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The small GTPase Arf6 regulates sea urchin morphogenesis

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ABSTRACT

The small GTPase Arf6 is a conserved protein that is expressed in all metazoans. Arf6 remodels cytoskeletal actin and mediates membrane protein trafficking between the plasma membrane in its active form and endosomal compartments in its inactive form. While a rich knowledge exists for the cellular functions of Arf6, relatively little is known about its physiological role in development. This study examines the function of Arf6 in mediating cellular morphogenesis in early development. We dissect the function of Arf6 with a loss-of-function morpholino and constitutively active Arf6-Q67L construct. We focus on the two cell types that undergo active directed migration: the primary mesenchyme cells (PMCs) that give rise to the sea urchin skeleton and endodermal cells that form the gut. Our results indicate that Arf6 plays an important role in skeleton formation and PMC migration, in part due to its ability to remodel actin. We also found that embryos injected with Arf6 morpholino have gastrulation defects and embryos injected with constitutively active Arf6 have endodermal cells detached from the gut epithelium with decreased junctional cadherin staining, indicating that Arf6 may mediate the recycling of cadherin. Thus, Arf6 impacts cells that undergo coordinated movement to form embryonic structures in the developing embryo.

Key words: primary mesenchyme cells; actin; skeleton; protein trafficking; endoderm; gut

Abbreviations: PMCs – primary mesenchyme cells, DVC rod – dorsoventral connecting rod, EMT – epithelial to mesenchymal transition, MASO – antisense morpholino oligonucleotide, GEF - guanine nucleotide exchange factors, GAPs - GTPase activating proteins

INTRODUCTION

The small guanine nucleotide-binding protein Arf6 is an evolutionarily conserved molecule that has been identified in a myriad of organisms, ranging from yeast to human. Important *in vitro* studies have revealed that Arf6 is capable of remodeling the actin cytoskeleton and mediating the recycling of cell surface proteins that can impact various signaling pathways and cell movement (Berndt et al., 2014; Blum et al., 2015; D'Souza-Schorey and Chavrier, 2006; Donaldson, 2003; Humphreys et al., 2013; Hunzicker-Dunn et al., 2002; Jacques-Fricke and Gammill, 2014; Powelka et al., 2004; Sabe, 2003; Santy et al., 2001). However, its *in vivo* functions, particularly during early development, have not been examined extensively.

The Arf family is composed of small guanine-nucleotide-binding proteins that can be separated into three different classes in mammals based on sequence homology: Class I (Arf1, Arf2 and Arf3), Class II (Arf4 and Arf5) and Class III (Arf6) (Gillingham and Munro, 2007). Arf6 activity is regulated by cycling between the plasma membrane in its active GTP bound form and endosomal compartments in its inactive GDP bound form (D'Souza-Schorey et al., 1995; Donaldson, 2002; Donaldson and Radhakrishna, 2001). Arf6 guanine nucleotide exchange factors (GEFs) activate Arf6 by catalyzing the dissociation of GDP to promote the binding of GTP. Conversely, Arf6 GTPase activating proteins (GAPs) bind to the GTP form of Arf6 to catalyze the hydrolysis of GTP to GDP (Bos et al., 2007).

Arf6 regulates a wide variety of cellular processes, including endosomal membrane traffic, phosphoinositide metabolism, regulated secretion, phagocytosis, disassembly of adherens junctions, cell motility, and cell signaling (Brown et al., 2001; D'Souza-Schorey et al., 1995; Humphreys et al., 2013; Radhakrishna et al., 1999; Santy et al., 2001; Zhang et al., 1998). The cellular functions of Arf6 are complex, but in general it is thought to act mainly through the

activation of lipid-modifying enzymes and modulation of actin structures (Donaldson, 2002).

Arf6-GTP is known to interact with several effectors that lead to the regulation of Rac1 activity, thus resulting in actin remodeling at the plasma membrane (Cotton et al., 2007; Humphreys et al., 2013; Kawaguchi et al., 2014; Koo et al., 2007).

In addition to the actin remodeling function, Arf6 mediates cell surface protein recycling and impacts various signaling pathways, such as β -integrin induced receptor tyrosine kinase signaling (Allaire et al., 2013; Heasman et al., 2000; Powelka et al., 2004), EGFR induced MEK-ERK pathway (Tague et al., 2004), the G-protein coupled receptors induced G protein signaling (Claing, 2011; Cotton and Claing, 2009), and cadherin recycling that affects adherens junctions as well as β -catenin abundance downstream of the Wnt signaling pathway (Dikshit et al., 2015; Egami et al., 2015; Grossmann et al., 2013). In the context of a developing embryo, proper regulation and integration of all these signaling pathways are essential for development.

While Arf6 shares conserved residues important for its membrane trafficking and actin remodeling functions, the Arf6 null phenotypes are variable in different organisms in terms of severity and the impact on development. Surprisingly, Arf6 is not essential in *Drosophila* and is only required during spermatogenesis with no other overt morphological abnormalities (Dyer et al., 2007). Systemic Arf6 loss-of-function in zebrafish caused a defect in epiboly (spreading of the blastoderm over the yolk) by regulating the recycling of syndecan that acts as a co-receptor for adhesion molecules and growth factors, such as FGFs and Wnt (Lambaerts et al., 2009). The *arf6*^{-/-} null mice resulted in embryonic lethality at midgestation stage (E13.5), with prominent defect in liver hepatic cord formation (Suzuki et al., 2006). The exact molecular mechanism of Arf6 mediated hepatic defects in the mouse is not known (Suzuki et al., 2006). Arf6 is not required for mouse early development, likely due to multiple endocytic pathways available to the

embryo (Doherty and McMahon, 2009). We examined the physiological function of Arf6 in the developing sea urchin embryo as we can perform both loss-of-function and gain-of-function experiments which circumvent embryonic lethality. The cell types of interest in this study are the primary mesenchyme cells (PMCs) that produce the embryonic skeleton spicules and the endodermal cells that form the gut.

The sea urchin PMCs are highly dynamic cells that exhibit many properties of an animal cell (reviewed in (Lyons et al., 2012). During development, the PMCs undergo epithelial to mesenchymal transition (EMT) to ingress into the blastocoel as individual cells (Ettensohn, 2013; Lyons et al., 2012). During gastrulation, they undergo cellular morphogenesis and cell fusion and form a subequatorial ring surrounding the embryonic gut followed by the formation of the two ventrolateral clusters (Ettensohn, 2013; Lyons et al., 2012). At this stage, they are connected by actin-based syncytial cables of filopodial extensions. Once the PMCs position into the subequatorial ring, they migrate anteriorly toward the anterior pole of the embryo guided by sensory filopodia. As they migrate anteriorly, they synthesize calcium carbonate deposits which constitute the skeleton of the embryo that supports the shape, swimming and feeding of the larvae (Hart and Strathmann, 1994; Pennington and Strathmann, 1990).

