogenesis, and wound repair (Hancock and Sahl 2006; Li et al. 2000; Shi et al. 1996; Rosenfeld et al. 2006; Veldhuizen et al. 2014). These potential antipathogen activities of AMPs have focused attention on their development for novel drugs.

Echinoderm Antimicrobial Peptides

Strongylocins and Centrocins

Several AMP families have been characterized in echinoderms (Table 6). These include the echinoid AMPs strongylocins and centrocins, which were originally characterized in the green sea urchin, *Strongylocentrotus droebachiensis* (Li et al. 2008, 2010b), with orthologues in the other sea urchins *S. purpuratus* (Li et al. 2010a), *Echinus esculentus* (Solstad et al. 2016), and *Arbacia lixula* (Perez-Portela et al. 2016). Strongylocins are cysteine-rich peptides, with six cysteines that may form three disulfide bridges to stabilize the peptide structure. Strongylocins have a leader that targets the peptide for secretion (Coleman et al. 1985; von Heijne 1990; Reddy et al. 2004), which is followed by a prosequence and the mature peptide (Li et al. 2008; Solstad et al. 2016). Both native and recombinant AMPs from echinoderms show antibacterial activity against both Gram-positive and Gram-negative bacteria. Recombinant (r)SpStrongylocins do not alter bacterial membrane permeability, suggesting intracellular targets or other means of microbial killing (Li et al. 2010a). SdStrongylocin 1 is expressed in phagocytes, vibratile cells, and colorless spherule cells, whereas SdStrongylocin 2 is expressed in phagocytes and red spherule cells (Li et al. 2014a). The diversity of the signal sequences and the expression profiles suggest that strongylocins likely have unique effects during immune responses among different echinoids.

The centrocin AMP family is present in several sea urchin species (Table 6) (Li et al. 2010b; Perez-Portela et al. 2016; Solstad et al. 2016). Centrocins are heterodimeric peptides consisting of a heavy chain plus a light chain, which is likely involved in the formation and stabilization of the active heterodimeric structure and resistance to bacterial proteases (Li et al. 2010b). Comparison of the deduced peptide sequences with native centrocin sequences indicates that the precursor peptide is cleaved from the preprosequence, the interchain, and the C-terminal dipeptide (Gly-Arg) after the posttranslational modification (Li et al. 2010b). In other species, the dimeric structure of these peptides enhances antimicrobial activity, solubility, and

Table 6 Antimicrobial peptides and proteins in echinoderms

Class and genus	Origin	Peptides	References
Asteroidea			
Asterias rubens	Coelomocytes	Fragments of actin, histone H2A, and filamin A[a]	Maltseva et al. (2007)
		Peptides	Maltseva et al. (2007)
Echinoidea			
Paracentrotus lividus	Coelomocytes	Fragments of β-thymosin[a]	Schillaci et al. (2010)
		Paracentrin 1	Schillaci et al. (2010)
Strongylocentrotus droebachiensis	Coelomocytes	SdStrongylocin	Li et al. (2008)
		SdCentrocin	Li et al. (2010b)
Strongylocentrotus purpuratus	Coelomocyte cDNA	SpStrongylocin[b]	Li et al. (2010a)
Echinus esculentus	Coelomocytes	EeStrongylocin 2 EeCentrocins	Solstad et al. (2016)
Arbacia lixula	Coelomocyte cDNA	AlStrongylocin	Perez-Portela et al. (2016)
	Gut and ovary cDNA	AlStrongylocin 2b	Perez-Portela et al. (2016)
	Coelomocyte, gut, testis, and ovary cDNA	AlCentrocin 1b	Perez-Portela et al. (2016)
Holothuroidea			
Cucumaria echinata	Whole body	Fragments of CEL-III protein[a,c]	Hatakeyama et al. (2004)
Cucumaria frondosa	Coelomic fluid	Sequence unknown	Beauregard et al. (2001)
Holothuria tubulosa	Coelomocytes	Holothuroidins[a,c]	Schillaci et al. (2013)

[a]Putative AMPs, derived from larger precursor molecules
[b]Recombinantly produced peptides
[c]Synthetic peptides

resistance to bacterial proteases (Dempsey et al. 2003; Pini et al. 2005; Lee et al. 2008; Dewan et al. 2009; Liu et al. 2010a, b; Shin et al. 2010).

Although both the strongylocin and centrocin families of AMPs are expressed in sea urchin coelomocytes, transcripts are also detected in digestive and reproductive tissues (Perez-Portela et al. 2016). Strongylocins and centrocins have different gene expression profiles in the four coelomocyte subclasses and are also expressed during embryogenesis (Li et al. 2014a, b). In larvae, centrocin 1 is present in the vesicles of blastocoelar cells that surround the stomach and esophagus and also present in the larval arms in locations that correspond to larval immune cells, which are central mediators of responses to ingested bacteria (see section "Blastocoelar Cells") (Ho et al. 2016). The identification and analyses of echinoderm AMPs indicate that they may be involved in various immune activities and that they respond to invading bacteria in both larvae and adult sea urchins.

Antibiofilm Activities of Antimicrobial Peptides from the Sea Urchin *Paracentrotus lividus*

In aquatic environments, bacteria may be present as single, free-swimming cells, which can form sessile, surface-associated multicellular communities called biofilms (Costerton et al. 1995; Hall-Stoodley et al. 2004; de la Fuente-Nunez et al. 2013). Biofilms occupy diverse environments and are highly resistant to antimicrobial therapies (Fey 2010; Spizek et al. 2010). Furthermore, damage to host tissues or microbiota allows access for secondary infections by opportunistic pathogens that can also form biofilms (Kostakioti et al. 2013). Almost all Gram-negative and Gram-positive bacteria form biofilms, and many are highly pathogenic when in this form. Biofilm formation is coordinated by a quorum-sensing communication system through small molecules to facilitate optimal cell density (Spoering and Gilmore 2006; Horswill et al. 2007; Brogden and Brogden 2011). Investigations of AMPs from a wide range of animals that may be used as novel drugs with activity against biofilms are of significant importance.

