

The evolutionary modifications of a GoLoco motif in the AGS protein facilitate micromere formation in the sea urchin embryo

Reviewed Preprint

v1 • August 21, 2024

Not revised

Natsuko Emura, Florence DM Wavreil, Annaliese Fries, Mamiko Yajima 

MCB Department, Brown University, Providence, USA

 https://en.wikipedia.org/wiki/Open_access
 Copyright information

Abstract

The evolutionary introduction of asymmetric cell division (ACD) into the developmental program facilitates the formation of a new cell type, contributing to developmental diversity and, eventually, to species diversification. The micromere of the sea urchin embryo may serve as one of those examples: An ACD at the 16-cell stage forms micromeres unique to echinoids among echinoderms. We previously reported that a polarity factor, Activator of G-protein Signaling (AGS), plays a crucial role in micromere formation. However, AGS and its associated ACD factors are present in all echinoderms and across most metazoans, leaving a question of what evolutionary modification of AGS protein or its surrounding molecular environment contributed to the evolutionary acquisition of micromeres only in echinoids. In this study, we learned that the GoLoco motifs at the AGS C-terminus play critical roles in regulating micromere formation in sea urchin embryos. Further, other echinoderms' AGS or chimeric AGS that contain the C-terminus of AGS orthologs from various organisms showed varied localization and function in micromere formation. In contrast, the sea star or the pencil urchin orthologs of other ACD factors were consistently localized at the vegetal cortex in the sea urchin embryo, suggesting that AGS may be a unique variable factor that facilitates ACD diversity among echinoderms. Consistently, sea urchin AGS appears to facilitate micromere-like cell formation and accelerate the enrichment timing of the germline factor Vasa during early embryogenesis of the pencil urchin, an ancestral type of sea urchin. Based on these observations, we propose that the molecular evolution of a single polarity factor facilitates ACD diversity while preserving the core ACD machinery among echinoderms and beyond during evolution.

Highlights

- Evolutionary modifications of GoLoco motifs are critical for AGS function in micromere formation in the sea urchin embryo.
- The chimeric AGS, which contains the C-terminus of AGS orthologs from various organisms, suggests that human LGN, pencil urchin AGS, and *Drosophila* Pins compensate for the activity of sea urchin AGS.

- Sea urchin AGS regulates the localization of the conserved asymmetric cell division (ACD) machinery members at the vegetal cortex.
- SpAGS is a variable factor facilitating ACD diversity during species diversification.

eLife assessment

This **important** study presents work on the molecular mechanism driving asymmetric cell division and fate decisions during embryonic development of echinoids. The evidence supporting the claims of the authors is **solid** overall but with some concerns about quantification and a lack of explanation for some of the findings. The work will be of interest to developmental biologists and cell biologists working in the field of self-renewal.

<https://doi.org/10.7554/eLife.100086.1.sa2>

Introduction

Asymmetric cell division (ACD) is a developmental process that facilitates cell fate diversification by distributing fate determinants differently between daughter cells. It is an essential process for multicellular organisms since it creates distinct cell types, leading to different tissues in an organism. For example, in *Drosophila*, embryonic neuroblasts divide asymmetrically to produce apical self-renewing neuroblasts and basal ganglion mother cells (Bate, 1978 [↗](#); Doe, 2008 [↗](#); Doe et al., 1988 [↗](#); Hartenstein & Campos-Ortega, 1984 [↗](#)). In *C. elegans*, the zygote divides asymmetrically to form a large anterior and a small posterior blastomere with distinct cell fates (Schnabel et al., 1996 [↗](#); Sulston et al., 1983 [↗](#); Watts et al., 1996 [↗](#)). In mammals, neuroepithelial cells undergo ACD to produce apical self-renewing stem cells as well as basal neural progenitor cells (Chenn & McConnell, 1995 [↗](#); Haydar et al., 2003 [↗](#); Konno et al., 2008 [↗](#); Noctor et al., 2004 [↗](#)). A set of polarity factors conserved across phyla regulates these highly organized ACD processes. However, the timing and location of such controlled ACD often occur randomly, even within the same phylum, providing uniqueness to the developmental program of each species. Therefore, we hypothesize that drastic changes in the ACD machinery are unnecessary. Instead, a slight modification in the ACD machinery may drive the formation of a new cell type and the change in the developmental program, contributing to species diversification in the process of evolution.

In this study, we use echinoderm embryos as a model system to test this hypothesis. Echinoderms are basal deuterostomes and include sea urchins, sea stars, and sea cucumbers, among others. In the well-studied echinoderm models, the sea urchin and sea star embryos, the first ACD or symmetry break occurs at the 8-cell stage, where a horizontal cell division separates animal and vegetal blastomeres that contribute to ectoderm and endomesoderm lineages, respectively (Fig. 1A [↗](#)). However, in the next cell cycle at the 16-cell stage, the sea urchin embryo undergoes an apparent unequal cell division, producing four micromeres at the vegetal pole. In contrast, the sea star embryo undergoes seemingly an equal cell division (Fig. 1B [↗](#)).

The micromere formation in the sea urchin embryo is a highly controlled ACD event since this cell lineage undergoes autonomous cell specification and functions as organizers as soon as it is formed at the 16-cell stage (Horstadius, 1928 [↗](#); Ransick & Davidson, 1993 [↗](#)). For example, micromeres autonomously divide asymmetrically again to produce large and small micromeres that are committed to two specific lineages of skeletogenic cells and the germline, respectively, at

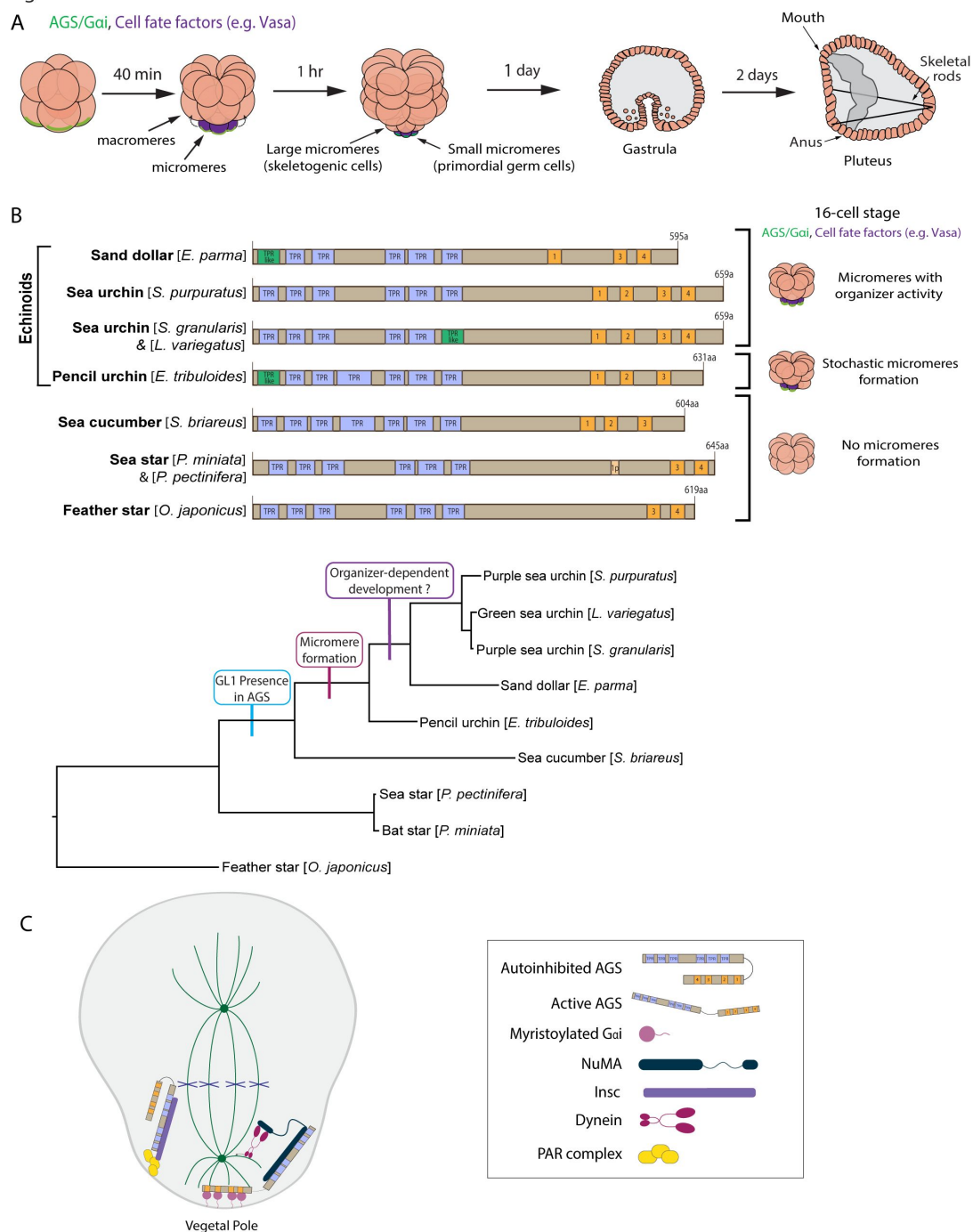


Figure 1.

The evolutionary modification of the SpAGS protein corresponds to the introduction of micromeres and inductive signaling during echinoid diversification.

A, Schema depicting sea urchin embryonic development from 8-cell stage to pluteus. Green represents the colocalization of AGS and Gai at the vegetal cortex, and purple represents the early segregation of fate factors such as Vasa. **B**, Comparative diagrams of predicted motifs of each echinoderm AGS protein, based on NCBI blast search results for AGS sequences. Conserved TPR motifs are indicated in blue, and GL motifs in orange. Green shows TPR-like motifs, which contain several amino acid changes. Lighter colors represent partial GL motifs. See [Fig. S2](#) for each echinoderm AGS sequence. The tree depicts SpAGS evolution among echinoderms with the introduction of the GL1 motif and micromeres. **C**, Working model of AGS mechanism in ACD based on fly and mammalian models.

the 32-cell stage (Okazaki, 1975 [↗](#); Yajima & Wessel, 2011 [↗](#)). This early segregation of the germline is unique to sea urchins among echinoderms (Juliano et al., 2009; Fresques et al., 2016). Further, micromeres induce endomesoderm specification (e.g., gastrulation) even when they are placed in the ectopic region of the embryo, such as the animal cap, suggesting they function as a major signaling center in this embryo (Horstadius, 1928 [↗](#); Ransick & Davidson, 1993 [↗](#)). The removal of sea urchin micromeres results in compromised or delayed endomesoderm development and compensatory upregulation of a germline factor, Vasa, to presumably start over the developmental program (Ransick & Davidson, 1993 [↗](#); Voronina et al., 2008 [↗](#)).

