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ATP-binding cassette (ABC) transporter expression and localization in sea urchin development

Lauren E. Shipp and Amro Hamdoun*

Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego

Abstract

Background—ATP-binding cassette (ABC) transporters are membrane proteins that regulate intracellular concentrations of myriad compounds and ions. There are >100 ABC transporter predictions in the *Strongylocentrotus purpuratus* genome, including 40 annotated ABCB, ABCC, and ABCG “multidrug efflux” transporters. Despite the importance of multidrug transporters for protection and signaling, their expression patterns have not been characterized in deuterostome embryos.

Results—Sea urchin embryos expressed 20 *ABCB*, *ABCC*, and *ABCG* transporter genes in the first 58 hours of development, from unfertilized egg to early prism. We quantified transcripts of *ABCB1a*, *ABCB4a*, *ABCC1*, *ABCC5a*, *ABCC9a*, and *ABCG2b*, and found that *ABCB1a* mRNA was 10–100 times more abundant than other transporter mRNAs. *In situ* hybridization showed *ABCB1a* was expressed ubiquitously in embryos, while *ABCC5a* was restricted to secondary mesenchyme cells and their precursors. Fluorescent protein fusions showed localization of ABCB1a on apical cell surfaces, and ABCC5a on basolateral surfaces.

Conclusions—Embryos utilize many ABC transporters with predicted functions in cell signaling, lysosomal and mitochondrial homeostasis, potassium channel regulation, pigmentation, and xenobiotic efflux. Detailed characterization of ABCB1a and ABCC5a revealed that they have different temporal and spatial gene expression profiles and protein localization patterns that correlate to their predicted functions in protection and development, respectively.

Keywords

ABC transporter; sea urchin; protection; signaling; cellular defenses; multidrug; embryo; gene expression

INTRODUCTION

ATP-Binding Cassette (ABC) transporters are a conserved family of membrane proteins that use ATP to move compounds across membranes in both adult and embryonic cells (Higgins, 1992; Elbling et al., 1993; Dean et al., 2001; Borst and Elferink, 2002). They transport peptides, metals, xenobiotics, and ions necessary for homeostasis, protection, and signaling. The ABC transporter family comprises eight subfamilies in sea urchins (ABCA to ABCH) and seven in mammals (ABCA to ABCG). Much of the functional information about these transporters pertains to the ABCB, ABCC, and ABCG “multidrug efflux” subfamilies and their roles in diseases including cancer, cholestasis, and cystic fibrosis. For example, increased expression of ABCB1 (P-glycoprotein, Pgp), ABCC1 (Multidrug resistance

*Correspondence to: Amro Hamdoun, UCSD-MBRD, 9500 Gilman Drive, MC 0202, La Jolla, CA 92093-0202. Phone: 858-822-4303. hamdoun@ucsd.edu.

protein 1, MRP1), and ABCG2 (Breast cancer resistance protein, BCRP) in cancer cells leads to acquired drug resistance (Borst and Elferink, 2002). In contrast, reduced surface expression of the ABC transporters ABCB11 (Bile salt export pump, BSEP) and ABCC7 (Cystic fibrosis transmembrane conductance regulator, CFTR) leads to cholestasis and cystic fibrosis, respectively (Dean et al., 2001; Borst and Elferink, 2002).

While ABC transporters are well studied in disease, relatively little is known about their functions in embryos. In the *Strongylocentrotus purpuratus* genome, there are >100 ABC transporter gene predictions (<http://spbase.org>). Whole-genome tiling arrays revealed that these genes are extensively expressed in the first five days of embryonic development. For example, >80% of *ABCC* genes were detected, a level that is ~30% higher than the overall level of gene utilization across the genome (Goldstone et al., 2006; Samanta et al., 2006). This high expression of transporters may be necessary to protect the embryo from xenobiotics. Consistent with this idea, sea urchin embryos possess both ABCB and ABCC transport activities that protect them from vinblastine (Hamdoun et al., 2004) and inorganic mercury (Bosnjak et al., 2009). Similarly, ABCB1 (Pgp) protects mouse embryos from xenobiotics such as doxorubicin and mitomycin C (Elbling et al., 1993).

This high utilization of ABC transporters could indicate that they also function in cell specification through efflux of morphogens. For example, *Drosophila melanogaster* *mdr49* (an ABCB transporter) protects flies from colchicine toxicity (Wu et al., 1991), and it also transports signaling molecules. *Dm-mdr49* is expressed in the embryonic mesoderm where it effluxes a chemoattractant that directs germ cell migration to the somatic gonad (Ricardo and Lehmann, 2009). Similarly, human ABCC1 (MRP1) is a xenobiotic transporter, but when expressed heterologously in *Caenorhabditis elegans*, it rescues defects in dauer larva formation induced by removal of the endogenous transporter (Yabe et al., 2005).

The goal of this study was to characterize the gene expression and protein localization of ABC transporters during embryonic development of sea urchins. Of the 40 manually annotated *ABCB*, *ABCC*, and *ABCG* genes, we found 20 to be expressed during the first three days of development. We quantified the number of transcripts per egg/embryo for six transporter genes and found that mRNA of *ABCB1a* was 10–100 times more abundant than that of other measured transporters. *In situ* hybridization of *ABCB1a* and *ABCC5a* revealed that *ABCB1a* was ubiquitously expressed in all cells, while *ABCC5a* was expressed only in a subset of mesodermal precursors. ABCB1a protein primarily localized to the apical membrane of polarized epithelial cells, while ABCC5a was found on basolateral membranes. The spatial gene expression and protein localization patterns of ABCB1a and ABCC5a are consistent with predicted differences in protection versus signaling, respectively. Our results highlight the importance of ABCB, ABCC, and ABCG transporters in a wide range of developmental functions, ranging from protection against xenobiotics to control of cell signaling and differentiation.