In contrast to the PMCs that undergo EMT as individual cells and then fuse together via syncytia, the endodermal cells migrate as a coherent sheet of cells during gut invagination (reviewed in (McClay et al., 1992; Wessel and Wikramanayake, 1999). Invagination of the archenteron starts with bending of the thickened vegetal plate of the embryo, followed by the archenteron elongation. Initially elongation is mediated by the rearrangement of the endodermal cells via convergent extension movements, when the endodermal cells locally rearrange (Burke et al., 1991). During gastrulation, these endodermal cells retained junctional cadherin and their

cell adhesive properties may be regulated by the interaction of cadherin with cadherin associated proteins that link to the actin cytoskeleton (such as β -catenin) (Miller and McClay, 1997) . As the gut elongates, the secondary mesenchyme cells, located at the tip of the archenteron (embryonic gut) extend filopodia to provide additional elongation force through the contact with the specific predefined target site on the ectoderm (Hardin and McClay, 1990). After fusion of the elongated gut with the ectoderm, the gut differentiates into three structurally distinctive domains of foregut, midgut, and hindgut (Burke, 1981; Wessel and Wikramanayake, 1999).

PMCs and endodermal cells exhibit very different behaviors and thus provide a unique opportunity to examine Arf6 function in different cell types. We tested the functions of Arf6 using antisense morpholino oligonucleotide (MASO) and constitutively active form of Arf6 (Arf6-Q67L). Results indicate that Arf6 is critical for the positioning and function of PMCs potentially through regulation of actin remodeling. Morphology of the gut epithelium is also negatively affected by Arf6 perturbations, potentially through Arf6-mediated recycling of cadherin. Overall, our results demonstrated the essential regulatory role of Arf6 in the development and function of the PMCs and morphology of the gut in the sea urchin embryo.

MATERIALS AND METHODS

Animals

Adult *Strongylocentrotus purpuratus* were received from California (Point Loma Marine Company). Gametes were collected by intracoelomic injection of 0.5 M KCl. All cultures are placed in 15°C incubator.

Constructs

To examine the localization of Arf6 in the sea urchin embryo, the sea urchin *Arf6-mcherry* was synthesized as a gBlocks gene fragment that contains the 5'UTR and coding sequence of *Arf6-WT* fused to *mCherry* with *Xenopus β -globin* 3'UTR (Idtdna.com) (Fig. S1A). This fragment was subsequently cloned into Zero-Blunt TOPO vector (Thermo Fisher Scientific, Waltham, MA) and sequenced (Genewiz Inc., South Plainfield, NJ). The construct was linearized with XhoI (Thermo Fisher Scientific, Waltham, MA) and *in vitro* transcribed with the Sp6 mMessage mMachine kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Microinjected embryos were collected at gastrula stage (48 hpf), and imaged live on LSM 780 confocal microscope (Carl Zeiss Inc., Thornwood, NY).

To perform Arf6 knockdown (KD) rescue experiment, the sea urchin Arf6 mRNA (*SpArf6*) was cloned into a plasmid containing *Xenopus β -globin* 5' and 3'UTRs that are known to function in the sea urchin embryo, ensuring the translation of Arf6 (Gustafson et al., 2011). To test the function of Arf6, we generated the constitutively active mutant *Arf6-Q67L*. Mutagenesis primers were designed using a primer design program provided by Stratagene (www.agilent.com/genomics/qcpd) (Arf6_Q67L_F: 5' ATGGGATGTTGGTGGTCTGGATAAAATTCGGCCTC 3', Arf6_Q67L_R: 5' GAGGCCGAATTTTATCCGAGACCACCAACATCCCAT 3'). Mutated Arf6 coding sequences were generated using the QuikChange Lightning mutagenesis kit according to manufacturer's specifications (Stratagene, La Jolla, CA). Positive clones were sequenced to check the fidelity of the mutagenesis (Genewiz Inc., South Plainfield, NJ). The sea urchin *SpArf6* and *Arf6-Q67L* were linearized with XhoI (Thermo Fisher Scientific, Waltham, MA) and *in vitro* transcribed with the Sp6 mMessage machine kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions.

To test if the human Arf6 mRNA can rescue the Arf6 KD in the sea urchin embryo, we subcloned the human Arf6 (*HsArf6*) that was received from Dr. Julie Donaldson (NIH) (Macia et al., 2004; Palacios et al., 2001; Peters et al., 1995) into pCR-Blunt vector (Thermo Fisher Scientific, Waltham, MA). The construct was linearized with SpeI (Thermo Fisher Scientific, Waltham, MA) prior to *in vitro* transcription using the T7 mMessage machine kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions.

GFP-LifeAct plasmid was received as a gift from Dr. Charles Shuster (New Mexico State University). The construct was linearized with NotI (Thermo Fisher Scientific, Waltham, MA) followed by *in vitro* transcription using the Sp6 mMessage machine kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions.

Embryo Injections

The newly fertilized eggs were microinjected with a Arf6 MASO 5' TCTTTGATAGTACCTTCCCCATCGT 3', negative control MASO 5' CCTCTTACCTCAGTTACAATTTATA 3' (range of 0.5 to 3 mM, with 1-1.5 mM stocks used for all phenotyping experiments) (Genetools, Philomath, OR), or mRNAs of *Firefly* negative control, constitutively active *Arf6-Q67L*, *GFP-LifeAct*, *Arf6-mCherry* or cytoplasmic *mCherry* (0.4 µg/µL, based on the empirically determined minimal amount injected to observe a phenotype or expression of the reporter construct). All *in vitro* transcribed mRNAs were purified using NucleoSpin RNA Clean-up kit (Macherey-Nagel, Bethlehem, PA) according to manufacturer's specifications and further purified with the Millipore spin columns prior to injections (EMD Millipore Corporation, Billerica, MA). Texas red dextran reporter was used as a

marker for injected embryos. Injections were performed as described previously (Stepicheva and Song, 2014). Embryos were cultured at 15°C and collected at various stages of development for further analyses.

Whole mount RNA *in situ* hybridization

The temporal and spatial distribution of *Arf6* were detected with the *Arf6 in situ* hybridization probe as previously described (Stepicheva et al., 2015; Stepicheva and Song, 2015). *Arf6* coding sequence (Fig. S1B) was cloned into a PCRII vector (Thermo Fisher Scientific, Waltham, MA) and used as an *in situ* probe. The primers used to generate the *Arf6 in situ* construct are the following: *Arf6_insitu_F*:

5' CATGGATCCATGGGGAAGGTACTATCAAA 3', *Arf6_insitu_R*:

5' ACAGTCTCGAGTCAGGGTTTATTATTAGATGTTA 3'. The construct was linearized with BamHI (Promega Corporation, Madison, WI) and labeled with DIG RNA labeling kit (T7 RNA polymerase) (Roche Life Science, Indianapolis, IN, USA). The negative control was transcribed off plasmid pSPT-18-Neo.

Real time, quantitative PCR (qPCR)

For the *Arf6* expression time course, 500 uninjected eggs or embryos were collected at the egg, 32-cell stage, early blastula, mesenchyme blastula, gastrula, and larval stages of development. Total RNA was extracted with the Qiagen microRNeasy kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's instructions and reverse transcribed with TaqMan Reverse Transcription Reagents kit (Thermo Fisher Scientific, Waltham, MA). The estimated numbers of *Arf6* transcripts are calculated based on the level of ubiquitin in various

developmental stages as previously described (Lowe et al., 2006; Materna and Davidson, 2012; Song and Wessel, 2012).