In contrast, other echinoderms undergo minor unequal cell divisions during early embryogenesis, yet they may not be linked to specific cell fate or function. For example, in sea star embryos, the removal of smaller cells does not impact embryonic patterning, and unequal cell divisions appear to be not necessarily linked to specific cell fate regulation or function (Barone et al., 2022). Similarly, even in sea urchin embryos, the non-micromere blastomeres at the 16-cell stage can change the cell fate in response to external cues, including the signaling from micromeres. Recent studies using single-cell RNA-seq analysis further support these observations by demonstrating the earlier molecular segregation of the micromere lineage, while other cell lineages appear to undergo more regulative development (Foster et al., 2019; Massri et al., 2021).

Fossil records and phylogenetic tree analysis suggest that sea urchins diverged relatively later from the common ancestor of echinoderms (Bottjer et al., 2006; Wada and Sato et al., 1994). Since micromeres are unique to echinoids (sea urchins, sand dollars, pencil urchins), they are considered to have emerged later during sea urchin diversification, which has dramatically changed the developmental style in the sea urchin embryo (Emura and Yajima, 2022). To understand how this unique lineage has emerged during evolution, we previously identified the Activator of G-protein signaling (AGS) (Pins in *Drosophila*; LGN in mammals) as a major regulator of micromere formation (Poon et al., 2019 [↗](#)). AGS is a polarity factor and plays a role in the ACD of many organisms (reviewed by di Pietro et al., 2016 [↗](#); Kotak, 2019 [↗](#); Rose & Gonczy, 2014; Siller & Doe, 2009 [↗](#); Wavreil & Yajima, 2020 [↗](#); Yu et al., 2006 [↗](#)). In the sea urchin (*S. purpuratus*; Sp), SpAGS localizes to the vegetal cortex before and during micromere formation, and its knockdown inhibits micromere formation (Poon et al., 2019 [↗](#)). On the other hand, in the sea star (*P. miniata*; Pm), PmAGS shows no cortical localization nor any significant role in ACD during early embryogenesis. Furthermore, the pencil urchin (*E. tribuloides*; Et) is an ancestral type of the sea urchin and diverged around 252 million years ago, which is located between the sea star and the sea urchin. The Et embryo exhibits an intermediate developmental program of the sea urchins and sea stars. It stochastically forms zero to four micromere-like cells (Fig. 1B [↗](#)). In these embryos, EtAGS localizes to the vegetal cortex only when the embryos form micromere-like cells (Poon et al., 2019 [↗](#)), suggesting a close correlation between cortical AGS localization and micromere-like cell formation.

Furthermore, the introduction of sea urchin AGS into sea star embryos induces random unequal cell divisions by recruiting the spindle poles to the cortex (Poon et al., 2019 [↗](#)), suggesting that SpAGS facilitates unequal cell divisions even in other echinoderm species. Phylogenetic analysis of AGS orthologs across taxa suggests that AGS orthologs increased the functional motif numbers over the course of evolution, likely allowing additional molecular interactions and mechanisms to modulate ACD in a more nuanced manner in higher-order organisms (Wavreil and Yajima, 2020 [↗](#)). Supporting this hypothesis, indeed, prior studies suggest that the higher-order mouse AGS ortholog (LGN) can substitute for its fly ortholog (Pins) in *Drosophila* cells, while the basal-order fly Pins cannot substitute its chick ortholog function in chick, the higher-order organism (Yu et al., 2003 [↗](#); Saadaouri et al., 2017). These observations led us to hypothesize that the molecular evolution of AGS orthologs drives ACD diversity across taxa, contributing to the developmental diversity within each phylum. In this study, through a series of molecular dissection experiments, we demonstrate that the AGS C-terminus is a variable region and creates its functional diversity in ACD control,

facilitating the developmental variations among echinoderms. This study provides insight into how the molecular evolution of a single polarity contributes to developmental diversity within each phylum.

Results

The N-terminal TPR domain is vital for restricting SpAGS localization and function at the vegetal cortex

AGS consists of two functional domains: the N-terminus contains tetratricopeptide repeats (TPR), and the C-terminus contains G-protein regulatory motifs (GoLoco, GL) (Bernard et al., 2001 [DOI](#)). AGS switches between a closed and open structure based on the intramolecular interaction between the TPR and GL motifs (Du & Macara, 2004 [DOI](#); Johnston et al., 2009 [DOI](#); Nipper et al., 2007 [DOI](#); Pan et al., 2013 [DOI](#)). The TPR motifs regulate protein-protein interaction with various partners such as Inscuteable (Insc) for its proper cortical localization or Nuclear Mitotic Apparatus (NuMA) for its microtubule-pulling force generation. In contrast, the GL motifs interact with the heterotrimeric G-protein subunit Gai for its anchoring to the cortex (Bowman et al., 2006 [DOI](#); Culurgioni et al., 2011 [DOI](#); Culurgioni et al., 2018 [DOI](#); Du & Macara, 2004 [DOI](#); Parmentier et al., 2000 [DOI](#); Wang et al., 2011 [DOI](#); Yu et al., 2000 [DOI](#)). Studies investigating AGS mechanisms in fly and mammals reveal that Pins/LGN (AGS orthologs) generally remain in the autoinhibited form in the cell (Du & Macara, 2004 [DOI](#); Johnston et al., 2009 [DOI](#); Nipper et al., 2007 [DOI](#)) (**Fig. 1C** [DOI](#)). At the time of ACD, Insc recruits Pins/LGN to the cortex through Gai. This Gai-binding releases Pins/LGN from its autoinhibition and allows it to interact with NuMA, which recruits the motor protein dynein to generate pulling forces on the microtubules and facilitate ACD (Bowman et al., 2006 [DOI](#); Culurgioni et al., 2011 [DOI](#); Izumi et al., 2006 [DOI](#); Parmentier et al., 2000 [DOI](#); Schaefer et al., 2001 [DOI](#); Siller et al., 2006 [DOI](#); Williams et al., 2014 [DOI](#); Yu et al., 2000 [DOI](#); Yuzawa et al., 2011 [DOI](#); Zhu et al., 2011a [DOI](#)).

To test whether sea urchin (*S. purpuratus*; Sp) AGS functions in ACD similarly to its orthologs, we first investigated the role of its N-terminus by constructing a series of GFP-tagged deletion mutants (**Fig. 2A** [DOI](#); **Fig. S1** [DOI](#)). AGS-1F is missing the first three TPR motifs, AGS-2F the first four, and AGS-3F the entire TPR domain of SpAGS Open Reading Frame (ORF). The mRNA for these deletion constructs was co-injected with 2x-mCherry-EMTB, a microtubule marker, to visualize the cell cycle phase, spindle location, and orientation. We counted the number of embryos with vegetal cortical localization and conducted a quantitative analysis by measuring the ratio of cortical and non-cortical signal intensity at the 16~32-cell stage (**Fig. 2B-C** [DOI](#)). Embryos injected with full-length SpAGS (Full AGS) or AGS-1F exhibited vegetal cortex-specific localization. In contrast, AGS-2F and AGS-3F showed uniform cortical localization (**Fig. 2B-C** [DOI](#)). These results suggest that TPR4-6 is necessary for restricting AGS to the vegetal cortex, whereas TPR1-3 appears to play a less critical role in controlling AGS localization.