RESULTS

ABC transporter genes expressed in early development

We measured the dynamics of *ABCB*, *ABCC*, and *ABCG* gene expression during the first three days of sea urchin development, from unfertilized egg through the early prism stage. Embryos expressed 20 transporters including those potentially responsible for xenobiotic efflux, ion movement, and transport of signaling molecules (Table 1). To assess similarity of the detected genes to other well-characterized ABC transporters, we used Blastp to compare their predicted peptide sequences (Spbase.org, genome version 3.1) to other proteins in NCBI. Embryos expressed eight *ABCB* transcripts including three genes related to multidrug pumps, one of which could be related to bile salt export pump (BSEP), four

mitochondrial transporters, and one transporter associated with antigen processing (Tap)-like gene. Among the eight *ABCC* genes detected, one was a homolog of a well-known multidrug pump, four genes encoded transporters with both xenobiotic and signaling molecule substrates, and three genes were similar to the potassium channel regulating protein, sulfonylurea receptor-2 (SUR2). Finally, four *ABCG* genes were expressed, including one encoding a xenobiotic pump, one similar to uncharacterized transporters from insects, and two transporters homologous to *Drosophila* White.

Temporal patterns of ABC transporter gene expression

All of the *ABCB*, *ABCC*, and *ABCG* mRNAs showed little change in abundance from the egg to early blastula stage (Fig. 1, Fig. 2, Supplemental Table 1). The first significant changes in expression (fold change <0.5 or >2) occurred at hatching, consistent with the large synthesis and turnover of mRNA that occurs at this time (Davidson, 1986). For example, transcripts of 13 ABC transporters decreased at hatching, presumably due to turnover of maternally derived mRNA (i.e. synthesized during oogenesis and present in the egg prior to fertilization).

All 20 ABC transporter genes were generally expressed in one of three temporal patterns: (1) transporter expression is present from egg on, decreases at hatching, then is restored thereafter (Fig. 2, blue lines), (2) transporter transcripts are absent in early development and rapidly appear at a distinct developmental time point (Fig. 2, orange lines), and (3) transporter expression is robust from egg on and increases steadily throughout development (Fig. 2, green lines). Thirteen genes showed Group 1 patterns (blue, Fig. 2A–H, J, N–P, R) including *ABCB1a*, *ABCB1b*, *ABCB4a*, *ABCB6*, *ABCB7*, *ABCB8*, *ABCB9a*, *ABCB10a*, *ABCC4a*, *ABCC9a*, *ABCC9b*, *ABCC9d*, and *ABCG9*. After hatching, *ABCB1b*, *ABCB4a*, *ABCB7*, *ABCB10a*, *ABCC9b*, *ABCC9d*, and *ABCG9* all increased >2 fold. The second most common expression pattern was the Group 2 pattern (orange, Fig. 2M, Q, S–T) found for *ABCC5a*, *ABCG2b*, *ABCG11*, and *ABCG12*. *ABCC5a*, *ABCG11*, and *ABCG12* were first detected at the hatching blastula stage while *ABCG2b* was not detected until the early prism stage. Group 3 (green, Fig. 2I, K–L) included three transporters, *ABCC1*, *ABCC4b*, and *ABCC4c*, that increased >2 fold by hatching (*ABCC1*, *ABCC4c*) or early gastrula (*ABCC4b*).

Temporal expression patterns of paralogs—Many *S. purpuratus* ABC transporters have multiple paralogs. For example, *ABCB1* has 10 paralogs, *ABCC5* has 16, *ABCC9* has 14, and *ABCG2* has five. To gain insight into the potential functions of these paralogs, we examined whether the expression patterns of detected paralogs were temporally synchronous. For *ABCB1a* and *ABCB1b*, expression was fairly uniform with both decreasing at hatching (Fig. 2A–B). In contrast, the *ABCC4* paralogs were asynchronous with *ABCC4a* decreasing at hatching then slowly restoring expression levels, while *ABCC4b* increased >2 fold at early gastrula, and *ABCC4c* increased >2 fold at hatching (Fig. 2J–L). Finally, while the *ABCC9* paralogs all decreased expression at hatching and restored expression levels later in development, only *ABCC9b* and *ABCC9d* increased >2 fold (Fig. 2N–P).

Quantification of ABC transporter mRNAs

We quantified six ABC transporter transcripts using cDNA standards (Fig. 3, Supplemental Table 1) to determine their abundance. These six genes represented each of the three common expression profiles. The genes included *ABCB1a*, *ABCB4a*, and *ABCC9a* from Group 1, *ABCC5a* and *ABCG2b* from Group 2, and *ABCC1* from Group 3. *ABCB1a* was the most abundantly expressed transporter, averaging 12,878 transcripts per egg/embryo and ranging from 6,223 copies in hatched blastula to 20,135 copies per late gastrula embryo

(Fig. 3A, Supplemental Table 1). *ABCB4a* ranged from 465 copies in hatching blastulae to 4,157 copies per early prism embryo with an average of 1,643 copies (Fig. 3B, Supplemental Table 1). *ABCC9a* had an average of 1,532 transcripts per egg/embryo, ranging from 468 copies in early gastrulae to 2,481 copies in 16-cell embryos (Fig. 3E, Supplemental Table 1). Both *ABCC5a* and *ABCG2b* could not be detected until they exceeded ~100 transcripts per embryo. *ABCC5a* reached this threshold with 343 copies at hatching, and it peaked at the early prism stage with 7,871 copies per embryo (Fig. 3D, Supplemental Table 1). *ABCG2b* transcripts could only be accurately quantified at the latest stage surveyed, when they reached 186 copies per early prism embryo (Fig. 3F, Supplemental Table 1). *ABCC1* transcripts were present at 590 copies per egg and 517 copies per 16-cell embryo, then increased through development peaking at 7,471 copies per late gastrula stage embryo (Fig. 3C, Supplemental Table 1).

Spatial patterns of ABC transporter gene expression

Next, we used whole mount *in situ* hybridization (WMISH) to characterize spatial expression of five ABC transporter genes. Two genes, *ABCB1a* and *ABCC5a*, showed clear localization patterns. *ABCB4a*, *ABCC1*, and *ABCC9a* were not detected, presumably because their messages were insufficiently abundant and/or widely dispersed. Temporal expression analyses described above showed that *ABCB1a*, which has predicted protective functions, was the most abundant mRNA (Fig. 3A). In contrast, *ABCC5a* mRNA was absent in early development but increased dramatically at a specific developmental stage (Fig. 3D), a pattern that is commonly observed for developmental genes such as *Nodal*, *HesC*, and *Delta* (Nam et al., 2007; Revilla-i-Domingo et al., 2007). Consistent with a predicted function in protection against toxicants, WMISH showed *ABCB1a* was ubiquitously expressed in all cells throughout development (Fig. 4E–H). At the gastrula stage, *ABCB1a* was detected with such intense staining on the ectoderm and endoderm that it was difficult to determine if there was uniform mesodermal expression (Fig. 4H).