To test the effect of Arf6 perturbations on genes important for biomineralization, fusion, PMC positioning, and endodermal specification, 100 embryos injected with control or Arf6 MASO were collected at 24 and 30 hpf. Total RNA was extracted using the NucleoSpin RNA XS kit (Macherey-Nagel, Bethlehem, PA) and reverse transcribed with iScript (BioRad, Hercules, CA). qPCR primers are listed in Table S1. The relative expression of transcripts in injected samples were analyzed using the $Ct^{-2\Delta\Delta}$ method.

Arf6 activity assay

Arf6 activity was detected from approximately 50,000 eggs or embryos collected from three different animals at the eggs, early blastula (15 hpf), mesenchyme blastula (24 hpf), early gastrula (30 hpf) and gastrula (48 hpf) stages of development, by using the absorbance-based G-LISA Arf6 activation assay biochem kit (Cytoskeleton Inc. Denver, CO, USA) according to the manufacturer's instructions. This method is based on preferential recognition of the active GTP bound form of Arf6 by the Arf family effector proteins. During the assay, the active Arf6-GTP binds to the Arf6 effector proteins and detected with an Arf6-specific antibody, followed by the incubation with the secondary antibody bound to HRP. The amount of the active Arf6-GTP is then obtained by measuring absorbance at 490 nm after adding the HRP detection reagent for 10 minutes.

The volume of the lysis buffer used to lyse each sample was 150 μ L. The protein concentration (in triplicates) was determined using the Precision Red protein assay at absorbance 600 nm using the Promega Glomax-Multi detection system (Promega, Madison, WI). All the

samples were adjusted to the total protein concentration 0.7 mg/mL. The Arf6 activity of each sample (1 technical replicate) was determined for at 490 nm using BioTek Synergy H1 microplate reader (BioTek, Winooski, VT).

Immunolabeling

Immunolabeling with PMC-specific 1D5 antibody (McClay et al., 1983) and endoderm-specific Endo1 antibody (Wessel and McClay, 1985) was performed as previously described (Stepicheva et al. 2015). For the double immunostaining with Endo1 and cadherin (Miller and McClay, 1997), embryos were fixed in 100% ice-cold methanol for 1 h at -20°C followed by four washes in 1X Phosphate Buffered Saline (BioRad, Hercules, CA) containing 0.05% Tween (PBST). Embryos were then blocked in 4% sheep serum (Sigma, St. Louis, MO) in PBST for 1 h at room temperature and then immunolabeled with Endo1 (1:200) and cadherin antibody (1:10) overnight at 4°C in a 72-well mini tray (Thermo Fisher Scientific, Waltham, MA) in a humid chamber. Embryos were washed three times with 1X PBST and incubated with goat anti-mouse Alexa Fluor 647 conjugated antibody at 1:1000 (Thermo Fisher Scientific, Waltham, MA) in blocking buffer (PBST 4% sheep serum) for 1 h at room temperature. Then the embryos were washed three times with 1X PBST and incubated with goat anti-guinea pig Alexa Fluor 488 conjugated antibody at 1:300 (Thermo Fisher Scientific, Waltham, MA) in blocking buffer (PBST 4% sheep serum) for 1 h at room temperature. Embryos were washed four times with PBST and then counterstained with Hoechst dye (Lonza, Walkersville, MD, USA) (1:1000) for 5 min to label DNA, followed by a final three washes with 1X PBST. Immunolabeled embryos were imaged on LSM 780 confocal microscope, or Zeiss Axio Observer Z.1 epifluorescent microscope (Carl Zeiss Inc., Thornwood, NY).

Alkaline phosphatase treatment

Larvae (72 hpf) embryos were fixed in MOPS-paraformaldehyde based fixative (4% paraformaldehyde, 100 mM MOPS pH 7, 2 mM MgSO₄, 1 mM EGTA, and 0.8 M NaCl) for 10 min at room temperature as previously described (Stepicheva et al., 2015). Embryos were then washed with alkaline phosphatase buffer 3 times (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween-20), followed by staining until the desired color was developed with the staining solution (0.1 M Tris pH 9.5, 50 mM MgCl₂, 0.1 M NaCl, 1 mM Levamisole, 10% Dimethylformamide, 45 µl of 75 mg/mL NBT and 35 µl of 50 mg/mL BCIP per 10 ml of solution). Staining was stopped with multiple washes of MOPS buffer (0.1 M MOPS pH 7, 0.5M NaCl, and 0.1% Tween-20). Images were acquired using Nikon D90 digital camera connected to a Zeiss Axio Observer Z.1 microscope.

Phenotyping and statistical analysis

For the measurements of the length of DVC rod and the blastopore width, the Z-stacks (20-40 slices of 1 µm distance apart) of DIC images of gastrulae were acquired with Zeiss Axio Observer Z.1 epifluorescent microscope (Carl Zeiss Inc., Thornwood, NY). The measurements were performed from 2-4 biological replicates using Axiovision 4.8.2 software (Carl Zeiss Inc., Thornwood, NY). The data were analyzed with Student T-test. The average spicule length or blastopore width and s.e.m. were plotted.

PMC positioning defects were assayed based on the 1D5 immunostaining. The gastrulae were considered to be normal if PMCs formed subequatorial ring, connected by syncytial cables,

and migrated anteriorly, with no more than two PMCs found at ectopic locations. The gastrulation defects were assayed based on the Endo1 immunostaining. The embryo was considered to be normal if the gut is fully extended anteriorly with no more than two Endo1-positive cells detached from the archenteron. The Z-stacks of 20-50 slices (1-2 μm distance apart) were imaged in DIC and fluorescent channels using Zeiss Axio Observer Z.1 epifluorescent microscope (Carl Zeiss Inc., Thornwood, NY). For gastrulae and larvae, 2-4 biological replicates were performed and analyzed with the Cochran-Mantel-Haenszel test. The average percentage of the normal embryos and s.e.m. were plotted on the graph. N represents the number of individual embryos.

To measure intensity of cadherin immunostaining in the gut, the Z-stacks of the gut (20-25 slices, 1 μm distance apart) were obtained on LSM 780 confocal microscope. Quantification of cadherin levels were performed using Axiovision 4.8.2 software (Carl Zeiss Inc., Thornwood, NY). Maximum intensity projection of cadherin fluorescence was made from 20 digital slices. Total pixel intensity of the gut and blastocoel (used as background) areas of the embryo were measured as the sum of the densitometric values of the selected regions. Measurement of the gut was normalized to the measurement of the blastocoel (background). The data were analyzed with Student T-test. The average sum of the densitometric values and s.e.m. were plotted.

RESULTS

Temporal and spatial expression of Arf6

We examined the temporal and spatial expression profile of Arf6 in embryos at various developmental stages. The spatial distribution of Arf6 transcripts was ubiquitous throughout early development (Fig. 1A). The level of accumulated Arf6 transcript was highest in the 32 cell

stage (6 hpf) among the developmental stages tested and began to decrease in the mesenchyme blastula stage (24 hpf) (Fig. 1A,B).