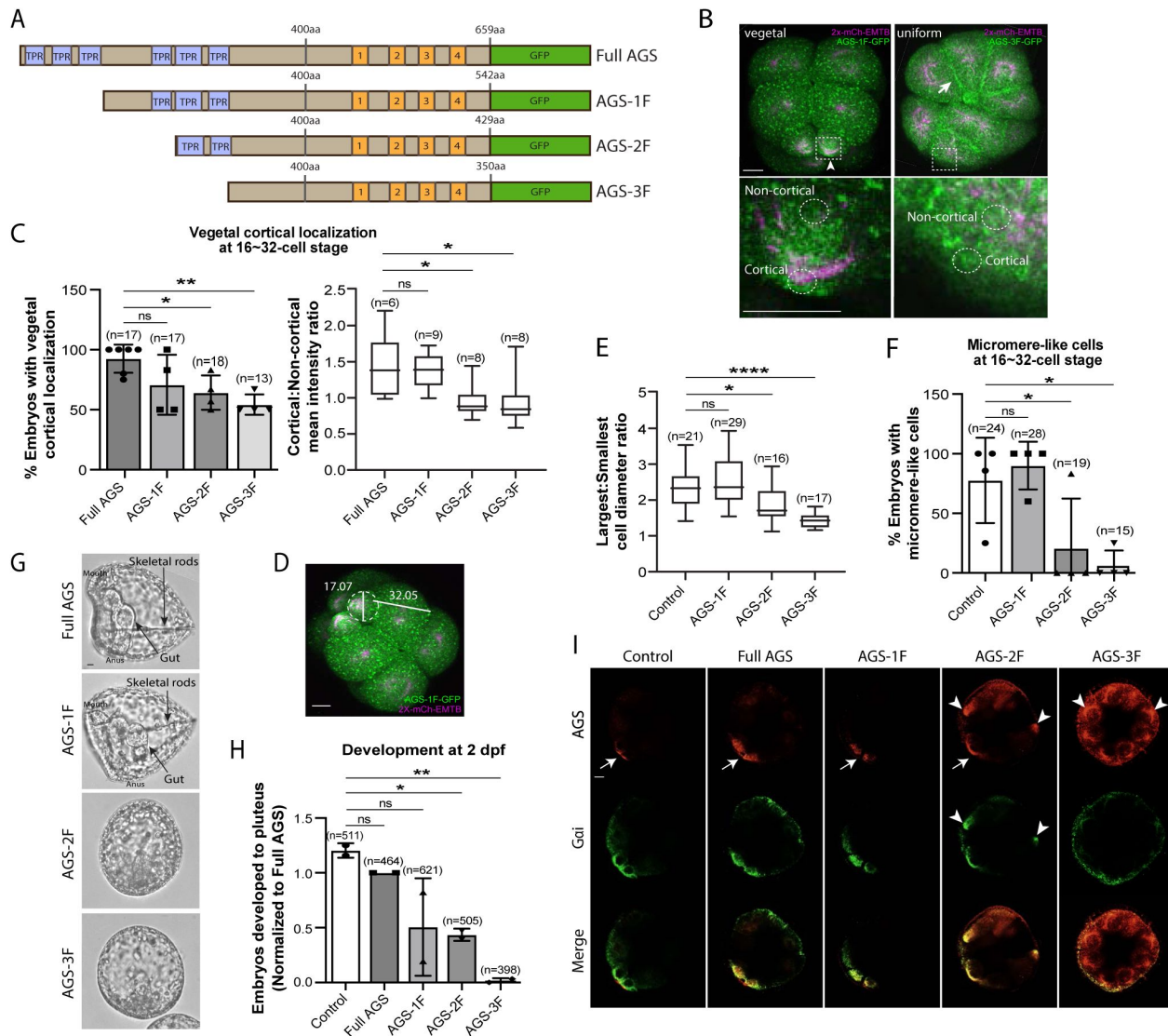


Figure 2.

The N-terminal TPR domain restricts SpAGS localization and function at the vegetal cortex.

A, Design of SpAGS-GFP N-terminal deletion constructs used in this study. TPR motifs are marked in blue, and GL motifs are in orange. **B**, Representative 2D-projection images of the embryo injected with AGS-3F and 2x-mCherry-EMTB, exhibiting vegetal (right panel, arrowhead) and uniform (left panel, arrow) cortical localization. The magnified images below each panel demonstrate how to measure the cortical and non-cortical mean intensities using *ImageJ*, as depicted in the corresponding graph (C). Embryos were injected with 0.15–0.3 µg/µl stock of SpAGS-GFP mRNA and 0.5 µg/µl stock of 2x-mCherry-EMTB mRNA. Z-stack images were taken at 1 µm intervals to cover a layer of the embryo. **C**, % of the embryos with vegetal cortical localization of SpAGS (left) and the ratio of the cortical-to-non-cortical mean intensity (right) at 16–32-cell embryos. Statistical analysis was performed against Full AGS by One-Way ANOVA. **D**, Representative 2D-projection confocal image of a 16-cell stage embryo injected with AGS-1F. The largest cell (macromere) and the smallest cell (micromere) diameters were measured using *ImageJ*. Z-stack images were taken at 1 µm intervals to cover a layer of the embryo. **E**, The diameter ratio of the smallest cell (micromere-like cell) over the largest cell (macromere-like cell) was quantified for the embryos injected with the SpAGS mutants or EMTB-only (control). **F**, % of the embryos forming micromere-like cells was scored for each SpAGS mutant and EMTB-only (control). “Micromere formation” is defined as the formation of a group of four cells that are smaller in size and made through a vertical cell division at the vegetal pole at the 16-cell stage. Since none of the AGS-3F-injected embryos formed normal micromeres, “micromere-like cells” were counted based on their vertical cell division, not relative to their size. Statistical analysis was performed against Control by One-Way ANOVA. **G–H** Brightfield images show the representative phenotypes scored in the corresponding graph (H) at 2 dpf. We categorized embryos into three groups, namely, “full development,” with embryos reaching the pluteus stage with complete gut formation and skeleton; “delayed development,” with some gastrulation but no proper skeleton; and “failed gastrulation.” As many of the abnormal-looking embryos fell into the median of the latter two categories, we scored only the embryos reaching full development in the graph. Control represents embryos injected with dye only. Statistical analysis was performed against Control by One-Way ANOVA. **I**, Single Z-slice confocal imaging was used to focus on the vegetal cortex. Embryos were stained with AGS (orange) and Gai (green) antibodies. White arrows and arrowheads indicate the signals at the vegetal cortex and ectopic cortical signals, respectively. Images represent over 80% of the embryos observed (n=30 or larger) per group. n indicates the total number of embryos scored. * represents p-value < 0.05, ** p-value < 0.01, and **** p-value < 0.0001. Each experiment was performed at least three independent times. Error bars represent standard error. Scale bars=10 µm.

In the control and the AGS-1F groups, micromeres were approximately half the size of the macromeres. In contrast, they were three-quarters the size in the AGS-2F group and almost the same size in the AGS-3F group (**Fig. 2D–E**), resulting in failed micromere formation even in the presence of the endogenous SpAGS (**Fig. 2F**). In these embryos, we also scored embryonic development at two days post fertilization (2 dpf) when gastrulation occurs. The AGS-1F mutant mostly showed normal development with extended skeletal rods, whereas AGS-2F and AGS-3F dramatically compromised development with incomplete skeleton extension or gut formation (**Fig. 2G–H**). Since these N-terminal deletions appear to cause a dominant negative phenotype, we did not knock down endogenous SpAGS in these experiments.

These results suggest that the N-terminal TPR domain is necessary to restrict SpAGS localization at the vegetal cortex. The TPR deletion disables AGS mutants from maintaining the autoinhibited form. It may thus induce their binding to Gai at every cortex and compete out the endogenous SpAGS at the vegetal cortex. Notably, Gai localization was also recruited to the exact ectopic location as AGS-2F and -3F mutants (**Fig. 2I**), suggesting that the SpAGS C-terminus is sufficient to control the Gai localization at the vegetal cortex. Protein sequences of AGS orthologs across

echinoderms are almost identical in their N-termini, suggesting that the AGS N-terminus serves as a core functional domain (**Fig. S2** [↗](#)). In contrast, the AGS C-terminus appears highly variable across echinoderms.

The C-terminal GL1 motif is essential for SpAGS localization and function in ACD

To test whether a variable AGS C-terminus creates functional diversity in ACD, we made a series of GFP-tagged C-terminus deletion SpAGS mutants that are missing different GL motifs (**Fig. 3A** [↗](#)). SpAGS mutants missing GL1 (Δ GL1), GL3 (Δ GL3) or all GL motifs (Δ GL1-4) failed to localize at the vegetal cortex compared to the Full AGS control (**Fig. 3B-D** [↗](#)), suggesting that GL1 and GL3 are essential for cortical localization of AGS. Next, we knocked down endogenous AGS by morpholino antisense oligonucleotides (MO), which was previously validated for the specificity (Poon et al., 2019 [↗](#)). We tested whether these deletion mutants could rescue micromere formation (**Fig. 3E** [↗](#)). The GL1 deletion significantly reduced micromere formation. In contrast, the GL2, GL3, or GL4 deletion showed no or little significant difference in micromere formation compared to the Full AGS control group (**Fig. 3F** [↗](#)). Consequently, the GL1 deletion showed significant disruption in embryonic development at 2 dpf, likely due to a lack of micromeres' inductive signaling at the 16-cell stage (**Fig. 3G-H** [↗](#)).

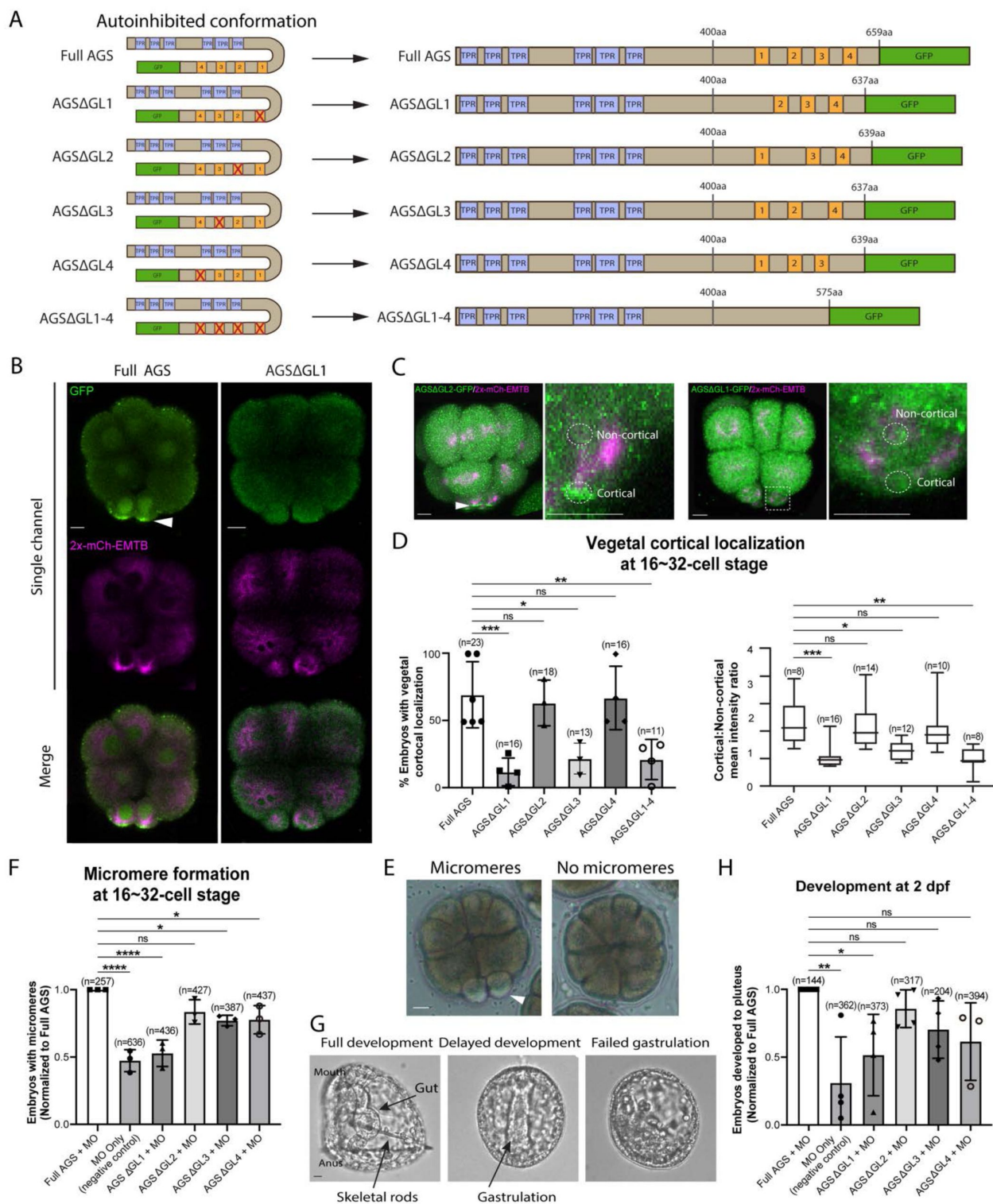


Figure 3.