In contrast, *ABCC5a* transcripts were undetectable early in development (Fig. 4I), then were expressed only in a subset of embryonic cells after hatching (Fig. 4J–L). *ABCC5a* was expressed in mesodermal cells (Fig. 4K–L) in a pattern consistent with the veg2 lineage and their descendants, the (non-skeletogenic) secondary mesenchyme cells (SMC) (Peter and Davidson, 2009a). In hatched mesenchyme blastulae, *ABCC5a* mRNA was detected in the vegetal pole of the embryo and was absent from primary mesenchyme cells (Fig. 4J). During gastrulation, *ABCC5a*-expressing cells dispersed and were ultimately incorporated into the ectoderm in a pattern similar to that of pigment cells (Fig. 4K–L) (Ransick et al., 2002; Peter and Davidson, 2009a). In addition, the temporal expression pattern of *ABCC5a* matches those of *ABCG11* and *ABCG12*, homologs of the transporter necessary for eye pigmentation in *Drosophila*, *Dm-White* (Ewart et al., 1994; Mackenzie et al., 2000). Thus, *ABCC5a* could be essential for the formation or function of pigment cells in sea urchin embryos.

Cellular localization of ABCB1a and ABCC5a proteins

We expressed fluorescent-protein fusions of ABCB1a and ABCC5a in the sea urchin embryo to determine their cellular localization (Fig. 5). ABCB1a-mCitrine protein localized to the apical membrane of the ectoderm in hatched mesenchyme blastulae (Fig. 5Aii). This transporter coated the outside surface of the embryo and was seen on the surfaces of apical microvilli (Fig. 5Bii). In contrast, ABCC5a-mCherry protein was absent from the apical cell surface and was instead localized on the basolateral cell membranes (Fig. 5Aiii, Biii). No co-localization of ABCB1a-mCitrine and ABCC5a-mCherry proteins was observed in polarized epithelial cells (Fig. 5Biv), except for some slight overlap at the vegetal pole of the embryo (Fig. 5Aiv).

Our findings from both WMISH and FP-overexpressions indicate that at the hatched blastula stage, endogenous *ABCB1a* transcripts are present in all embryonic cells (Fig. 4F, Fig. 6A), while ABCB1a-mCitrine protein localizes on the apical membrane of all ectodermal cells (Fig. 5Aii). This indicates that in blastulae, endogenous ABCB1a protein is also present on the apical side of all ectodermal cells (Fig. 6B, green). This places ABCB1a proteins in direct contact with the environment, where they can directly efflux unwanted chemicals from the embryo.

In contrast, at the same developmental stage, *ABCC5a* transcripts are detected exclusively in a subset of vegetal cells (Fig. 4J, Fig. 6A) likely to be part of the Veg2 lineage (Peter and Davidson, 2009a). ABCC5a-mCherry protein is not detected at the apical surface of the embryo but is instead localized on basolateral membranes of polarized cells, which are not in direct contact with the environment (Fig. 5Aiii). Thus, we propose that endogenous ABCC5a protein is expressed on basolateral membranes of vegetal cells in hatched blastulae (Fig. 6B, red). This position in the embryo suggests that ABCC5a is not involved in protective efflux of environmental chemicals.

DISCUSSION

Our results demonstrate that sea urchin embryos utilize many ABC transporters in early development. In the first three days of development, 20 ABC transporters were expressed including those with predicted functions in cell signaling, mitochondrial and lysosomal homeostasis, potassium channel regulation, pigmentation, and xenobiotic efflux (Table 1). *In situ* hybridization and fluorescent protein fusion expression of ABCB1a and ABCC5a revealed significant differences in location and timing of expression of these two transporters that correlate with predicted differences in their functions.

Xenobiotic transport

Sea urchin embryos expressed homologs of all three major types of multidrug transporters including ABCB1, ABCC1, and ABCG2. Among the detected ABCB transporters, *Sp-ABCB1a*, *Sp-ABCB1b*, and *Sp-ABCB4a* are similar to the human multidrug resistance transporter, P-glycoprotein (Pgp). This transporter protects cells by effluxing a wide range of mildly hydrophobic molecules (Dean et al., 2001; Fletcher et al., 2010), and its substrate poly-specificity is mediated by a large binding pocket with multiple drug binding sites (Aller et al., 2009). ABCB1/Pgp-mediated xenobiotic efflux activity is essential for protecting hematopoietic stem cells (Smeets et al., 1997) and various mammalian (Elbling et al., 1993) and invertebrate embryos including sea urchins (Toomey and Epel, 1993; Hamdoun et al., 2004).

Previous studies indicated that sea urchins have high levels of ABCC-like multidrug efflux activity (Hamdoun et al., 2004). We found that embryos expressed *Sp-ABCC1*, a homolog of Multidrug resistance protein 1 (MRP1), which could mediate this activity. In addition to direct efflux of toxic molecules, ABCC1/MRP1 effluxes glutathione-conjugates and therefore can also transport hydrophilic toxicants such as metals (Leslie et al., 2005; Fletcher et al., 2010; Chen and Tiwari, 2011; He et al., 2011). ABCC1 can also maintain cellular redox homeostasis via GSH and GSSG transport (Leslie et al., 2001), and it regulates inflammation and dendritic cell migration via transport of leukotriene LTC₄ (Leslie et al., 2005). Since ABCC1 performs both protective and signaling functions in mammals, it could also be a dual-functioning transporter in sea urchin development.

Finally, sea urchin embryos expressed *Sp-ABCG2b*, a homolog of human Breast cancer resistance protein (BCRP). ABCG2/BCRP is a xenobiotic transporter known to mediate drug resistance by effluxing anticancer drugs such as mitoxantrone (Leslie et al., 2005;

Krishnamurthy and Schuetz, 2006). In addition to protection, ABCG2 may also maintain multipotency of hematopoietic stem cells (Zhou et al., 2001; Bunting, 2002). Consistent with this hypothesis, ABCG2 is involved in porphyrin homeostasis and contributes to self-renewal of mouse embryonic stem cells (Susanto et al., 2008).