To examine the protein localization of the sea urchin Arf6, we constructed an *Arf6* - *mCherry* chimera reporter (Figs. 1C, S2). We observed that the exogenously expressed sea urchin Arf6 had subcellular enrichment in the plasma membrane and presumptive endosomal compartments in all cells (Fig. 1C). In contrast, the control mCherry protein without fusion with Arf6, had a ubiquitous localization.

To examine the dynamics of Arf6 activity throughout early development of the sea urchin, we performed G-LISA Arf6 activation assay (Fig. 1D). We observed that Arf6 activity peaked at early blastula stage of development, at a time of active cellular morphogenesis. However, this increase of Arf6 activity at early blastula stage was not significantly higher compared to the egg stage (Student T-test; $p=0.23$). Consistent with the Arf6 mRNA expression detected by qPCR and *in situ* hybridization, Arf6 activity decreased after blastula stage.

Arf6 KD led to defects in PMC development and function

To test the function of Arf6 in the early embryo, we injected Arf6 MASO into newly fertilized eggs to inhibit newly translated Arf6, leading to a knockdown of the Arf6 protein. 87% of control MASO injected embryos survived at 24 hpf, whereas 66% of the Arf6 MASO injected embryos survived at 24 hpf. We assayed for percentage of healthy or dead embryos over time and found that overall the percentage of healthy embryos in control embryos is much higher than in the Arf6 KD embryos by 72 hpf (69% vs. 12%), indicating that Arf6 KD embryos had lower survival rate (Fig. S4A).

We did not detect any significant defects in the PMC positioning or archenteron invagination in the *Arf6* KD embryos at blastula (24 hpf) or early gastrula (30 hpf) stages of development (Fig. S3); however, by gastrula stage (48 hpf), the morphological defects become apparent. We assessed PMC function by measuring the length of the dorsoventral connecting (DVC) rods at the gastrula stage, PMC positioning with an 1D5 antibody against the membrane protein present on PMCs, and their actin-based filopodial connections (Fig. 2A). We observed that *Arf6* KD gastrulae had a dose-dependent severity of defects in both the length of the DVC rods and the positioning of the PMCs (Fig. 2B,C). The aberrant PMC patterning for both control and *Arf6* MASO at 3mM is likely a result of non-specific morpholino toxicity, since the control embryos have only 40% of embryos having normal PMC patterning (Fig. 2B). At the same 3mM concentration, we observed that control MASO embryos had normal DVC rod length and a significantly shorter DVC rod length with the *Arf6* MASO (Fig. 2C). This discrepancy is likely due to the different batches of embryos assayed for PMC patterning and for spicule measurement. PMCs in *Arf6* KD gastrulae were able to form a subequatorial ring around the gut, but consistently failed to migrate anteriorly (Fig. 2A). Further, KD of *Arf6* has persistent effect on PMC development in that the *Arf6* KD larvae (plutei at 72 hpf) were smaller and developmentally delayed with mispositioned PMCs compared to the control (Fig. 2D,E). Importantly, both the PMC skeleton and positioning defects were significantly rescued by the co-injection of *Arf6* MASO with either the sea urchin (*SpArf6*) or the human (*HsArf6*) *Arf6* mRNA, indicating that *Arf6* KD phenotypes were specifically induced by *Arf6* MASO treatment (Fig. 2F-H). The injected *Arf6* mRNA would be recalcitrant to the *Arf6* MASO, since it lacks the *Arf6* 5'UTR that is complementary to the *Arf6* MASO sequence. The sea urchin *Arf6* shares 91.4% protein identity with the human *Arf6*, so they are highly conserved at the protein sequence level

(Fig. S1C). The fact that the *HsArf6* mRNA can rescue the sea urchin Arf6 KD phenotypes indicated that Arf6 is also functionally conserved between the sea urchin and the human.

To identify the molecular mechanism that underlies the Arf6 KD induced phenotypes, we tested the transcript levels of genes involved in biomineralization, fusion or PMC positioning in Arf6 KD embryos at mesenchyme blastula (24 hpf) and early gastrula (30 hpf) stages. None of the tested genes had more than a 2-fold change at the mRNA level in the Arf6 KD embryos compared to the control (Fig. S6A,B), indicating that Arf6 is likely to regulate PMC development at the post-transcriptional or post-translational levels.

Arf6 is known to play an important role in actin remodeling (Cotton et al., 2007; Humphreys et al., 2013; Kawaguchi et al., 2014; Koo et al., 2007). We observed that the Arf6 KD gastrulae have less filopodia extending from the PMCs that form a subequatorial ring compared to the control (Fig. 2A). To test how actin remodeling is impacted by Arf6 KD, we microinjected the newly fertilized eggs with the *GFP-LifeAct* reporter mRNA (Riedl et al., 2008) along with the control or Arf6 MASO to examine actin distribution in living embryos (Fig. 2I). We observed that Arf6 KD gastrulae had shorter and less abundant actin-based protrusions in cells surrounding the skeleton spicule in comparison to the control gastrulae (Fig. 2I, insets), indicating that Arf6 plays an important role in actin remodeling in the developing embryo. The shortened actin protrusions observed in the Arf6 KD embryos seem to be specific to the PMCs, since we did not observe obvious differences in the structure of actin-based apical microvilli of the ectoderm with GFP-LifeAct (Fig. 2I).

Arf6 activity is important for skeletogenesis and PMC positioning

The sea urchin Arf6 contains conserved residues for activation with GTP binding and inactivation with GTP hydrolysis (Fig. S1B, C). Thus, to test the effect of Arf6 activity on PMC function and development, we microinjected newly fertilized eggs with constitutively active *Arf6-Q67L* mRNA that encodes Arf6 that is unable to hydrolyze GTP (Macia et al., 2004; Palacios et al., 2001; Peters et al., 1995). 92% of *Firefly* mRNA-injected embryos survived at 24 hpf, whereas 67% of the *Arf6-Q67L* mRNA-injected embryos survived at 24 hpf (Fig. S4B). By 72 hpf, 73% and 25% of the *Firefly* mRNA and *Arf6-Q67L* mRNA-injected embryos remained healthy, respectively.

Similar to the Arf6 KD embryos, no significant defects in the PMC positioning or archenteron invagination were observed in *Arf6-Q67L* mRNA-microinjected embryos at blastula (24 hpf) or early gastrula (30 hpf) stages of development (Fig. S5). By gastrula stage (48 hpf), embryos injected with the constitutively active *Arf6-Q67L* mRNA had significantly shorter DVC rod and mispositioned PMCs compared to the control (Fig. 3A-D). The PMCs of *Arf6-Q67L* mRNA-injected embryos were connected by thick, extensive filopodial cables (Fig. 3D), consistent with its role in actin remodeling observed in the case of Arf6 KD (Fig. 2I). By larval stage (72 hpf), the embryos injected with the constitutively active *Arf6-Q67L* mRNA were developmentally delayed and displayed abnormal PMC positioning and skeletal development (Fig. 3E,F). PMC defects induced by the microinjection of *Arf6-Q67L* mRNA were not due to the nonspecific overexpression of Arf6 protein, but to the specific blockade of Arf6 inactivation. This is supported by the lack of defects in *SpArf6* mRNA-injected embryos compared to the *Firefly* mRNA-injected embryos (Fig. S7). Thus, the active form of Arf6 impacts skeleton spicule formation, PMC positioning, and actin-based filopodia formation.