GL1 is essential for vegetal cortical recruitment of SpAGS at the 8~16-cell stage of the sea urchin embryo.

A, Design of SpAGS-GFP C-terminal deletion mRNAs tested in this study. TPR motifs are marked in blue, and GL motifs are in orange. See [Fig. S2](#) for protein sequence. **B**, Single Z-slice confocal imaging was used to focus on the vegetal cortex. Representative embryos injected with SpAGS-GFP or SpAGSΔGL1-GFP are shown. Embryos were injected with 0.3μg/μl stock of SpAGS-mutant-GFP mRNA (green) and 0.5μg/μl stock of 2x-mCherry-EMTB mRNA (magenta). The white arrowhead indicates vegetal cortical localization of AGS-GFP. **C-D**, Representative 2D-projection images of the embryo injected with SpAGS-GFP mRNAs and 2x-mCherry-EMTB, exhibiting vegetal cortical (left panel, AGSΔGL2, arrowhead) and uniform cytoplasmic (right panel, AGSΔGL1) localization. The magnified images right each panel demonstrate how to measure the cortical and non-cortical mean intensities using *ImageJ*, as depicted in the corresponding graph (D). Z-stack images were taken at 1μm intervals to cover a layer of the embryo. % of the embryos that had the GFP signal at the vegetal cortex (left) and the ratio of the cortical-to-non-cortical mean intensity (right) during the 16~32-cell stage were scored in the graphs. Statistical analysis was performed against Full AGS by One-Way ANOVA. **E-F**, Brightfield images show the representative phenotypes scored in the corresponding graph (F) at the 16-cell stage. White arrowhead shows micromeres. Embryos were injected with 0.15μg/μl stock of SpAGS-GFP mRNAs and 0.75mM SpAGS MO. The number of embryos forming micromeres was scored and normalized to that of Full AGS in the graph. Statistical analysis was performed by One-Way ANOVA. **G-H**, Brightfield images show the representative phenotypes scored in the corresponding graph (H) at 2 dpf. Embryos were injected with 0.15μg/μl stock of SpAGS-GFP mRNAs and 0.75mM SpAGS MO. The number of embryos developing to the pluteus stage was scored and normalized to that of Full AGS in the graph. Statistical analysis was performed by One-Way ANOVA. n indicates the total number of embryos scored. * represents p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001. Each experiment was performed at least three independent times. Error bars represent standard error. Scale bars=10μm.

These results suggest GL1 is critical for both AGS localization and function at the vegetal cortex for micromere formation. GL3 and GL4 are important for intramolecular binding to the TPR domain in other organisms, which may impact the proper open-close control of AGS protein (Du & Macara, 2004; Johnston et al., 2009; Nipper et al., 2007; Pan et al., 2013).

The position of GL1 is important for SpAGS function in ACD

To determine whether the sequence or positioning of GL1 is essential for the SpAGS function, we next made a series of mutants where the GL motifs were interchanged or replaced ([Fig. 4A](#)). For instance, AGS1111 has all GL motifs replaced with the sequence of GL1, whereas AGS4234 has the sequence of GL1 replaced with that of GL4. Most embryos that formed micromeres displayed vegetal cortical localization for all mutants except for AGS1111 and AGS2222, which severely inhibited micromere formation ([Fig. 4B-C](#)). A small portion ($4.14\% \pm 2.65$, n=170) of AGS2222 embryos formed micromeres and always showed vegetal cortical localization, suggesting that AGS localization and micromere formation are closely linked to each other. Additionally, most of all AGS1111 embryos ($99.36\% \pm 0.64$, n=182) and of AGS2222 ($98.06\% \pm 1.94$, n=170) displayed ectopic cortical localization around the entire embryo ([Fig. 4B](#)). We did not observe this phenotype in the Full AGS control nor the other two mutants (AGS2134 and AGS4234).

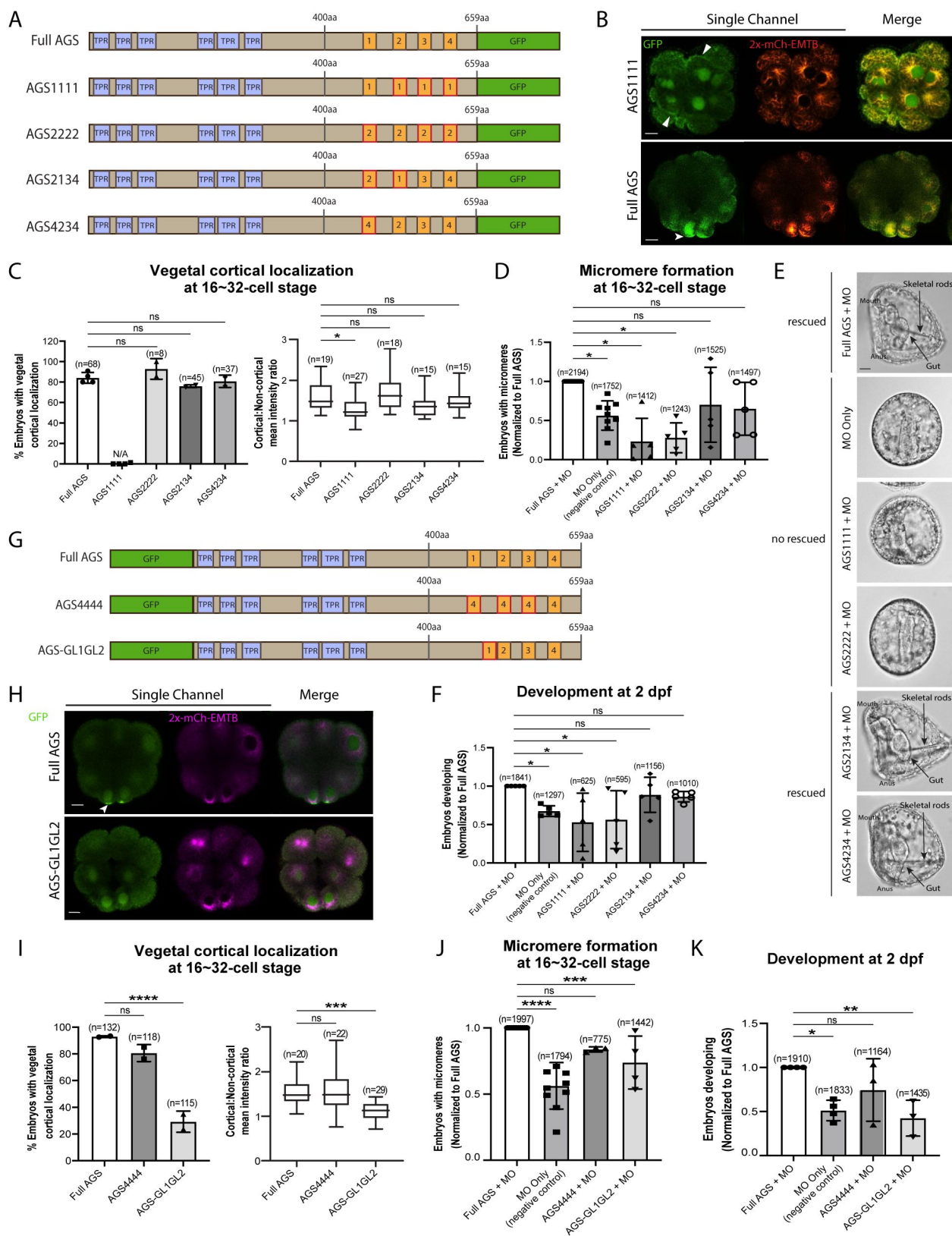


Figure 4.

The position of GL1 and the sequences of GL3 and GL4 are important for SpAGS localization and function.