Mitochondrial transport

An interesting finding of our study was the expression of four homologs of mitochondrial transporters, *Sp-ABCB6*, *Sp-ABCB7*, *Sp-ABCB8*, and *Sp-ABCB10a*. In sea urchin embryos, mitochondria are important for both energetics and oral-aboral specification (Coffman et al., 2009). The genes expressed are homologous to mammalian mitochondrial half-transporters likely to function in Fe/S cluster transport, iron homeostasis, heme biosynthesis, peptide transport, and oxidative stress protection (Zutz et al., 2009; Herget and Tampé, 2007; Chen et al., 2009). In mammals, three of these transporters (ABCB7, ABCB8, and ABCB10) are expressed on the inner mitochondrial membrane, while ABCB6 is thought to localize to the outer mitochondrial membrane (Zutz et al., 2009).

Lysosomal transport

The final ABCB gene expressed was *Sp-ABCB9a*, which is homologous to the lysosomal Tap-like (TAPL) protein, a transporter of peptides from cytosol into the lysosome. ABCB9/TAPL may perform a homeostatic role such as disposing of accumulated cytosolic peptides (Zhao et al., 2006; Bangert et al., 2011), and it is possibly also involved in antigen processing (Bangert et al., 2011).

Multifunctional transport

Several of the expressed transporters could have dual functions in the embryo, including the aforementioned example of *Sp-ABCC1*. We detected other predicted multifunctional transporters including *Sp-ABCC4a*, *Sp-ABCC4b*, *Sp-ABCC4c*, and *Sp-ABCC5a*, which are homologs of mammalian transporters with broad specificity for both signaling molecules (e.g. cGMP) and xenobiotics. Like ABCC1, ABCC4/MRP4 transports leukotrienes and is necessary for human dendritic cell migration (Fletcher et al., 2010).

One of the central transporters in this study was *Sp-ABCC5a*, whose homologs remain relatively poorly characterized in any organism. In humans, ABCC5/MRP5 localizes to basolateral membranes of polarized cells, and its mRNA is ubiquitously expressed in adult tissues, though it is highest in skeletal muscle, heart and brain (Chen and Tiwari, 2011). It has been suggested to be a cGMP transporter (Jedlitschky et al., 2000), although its affinity for this substrate is relatively low (de Wolf et al., 2007). Since *Hs-ABCC5* may export cyclic nucleotides, one possibility is that *Sp-ABCC5a* plays some role in signaling necessary for morphogenesis or migration of the mesenchyme cells. Alternatively, it may function in xenobiotic efflux, though in other systems it has yet to be demonstrated as toxicologically important (Leslie et al., 2001; Chen and Tiwari, 2011). One possibility is that *Sp-ABCC5a* may be involved in protection against some endogenously produced toxic metabolite.

Potassium channel conductance regulators

Three of the expressed transporter genes, *Sp-ABCC9a*, *Sp-ABCC9b*, and *Sp-ABCC9d*, are homologous to sulfonyl urea receptor 2 (SUR2), which associates with inwardly rectifying potassium channels to regulate insulin secretion in humans (Bryan et al., 2007). Little is presently known about the functions of these channels in development. In *Drosophila*, ABCC9/SUR2 is expressed in trachea and dorsal vessels and is potentially involved in cell migration (Nasonkin et al., 1999).

White transporter homologs

Finally among the genes expressed, *Sp-ABCG11* and *Sp-ABCG12* are homologs of the White half-transporter, which transports precursors or metabolic intermediates of pigment to control eye color in *D. melanogaster* (Ewart et al., 1994; Mackenzie et al., 2000). Like the ABCB half-transporters, White protein is not localized on the cell membrane, but instead is found on intracellular vesicles (Mackenzie et al., 2000; Evans et al., 2008) where it may also transport cGMP (Evans et al., 2008). In *Drosophila* embryos, *white* transcripts are detected in the Malpighian tubules coincident with the onset of cell differentiation (Fjose et al., 1984).

Transporters with unresolved classification

Interestingly, we found two genes that may have different homologs than those indicated by their original names. For example, although the name *Sp-ABCB1b* indicates similarity to human ABCB1/Pgp, the top NCBI Blastp hits were chicken ABCB1 (CMDR1) (e=0.0) and human ABCB11 (Bile salt export pump, BSEP) (e=0.0). BSEP transports bile salts across the canalicular plasma membrane (Stieger et al., 2007). Chicken *Mdr1* is expressed in the thymus and bursa of embryos and may participate in lymphoid differentiation of T and B cells (Petrini et al., 1995). Given that sea urchins lack a direct ABCB11 homolog, it is possible that *Sp-ABCB1b* transports sterols similar to bile salts.

Similarly, although *Sp-ABCC9b* is homologous to SUR2 (e=0.0), Blastp indicates it is equally similar to Multidrug resistance protein 2 (MRP2) (e=0.0). MRP2 is related to MRP1, and as such it effluxes both signaling molecules and xenobiotics (Leslie et al., 2001; Fletcher et al., 2010). Thus, one possibility is that *Sp-ABCC9b* has different functions than those predicted by its designation as an SUR.

Differential regulation of transporters

Given this great diversity of ABC transporters present in embryos, it seems plausible that multiple modes of regulation are employed to maintain and modulate their membrane activity through development. For example, our results with ABCB1a and ABCC5a indicate that they are likely to be under different modes of regulation.

ABCB1a transcripts are abundant throughout development (Fig. 3A, Supplemental Table 1) and are strongly detected in all cells of embryos including the primary mesenchyme (Fig. 4F). Yet, while ABCB1a-mCitrine accumulates to high levels on surfaces of ectodermal cells, it is expressed weakly on surfaces of primary mesenchymal cells (Fig. 5Aii, Bii). This could indicate that ABCB1a is post-transcriptionally regulated and that levels of its mRNA do not necessarily correlate to surface levels of the protein. Additional evidence for post-transcriptional regulation of ABCB1a comes from the observation that efflux activity increases after fertilization of sea urchin eggs, even with exposure to inhibitors of transcription and translation (Hamdoun et al., 2004). This indicates that in very early development, efflux activity is post-translationally controlled, and it is possible that ABCB1a is similarly regulated throughout development. Alternatively, primary mesenchyme cells may have less ABCB1a-mCitrine due to membrane turnover associated with the epithelial to mesenchymal transition (EMT) (Wu et al., 2007).