Arf6 perturbation results in endodermal defects

In contrast to the PMCs that undergo EMT and migrate as individual cells, endodermal cells migrate as a coherent sheet of cells, enabling us to study the regulatory role of Arf6 in a different cell type. At gastrula stage, Arf6 KD embryos exhibited a range of defects in gastrulation, including morphological disorganization of the gut epithelium, lack of gut formation, and exogastrulation (protruding gut) (Fig. 4A,B). By larval stage, Arf6 KD embryos displayed phenotypes such as delayed development, defective gut that clearly lacked distinct foregut, midgut, and hindgut structures, and disconnected endodermal cells (Fig. 4C,D). The defective gut phenotype in Arf6 KD embryos was significantly rescued with co-injection with *SpArf6* or *HsArf6* mRNA, indicating that the gastrulation defect is specifically induced by the Arf6 MASO and that Arf6 is functionally conserved between the sea urchin and the human (Fig. 4A,B).

To test if endodermal specification is affected by Arf6 KD, we measured transcript level changes in transcription factors and signaling molecules known to be critical for endodermal specification (Fig. S6C). We performed qPCR at mesenchyme blastula (24 hpf) and early gastrula (30 hpf) stages. All the genes tested were not altered more than 2-fold in the Arf6 KD embryos compared to the control. Thus, Arf6 is likely to regulate gut development at the post-transcriptional or post-translational levels.

In addition, we observed that Arf6 KD gastrulae had significantly wider blastopore compared to the control (Fig. 5A,B). Similarly, embryos with exogenous constitutively active *Arf6-Q67L* mRNA also displayed a similar phenotype, suggesting that both the levels and activity of Arf6 are important for blastopore formation and gut elongation process (Fig. 5A,B). In addition to the widening of blastopore, microinjection of constitutively active *Arf6-Q67L*

mRNA into zygotes resulted in detached Endo1-positive cells from the gut epithelium (Fig. 5C). The detached endodermal cells had aberrant filopodial projections, in comparison to cells that are part of the gut epithelium with no filopodial projections (Fig. 5D). The defects of the gut persisted to the larval stage, where *Arf6-Q67L* mRNA-injected embryos were developmentally delayed and exhibited disorganized and defective gut morphology (Fig. 5E,F).

We also examined *Arf6* perturbed embryos with tissue-specific endodermal differentiation marker alkaline phosphatase, which in sea urchin larvae is expressed only in the gut epithelium (Drawbridge, 2003; Kumano and Nishida, 1998; Nishida and Kumano, 1997; Whittaker, 1990). Although the *Arf6* KD larvae stained for alkaline phosphatase (Fig. 5G), these embryos were significantly delayed in development and had aberrant larval gut structures. The gastrulae injected with *Arf6-Q67L* mRNA had much less alkaline phosphatase staining, indicating that their gut potentially did not differentiate properly. Thus, *Arf6* regulates various aspects of gut development.

Arf6 may regulate junctional cadherin

Arf6 KD and *Arf6-Q67L* mRNA injected embryos had disorganized endodermal epithelial cells that sometimes detached from the main gut epithelium, suggesting potential defects in cell-to-cell adhesion. To test this possibility, we examined cadherin in *Arf6* perturbed embryos (Fig. 6). In wild type embryos, cadherin in the gut epithelium localizes to the apical and lateral membranes (Miller and McClay, 1997). We observed that *Arf6* perturbations resulted in significant decrease (2.5 fold for *Arf6* KD and 1.3 fold for *Arf6-Q67L*) in the levels of cadherin staining in the gut compared to the control (Fig. 6B). Further, endodermal cells that were detached from the basal lamina of the gut in the *Arf6-Q67L* injected embryos lacked polarized

stain of cadherin and had much lower level of cadherin staining at their cell membrane compared to the endodermal cells of the gut epithelium in either control or *Arf6-Q67L* injected embryos (Fig. 6C). Interestingly, Arf6 perturbations do not seem to affect cadherin expression that would affect cell-to-cell adhesion of ectodermal cells (Fig. S8). These results suggest that Arf6 may directly or indirectly mediate cadherin levels at the plasma membrane of endodermal cells that potentially impacts gastrulation and the morphology of the embryonic gut.

DISCUSSION

Arf6 is a key small GTPase that regulates endocytosis, cortical actin rearrangements, cytokinesis, and recycling of endosomal membrane proteins (Donaldson, 2003). Even though the cellular functions of Arf6 are well understood, the physiological role and regulatory mechanism of Arf6 in development has not been examined extensively in the context of a developing embryo. To examine the physiological function of Arf6, we took advantage of the different morphogenic behavior of the two cell types in the sea urchin embryos: PMCs that undergo EMT as they ingress into the blastocoel as individual cells to form the skeleton and endodermal cells that invaginate as a sheet of cells to form the embryonic gut. Results from this study indicate that Arf6 may mediate proper skeleton formation via remodeling actin and maintain the gut epithelium through regulating junctional cadherin .

In the sea urchin embryo, both Arf6 KD and overexpression of constitutively active *Arf6-Q67L* resulted in the failure of PMCs to migrate anteriorly and shortened DVC skeletal rods (Figs 2, 3). This may be due to defects in the ability of PMCs to receive chemotactic cues, to form a syncytium, or to migrate in general. To address these questions, we first assayed for the transcript level changes of *VegfR10*, *Vegf3* and *Prestin* that had previously been shown to be

important for directed migration of PMCs (Adomako-Ankomah and Etensohn, 2013; Duloquin et al., 2007; Lapraz et al., 2006; Piacentino et al., 2016; Sodergren et al., 2006), as well as genes *Kirrell* and *Hypp1164* that may regulate PMC fusion (Rafiq et al., 2014) (Fig. S6A). The transcripts of these genes in Arf6 KD embryos were not altered greater than 2-fold compared to the control, suggesting that depletion of Arf6 did not impact transcript levels of these genes. Arf6 may regulate PMC development via its recycling of target proteins, such as the receptors on PMCs that would be important for receiving migratory cues. However, we do not have direct evidence for this possibility.

In addition to the PMC positioning defects, we observed that Arf6 perturbed embryos had defects in actin remodeling. Arf6 KD gastrulae had shorter and less abundant actin protrusions compared to the control (Fig. 2I), indicating that the level of Arf6 protein is important for the proper actin remodeling. The shortened actin-based extensions may directly contribute to the PMC positioning defects. During PMC migration, sensory filopodia make contact with the ectoderm to provide directional cues for PMC migration (Lyons et al., 2012; Malinda et al., 1995; Miller et al., 1995). Thus, lack of proper sensory filopodia may impact defective PMC migration in the Arf6 KD embryos.