A, Design of SpAGS-GFP C-terminal mutant constructs tested in this study. TPR motifs are marked in blue, and GL motifs are in orange. Red boxes show interchanged GL motifs. **B**, Single Z-slice confocal images of sea urchin embryos at the 8~16-cell stage showing localization of SpAGS1111-GFP mutant. Embryos were injected with 0.3µg/µl stock of SpAGS-mutant GFP mRNAs and 0.25µg/µl stock of 2x-mCherry-EMTB mRNA. White arrowheads indicate vegetal cortical localization of AGS-GFP proteins and ectopic localization of AGS1111 mutant. **C**, % of the embryos with vegetal cortical localization of SpAGS mutants (left) and the ratio of the cortical-to-non-cortical mean intensity (right) in 16~32-cell embryos. Statistical analysis was performed against Full AGS by One-Way ANOVA. **D-F**, Embryos were injected with 0.15µg/µl stock of SpAGS-GFP mRNAs and 0.75mM SpAGS MO. The number of embryos making micromeres (D) and developing to gastrula or pluteus stage (F) were scored and normalized to that of the Full AGS control group. Brightfield images (E) show the representative phenotypes scored in the corresponding graph (F) at 2 dpf. Of note, AGS1111 and AGS2222 mutants caused substantial toxicity, degrading many embryos by 2 dpf and resulting in inconsistent scoring. Thus, we scored embryos reaching the pluteus stage, which revealed delayed development in this analysis. Statistical analysis was performed by One-Way ANOVA. **G**, Design of GFP-SpAGS C-terminal mutant constructs tested in this study. In AGS4444, we replaced all GL motifs with GL4. In AGS-GL1GL2, GL1 is shifted adjacent to GL2. TPR motifs are marked in blue, and GL motifs are in orange. Red boxes show modified GL motifs. **H**, Single Z-slice confocal images of sea urchin embryos at 8~16-cell stage showing localization of GFP-SpAGS-GL1GL2 mutant. Embryos were injected with 0.3µg/µl stock of SpAGS-GFP mRNA and 0.25µg/µl stock of 2x-mCherry-EMTB mRNA. The white arrowhead indicates the vegetal cortical localization of GFP-AGS. **I**, % of the embryos with vegetal cortical localization of SpAGS mutants (left) and the ratio of the cortical-to-non-cortical mean intensity (right) in 16~32-cell embryos. Statistical analysis was performed against Full AGS by One-Way ANOVA. **J-K**, Embryos were injected with 0.15µg/µl stock of GFP-SpAGS mRNAs and 0.75mM SpAGS MO. The number of embryos making micromeres (J) and developing to gastrula or pluteus stage (K) were scored and normalized to that of the Full AGS. Statistical analysis was performed by One-Way ANOVA. n indicates the total number of embryos scored. * represents p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001. Each experiment was performed at least two independent times. Error bars represent standard error. Scale bars=10µm.

We quantified the function of these AGS mutants in the endogenous AGS-knockdown background. AGS1111 and AGS2222 mutants failed to restore micromere formation at the 16-cell stage, while AGS4234 and AGS2134 mutants rescued micromere formation similarly to Full AGS (**Fig. 4D**). Furthermore, Full AGS, AGS2134, and AGS4234 showed comparable development at 2 dpf. In contrast, the AGS1111 and AGS2222 showed disrupted development (**Fig. 4E-F**). These results suggest that the GL1 sequence is not essential, but its position is vital. In contrast, the sequence of GL3 or GL4 appears to be critical for restricting AGS localization to the vegetal cortex, perhaps by maintaining the autoinhibited form of AGS through their interaction with the TPR domains. AGS1111 and AGS2222 mutants were thus unable to sustain a closed/inactive state, resulting in a constitutively active form all around the cortex.

To test this model further, we made two additional SpAGS mutants, AGS4444 and AGS-GL1GL2 (**Fig. 4G**). AGS4444 localized properly at the vegetal cortex, whereas AGS-GL1GL2 showed significantly fewer embryos with vegetal cortical localization (**Fig. 4H-I**). Furthermore, AGS-GL1GL2 showed impaired function in micromere formation and development compared to Full AGS control (**Fig. 4J-K**). On the other hand, AGS4444 showed no significant difference in the proportion of embryos with micromeres at the 16-cell stage and normal development at 2 dpf compared to the Full AGS control. These results further support the contention that GL3 and GL4 are essential for maintaining the SpAGS in a closed form. Additionally, the position of GL1 is critical for SpAGS localization and function.

The molecular evolution of the AGS C-terminus facilitates the ACD diversity among AGS orthologs

To understand if/how SpAGS functions uniquely compared to other echinoderm AGS orthologs, we cloned sea star (*P. miniata*; Pm) and pencil urchin (*E. tribuloides*; Et) AGS into the GFP-reporter construct (**Fig. 5A** [↗](#)) and introduced them into the sea urchin zygotes. EtAGS showed no significant difference compared to the SpAGS control, while PmAGS failed in vegetal cortical localization and micromere formation and function (**Fig. 5B-E** [↗](#)). Hence, PmAGS is incapable of inducing micromere formation.

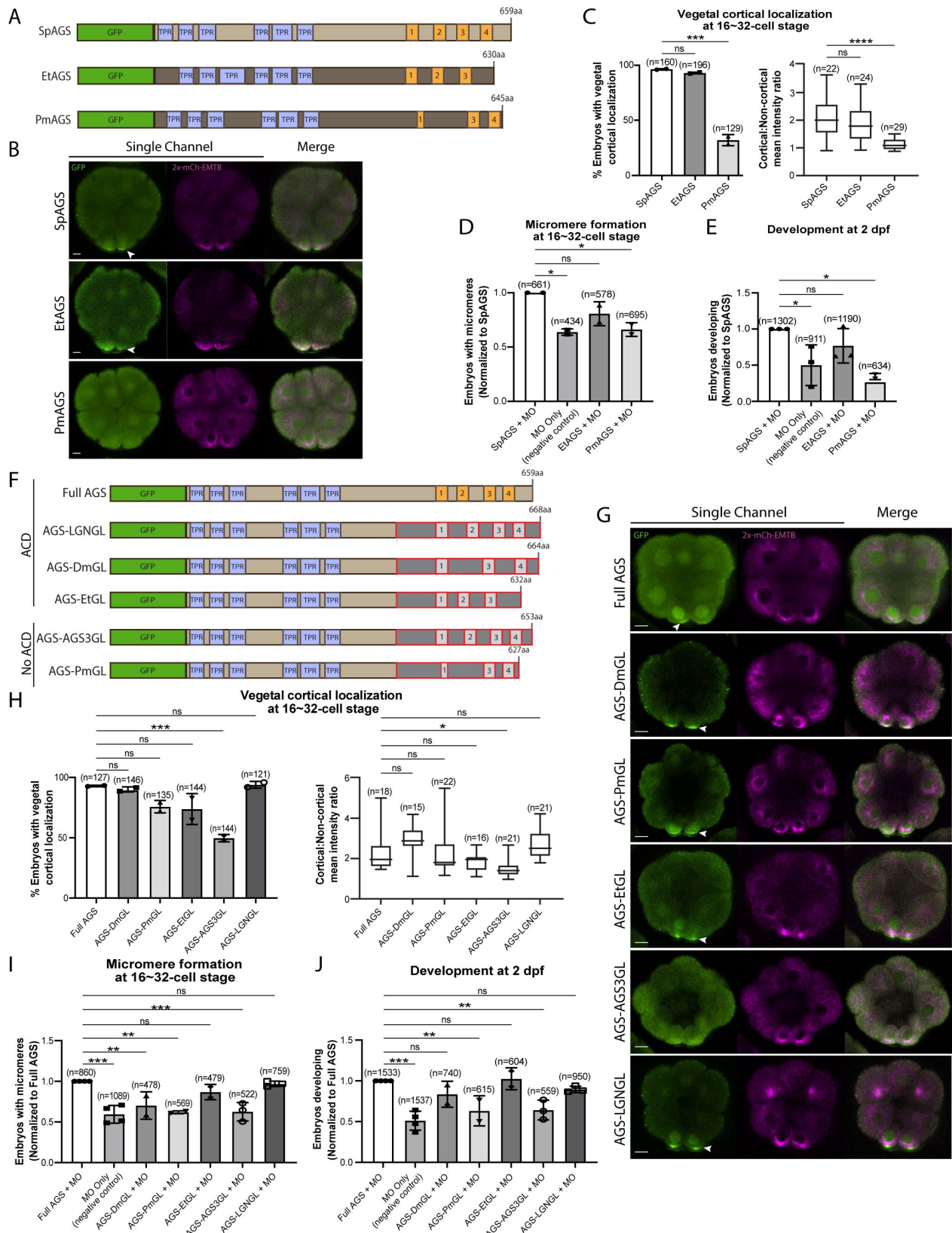


Figure 5.

Molecular evolution of AGS C-terminus facilitates micromere formation

A, Design of GFP-AGS constructs from three different species tested in this study, namely, *S. purpuratus* (Sp), *E. tribuloides* (Et), and *P. miniata* (Pm). TPR motifs are marked in blue, and GL motifs are in orange. **B**, Single Z-slice confocal images of sea urchin embryos at 16-cell stage showing localization of each GFP-AGS. Embryos were injected with 0.3µg/µl stock of GFP-AGS mRNAs and 0.25µg/µl stock of 2x-mCherry-EMTB mRNA. The white arrowhead indicates vegetal cortical localization of GFP-AGS. **C**, % of embryos with vegetal cortical localization of GFP-AGS (left) and the ratio of the cortical-to-non-cortical mean intensity (right) in 16~32-cell embryos. Statistical analysis was performed against SpAGS by One-Way ANOVA. **D-E**, Embryos were injected with 0.15µg/µl stock of GFP-AGS mRNAs and 0.75mM SpAGS MO. The number of embryos making micromeres (D) and developing to pluteus stage (E) were scored and normalized to that of the Full AGS. Statistical analysis was performed by One-Way ANOVA. **F**, Design of GFP-AGS C-terminal chimeric mutant constructs tested in this study. TPR motifs are marked in blue, and GL motifs are in orange. The brown section shows the SpAGS portion, and the red and dark grey boxes show the non-sea urchin (non-SpAGS) C-terminal sequence introduced. Protein sequences used include *Drosophila* Pins (Dm), *P. miniata* AGS (Pm), *E. tribuloides* AGS (Et), *H. sapiens* AGS3 (AGS3) and *H. sapiens* LGN (LGN). **G**, Single Z-slice confocal images of sea urchin embryos at 8~16-cell stage showing localization of each GFP-AGS. Embryos were injected with 0.3µg/µl stock of GFP-AGS mRNA and 0.25µg/µl stock of 2x-mCherry-EMTB mRNA. The white arrowheads indicate vegetal cortical localization of GFP-AGS. **H**, % of the embryos with vegetal cortical localization of GFP-AGS chimeric mutants (left) and the ratio of the cortical-to-non-cortical mean intensity (right) in 16~32-cell embryos. Statistical analysis was performed against Full AGS by One-Way ANOVA. **I-J**, Embryos were injected with 0.15µg/µl stock of GFP-AGS mRNAs and 0.75mM SpAGS MO. The number of embryos making micromeres (I) and developing to gastrula or pluteus stage (J) were scored and normalized to that of the Full AGS. Statistical analysis was performed by One-Way ANOVA. n indicates the total number of embryos scored. * represents p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001. Each experiment was performed at least two independent times. Error bars represent standard error. Scale bars=10µm.