In contrast, *ABCC5a* expression is tightly temporally and spatially controlled (Fig. 3D, Fig. 4J), expressed only after hatching and exclusively in a subset of veg2 cells and their descendants. This suggests that *ABCC5a* is transcriptionally regulated. Consistent with this hypothesis, in MCF7 cells, *Hs-ABCC5* expression is regulated by the EMT-inducing transcription factors Snail, Twist and FOXC2 (Saxena et al., 2011). These are important developmental transcription factors that could interact with *Sp-ABCC5a*. Our future studies

will address this possibility and probe the role of *Sp*-ABCC5a in protection, specification, and/or functions of mesodermal cells.

Conclusions

Collectively, our results highlight the diversity of ABC transporters necessary for sea urchin development and provide a foundation for exploring their biology. The characterization of ABCB1a and ABCC5a emphasizes differences in spatial and temporal expression of ABC transporters, and the relation of these differences to predicted functions. Clearly, ABC transporters are more than protective transporters in embryogenesis, and exquisite regulation of membrane function by expression of transporters is likely central to homeostasis, protection, and signaling during development. Our future work will focus on major developmental transitions to address the regulation and function of ABCC5a in protection and/or specification of embryonic cells.

EXPERIMENTAL PROCEDURES

Animals

Purple sea urchins, *Strongylocentrotus purpuratus*, were collected off the coast of San Diego, CA, USA, kept in 12°C running seawater, and fed *Macrocystis pyrifera*. Gametes were collected according to standard procedures (Foltz et al., 2004). Eggs were collected in raw seawater, passed through a 120 µm nitex filter, and washed in filtered seawater (FSW). For RNA isolation and *in situ* analyses, a 500 ml solution of 1% packed eggs in FSW was fertilized with 5 µl sperm (in FSW). Fertilization was visually confirmed, and only batches with >90% fertilization were used for experiments. Embryos were washed twice in FSW to remove excess sperm, and the culture was grown at a concentration of 500 embryos/ml FSW at 12°C.

RNA isolation

For all gene expression experiments, total RNA was isolated at nine developmental stages (approximate hours post-fertilization, hpf): 1. Unfertilized egg, 2. 16-cell (~6 hpf), 3. 60-cell (~8.5 hpf), 4. early blastula (12–13 hpf), 5. hatching blastula (21–23 hpf), 6. hatched blastula (27–29 hpf), 7. early gastrula (33–35 hpf), 8. late gastrula (50–52 hpf), 9. early prism (55–58 hpf). Experiments were replicated four times with progeny of four females.

Aliquots of cultures were hand-centrifuged to pellet eggs/embryos, and RNA was isolated using a Nucleospin RNA II isolation kit (Macherey-Nagel, Bethlehem, PA, USA) according to the manufacturer's protocol. Yields varied (depending on the density of the pellet) from 3–56 µg total RNA. RNA concentration and purity was determined by spectrophotometry and agarose gel electrophoresis. Only samples with absorbance ratios of ~2.0 (260/280) and ~2.0–2.2 (260/230) with clear major ribosomal subunit bands on gel visualization were used for experiments.

cDNA synthesis

Reverse transcription was performed using 500 ng total RNA, 1.5 µM random primer (New England Biolabs, Ipswich, MA, USA), 0.5 mM dNTPs (Fermentas, Glen Burnie, Maryland, USA), M-MuLV Reverse Transcriptase (New England Biolabs), and RNasin (Promega, Madison, WI, USA) at a final volume of 20 µl.

Quantitative real-time PCR (qPCR)

qPCR was performed on a Stratagene MX3000p thermal cycler (Agilent, Santa Clara, CA, USA) with EVA QPCR SuperMix Kit (Biochain, Hayward, CA, USA) according to the

manufacturer's protocol. All reactions were run in duplicate. To each 20 μ l cDNA synthesis reaction, 200 μ l nuclease-free water was added for analysis with qPCR. A volume of 3.5 μ l was used as template for qPCR with 625 nM of each (forward and reverse) primer in a total reaction volume of 20 μ l per well.

Primer design and testing

We designed a total of 76 primer pairs: 24 in the ABCB family, 38 in the ABCC family, and 14 in the ABCG family. For each ABC transporter, primers were designed to avoid conserved regions (i.e. nucleotide binding domains). Two primer pairs (forward and reverse) were designed for each transporter gene, and the best pair was selected for each gene. Selected primer pairs are listed in Supplemental Table 2. Primers for control genes *Nodal*, *Nanos2*, and *Spz12* were generically designed from existing cDNA annotations. The *Ubiquitin* control primer pair was taken from <http://sugp.caltech.edu/SUGP/resources/methods/q-pcr.php>.

Primers were tested by amplification with serial dilutions of stock cDNA using the following criteria: confirmation of a "steep" amplification curve, single peak dissociation curve, and correct length (~100 bp) of a single amplicon on an agarose gel (Schmittgen and Livak, 2008). Serial dilutions of cDNA were made in 1x, 4x, 64x, 256x, and 1024x dilutions with water. Each primer was tested with these dilutions from eggs, 24 hpf and/or 55 hpf embryos to confirm that threshold cycle (Ct) increased 2 units for each dilution. Primer pairs that did not meet the above criteria were rejected from the study.