In contrast to the shortened filopodia in Arf6 KD PMCs, we observed the formation of aberrantly thick and long actin-based filopodial extensions in PMCs of the embryos microinjected with constitutively active *Arf6-Q67L* mRNA (Fig. 3D). This result indicates that exogenously overexpressed *Arf6-Q67L* may remodel and recruit more actin to promote the formation of these actin-based filopodia, possibly through interactions with a number of effectors that promote Rac1 activity, thus resulting in actin remodeling at the plasma membrane (Cotton et al., 2007; Humphreys et al., 2013; Kawaguchi et al., 2014; Koo et al., 2007). Changes in the

filopodial structures upon perturbation of Arf6 activity are reminiscent of Arf6-Q67L phenotypes in the rat hippocampal neurons, where Arf6 regulated the branching of dendrites and axons (Choi et al., 2006; Hernandez-Deviez et al., 2004). The rat hippocampal neurons that were transfected with the *Arf6-Q67L* promoted the formation of thick dendritic spines (Choi et al., 2006; Hernandez-Deviez et al., 2004). Thus, Arf6 seems to play an evolutionarily conserved role in mediating actin-based structures. Further, these results indicate that it is likely the overall balance of Arf6-GDP and Arf6-ATP forms in the embryo that mediate proper actin-based filopodial structures to enable proper PMC anterior migration.

In addition to PMC positioning defects and formation of abnormally thick filopodial structures, Arf6-perturbed gastrulae exhibited a significant reduction in the length of DVC skeletal rods. The transcripts of biomineralization genes in Arf6 KD embryos were not altered greater than 2-fold compared to the control embryos, indicating that Arf6 does not regulate these genes at the transcriptional level (Fig. S6A). However, Arf6 may contribute to biomineralization defects by mediating endocytosis of calcium carbonate granules that are necessary to form the single calcite crystal that make up the skeleton (Beniash et al., 1999; Vidavsky et al., 2014). Since we observed Arf6-mCherry localized in PMCs and along the skeleton spicules (Fig. 1C) and GFP-LifeAct localized to the skeleton spicules (Fig. 2I), Arf6 may also be involved in assembling the actin-containing PMC filopodial cables. Because the PMCs use the syncytium as a mold to deposit calcium carbonate to synthesize the skeleton (Wilt, 2005), defects in Arf6-mediated formation of syncytium may indirectly result in the reduction of DVC length and defective larval skeletal structures.

In addition to the skeletogenic defects, Arf6 perturbation also resulted in gastrulation and gut defects (Figs. 4,5). One of the observed defects was widening of the blastopore (Fig. 5A,B).

One possible explanation for the widened blastopore phenotype is the role of Arf6 in actin rearrangements in the cells that form the lip of the blastopore. In the sea urchin species *L. pictus*, blastopore formation is mediated by cells that are constricted apically (Hardin, 1989; Kimberly and Hardin, 1998). It is proposed that the morphological changes of these cells drive the forces required for invagination (Nakajima and Burke, 1996). Thus, defects in actin remodeling in the Arf6 perturbed embryos may hinder the morphogenic movement of cells of the blastopore during gastrulation.

We also observed that embryos with overexpressed *Arf6-Q67L* had endodermal cells detached from the elongated archenteron (Fig. 5). Interestingly, similar to the PMCs, the detached endodermal cells in the *Arf6-Q67L* injected gastrulae exhibited unusual thick extensive filopodia (Figs. 3D and 5B) which may serve as an indication of the abnormal actin remodeling in the *Arf6-Q67L* injected embryos. Reorganization of actin cytoskeleton has been a well-known mediator of EMT (Shankar and Nabi, 2015; Wu and McClay, 2007), a process which directly correlates with the detachment of the endodermal cells. However, it is not clear if the detached Endo-positive cells acquired migratory ability due to these aberrant filopodia or that these filopodial extensions resulted from their ability to move.

To explore if cadherin was regulated by Arf6, we examined the localization and levels of cadherin in Arf6 perturbed embryos (Fig. 6). Endodermal cells have intact junctional cadherin that does not seem to alter during gastrulation (Miller and McClay, 1997). Thus, Arf6 KD embryos with decreased cadherin staining may indicate that these cells did not adhere to each other properly, leading to severe gastrulation defects with higher dosage of Arf6 MASO (Fig. 4A and 6A).

Overexpression of the constitutively active *Arf6-Q67L* in embryos, in turn, had a less severe impact on the levels of the cadherin on the cell surface of the endodermal cells of the gut epithelium (2.5 fold decrease in the *Arf6* KD embryos vs. 1.3 fold decrease in the *Arf6-Q67L* injected embryos, compared to the control) (Fig. 6B). However, detached Endo1-positive cells from these embryos had relatively little membrane cadherin (Fig. 6C), indicating that *Arf6* is likely to mediate cadherin dynamics at the cell surface. These results are consistent with previous studies that demonstrated *Arf6* to mediate the recycling of E-cadherin of mammalian epithelial cells (Palacios et al., 2001; Paterson et al., 2003). Together with the *Arf6*-mediated changes in actin remodeling in the *Arf6* perturbed embryos, internalization of cadherin may contribute to the EMT of endodermal cells and their migration to ectopic locations.

These phenotypes caused by *Arf6* perturbation that were observed in our study are reminiscent to those caused by *Par6* knockdown in the sea urchin embryos, including impaired skeletogenesis and gut differentiation (Shiomi et al., 2012). In sea urchin embryos, *Par6*-aPKC complex mediates cell polarity in the early embryo which impact later developmental stages (Alford et al., 2009; Moorhouse et al., 2015). *Arf6*-dependent vesicular trafficking has been shown to control Cdc42-mediated polarity signaling pathway via recruitment of *Par6* and aPKC to the leading edge of the migrating astrocytes (Osmani et al., 2010). Thus, the membrane trafficking function of *Arf6* may regulate Cdc42 to recruit *Par6* and aPKC complexes to mediate PMC migration and skeletogenesis.

Taken together, this research has demonstrated that *Arf6* is essential for early development and plays a critical role in regulating cellular morphogenesis of PMCs and endodermal cells (Fig. 7). The results from this study revealed important regulatory mechanism of *Arf6* in remodeling actin in PMCs and their filopodial connections, and *Arf6* in regulating

cadherin to impact gut morphology and potentially its function. For the future studies, it would be important to identify cargos of Arf6 that contributes to the directed migration of PMCs.

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FIGURE LEGENDS

Fig. 1 Temporal and spatial expression of Arf6. (A) RNA *in situ* hybridization of Arf6 indicated ubiquitous expression. Scale bar is 50 μ m. (B) The transcript level of Arf6 peaked at 32 cell stage and decreased later in development as indicated by qPCR. The number of Arf6 transcripts was calculated based on the number of internal control ubiquitin mRNAs in 3 biological replicates. (C) The *SpArf6-mCherry* or cytoplasmic *mCherry* control reporters were *in vitro* transcribed and microinjected into the newly fertilized eggs. Gastrulae (48 hpf) were imaged live using confocal microscopy. Exogenously expressed sea urchin Arf6 protein was enriched at the plasma membrane, in punctate structures that are likely to be endosomes, and in the skeleton spicules. Scale bar is 10 μ m. (D) The level of active form of Arf6 was measured in various developmental stages. All Arf6 activity was normalized to the Arf6 activity in the egg stage. Arf6 activity is highest in the early blastula stage.