Since the N-terminal sequences of SpAGS and PmAGS are almost identical (Fig. S2), we hypothesize that the variable C-terminus made a difference in AGS localization and function at the vegetal cortex. To test this hypothesis, we constructed a series of chimeric SpAGS mutants that replaced its C-terminus with that of other AGS orthologs (Fig. 5F). These AGS orthologs include human LGN, *Drosophila* (Dm) Pins, and EtAGS, which are all involved in ACD (Gonczy, 2008; Schaefer et al., 2000; Wavreil & Yajima, 2020; Zhu et al., 2011a, 2011b) as well as human AGS3 and PmAGS, neither of which is involved in ACD (Saadaoui et al., 2017).

The chimeras of ACD-facilitating orthologs (EtGL, LGNGL, DmGL) showed no significant difference in the vegetal cortical localization and micromere function compared to the SpAGS control (Fig. 5G-J). In contrast, chimeras of no-ACD-facilitators (AGS3GL and PmGL) failed in micromere formation and function. These results suggest that the AGS C-terminus creates ACD diversity by largely reflecting the original function of each ortholog in the host species. Of note, *Drosophila* Pins chimera (DmGL) showed reduced micromere formation (Fig. 5I), which may be due to fewer functional domains with reduced efficacy in the higher-order organism (Wavreil and Yajima, 2020).

Additionally, AGS-PmGL unexpectedly showed cortical localization (Fig. 5G), while PmAGS showed no cortical localization (Fig. 5B). This result suggests that other elements of SpAGS outside of its C-terminus can drive its vegetal cortical localization but not function. Aurora A phosphorylates the linker serine region, enabling Dlg to bind and activate Pins in *Drosophila* (Johnston et al., 2009). To test if this serine is essential for SpAGS localization, we mutated it to alanine (AGS-S389A in Fig. S3A). Compared to the Full AGS control, the mutant AGS-S389A showed reduced vegetal cortical localization (Fig. S3B-C) and function (Fig. S3D-E).

Furthermore, we replaced the linker region of PmAGS with that of SpAGS (PmAGS-SpLinker in **Fig. S4A-B**). However, this mutant did not show any cortical localization nor proper function in ACD (**Fig. S4C-F**). Therefore, the SpAGS C-terminus is the primary element that drives ACD, while the linker region serves as the secondary element to help cortical localization of AGS.

Lastly, in humans, it is proposed that the interdomain sequence between GL2 and GL3 is important for intramolecular interaction with TPR through phosphorylation, mediating the autoinhibitory state of LGN differently from that of AGS3 (Takayanagi et al., 2019). To test this possibility, we made mutants targeting the residues unique to the AGS3 GL2-GL3 interdomain region (green and red residues in **Fig. S5**): three serine residues were mutated to alanine, and three other residues (G, N, Y) were replaced with the corresponding residues of LGN. Consistent with our hypothesis, the chimera replaced with the LGN residues (AGS3GL-GL2GL3) gained the proper localization and function, while the chimera with serine alterations (AGS3GL-3S/A) failed to function in ACD (**Fig. S3C-E**). These results suggest that specific amino acid residues within the GL3 motif are critical, likely mediating interaction with TPR domains and the autoinhibited state of AGS. This result aligns with the earlier results of AGS1111 and AGS2222, which failed in ACD. On the other hand, potential serine phosphorylation between GL2-GL3 motifs appears to be irrelevant to the AGS function.

Overall, we conclude that the variable C-terminus of AGS orthologs facilitates ACD diversity. At the same time, the N-terminus and the linker region of AGS appear to help mediate its autoinhibited state, which regulates its cortical localization (summary diagrams in **Fig. 6**).

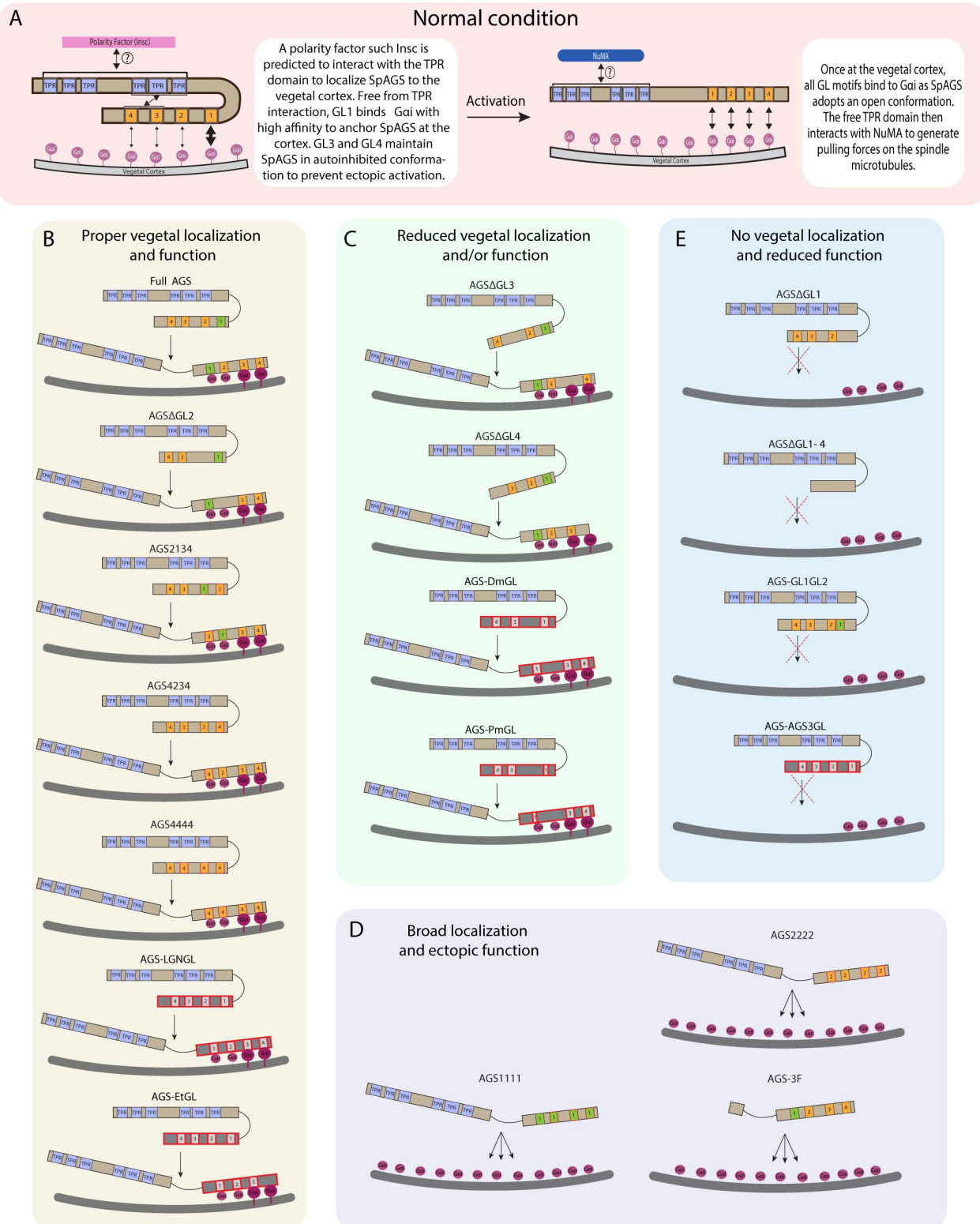


Figure 6.

Summary diagrams of SpAGS dissection experiments.

A, A model for the mechanism of SpAGS localization and function at the vegetal cortex. In a closed conformation, GL1 is critical for SpAGS recruitment and anchoring at the cortex through Gai binding, while GL3 and GL4 maintain the autoinhibition. The TPR domain is hypothesized to interact with a polarity factor such as Insc to restrict SpAGS localization to the vegetal cortex. Upon Gai binding, SpAGS adopts an open conformation, allowing all four GLs to bind to Gai and the TPR domain to interact with NuMA for force generation on the astral microtubules. **B**, A series of mutants that showed normal vegetal localization and functions. The position of GL1 is a more determining factor since mutants with GL1 replaced with other GL sequences localized and functioned properly. **C**, A series of mutants that showed a reduced vegetal localization and/or function. The GL3 and GL4 are necessary to regulate AGS localization and function, likely by mediating its autoinhibitory mechanism through their binding to TPRs. Furthermore, AGS-DmGL and PmGL were categorized in this group due to the reduced number of GL motifs. **D**, A series of mutants that showed broad AGS localization and ectopic function. The TPR domain is critical for restricting AGS localization at the vegetal cortex since its removal spreads the AGS signal around all cortices. The sequences of GL3 and GL4 are also crucial for the SpAGS function. **E**, A series of mutants that showed neither vegetal localization nor function. Removing or displacing GL1 led to significant disturbances in AGS localization and function, suggesting that having a GL motif at this specific position is critical for AGS' interaction with Gai and its anchoring to the cortex.

SpAGS is a dominant factor for micromere formation

Since AGS is a part of the conserved ACD machinery, we next sought to understand how dominant SpAGS is for micromere formation. The other conserved ACD factors include Insc, Dlg, NuMA, and Par3 (**Fig. 1C**). Insc controls cortical localization of Pins and LGN in flies and humans, respectively (Schaefer et al., 2000; Williams et al., 2014; Yu et al., 2000; Culurgioni et al., 2011; Culurgioni et al., 2018). Dlg appears to bind to the phosphorylated linker domain of Pins, which recruits microtubules to the cortex in flies (Johnston et al., 2009; Siegrist & Doe, 2005). NuMA (Mud in *Drosophila*) interacts with LGN/Pins to generate pulling forces on the microtubules in humans and flies. Par3 (Baz in *Drosophila*) is part of the PAR complex with Par6 and aPKC and binds to Insc to help localize LGN/Pins at the cortex (Culurgioni et al., 2011; Parmentier et al., 2000; Schaefer et al., 2000; Schober et al., 1999; Wodarz et al., 2000; Yu et al., 2000).