Confirmation of amplicon specificity to targeted genes

Amplicon specificity was confirmed for a subset of primers (*ABCB1a*, *ABCB4a*, *ABCC1*, *ABCC5a*, *ABCC9a*, and *ABCG2b*) by cloning and sequencing the qPCR products from embryos of two females. qPCR amplicons were purified and cloned into a pCR4-TOPO vector (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol, then sequenced (Retrogen, San Diego, CA, USA). The resulting sequences were searched using the Blastn algorithm on SpBase.org in the Sp genome v3.1 database. All inputs mapped exclusively to the targeted gene, confirming the qPCR primers were specific to individual targeted genes.

qPCR Analyses

Gene expression changes are reported as fold differences with respect to the unfertilized egg. For *ABCC5a*, *ABCG2b*, *ABCG11*, and *ABCG12*, egg transcript levels were too low to quantify so expression is reported with respect to the earliest developmental stage at which quantification was possible (hatching blastula for *ABCC5a*, *ABCG11*, and *ABCG12*, and early prism for *ABCG2b*). The formula 2^x was used, where x is the threshold cycle (Ct) number difference between the reference stage (egg, hatching blastula or early prism) and the other stages of development ($\Delta\Delta$ Ct method). For example, the pre-normalized fold change for *ABCB1a* gene expression at the late gastrula stage is:

$$2^x_{ABCB1a} = 2^{(Ct_{egg} - Ct_{late\ gastrula})_{ABCB1a}}$$

Results were normalized to *Ubiquitin* according to (Nemer et al., 1991; Juliano et al., 2006; Peter and Davidson, 2009b) such that the reported value reflects the formula:

$$\text{Fold change} = 2^x_{ABCB1a} - 2^x_{ubiquitin}$$

Reported data are an average of four females. Fold changes are significant if they are ± 2 -fold change from the reference point ($0.5 < \text{not significant} < 2$) (Peter and Davidson, 2009b).

Transcript copy number calculations

Transcript copy numbers per egg/embryo were calculated by quantifying the reference time point against a standard curve generated with dilutions of the sequenced TOPO-cloned qPCR amplicons. We calculated that each qPCR well contained the equivalent of 1.27 eggs/embryos per well based on the following: each *S. purpuratus* egg/embryo contains between 3.3 ng (Brandhorst, 2004) and 3.0 ± 0.2 ng total RNA through 60 hpf (Nemer et al., 1984). Using an average of these values (3.15 ng per egg/embryo) and the assumption that 50% of material is lost in converting mRNA to cDNA (Ransick, 2004), we converted 0.5 μ g total RNA to cDNA to achieve ~80 embryos worth of cDNA in a 220 μ l volume. We used 3.5 μ l per qPCR well, corresponding to 1.27 eggs/embryos per well.

Using a DNA molecular weight calculator (www.bioinformatics.org), we determined the molecular weight of each TOPO-cloned qPCR amplicon. We made 4x serial dilutions of these plasmids in water at concentrations equivalent to 1–1,048,576 copies per egg/embryo. We repeated qPCR with the serial dilution series and the reference time point cDNA samples. From the dilution series, we generated a standard curve by applying a nonlinear regression trendline fit (Microsoft Excel) in the format: $Y = ae^{-bX}$ where Y is copies per egg/embryo and X is Ct. Transcript numbers were calculated from these equations.

ABC transporter protein expression

Sp-ABCB1a and *Sp-ABCC5a* cDNAs were cloned from egg and gastrula stage RNA, respectively, by Rapid Amplification of cDNA Ends (RACE; Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Phusion High-Fidelity DNA polymerase (New England BioLabs) was used for all PCR reactions. Fluorescent proteins mCitrine and mCherry were subcloned into a modified pCS2 vector, and fusions were generated by inserting transporter cDNAs using XhoI for an N-terminal FP-ABCB1a fusion (ABCB1a-mCitrine), and SpeI for a C-terminal FP-ABCC5a fusion (ABCC5a-mCherry). All constructs were sequenced after cloning (Retrogen). Capped mRNA was made using the mMessage mMachine SP6 kit (Ambion, Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. mRNA was injected into fertilized eggs at 2–5% egg volume in a final concentration of 1 mg/ml in water (Lepage and Gache, 2004). Fluorescent protein localization was visualized on a Zeiss LSM-700 laser scanning confocal microscope using a Zeiss Plan APOChromat 20x air objective (numerical aperture, 0.8) (Zeiss, Thornwood, NY, USA). All images were captured using the Zen software suite (Zeiss, revision 5.5) and processed with ImageJ freeware (NIH, Bethesda, MD, USA).

Whole Mount *In Situ* Hybridization (WMISH)

Templates for *in situ* probes were generated by cloning ~1.5kb of *ABCB1a* or *ABCC5a* into dual promoter pCRII-Topo TA vector (Invitrogen) according to the manufacturer's protocol. PCR was carried out with Phusion High-Fidelity DNA polymerase (New England BioLabs) using ABCB1a-mCitrine and ABCC5a-mCherry as templates. Primers and probe sequences are listed in Supplemental Table 2. Probe templates were sequenced after cloning (Retrogen).

WMISH was performed following a modified protocol (Ransick, 2004). Briefly, hatched blastula and later stage embryos were cultured as described. Cleavage-stage embryos were fertilized in 1 mM para-aminobenzoic acid (PABA) and passed through a 60 μ m filter to remove the fertilization envelope, then cultured as described. Swimming embryos were pelleted by cooling and gentle centrifugation. A dense aliquot of eggs or embryos was

distributed among wells of plastic round-bottom plates to form a monolayer at the base of each well. Specimens were fixed on ice in two steps: 1) 20 minutes with 0.625% glutaraldehyde in Fixation Buffer (FB: 32.5 mM MOPS buffer, pH 7.0; 162.5 mM NaCl; 32.5% FSW), then transferred to 2) 1.25% glutaraldehyde in FB overnight at 4°C.

Specimens were washed once in FB without glutaraldehyde then three times in Tris-buffered Saline + Tween-20 (TBST: 0.1 M Tris buffer, pH 7.5; 0.15 M NaCl; 0.1% Tween-20). Proteinase K was applied for 10–15 minutes at 50 ng/μl in TBST. The digestion was stopped with 25 mM glycine in TBST, then specimens were washed two times in TBST and post-fixed for 30 minutes at room temperature with 4% paraformaldehyde in 50 mM MOPS buffer, pH 7.0 + 150 mM NaCl. Preceding hybridization, specimens were washed three times in TBST then transitioned into Hybridization Buffer (HB: 50% formamide; 5x Saline-Sodium Citrate (SSC); 20 mM Tris-base, pH 7.5; 5 mM EDTA; 0.1% Tween-20; 2x Denhardt's Solution; 50 μg/ml Heparin; 500 μg/ml yeast tRNA) in three steps: 30% HB in TBST, 60% HB in TBST, then 100% HB. Specimens were incubated in HB for 1 h at 60°C to pre-hybridize.