Novel cationic peptides from the sea urchin *Paracentrotus lividus* inhibit biofilm activity (Schillaci et al. 2010), as well as the growth of the pathogens *Staphylococcus aureus*, *S. epidermidis*, and *Pseudomonas aeruginosa* (Schurr et al. 1994; Schillaci et al. 2010, 2013, 2014). The acid extract of coelomocyte lysates from *P. lividus* shows antimicrobial activity for peptides of less than 5 kDa (collectively called 5-CC). 5-CC has a range of minimal inhibitory concentrations (MICs), depending on the bacterial species, and blocks biofilm formation effectively for *S. epidermidis* and *S. aureus* at sub-MIC concentrations (Fig. 20a–d). The biological activity of 5-CC is due to the peptide Paracentrin 1 (SP1; 1.251 kDa), which shares a short region of sequence identity with β thymosin from *P. lividus*. The structural properties of SP1 and the thymosin fragment, $hT\beta_4$, based on molecular dynamics, show that both have a central hydrophobic core and peripheral charged amino acids (Fig. 21). β thymosin has numerous biological effects such as induction of chemotaxis, angiogenesis, expression of metalloproteinases, and inhibition of inflammation (Huff et al. 2001). Chemically synthesized SP1 is active against the aquatic form and biofilms of several staphylococcal strains, in addition to *P. aeruginosa* (Fig. 20e, f) (Schillaci et al. 2014).

Holothurian Antimicrobial Peptides

Holothurians also express proteins and peptides with antimicrobial activity (e.g., see Kuznetsova et al. (1982), Ridzwan et al. (1995), Mohammadizadeh et al. (2013), Kiani et al. (2014)). For example, a lectin with antimicrobial activity against Gram-positive and Gram-negative bacteria is induced in the sea cucumber *Holothuria scabra* by bacterial challenge (Gowda et al. 2008). Immune cell lysates from the congeneric sea cucumber *H. tubulosa* contain two novel peptides, Holothuroidin 1 (H1; 1.389 kDa) and Holothuroidin 2 (H2; 1.547 kDa), which have characteristics of cationic AMPS (Schillaci et al. 2013). Both H1 and H2 are active against human bacterial pathogens and inhibit biofilm formation (Schillaci et al. 2013). H1 and H2 are ~30% hydrophobic with amphipathic α helices arranged so that the peptides have a hydrophilic and a hydrophobic face. This amphipathic folding characteristic

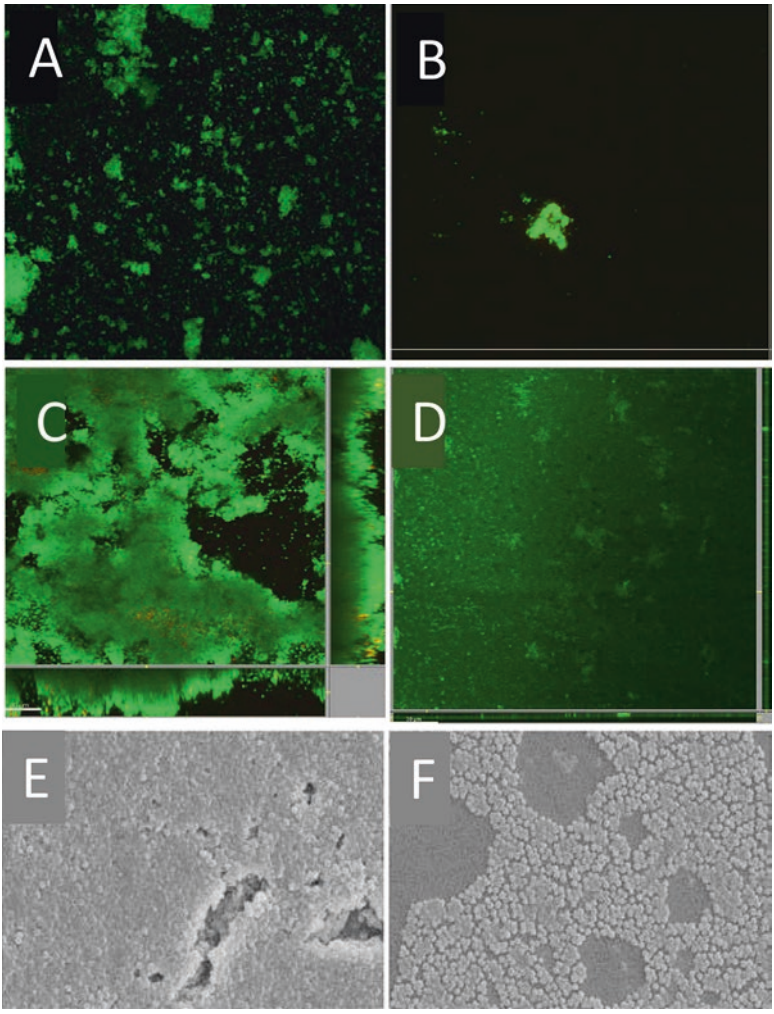

Fig. 20 *Peptides from the sea urchin Paracentrotus lividus have antibacterial activity*. (**a**–**d**) Biofilms of *Staphylococcus epidermidis* (strain 1457) were incubated for 6 or 24 h with the AMP mixture of Holothuroidin 1 (H1) and H2 (originally defined as 5-CC) (Schillaci et al. 2010). Dead cells were stained with propidium iodide (red) and live cells were stained with SYTO9 (green), followed by evaluation by laser scanning microscopy. The assays were repeated at least twice, with similar results. (**a**) After 6 h in the absence of 5-CC. (**b**) After 6 h with 5-CC. (**c**) After 24 h in the absence of 5-CC. (**d**) After 24 h with 5-CC. (**e**, **f**) Paracentrin 1 (SP1) inhibits growth and biofilm formation of *S. epidermidis* (strain RP62A) at subminimum inhibitory concentrations. (**e**) *S. epidermidis* grown without SP1. (**f**) *S. epidermidis* grown in the presence of SP1. Images (**e**) and (**f**) were captured by scanning electron microscopy. (Modified from Schillaci et al. (2014) in AMB Express)