We cloned the sea urchin orthologs of these ACD factors and tagged each ORF with a GFP reporter. All ACD factors showed precise vegetal cortical localization during or upon micromere formation by GFP live imaging or immunofluorescence (**Fig. 7A** and S6). Furthermore, we tested for physical interaction by performing the proximity ligation assay (PLA) for AGS and ACD factors (Insc, NuMA, Dlg). The results suggest these factors physically interact with AGS at the vegetal cortex (**Fig. 7B**). Hence, the core ACD machinery is present at the vegetal cortex and interacts with AGS. We also observed AGS interacting with a fate determinant, Vasa, that is known to be enriched in micromeres at the vegetal cortex (**Fig. 7B**) (Voronina et al., 2008). These results indicate that AGS may recruit both ACD factors and fate determinants to the vegetal cortex, directly facilitating rapid lineage segregation of micromeres.

Consistent with this observation, SpAGS knockdown reduced the signal enrichment of ACD factors and another fate determinant of micromeres, β -catenin (Logan et al., 1999) (**Fig. 7C-H**). Furthermore, in our previous study (Poon et al., 2019), we identified that SpAGS recruits the spindle poles to every cortex when overexpressed (**Fig. 57A**, arrows). Similarly, SpAGS at least partially recruits its partner proteins to the ectopic cortical region, which we never observed in the control group (**Fig. 57B-C**, arrows). These results support the idea that SpAGS directly recruits the molecules essential for micromere lineage segregation. Indeed, *in situ hybridization*

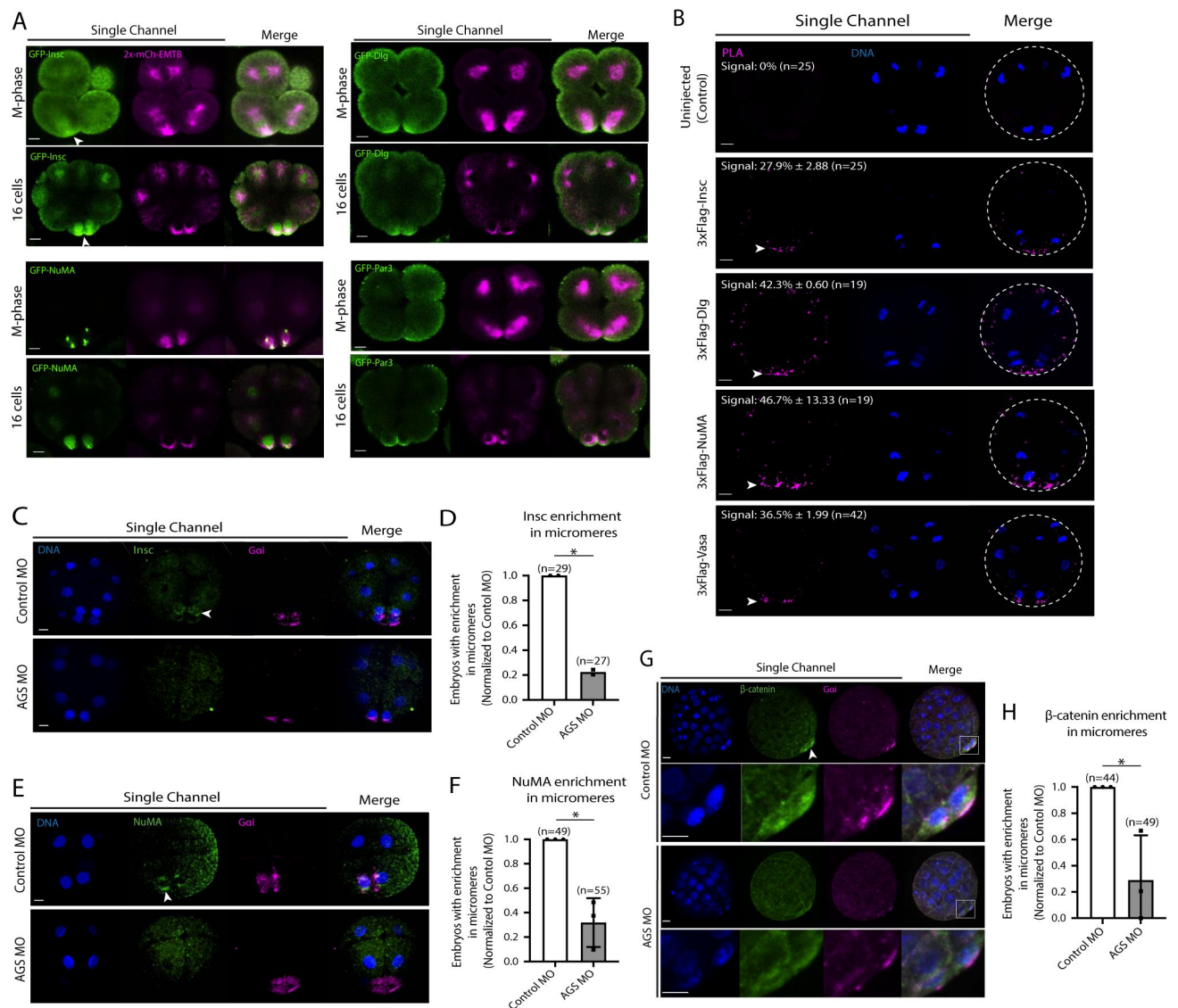


Figure 7.

SpAGS is critical for the proper localization of ACD factors and fate determinants.

A, Single Z-slice confocal imaging was used to focus on the vegetal cortex. Representative images of embryos during the metaphase and at the 16-cell stage show localization of each GFP-ACD factor, SpInsc, SpDlg, SpNuMA, and SpPar3. Embryos were injected with 0.5µg/µl stock of GFP-ACD factor mRNAs and 0.25µg/µl stock of 2x-mCherry-EMTB mRNA. White arrowheads indicate vegetal cortical localization of GFP constructs. Images represent over 80% of the embryos observed (n=30 or larger) per group across at least two independent cycles of experiments. **B**, Single Z-slice confocal images of sea urchin embryos at the 8~16-cell stage showing the signals at the vegetal cortex by PLA assay with Flag and AGS antibodies. Embryos were injected with 0.3-1µg/µl stock of 3xFlag-ACD factor mRNA. White arrowheads indicate the colocalization of AGS and another ACD factor at the vegetal cortex. The average % of the 8-cell and 8~16-cell embryos with the PLA signal across two independent cycles of experiments is indicated in each image. All embryos were scored independently of the angle since it was hard to identify the angle at the 8-cell stage. **C-F**, Representative 2D-projection images of the embryo stained with Insc (C), NuMA (E), and β-catenin (G) antibodies (green) by immunofluorescence. Embryos were stained with Gai antibody (magenta) and Hoechst dye (blue) as well. Z-stack images were taken at 1µm intervals to cover a layer of the embryo. White arrowheads indicate the signal in micromeres. Embryos were injected with 0.75mM Control MO or 0.75mM SpAGS MO. The number of embryos showing the localization of Insc (D), NuMA (F), and β-catenin (H) in micromeres were scored and normalized to that of the Control MO. Statistical analysis was performed by *t*-test. *n* indicates the total number of embryos scored. * represents *p*-value<0.05. Each experiment was performed at least two independent times. Error bars represent standard error. Scale bars=10µm.

(ISH) analysis suggests that the downstream genes regulated by micromere signaling, such as endomesoderm marker genes (*wnt8*, *foxa*, *blimp1b*, and *endo16*), decreased their expression territories in the AGS-knockdown embryos (**Fig. 8** [↗](#)). In contrast, ectoderm (*foxq2*) and skeletogenic mesoderm (*ets1*, *alx1*, *tbr1*, and *sm50*) marker genes showed no significant change in their expressions by AGS knockdown. Overall, these results suggest that SpAGS directly recruits multiple ACD factors and fate determinants necessary for micromere formation and function as an organizer, facilitating the downstream gene expressions necessary for endomesoderm specification.