Digoxigenin (DIG)-labeled antisense probes were made by *in vitro* transcription using Sp6 or T7 RNA polymerase (New England Biolabs) with DIG RNA Labeling Mix (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Sense probes were used as negative controls. Probes were diluted to 1 ng/μl in HB and heated to 70°C for 5 min, then added to specimens for 12–16 h hybridization at 60°C. Post-hybridization washes included 15–20 min incubations at 60°C in the following solutions: HB; 50% HB + 50% 2X SSCT (2X SSC + 0.2% Tween-20); 2X SSCT; 0.5X SSCT; 0.2X SSCT. Specimens were then returned to room temperature, transferred to clean wells, and washed three times in TBST.

Specimens were blocked for 30 min at room temperature with 10% sheep serum + 1 mg/ml Bovine Serum Albumin (BSA) in TBST. Anti-digoxigenin-AP, Fab fragments from sheep (Roche) were added in a 1:1000 dilution in 5% sheep serum + 1 mg/ml BSA in TBST, then incubated for 1 h at room temperature. Specimens were washed three times in TBST, then two times in Alkaline Phosphate Buffer (APB: 100 mM Tris-base pH 9.5; 100 mM NaCl; 50 mM MgCl₂; 1 mM levamisole; 0.1% Tween-20). Stain was developed in 0.3375 mg/ml NBT + 0.175 mg/ml BCIP in APB for 1–24 h depending on the probe and transcript abundance. Reactions were quenched with 50 mM EDTA in TBST. Specimens were transitioned into 50% glycerol in TBST with 5 mM Na-azide, and then photographed with a Canon EOS 60D camera (Canon, Lake Success, NY, USA) through a 40x air (0.75NA) Neofluar objective on a Zeiss Axiovert S100 microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- 20 *ABCB*, *ABCC*, and *ABCG* genes are expressed during the first 58 hours of sea urchin development.
- Expressed ABC transporter genes have predicted functions in cell signaling, lysosomal and mitochondrial homeostasis, potassium channel regulation, pigmentation, and xenobiotic efflux.
- *ABCB1a* mRNA is 10–100 times more abundant than mRNA of other ABC transporters.
- *ABCB1a* is expressed in all cells of the embryo, while *ABCC5a* is expressed only in mesodermal precursors.
- ABCB1a protein localizes to the apical membrane of the embryo, while ABCC5a is expressed on basolateral membranes of polarized cells.

Fig. 1. Developmental stages surveyed and relative ABC transporter gene expression
(A) DIC micrographs depict the developmental stages included in the gene expression survey. (B) Heat map of quantitative real-time PCR data expressed as fold change from the reference stage (i.e. earliest detectable stage). Reference stage is egg in all transporters except *ABCC5a*, *ABCG2b*, *ABCG11*, and *ABCG12*. Reference stage is hatching blastula for *ABCC5a*, *ABCG11*, and *ABCG12*. Reference stage is early prism for *ABCG2b*. All data represent the average of progeny from four females (N=4).

Fig. 2. Relative ABC transporter gene expression during sea urchin development

Data depicted in Fig. 1 is presented as individual graphs on a logarithmic scale. Expression profiles are shown in three color-coded groups. Group 1 is shown in blue, Group 2 in orange, and Group 3 in green. N = 4, error bars represent standard error.

Fig. 3. Number of ABC transporter transcripts per egg/embryo

Number of mRNA copies per egg/embryo is shown. Transcript copies were determined using a standard curve to quantify the reference point (egg for *ABCB1a*, *ABCB4a*, *ABCC1*, and *ABCC9a*; hatching blastula for *ABCC5a*; early prism for *ABCG2b*), and by applying fold change values (Fig. 1, Fig. 2, Supplemental Table 1) to quantify all other stages. Dashed lines (**D**, **F**) indicate where transcripts were below the threshold of detection using qPCR (i.e. <100 copies per egg/embryo). N=4, bars represent standard error.

Fig. 4. Spatial expression of ABC transporter genes

Whole mount *in situ* hybridization depicts endogenous expression of *ABCB1a* and *ABCC5a*. (A–D) For controls, DIG-labeled sense probe was used. (E–H) *ABCB1a* is expressed in all cells of embryos. (I) *ABCC5a* mRNA is not detected prior to hatching, after which it is detected (J) in the vegetal pole of hatched mesenchyme blastulae, and (K, L) in mesodermal cells in later embryos. (A, E, I) 16-cell embryos were fixed at 7 hpf, (B, F, J) hatched mesenchyme blastulae at 36 hpf, (C, G, K) early gastrulae at 42 hpf, and (D, H, L) late gastrulae at 48 hpf.

Fig. 5. Localization of ABCB1a and ABCC5a proteins

Micrographs show exogenous ABCB1a and ABCC5a localization from expression of fluorescent-protein fusions. Representative embryos are shown. **B** is an inset from the embryo shown in **A**. mRNA from ABCB1a-mCitrine (**ii**, green) and ABCC5a-mCherry (**iii**, red) were injected into fertilized eggs, then embryos were grown to mesenchyme blastulae for imaging. (**iv**) Merged channel includes Histone H2B-CFP (blue), injected as a nuclear marker. ABCB1a-mCitrine protein localizes to the apical surface of the embryo, while ABCC5a-mCherry localizes on the basolateral cell surfaces.

Fig. 6. Model of endogenous ABCB1a and ABCC5a protein localization
 (A) A cartoon represents WMISH transcript localization shown in Fig. 4F, 4J. By combining this with FP-fusion ABCB1a and ABCC5a protein localization data shown in Fig. 5A, we propose (B) a model for endogenous ABCB1a (green) and ABCC5a (red) expression. ABCB1a is primarily expressed on the apical membrane of polarized epithelial cells, while ABCC5a is expressed on the basolateral membranes of secondary (non-skeletogenic) mesenchyme cells.

TABLE 1
ABC transporter genes detected in sea urchin development and functions of their homologs

Blastp score indicates E value.