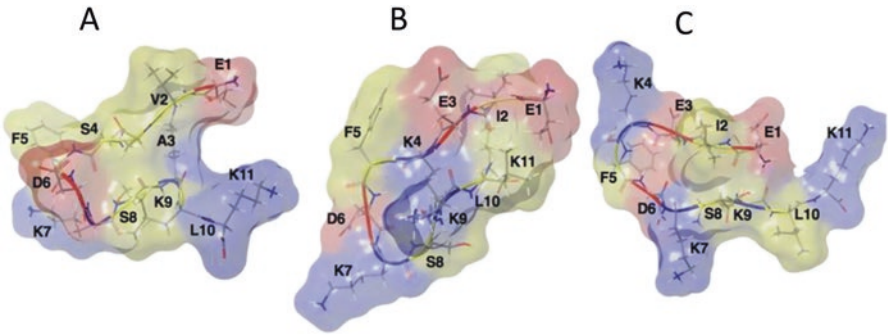

Fig. 21 *Molecular structure of the most stable conformations of SP1 and hTβ₄.* (**a**) SP1. (**b**) hTβ$_4$. (**c**) hTβ$_4$ rotated. The locations of some amino acids are labeled with colors to indicate acidic residues (red), basic residues (blue), and noncharged residues (yellow). (Reprinted from Schillaci et al. (2016), with permission from Springer Publishing)

facilitates interactions between the hydrophilic face of the peptides with the polar edges of bacterial membranes, and engages the nonpolar face with the hydrophobic core of membranes (Schillaci et al. 2013). Chemically synthesized H1 and H2 show broad-spectrum activity against most of the tested Gram-positive and Gram-negative strains, and they inhibit biofilm formation for a significant percentage of staphylococcal and *P. aeruginosa* strains (Fig. 22). Other AMPs from the Holothuroidea include one isolated from egg homogenates and CF from the sea cucumber *Cucumaria frondosa*, which has antibacterial activity against Gram-positive bacteria (Haug et al. 2002; Beauregard et al. 2001), and another that is a synthetic peptide of the α helical region of CEL-III from a congeneric sea cucumber, *C. echinata*, that also has antibacterial activity (Hatakeyama et al. 2004; Hisamatsu et al. 2008).

Although the results described here represent only a few AMPs from a few species, echinoderms are a potentially rich resource for the discovery of new AMPs. These natural compounds may offer novel and alternative strategies with applications in biotechnology and medicine to prevent and treat bacterial infections, including those associated with biofilm formation (Zilberman and Elsner 2008; Shukla et al. 2010; Glinel et al. 2012). The identification of novel AMPs from invertebrates is likely to provide innovative approaches for the design of new synthetic or recombinant derivatives with modified chemical–physical properties to improve antimicrobial activity against pathogens (Huang et al. 2010; Brogden and Brogden 2011).

Proteomics of Echinoderm Immune Responses

Cell processes are governed not only by gene expression or protein abundance but also by rapid variations in protein activity, localization, and interactions with other proteins, DNA, and RNA. Data generated through proteomics can be used to

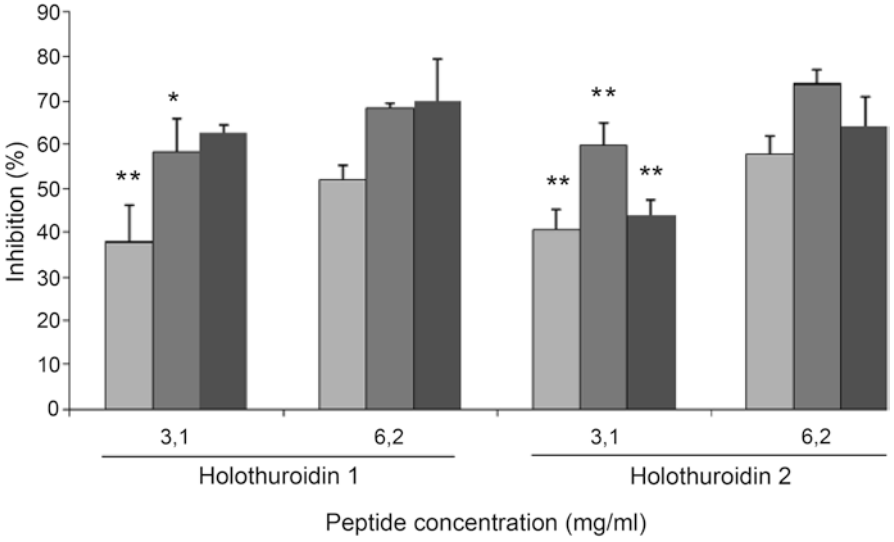

Fig. 22 *Holothuroidin 1 (H1) and Holothuroidin 2 (H2) from the sea cucumber Holothuria tubulosa have antibiofilm activity*. H1 and H2 have inhibitory activity against biofilms of *Staphylococcus aureus* (ATCC 25923) (light bars), *S. epidermidis* (ATCC 35984) (medium bars), and *Pseudomonas aeruginosa* (ATCC 15442) (dark bars). The data are expressed as the mean (± SD) of three experiments. $*p < 0.05$; $**p < 0.01$. (Reprinted from Schillaci et al. (2013) in AMB Express)

evaluate simultaneously all proteins involved in host defense mechanisms in a slice of time, which can be used to predict biological functions. Most proteomic approaches rely on fractionation of protein mixtures and the subsequent identification of separated proteins by mass spectrometry (MS). The process includes an enzymatic digestion of the proteins to produce peptides that are separated using liquid chromatography (LC) followed by ionization for MS analysis. Peptide mass fingerprinting is employed to search databases of protein sequences for predicted masses that would arise from peptide digestion. Tandem MS (MS/MS) evaluates the outcomes of additional fragmentation and also gives rise to a list of masses for each peptide fragment. The masses for each peptide and its fragments are also used to search databases of predicted masses that would arise from fragmentation of all peptides. The outcome from this analytic approach is the identification of the proteins in a sample.

Proteomic analyses rely heavily on the availability of reference genomes and transcriptomes to generate reference protein databases, and improved sequencing technologies have been a powerful engine for generating these databases. Proteomic strategies are now theoretically accessible for any species of interest (Dheilly et al. 2014) but have not been used extensively to investigate the molecular mechanisms underlying echinoderm immune responses. In addition to the genome resources described above, de novo transcriptomes are available for 23 species from all five echinoderm classes (Reich et al. 2015). Proteomic evaluation of coelomocytes shows changes in protein synthesis before and during an infection by various pathogenic microorganisms in the sea urchins *S. purpuratus* and *H. erythrogramma*, the

sea star *Marthasterias glacialis*, the sea cucumber *Apostichopus japonicus*, and the sunflower star, *Pycnopodia helianthoides* (see section "Sea Star Wasting Disease"). The functions of many potential immune response proteins in echinoderms remain unknown, but the currently available echinoderm genomes and transcriptomes can be used to generate reference databases for functional proteomics.