Fig. 2 Arf6 knockdown resulted in skeletogenesis and PMC positioning defects. (A) Control or Arf6 MASO was microinjected into the newly fertilized eggs. Gastrulae were immunolabeled using 1D5 antibody against PMCs. The gut is outlined in the DIC image. The maximum intensity projection of 4 digital slices is shown for the PMC immunostaining. Scale bar is 50 μ m. (B) The percentage of embryos with normal PMC positioning in Arf6 MASO injected embryos is significantly lower than in control MASO injected embryos in a dose-dependent manner (Cochran-Mantel-Haenszel test). (C) The lengths of DVC rod were significantly decreased in the Arf6 KD embryos compared to the control in a dose-dependent manner (Student's T-test). (D) Skeletogenic defects caused by Arf6 KD (1-1.5 mM MASO) persisted to the larval stage (72 hpf). The maximum intensity projection of 10 digital slices is shown. Scale bar is 50 μ m. (E) The percentage of larvae at 72 hpf with normal PMC positioning was significantly reduced upon Arf6

KD (1 mM MASO) (Cochran-Mantel-Haenszel test). The delayed category is defined as embryos that were developmentally delayed with normal PMC positioning. (F) 1 mM Arf6 MASO was microinjected into the newly fertilized eggs alone or co-injected with either sea urchin Arf6 (*SpArf6*) or human Arf6 (*HsArf6*) mRNA (0.4 $\mu\text{g}/\mu\text{L}$), which significantly rescued the Arf6 KD phenotype. Gastrulae were immunolabeled using 1D5 antibody against PMCs. Scale bar is 50 μm . (G) PMC positioning defects and (H) shortened DVC rods induced by Arf6 KD (1 mM MASO) were rescued with either co-injection with *SpArf6* or *HsArf6* mRNA. (I) The *GFP-LifeAct* was co-injected with either Arf6 MASO or control MASO (1 mM MASO). Dashed areas indicate the enlarged area of the embryo. Arf6 KD embryos had less abundant and shorter filopodia extending from the cells surrounding the skeleton compared to the control. The maximum intensity projection of 5 digital slices is shown, scale bar is 50 μm for the whole embryos and 10 μm for the inset. *** $p < 0.0001$; * $0.01 < p < 0.05$. NS indicates not significant ($p > 0.05$).

Fig. 3 Embryos injected with constitutively active *Arf6-Q67L* mRNA resulted in PMC positioning and actin remodeling defects. (A, B) The skeleton spicule length was significantly shorter in the *Arf6-Q67L* mRNA-injected (0.4 $\mu\text{g}/\mu\text{L}$) embryos compared to the control (Student's T-test). Arrows indicate the length of DVC rod measured. Scale bar is 50 μm (C) The percentage of the embryos with the normal PMC positioning was significantly reduced in the *Arf6-Q67L* injected embryos compared to the control (Cochran-Mantel-Haenszel test). (D) Embryos injected with control or *Arf6-Q67L* mRNA were immunolabeled using PMC-specific antibody 1D5 (red) and counterstained with Hoechst dye for DNA (blue). The *Arf6-Q67L* mRNA injected embryo contains a normal gut; this is not seen because the gut is in a different plane as the PMCs. In addition to PMC positioning defects, exogenous *Arf6-Q67L* mRNA in embryos

resulted in thick and extensive filopodial structures. Dashed areas indicate the enlarged area of the embryo. The maximum intensity projections of 10 digital slices and 5 digital slices are shown for the whole embryos and insets, respectively. Scale bars are 50 μm for the whole embryo images and 10 μm for the insets. (E,F) Defects in PMC positioning persisted to the larval stage (72 hpf) (Cochran-Mantel-Haenszel test). The maximum intensity projection of 10 digital slices is shown, scale bar is 50 μm . The delayed category indicated that embryos had developmental delay with normal PMC positioning.

Fig. 4 Arf6 KD embryos have gastrulation and endodermal defects. (A) Gastrulae (48 hpf) were collected and immunolabeled with Endo1 antibody against the midgut and hindgut. Arf6 KD (1 mM MASO) resulted in a range of defects, including mild phenotype of disorganized endodermal cells (shown by arrow) that line the gut or severe phenotype of exogastrulated (protruding gut) embryos. Embryos co-injected with 1 mM Arf6 MASO with 0.6 $\mu\text{g}/\mu\text{L}$ of either *SpArf6* or *HsArf6* had normal gut, indicating that the KD phenotype is specific to the Arf6 KD. Dashed regions indicate the enlarged area of the gut. The maximum intensity projection of 10 digital slices is shown for the enlarged image of the exogastrulated embryo to visualize all the disorganized endodermal cells. Scale bar is 50 μm for the whole embryos and 10 μm for the enlarged gut. (B) *SpArf6* or *HsArf6* significantly rescued the Arf6 KD phenotype. *** $p < 0.0001$. NS indicates not significant ($p > 0.05$). (C,D) Arf6 KD larvae exhibited lack of the proper gut structures including detached endodermal cells (red arrows) and narrow hindgut (hindgut is indicated by yellow arrows). Scale bar is 50 μm .

Fig. 5 Embryos overexpressing constitutively active Arf6-Q67L have endodermal defects.

(A,B) The blastopore opening (shown by arrows) is significantly larger in gastrulae injected with the 1-1.5 mM Arf6 MASO or 0.4 $\mu\text{g}/\mu\text{L}$ *Arf6-Q67L* mRNA in comparison to the control

(Student's T-test). Scale bar is 50 μm . (C) Injection of *Arf6-Q67L* mRNA into the newly fertilized eggs resulted in endodermal cells detached from the gut. Scale bar is 50 μm . (D) The endodermal cells detached from the gut epithelium in the *Arf6-Q67L* mRNA injected gastrulae had multiple filopodial projections (shown by the arrows). The endodermal cells that are part of the gut do not have such filopodial projections. The maximum intensity projection of 5 digital slices is shown. Scale bar is 10 μm . (E,F) By larval stage, embryos injected with *Arf6-Q67L* mRNA exhibited a clear lack of proper gut structures with multiple detached endodermal cells (arrows) (Cochran-Mantel-Haenszel test). (G) *Arf6* perturbation results in endodermal differentiation defects, as detected with alkaline phosphatase stain. Embryos injected with active *Arf6-Q67L* mRNA have significant developmental delay as well as reduced endogenous alkaline phosphatase. Although the *Arf6* KD embryos stained for alkaline phosphatase, these embryos are significantly delayed in development. f=foregut, m=midgut, and h=hindgut.

Fig. 6 *Arf6* perturbations resulted in defective cadherin levels and localization. (A) Control or *Arf6* MASO were microinjected into newly fertilized eggs. Gastrulae were collected and immunostained against cadherin (green) and Endo1 (magenta) and counterstained with Hoechst dye for DNA (blue). Scale bar is 10 μm . (B) Embryos injected with *Arf6* KD or *Arf6-Q67L* had significant decrease in the cadherin staining at the apical and lateral membranes in the gut epithelium (Student's T-test). (C) Detached endodermal cells in the *Arf6-Q67L* injected embryos lacked membrane cadherin staining (arrows). Dashed areas are enlarged with a single digital slice shown. The maximum intensity projection of 5 digital slices is shown for the whole embryos. Scale bars are 50 μm for the whole embryos and 10 μm for the insets.