Gene	Gene ID	Annotated peptide length (aa)	Homologs	Blastp score	Also known as	Cellular membrane localization	Function (substrates)	References
<i>Sp-ABCB1a</i>	<u>SPU_001752</u>	1329	<i>H. sapiens</i> ABCB1	0.0	CLCS; MDR1; P-GP; PGY1	Apical	Xenobiotic efflux (anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, mitoxantrone)	(Dean et al., 2001; Leslie et al., 2005; Fletcher et al., 2010)
<i>Sp-ABCB1b</i>	<u>SPU_002431</u>	1079	<i>H. sapiens</i> ABCB11	0.0	BSEP; PGY4; SPGP	Apical	Bile salt transport (Taurochenodeoxycholate)	(Stieger et al., 2007)
<i>Sp-ABCB4a</i>	<u>SPU_007014</u>	1235	<i>H. sapiens</i> ABCB1	0.0	CLCS; MDR1; P-GP; PGY1	Apical	Xenobiotic efflux (anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, mitoxantrone)	(Dean et al., 2001; Leslie et al., 2005; Fletcher et al., 2010)
			<i>H. sapiens</i> ABCB4	0.0	GBD1; MDR2; MDR3; PGY3	Apical	Phospholipid flippase (phosphatidylcholine); Bile salt transport; Xenobiotic efflux (Anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, mitoxantrone)	(Dean et al., 2001; Borst and Elferink, 2002; Fletcher et al., 2010)
<i>Sp-ABCB6</i>	<u>SPU_018342</u>	776	<i>H. sapiens</i> ABCB6	0.0	ABC; PRP; MTABC3	Outer mitochondrial	Iron transport	(Dean et al., 2001; Zutz et al., 2009)
<i>Sp-ABCB7</i>	<u>SPU_003241</u>	651	<i>H. sapiens</i> ABCB7	0.0	ABC7; ASAT; Atm1p	Inner mitochondrial	Cytosolic Fe/S transport; Iron homeostasis	(Dean et al., 2001; Zutz et al., 2009)
<i>Sp-ABCB8</i>	<u>SPU_024666</u>	469	<i>H. sapiens</i> ABCB8	4e-97	MABC1; M-ABC1	Inner mitochondrial	Oxidative stress protection	(Zutz et al., 2009)
<i>Sp-ABCB9a</i>	<u>SPU_026825</u>	398	<i>H. sapiens</i> ABCB9	8e-160	TAPL	Lysosomal	Peptide transport	(Zhao et al., 2006; Bangert et al., 2011)
<i>Sp-ABCB10a</i>	<u>SPU_016850</u>	623	<i>H. sapiens</i> ABCB10	0.0	M-ABC2; MTABC2	Inner mitochondrial	Iron transport; possible peptide transport & antigen processing	(Herget and Tampe, 2007; Chen et al., 2009; Zutz et al., 2009)
<i>Sp-ABCC1</i>	<u>SPU_026395</u>	1025	<i>H. sapiens</i> ABCC1	0.0	MRP; ABCC; GS-X; MRP1	Basolateral	Xenobiotic efflux (GSH-conjugates, anthracyclines, mitoxantrone, vinca	(Leslie et al., 2005; Fletcher et al., 2010;

Gene	Gene ID	Annotated peptide length (aa)	Homologs	Blastp score	Also known as	Cellular membrane localization	Function (substrates)	References
<i>Sp-ABCC4a</i>	SPU_020669	1411	<i>H. sapiens</i> ABCC4	0.0			alkaloids, imatinib, epipodophyllotoxins, camptothecins, colchicines, metals, methotrexate, Etoposide-glucuronide, Doxorubicin-GS, glutathione disulfide (GSSG); Signaling & homeostasis (GSH-conjugates, leukotrienes, prostaglandins, sphingosine-1-phosphate, bilirubin, estradiol 17 β -D-glucuronide)	Chen and Tiwari, 2011; He et al., 2011)
<i>Sp-ABCC4b</i>	SPU_024191	1214	<i>H. sapiens</i> ABCC4	1e-103			Xenobiotic efflux (nucleosides, thiopurines, PMEA, methotrexate, anti-HIV nucleoside analogues, camptothecins); Signaling & homeostasis (leukotrienes, prostaglandins, thromboxane, cyclic nucleotides)	(Fletcher et al., 2010; Chen and Tiwari, 2011)
<i>Sp-ABCC4c</i>	SPU_002411	1174	<i>H. sapiens</i> ABCC4	0.0	MRP4; MOATB	Apical or Basolateral (tissue dependent)		
<i>Sp-ABCC5a</i>	SPU_023723	1424	<i>H. sapiens</i> ABCC5	0.0	MRP5; SMRP; MOATC	Basolateral	Organic anion transport (acidic organic dyes, dinitrophenylglutathione); Xenobiotic efflux (methotrexate, cisplatin, PMEA, AZT, daunorubicin, doxorubicin, gemcitabine); Signaling & homeostasis (cyclic nucleotides)	(Borst and Elferink, 2002; Fletcher et al., 2010; Chen and Tiwari, 2011)
<i>Sp-ABCC9a</i>	SPU_025903	1585	<i>H. sapiens</i> ABCC9	0.0			Potassium channel regulation	(Bryan et al., 2007)
<i>Sp-ABCC9b</i>	SPU_028797	1497	<i>H. sapiens</i> ABCC9	0.0	SUR2			
<i>Sp-ABCC9d</i>	SPU_007764	1481	<i>H. sapiens</i> ABCC9	0.0				
<i>Sp-ABCC2b</i>	SPU_014013	448	<i>H. sapiens</i> ABCC2	7e-138	MRX; MXR; ABCP; BCRP; BMDP	Apical	Xenobiotic efflux (mitoxantrone, camptothecins, anthracyclins, bisantrene, imatinib, methotrexate, flavopiridol, epipodophyllotoxins); Stem cell protection & maintenance	(Leslie et al., 2005; Krishnamurthy and Schuetz, 2006; Fletcher et al., 2010)
<i>Sp-ABCC9</i>	SPU_012874	485	<i>D. melanogaster</i> E23	1e-81	Early gene at 23		Ecdysone signaling, circadian rhythm	(Itoh, Tanimura, and Matsumoto, 2011)

Gene	Gene ID	Annotated peptide length (aa)	Homologs	Blastp score	Also known as	Cellular membrane localization	Function (substrates)	References
<i>Sp-ABCG11</i>	<u>SPU_020849</u>	590	<i>D. melanogaster</i> White	5e-109	white; DMWHITE; EG:BACN33B1	Pigment granular	Eye color determinant (pigment metabolites)	(Ewart et al., 1994; Mackenzie et al., 2000)
<i>Sp-ABCG12</i>	<u>SPU_015080</u>	677	<i>D. melanogaster</i> White	0.0				