Proteomics of Immune Responses in Sea Stars and Sea Cucumbers

The proteome of the sea star *Marthasterias glacialis* was first characterized using a combination of one-dimensional (1D) SDS-PAGE followed by in-gel trypsin digestion and nanoliquid chromatography (LC) separation of peptides, plus two-dimensional (2D) SDS-PAGE followed by in-gel trypsin digestion before MS analysis (Franco et al. 2011). At the time, next-generation sequencing approaches were not developed sufficiently and protein characterization was based on the echinoderm database at GenBank (www.ncbi.nlm.nih.gov). The absence of a reference database for *M. glacialis* limited the efficiency and accuracy of protein identification and was likely the basis for why no sea star complement homologues were identified despite their known presence in members of other classes (Al-Sharif et al. 1998; Smith et al. 1996, 1998, 1999, 2001; Xue et al. 2015). However, this approach identified an abundance of homologous proteins involved in cytoskeleton regulation, cell adhesion, signaling, regulation, proliferation, and regeneration. Similarly, comparative proteomics for coelomocytes from the sea cucumber *Apostichopus japonicus* before and after response to a challenge from the Gram-negative bacteria *Vibrio splendidus* identified 40 proteins, of which 32 are upregulated (e.g., calreticulin, calumenin, ficolin, and NIPSNAP1) and eight are downregulated (Zhang et al. 2014). Of these differentially expressed proteins, approximately a third are immune response proteins. Despite the shortcoming of this study, based on the lack of an appropriate database, several proteins with predictions of crucial functions in sea cucumber immune responses were first identified by this proteomic approach (reviewed in Xue et al. (2015)).

Proteomics of Immune Responses in Sea Urchins

Similar proteomes are observed from the CF of the sea urchins *S. purpuratus* and *H. erythrogramma,* using an approach based solely on proteins separated by 1D SDS-PAGE, followed by in-gel trypsin digestion, nano-LC peptide separation, and MS analysis (Dheilly et al. 2012, 2013). A total of 323 unique proteins could be identified from *S. purpuratus*, with matches to 236 homologues in *H. erythrogramma*. Although the *S. purpuratus* genome was used as a reference, it was probable that some proteins in *H. erythrogramma* could not be identified because of sequence divergence between the species rather than absence. Proteins associated with cytoskeletal regulation and cell adhesion are the most abundant in both proteomes, which is consistent with their prevalence in highly mobile amoeboid

coelomocytes. These cells have previously been implicated in clotting reactions and phagocytosis, and express the complement component SpC3 (Al-Sharif et al. 1998; Gross et al. 1999, 2000). It is noteworthy that the complement component C3 and the complement receptor type 2 (CR2) are among the most abundant proteins in sea urchin CF, suggesting their involvement in the echinoderm immune system (Dheilly et al. 2013). Major yolk protein (MYP) is another abundant protein in the CF of sea urchins (Giga and Ikai 1985a; Dheilly et al. 2013). An isoform of MYP, a major constituent of sea urchin egg yolk, functions as an ironless calcium-binding transferrin with activities in cell–cell adhesion and positional information during embryonic development (Noll et al. 1985, 2007). The MYP isoform in the CF is larger and has greater zinc-binding capacity and greater glycosylation, and requires higher concentrations of Ca^{2+} for successful binding to liposomes (Giga and Ikai 1985a, b; Unuma et al. 2007; Dev and Robinson 2014). However, despite its extreme abundance, the functions of MYP in the CF in vivo are not known. Like the proteome of sea star CF (see section "Proteomics of Immune Responses in Sea Stars and Sea Cucumbers"), other proteins in the CF of sea urchins are predicted to have functions related to cellular regulation and proliferation, lysosomes, proteases, peptidases, stress responses, and detoxification (Dheilly et al. 2013).

Comparative proteomics can be used to predict the functions of proteins in the CF of echinoderms that are involved in antipathogen responses. Predictions are based, in part, on the assumption that variations in protein abundance in response to immune challenge indicate involvement in immune activities. For example, there is a substantial change in the proteome of the CF from the sea urchin *H. erythrogramma* after challenge with bacteria or LPS in comparison with the proteome after injection of buffer, which suggests differences in proteins involved with immune responsiveness versus injury repair (Dheilly et al. 2011b, 2012). The results imply significant changes in energy metabolism and cell signaling, which could ultimately regulate the recruitment of coelomocytes to the site of injury for wound repair (Dheilly et al. 2011b). Furthermore, a discrete modification of the coelomocyte proteome occurs in response to the injection of bacteria with increased expression of apextrin and calreticulin. Apextrin is found in the secretory vesicles of sea urchin eggs, is involved in cell adhesion during embryonic development (Haag et al. 1999), and has an MACPF domain that may be associated with cell lysis (Miller et al. 2007; Rosado et al. 2008). Similarly, expression of apextrin in the protochordate *Branchiostoma belcheri* is induced during acute immune responses (Huang et al. 2007), acts as an extracellular effector for bacterial agglutination and intracellular bacterial recognition, and activates signaling mediated by NFκB (Huang et al. 2014). Calreticulin is a multifunctional Ca^{2+}-binding protein, which regulates intracellular Ca^{2+} homeostasis and storage in the endoplasmic reticulum (Gelebart et al. 2005). Its increase in abundance following bacterial challenge demonstrates the importance of calcium signaling in immune cells of echinoderms.

Proteomics are also used to evaluate temporal responses of sea urchins to immune challenge. When the CF is collected over time from the sea urchin *H. erythrogramma* after injection with LPS or buffer, and is evaluated by 1D SDS-PAGE, in-gel trypsin digestion, and LC-MS, 345 proteins are identified and their quantification provides a picture of the successive steps in sea urchin responses to

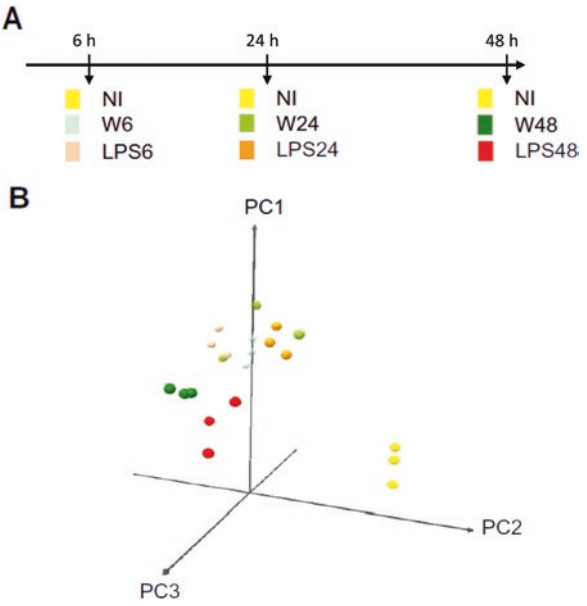

Fig. 23 *Temporal changes in the coelomic fluid proteome of the sea urchin Heliocidaris erythrogramma, responding to LPS*. (**a**) The experimenal design is illustrated showing the timeline over which samples were collected. Coelomic fluid samples from 21 sea urchins were collected for 48 h after treatment. Samples (n = 9) were collected from three sea urchins that were not injected (NI), including three samples after 6 h (NI6), 24 h (NI24), and 48 h (NI48). Samples (n = 9) were also collected after wounding (W), including three samples after 6 h (W6), 24 h (W24), and 48 h (W48). Additional samples (n = 9) were collected after the injection of LPS, including three samples after 6 h (LPS6), 24 h (LPS24), and 48 h (LPS48). (**b**) The three-dimensional score plot of the principal component (PC) analysis shows the cumulative data for all proteins identified in the CF of *H. erythrogramma*. (Reprinted from Dheilly et al. (2012) in Developmental and Comparative Immunology, with permission from Elsevier)