Fig. 7 Model of *Arf6* functions in the embryo. (A) The gut of the WT gastrulae consists of a single layer of endodermally-derived epithelial cells that adhere to each other in an organized

pattern (orange). The PMCs (red) migrated anteriorly and produced skeleton rods (red line) within their filopodial cables. (B) Arf6 perturbed embryos result in endodermal and skeletogenesis defects. Arf6 KD gastrula (left side of the embryo) has gastrulation defects, possibly due to the decrease of the junctional cadherin of the endodermal cells. Arf6 KD gastrula also has clumped PMCs that fail to migrate anteriorly, potentially due to decreased Arf6 that remodel actin. Gastrula injected with *Arf6-Q67L* mRNA (right side of the embryo) has detached endodermal cells, potentially due to the increase in actin remodeling and decrease in the levels of membrane cadherin, which may promote EMT of the endodermal cells. PMCs in the gastrula injected with *Arf6-Q67L* mRNA are connected by extensive thick filopodia that may interfere with the proper skeletogenesis, potentially due to the excessive actin remodeling.

Fig. S1 Sequence of the sea urchin Arf6. (A) The Arf6-mCherry reporter contained the Arf6 5'UTR and coding region (highlighted in yellow) fused with mCherry (red font) and β -globin 3' UTR (underlined). (B) The mRNA sequence of *SpArf6*. The position of the amino acid important for the GTP hydrolysis (Q67) is highlighted in light blue. (C) The SpArf6 protein is 91.4% identical to the HsArf6 protein using LALIGN program. Amino acid differences between SpArf6 and HsArf6 are highlighted in yellow. The conserved amino acid important for the GTP hydrolysis (Q67) is highlighted in light blue.

Fig. S2. The SpArf6-mCherry reporter construct is expressed in all cell types. The *SpArf6-mCherry* or cytoplasmic *mCherry* control reporters were *in vitro* transcribed and microinjected into the newly fertilized eggs. Living gastrula stage embryos were imaged using confocal microscopy. Exogenously expressed mCherry fused to the sea urchin Arf6 was enriched at the plasma membrane in all cells. Scale bar is 50 μ m.

Fig. S3. Arf6 KD embryos did not have apparent developmental defects prior to the gastrula stage. 1 mM of control MASO or Arf6 MASO were microinjected into the newly fertilized eggs. The blastula (24 hpf) and early gastrula (30 hpf) stage embryos were collected to assess PMC and archenteron development. (A) Blastulae were fixed and immunostained with PMC-specific marker 1D5. About 20% of the Arf6 KD embryos exhibited mislocalized PMCs; however, this percentage was not significantly different than the control embryos (Cochran-Mantel-Haenszel test, 2 biological replicates). (B) 15% of the Arf6 KD blastulae exhibited delay in development; however, this delay was not statistically significant compared to the control (Cochran-Mantel-Haenszel test, 2 biological replicates). (C) PMC patterning was not disrupted by Arf6 KD assessed at the early gastrula stage (30 hpf) (Cochran-Mantel-Haenszel test, 3 biological replicates). Scale bar is 50 μ m. (D) Archenteron invagination was evaluated by ratios of the gut length and the embryo height. To account for the differences in the development of embryos from different biological replicates, the gut/height ratios in the Arf6 KD embryos were normalized to the ratios obtained for the control embryos. Archenteron formation was not affected by Arf6 KD (Student's T-test, 3 biological replicates). N is the number of embryos evaluated.

Fig. S4 Perturbations in the levels of activity of Arf6 negatively affects the survival and health of the embryos. The newly fertilized eggs were microinjected with either (A) 1 mM control or Arf6 MASO or (B) 0.4 μ g/ μ L *Arf6-Q67L* or *Firefly* control mRNA. The same embryos were tracked throughout development and evaluated for survival (solid lines). The embryos were assessed based on their morphology and ability to swim (dashed lines) at three developmental stages: blastula (24 hpf), gastrula (48 hpf) and pluteus (72 hpf). Both perturbations in Arf6 levels and activity resulted in almost 40% drop in survival after the blastula

stage. About 40-50% of Arf6 perturbed embryos were still alive by pluteus stage, of which approximately half of the survived embryos were healthy.

Fig. S5. Constitutive activation of Arf6 does not lead to developmental defects early in development. 0.4 $\mu\text{g}/\mu\text{L}$ of *Arf6-Q67L* or control *Firefly* mRNA were microinjected into the newly fertilized eggs and the embryos were collected at blastula stage (24 hpf) and early gastrula stage (30 hpf) of development. (A,B) Blastulae were fixed and immunostained with PMC-specific marker 1D5. About 40% of the Arf6 KD embryos exhibited mislocalized PMCs; however, this percentage was not significantly different from the control embryos (Fisher's exact test of independence). (C, D) PMC patterning was also not disrupted by Arf6 KD by early gastrula stage (30 hpf) (Fisher's exact test of independence). Scale bar is 50 μm . (E) Archenteron invagination was evaluated by measuring the gut length and the embryo height and obtaining the ratios between them. Archenteron formation was not affected by Arf6 KD (Student's T-test, 1 biological replicate). N is the number of embryos evaluated.

Fig. S6 Transcript levels of various genes detected by qPCR. 100 embryos injected with either control MASO or Arf6 MASO were collected at various time points. (A) Embryos at early gastrula stage (30 hpf) were assayed for the transcript levels of biomineralization genes (brown) and fusion genes (green). (B) Embryos at mesenchyme blastula (24 hpf) or early gastrula stage (30 hpf) were collected and assayed for their transcript levels of genes that had been shown to be important for PMC positioning and migration. (C) Embryos at mesenchyme blastula and early gastrula stages were collected and assayed for genes expressed in the ectoderm (blue) and endoderm (orange).

Fig. S7 The DVC rod length was not affected by the microinjection of *SpArf6* compared to the *Firefly* control. This result indicates that skeletogenesis defects induced by the microinjection of *Arf6-Q67L* mRNA were not due to the nonspecific overexpression of Arf6 protein, but to the specific blockade of Arf6 activation or inactivation.

Fig. S8 Arf6 perturbations did not result in defective cadherin levels and organization of ectodermal cells. Control vs Arf6 MASO (1 mM) or *Arf6-Q67L* vs Firefly control mRNA (0.4 µg/µL) were microinjected into newly fertilized eggs. Gastrulae were collected and immunostained against cadherin. No significant changes in cadherin localization or the organization of ectodermal cells were detected in Arf6 perturbed embryos compared to the control. The gut failed to invaginate in the Arf6 KD embryo. The maximum intensity projection of 10-20 digital slices is shown. Scale bar is 50 µm.

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Figure 1

Figure 2

Figure 2 cont.

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G.

H.

I.

Figure 3

A.

B.

C.

D.

E.

F.

Figure 4

Figure 5

Figure 6

Figure 7

A.

Endodermal cells
PMCs

B.