immunological challenge compared with injury (Dheilly et al. 2012). The injury response consists of an initial upregulation of cytoskeletal proteins consistent with an increase in cell motility, followed by an increase in MYP plus members of the large lipid transfer protein (LLTP) family, which are iron-binding proteins. The late-stage cellular response shows an induction of von Willebrand Factor, cyclophilin B, and selectins, suggesting cell migration to the site of injury and adhesion to collagen. The principal component analysis of the LPS response shows that most changes in protein abundances occur after 48 h, and that responses to injury are quite different (Fig. 23) (Dheilly et al. 2012). After LPS injection, there are relative increases in vesicular transport proteins such as coatomer proteins, receptor activated C kinase (RACK), and cellular signaling molecules such as G protein (q) and mitogen-activated protein kinase (MAPK). Many immune response proteins such as the sea urchin complement homologue SpC3, dual oxidase maturation factor, dual oxidase 1, α2-macroglobulin, and SRCRs are also more abundant 48 h after injection of LPS compared with injury, suggesting that these proteins are important in inducible immune responses in sea urchins.

Fibrocystin L and aminopeptidase N are identified only 48 h after LPS injection, indicating that these proteins are expressed only during late stages of PAMP-induced immune responses. In vertebrates, fibrocystin L is a lectin receptor expressed on immune responsive cells and participates in regulating phagocytosis (Hogan et al. 2003). Aminopeptidase N (also called CD13) is involved in immunomodulatory peptide degradation, antigen trimming, and antigen processing (Riemann et al. 1999). This approach for analyzing immune/injury response proteins in the sea urchin *H. erythrogramma* is sufficiently sensitive to detect the over expression of highly variable proteins in response to LPS, such as those of the diverse SRCR family. For example, 14 different SRCRs are present in the CF of *S. purpuratus* according to results from shotgun proteomics (Dheilly et al. 2013) in agreement with cDNA sequences and specific patterns of SRCR expression with respect to individual sea urchins (Pancer 2000). Similarly, shotgun proteomics of *H. erythrogramma* CF show that three SRCRs are expressed specifically in response to LPS challenge (Dheilly et al. 2012). The inducible expression of these cell surface proteins on sea urchin coelomocytes and those that are secreted into the CF suggests their involvement in the recognition of, or response to, pathogens.

Proteomic studies of echinoderm immune systems have yielded important results, and the lower cost and higher accessibility of next-generation sequencing approaches have provided genomes and transcriptomes of diverse sea urchins, brittle stars, sea stars, and sea cucumbers (see www.echinobase.org). This expansion to any echinoderm species is expected to revolutionize the proteomics of the immune system of echinoderms and to allow in-depth analyses of this complex system. In addition, reanalysis of previously published MS results, using newly sequenced genomes and transcriptomes as references, will increase significantly the amount of information extracted from previous studies (Franco et al. 2011; Xue et al. 2015). Applications of proteomics have also been used to evaluate the diversity of highly variable proteins that function in the immune response, and investigations of immune biomarkers (Fulton and Twine 2013) will be used to characterize the protein antigens targeted by the diverse families of PRRs that function in the sea urchin immune system. Such studies may enable an understanding of the extraordinary expansion of immune response gene families from a range of echinoderms, including TLRs, NLRs, SRCRs, lectins, and Trf proteins (see section "Immunogenomics: Immune Genes Encoded in Echinoderm Genomes") (Rast et al. 2006; Buckley and Rast 2012).

Negligible Senescence and the Immune System of Sea Urchins

The term "negligible senescence" is used to describe animals that do not show an association between increased age and an increased mortality rate or decreased fertility, physiological function, or disease resistance (Finch 1990; Finch and Austad 2001). Sea urchins appear to exhibit negligible senescence with indeterminate

Table 7 Estimated maximum life-spans for selected sea urchins

Species	Maximum life span (years)	References
Tripneustes ventricosus	3	Pena et al. (2010)
Lytechinus variegatus	3–10	Moore et al. (1963), Beddingfield and McClintock (2000), Hill et al. (2004), Russell et al. (2012)
Echinometra lucunter	≥40	Ebert et al. (2008)
Strongylocentrotus purpuratus	≥50	Ebert (2007, 2010)
Mesocentrotus franciscanus	>100	Ebert and Southon (2003), Ebert (2007)

growth, sustained ability to regenerate external appendages, lifelong reproduction, few reported cases of neoplasm, and no increase in the mortality rate at advanced ages (Jangoux 1987; Robert 2010; Ebert 2008; Bodnar and Coffman 2016). Despite these properties, different sea urchin species have very different life-spans that range over nearly two orders of magnitude (Table 7). Characterization of immune cell functions in sea urchins may help us to understand the contribution of the immune system to the negligible senescence and increased life-span that have been observed for many members of the echinoid lineage.

The accumulation of damage in somatic tissues with age is thought to contribute to a reduction in cell function, cell death, or cancer progression (Martin and Grotewiel 2006). Sea urchin species with life-spans ranging from ~3 to >100 years (Table 7) show no age-related increase in indicators of oxidative damage in coelomocytes and other somatic tissues such as protein carbonyls and 4-hydroxynonenal, indicating effective preservation with age. A marker for oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, measured in cell-free CF from sea urchins, also does not increase with age (Du et al. 2013). Antioxidant activities (e.g., superoxide dismutase and total antioxidant capacity) are largely maintained with age in coelomocytes and other somatic tissues, which may contribute to the lack of accumulated damage. Interestingly, coelomocytes from longer-lived species (*S. purpuratus* and *M. franciscanus*) have greater antioxidant properties than shorter-lived *L. variegatus*, suggesting that the immune cells of the long-lived animals are better equipped to mitigate oxidative damage.

Although the sea urchin *L. variegatus* is short-lived, coelomocytes from this species are highly resistant to a variety of DNA-damaging agents including ultraviolet radiation (UV), hydrogen peroxide (H_2O_2), methyl methanesulfonate (MMS), benzo[a]pyrene, and bleomycin (Loram et al. 2012; Reinardy and Bodnar 2015). The LD_{50} values calculated for coelomocytes 24 h after ex vivo exposure to these agents are many times higher than those for mesenchyme cells of other marine invertebrates (including larvae from *L. variegatus*) and values reported for cultured mammalian cells (Loram et al. 2012; El-Bibany et al. 2014; Reinardy and Bodnar 2015). Apoptosis is not detected in *L. variegatus* coelomocytes even at high doses

of these damaging agents (Loram et al. 2012), which is consistent with reports of low levels of apoptosis in coelomocytes of the sea urchin *Paracentrotus lividus* after exposure to UV-B (Matranga et al. 2006). Effective DNA repair is evident in *L. variegatus* coelomocytes following acute ex vivo exposures and is accompanied by an upregulation in the expression of genes encoding components of DNA repair pathways (Reinardy and Bodnar 2015). The DNA repair capacity of coelomocytes is maintained with age in the sea urchin *L. variegatus* and in the rock-boring sea urchin, *Echinometra lucunter*, following H_2O_2-induced DNA damage; however, the sample sizes were small in this study (El-Bibany et al. 2014). There is a general correlation between the DNA repair capacity of coelomocytes and the life-span of different sea urchin species (*Echinometra lucunter* > *Lytechinus variegatus* > *Tripneustes ventricosus*), with longer-lived species showing higher levels of repair 24 h after exposure to DNA damage (El-Bibany et al. 2014). This observation supports the notion that longer-lived species invest greater resources in cellular maintenance and repair (Kirkwood 2005).

The ability to protect the genome from harmful DNA damage is critical for maintaining genome stability and protection against neoplastic disease (Lombard et al. 2005). Sea urchins are noted for the lack of reported cases of neoplasm (Jangoux 1987; Robert 2010), although further study is needed to determine whether the high resistance to DNA damage correlates with effective DNA repair in sea urchins and if this contributes to a low incidence of neoplastic disease. Interestingly, DNA damage induced in somatic tissues following intracoelomic injection of the DNA-alkylating agent MMS is accompanied by increased immune gene expression in coelomocytes (Reinardy et al. 2016). This suggests a link between the DNA damage response and activation of the echinoid immune system and possible activities in the surveillance and removal of damaged cells.

Systematic studies that compare immune function with age across sea urchin species with different life-spans will be important for discerning the contribution of the immune system to the long-term maintenance of tissue homeostasis and resistance to disease that defines negligible senescence. Interspecies genomic comparisons indicate that the immune gene repertoire is more complex in long-lived sea urchin species (*S. purpuratus*, *M. franciscanus*) than in short-lived species (*L. variegatus*) (Buckley and Rast 2012). Aging is typically accompanied by a decline in immune function and increased vulnerability to infectious, inflammatory, and neoplastic disease. Understanding the extent and mechanisms by which sea urchins avoid age-related decline in immune function will provide valuable insight into the underpinnings of negligible senescence.

Toxicology and Effects of Pollution on Echinoderm Immunity

Marine ecosystems are vulnerable to anthropogenic stress because of their interface with terrestrial environments and the impacts of coastal city development related to urban runoff, industrial effluents, antifouling paints on boats, mining operations,

and atmospheric particulate pollutants (Islam and Tanaka 2004; Li et al. 2014b). Pollutants include heavy metals, insecticides and herbicides, waste from food processing, pollutants from livestock operations, volatile organic compounds, and chemical waste. Despite the continuous use and release of such substances, surprisingly little is known about the toxicological risk faced by marine organisms for many of these compounds. An increasing number of studies are combining approaches for monitoring chemical contaminant levels with measurements of biological responses to assess the environmental status across marine regions (Lyons et al. 2010; Connon et al. 2012; Andersen et al. 2016).

Pollutants affect survival, growth, reproduction, metabolism, and immunity in marine invertebrates (Ellis et al. 2011; Gallo and Tosti 2013; Ray et al. 2015). In echinoderms, heavy metals in contaminated sites alter the immune responses of the sea star *Asterias rubens* (Coteur et al. 2003a, b). In response to manganese, *A. rubens* induces the proliferation of hematopoietic cells and shows an increase in the number of coelomocytes (Oweson et al. 2008, 2010). In the sea star *Marthasterias glacialis*, zinc inhibits the lysozyme-like activity of the mucus (Stabili and Pagliara 2009), which may lead to increased disease susceptibility. The use of immunological parameters such as lysozyme activity has been proposed for risk assessment in echinoderms exposed to chemical contamination. In the sea urchin *Paracentrotus lividus*, zinc treatment causes phagocytes to change shape from petaloid to filopodial and increases the number of the red spherule cells (or red amoebocytes) (Fig. 24) (Pagliara and Stabili 2012). Coelomocytes respond to stress conditions such as temperature shock and pollution in both laboratory experiments and field studies (Matranga et al. 2000), and their use as sentinels of environmental stress was first proposed by Matranga et al. (2005). Short-term treatment with zinc also affects humoral parameters, causing decreases in both lysozyme-like activity and antibacterial activity against *Vibrio alginolyticus* (Fig. 25a, b) (Pagliara and Stabili 2012). The impacts of eight divalent heavy metal ions alter the activities of immune-related enzymes, including superoxide dismutase, phenoloxidase, acid phosphatase, alkaline phosphatase, and myeloperoxidase in CF from the sea cucumber *Apostichopus japonicus* (Jiang et al. 2016). Lead inhibits the activities of most immune-related enzymes in *A. japonicus*, whereas cadmium strongly inhibits myeloperoxidase. The results suggest that heavy metals have significant impacts on *A. japonicus* immunity and that metals are likely important stressors that modulate responses in echinoderm immune systems.

The sea urchin *Paracentrotus lividus* is a dominant predator in Mediterranean rocky reef ecosystems and serves as an important model organism for monitoring of the state of marine environmental health (Pinsino and Matranga 2015). Sea urchins are capable of adjusting to environmental changes, which enables investigations to uncover the conserved molecular signaling pathways involved in the protection, robustness, resistance, and plasticity of this invertebrate innate immune system (Rast et al. 2006). Immunological and eco-toxicological analyses of coelomocytes from *P. lividus* have been used to assess pollution in a marine coastal area of the

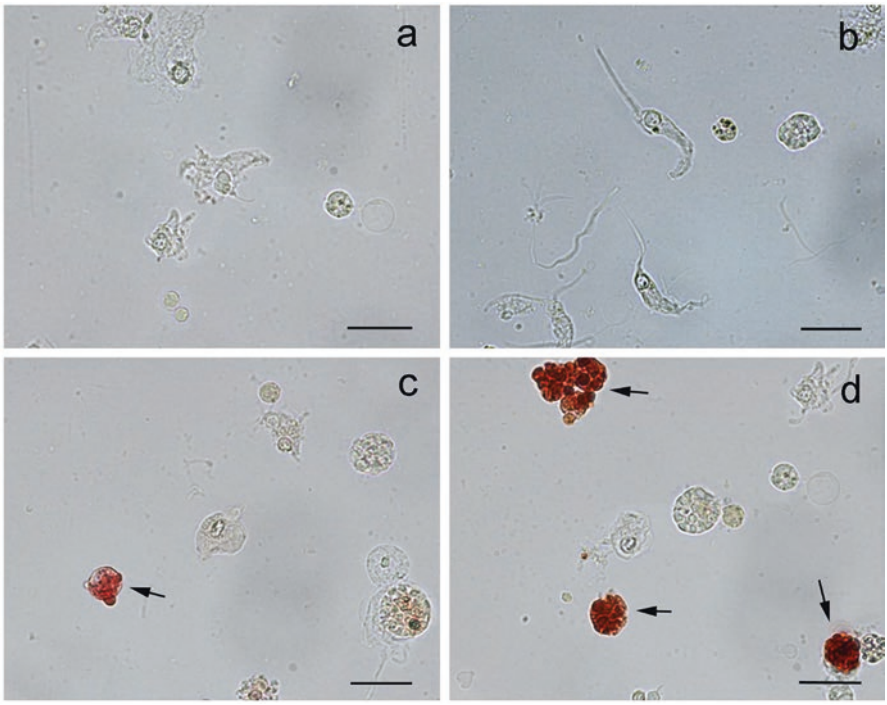

Fig. 24 *Coelomocytes from the sea urchin Paracentrotus lividus respond to zinc*. Sea urchins were housed at 20°C in filtered seawater at 37‰ salinity. Experimental animals were exposed to 18.4 μM $ZnCl_2$ in the seawater. Coelomocytes were collected from treated and untreated animals after 24 h. (**a**) Phagocytes from untreated sea urchins. (**b**) Phagocytes from sea urchins exposed to zinc. (**c**) Red spherule cells (arrow) from untreated sea urchins. (**d**) Red spherule cells (arrows) from sea urchins exposed to zinc. The scale bar is 5 μm. (Reprinted from Pagliara and Stabili (2012) in *Chemosphere*, with permission from Elsevier)

northern Adriatic Sea (Matranga et al. 2000). Coelomocytes isolated from animals collected from urban and industrially contaminated sites (in Rovinj, Croatia) exhibited increased numbers of the red spherule cells (or red amoebocytes) in comparison with animals collected from unpolluted sites or subjected to accidental injuries (Matranga et al. 2000; Pinsino et al. 2008). Under normal, nonpolluted physiological conditions, the relative percentage of red spherule cells from *P. lividus* constitutes only 4.70 ± 1.48% (mean ± SE) of the total cell population (Matranga et al. 2006). However, under stressful conditions, the percentage increases to 11.7 ± 0.99% (≥50% increase) although the total number of coelomocytes in the CF remains constant (Matranga et al. 2006; Pinsino et al. 2008). Immune cells harvested from animals from polluted seawater exhibit increased levels of the constitutive form of the 70kDa heat shock cognate (Hsc70) protein (Matranga et al. 2000; Pinsino et al. 2008). Similar increases in the Hsc70 concentration are observed in in vitro studies

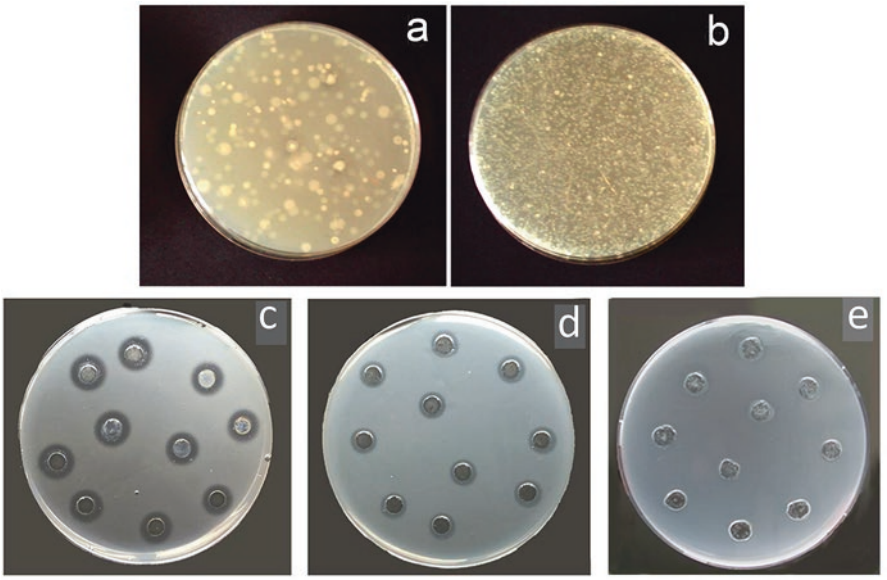

Fig. 25 *Antibacterial activity in coelomocyte lysates from Paracentrotus lividus inhibits growth of Vibrio alginolyticus*. Sea urchins were housed in filtered seawater or seawater with 18.4 μM $ZnCl_2$ as described in the legend for Fig. 24. *Vibrio alginolyticus* bacteria were incubated for 30 min with coelomocyte lysate from untreated sea urchins (**a**) or zinc-treated sea urchins (**b**) and evaluated for bacterial proliferation. (**c**–**e**) Coelomocytes from the sea urchin *Paracentrotus lividus* have lysozyme-like activity. Sea urchins were housed at 20 °C in filtered seawater of 37‰ salinity, and experimental animals were exposed to 0.5 mg/L lindane. Coelomocytes were collected after 24 or 48 h of treatment, and lysates were evaluated for lysozyme activity in a standard growth assay of *Micrococcus lysodeikticus*. The diameter of the cleared zone around each well filled with 30 μL of coelomocyte lysate was due to the lysis of bacterial cell walls and was recorded after overnight incubation at 37 °C. The diameter of the cleared zone was compared with a reference sample of hen egg white lysozyme. (**c**) *M. lysodeikticus* plus coelomocyte lysate from untreated sea urchins after 24 h. (**d**) *M. lysodeikticus* plus coelomocytes lysate from sea urchins treated with lindane for 24 h. (**e**) *M. lysodeikticus* plus coelomocyte lysate from sea urchins treated with lindane for 48 h

in which *P. lividus* coelomocytes are exposed to temperature shock, decreased pH, UV-B radiation, or heavy metals (Matranga et al. 2002, 2005, 2006). Hsc70 serves as an indicator of changes in the steady-state homeostasis with key activities in mediating stress resistance, immune resistance, and apoptosis (Mosser et al. 2000). Accordingly, the results demonstrate the utility of Hsc70 as a general stress response marker for reliable monitoring of both acute and chronic stresses in *P. lividus* immune cells.

Sea urchin coelomocytes are useful for evaluating the toxicity of nanoparticles (NPs) through changes in expression patterns of genes and proteins and alterations to cellular morphology. Coelomocyte exposure to a range of NPs has variable effects

on Hsc70 protein levels, and when taken up by the cells, NPs cause modifications to the trans face of the Golgi apparatus and to the endoplasmic reticulum (Falugi et al. 2012). Coelomocytes exposed to tin dioxide (SnO_2) NPs do not show altered Hsc70 levels relative to controls, whereas immune cells exposed to cerium dioxide (CeO_2) or iron oxide (Fe_3O_4) NPs show decreased Hsc70 protein levels. Furthermore, the activity of acetylcholinesterase (AChE), butirylcholinsterase (BChE), and propionylcholinesterase (PrChE), as well as the basal protein levels of glucose-regulated protein 78 (GRP78), are significantly inhibited in immune cells exposed to all of these NPs. Thus, as in human immune cells, coelomocytes from *P. lividus* exposed to NPs show selective changes to specific pathways and biomarkers (Boraschi et al. 2012). Accordingly, phagocytes from *P. lividus* that interact with titanium dioxide (TiO_2) NPs elicit a receptor-mediated phagocytic mechanism that involves the TLR/p38 MAPK signaling pathway but does not activate an inflammatory response or the Hsc70-dependent stress response (Pinsino et al. 2015). In general, these findings demonstrate that sea urchin immune cells can be employed as a tool for analysis of toxicity and safety of NPs, and can also be used to provide useful data on the pollution status of marine ecosystems.

Unlike many other chemicals, phosphate pollutants and some pesticides are deliberately added to the environment to kill selective organisms (Vijgen et al. 2011; Szabo and Loccisano 2012). Highly toxic, persistent, and bioaccumulating pollutants (e.g., polychorobiphenyls (PCBs)) may have major ecological consequences. For the sea star *Asterias rubens*, PCB exposure results in increased production of reactive oxygen species (ROS) (Coteur et al. 2002b, 2003a; Danis et al. 2004a, b, 2006). Similarly, chronic exposure of the sea urchin *L. variegatus* to phosphate decreases bactericidal activity against pathogenic *Vibrio* sp. (Böttger and McClintock 2009). Exposure to subchronic concentrations of the pesticide lindane alters both cellular and humoral immune responses in *P. lividus*, decreasing total coelomocyte numbers in the CF and increasing the proportion of red spherule cells (Stabili and Pagliara 2015). Hemolytic and lysozyme-like activities, as well as antibacterial activity toward *Vibrio alginolyticus*, decrease in sea urchins treated with lindane (Fig. 25c–e). These immunological changes in response to lindane exposure highlight the use of coelomocytes from *P. lividus* and other echinoderms as novel biosensors to assess the impact of pollutants on invertebrate health and to provide invaluable information on marine ecology (Stabili and Pagliara 2009; Luna-Acosta et al. 2010, Pinsino and Matranga 2015).

Conclusion

Elia Metchnikoff opened the field of immunology through his investigations of blastocoelar cell chemotaxis and encapsulation of the tip of a rose prickle with which he had impaled a sea star larva (Metchnikoff 1893). This first report and subsequent work on larvae, plus tissue rejection and clearance of pathogens from the adult body cavity, demonstrated the presence of an echinoderm immune system. Clear echinoderm larvae and observations of larval immune cells, particularly

after manipulating the expression levels of transcription factors that function in hematopoiesis, demonstrate the conservation and ancient ancestry of the transcriptional circuitry required to differentiate an immune system in deuterostomes. Although the echinoderm phylum is ancient, which is consistent with species diversity, phagocytic coelomocytes are pervasive within the classes and indicate that phagocytosis and encapsulation are essential and basal attributes of immunity. The genome of the purple sea urchin was the first basal deuterostome to be analyzed and provided information to identify and characterize the astounding numbers of immune system genes that function in the echinoderm immune system (Sodergren et al. 2006; Rast et al. 2006; Hibino et al., 2006). It was the first assessment of the entire system, which documented it as surprisingly complex, robust, and likely highly sophisticated.

Many humoral factors that are secreted into the CF—including antipathogen proteins, complement components, and some AMPs—augment phagocytosis through opsonization, whereas other humoral factors, including other AMPs, are microbicidal. The involvement of large protein families that function in immune detection and response, plus the possibility of regulated mechanisms for diversifying immune genes, suggest that many or perhaps all multicellular organisms have molecular mechanisms for immune gene sequence diversification. Although the echinoderm mechanisms for immune diversification are unknown and may be unlike mechanisms in other groups, gene sequence diversity illustrates the robustness of the echinoderm immune response. Echinoderms inhabit a wide range of ecological niches from the abyssal depths of the oceans to the intertidal zone, are often key, long-lived species that shape and maintain the status of many marine ecosystems, and can be employed as sentinels of change in ecological health. The marine habitats in which echinoderms live are shared with multitudes of microbes, many of which are opportunist pathogens that can complicate and exacerbate infections initiated by true pathogens. Understanding the flexibility of the echinoderm immune response in its arms race for survival against ever changing pathogens will be the challenge for the future.

Dedication This work is dedicated to Valeria Matranga who passed away too young in April 2016 after a long and courageous battle against cancer. Valeria contributed immensely to our understanding of cellular and molecular immune processes in the sea urchin, *Paracentrotus lividus*. Her dedicated research on echinoderms led to an understanding of how they interact with their environment and how coelomocytes can be employed to evaluate environmental toxins and pollutants. She and her insight for creative approaches in eco-immuno-toxicology will be missed because her approach to thinking about how to answer difficult scientific questions would have been more and more valuable in the future.

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