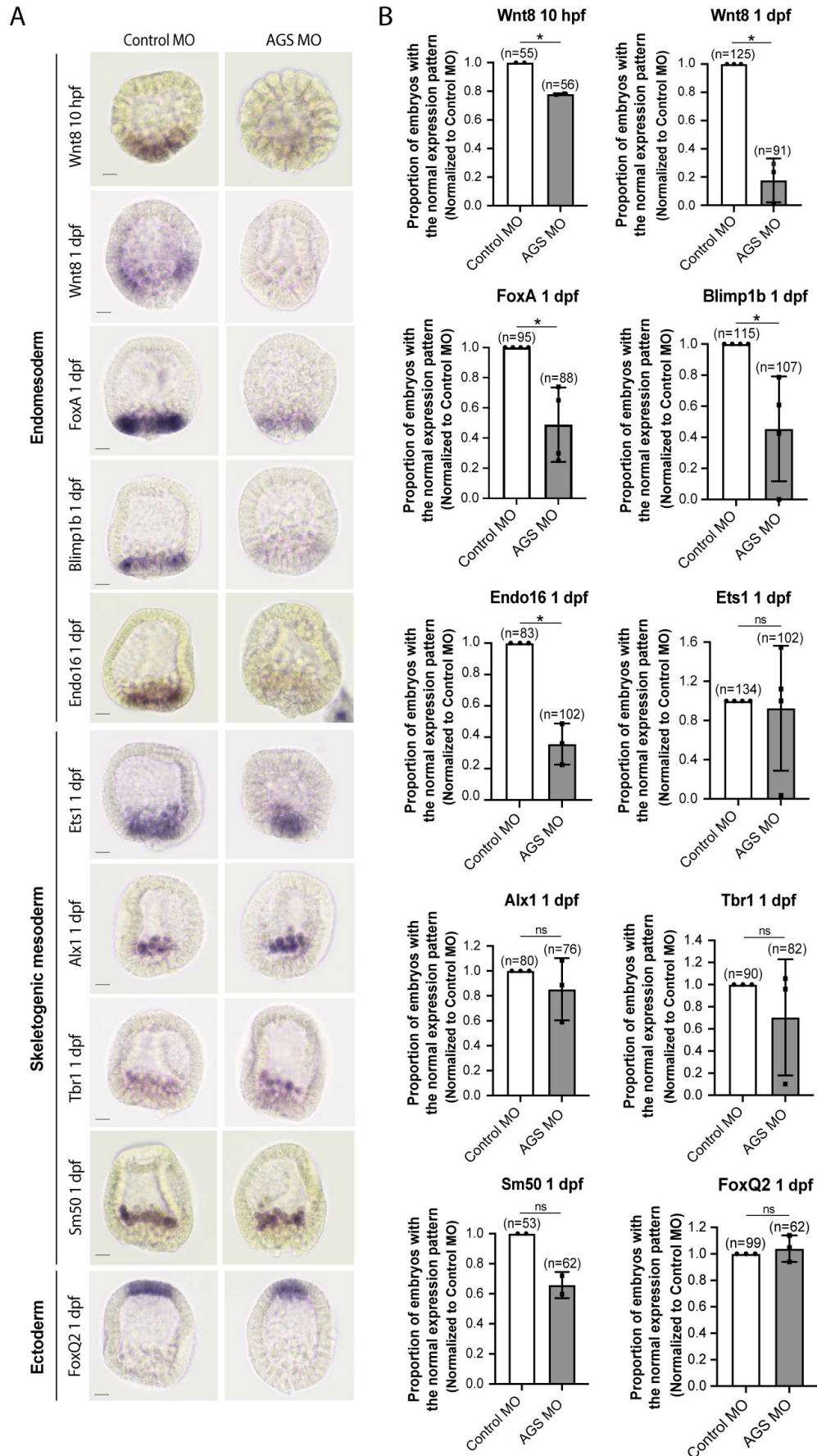


Figure 8.

SpAGS is critical for the downstream gene expressions facilitated by micromere signaling.

Embryos were injected with 0.75mM Control MO or 0.75mM SpAGS MO. Brightfield images (A) show the representative ISH staining for each cell lineage marker scored in the corresponding graph (B). The number of embryos showing the normal signal patterns of each marker gene was scored and normalized to that of the Control MO. Statistical analysis was performed by *t*-test. *n* indicates the total number of embryos scored. * represents *p*-value < 0.05. Each experiment was performed at least two independent times. Error bars represent standard error. Scale bars=20μm.

AGS serves as a variable factor in the conserved ACD machinery

AGS shows a variable C-terminal domain and appears to be a primary factor for facilitating ACD diversity. However, is AGS the only variable factor among the ACD machinery? To test this question, we cloned and injected orthologs of other ACD factors, such as Insc and Dlg, from pencil urchins (Et) or sea stars (Pm) into sea urchins. Both Insc and Dlg showed relatively conserved functional domains among three echinoderms with an extra PDZ domain present in PmDlg (**Fig. 9A-B**; S8-9). Importantly, these Pm and Et ACD factors showed cortical localization at the vegetal cortex in the sea urchin embryo (**Fig. 9C-F**). These results are in stark contrast to the earlier results of Pm/Et AGS, which showed varied localization and function in ACD. Therefore, Insc and Dlg might not be the significant variable factors.

To determine how dominantly SpAGS facilitates ACD diversity, we introduced SpAGS, EtAGS, or PmAGS into the pencil urchin, an ancestral type of sea urchin, and compared their function. We co-introduced Vasa-mCherry to identify the development of the germline, which is one of the micromere descendants. Pencil urchin embryos typically form 0-4 micromere-like cells randomly (**Fig. 10A**). Notably, only SpAGS injection increased the formation of micromere-like cells in the resultant pencil urchin embryos. In contrast, EtAGS and PmAGS showed no significant difference from the negative control (Vasa-mCherry only, **Fig. 10B**). This result suggests that SpAGS increases the frequency of micromere-like cell formation in pencil urchin embryos.

Sea urchin embryos show Vasa enrichment in micromeres at the 16-cell stage. In contrast, pencil urchin embryos show such enrichment later in the larval stage (3-4 dpf), which is more similar to the timing of the germline segregation in sea star embryos (Juliano & Wessel, 2009). We observed that SpAGS increased the Vasa signal enrichment in micromere-like cells compared to the control (Vasa-mCherry only) at the 16-cell stage. On the other hand, other AGS orthologs showed no significant difference from the control (**Fig. 10C-D**). Nearly 80% (80.12% ± 3.75) of the SpAGS-introduced embryos showed co-enrichment of AGS and Vasa in micromere-like cells, while the EtAGS and PmAGS groups showed only 49.2% ± 8.94 and 43.37% ± 3.94 enrichment, respectively (**Fig. 10E**). Consistently, the SpAGS group showed the earlier segregation of Vasa-positive cells similar to sea urchin embryos at 1 dpf (**Fig. 10F-G**), potentially accelerating the lineage segregation of the pencil urchin embryo.

Discussion

The introduction of ACD in early embryogenesis of the sea urchin led to the formation of a new cell type, micromeres, with a critical organizer function. In the sea urchin, SpAGS is essential for micromere formation, while other echinoderm embryos show no cortical AGS localization (Poon

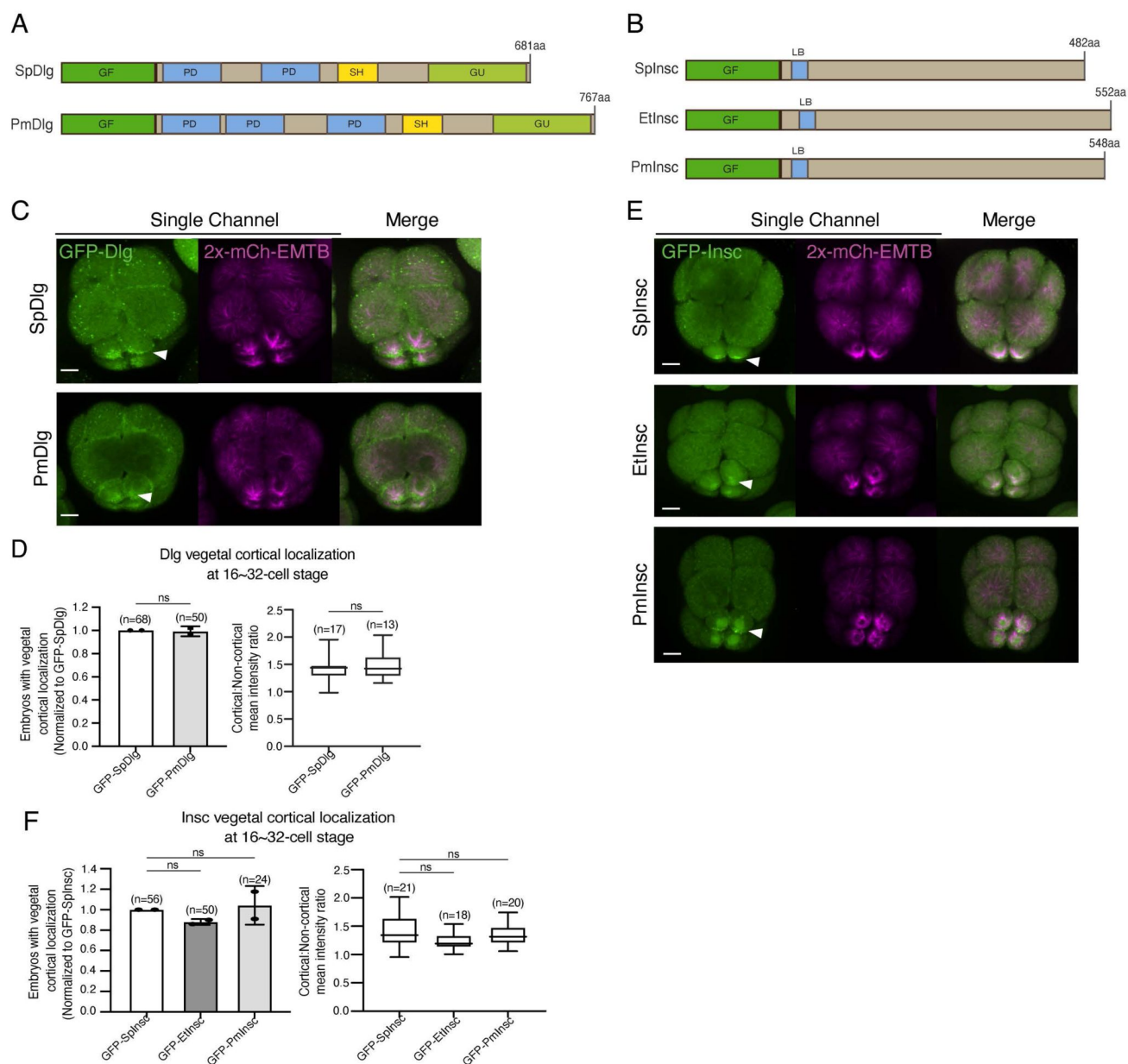


Figure 9.

Dlg and Insc are not the variable factors of the ACD machinery in evolution.

A-B, The design of GFP-Dlg (A) and GFP-Insc (B) constructs that were tested in this study. Of note, EtDlg was unavailable in the database due to the limited genomic information available for this species. **C-F**, Representative 2D-projection images of sea urchin embryos at 8~16-cell stage showing localization of each echinoderm GFP-Dlg (C) and GFP-Insc (E). Z-stack images were taken at 1μm intervals. Embryos were injected with 0.5μg/μl stock of GFP-Dlg or GFP-Insc mRNAs and 0.25μg/μl stock of 2x-mCherry-EMTB mRNA. White arrowheads indicate vegetal cortical localization of GFP constructs. The number of embryos with vegetal cortical localization of GFP-Dlg (D) and GFP-Insc (F) in 16~32-cell embryos was scored and normalized to that of the GFP-SpDlg or GFP-SpInsc (left graph). Right graph shows the ratio of the cortical-to-non-cortical mean intensity. Statistical analysis was performed by *t*-test or One-Way ANOVA. *n* indicates the total number of embryos scored. Each experiment was performed at least two independent times. Error bars represent standard error. Scale bars=10μm.

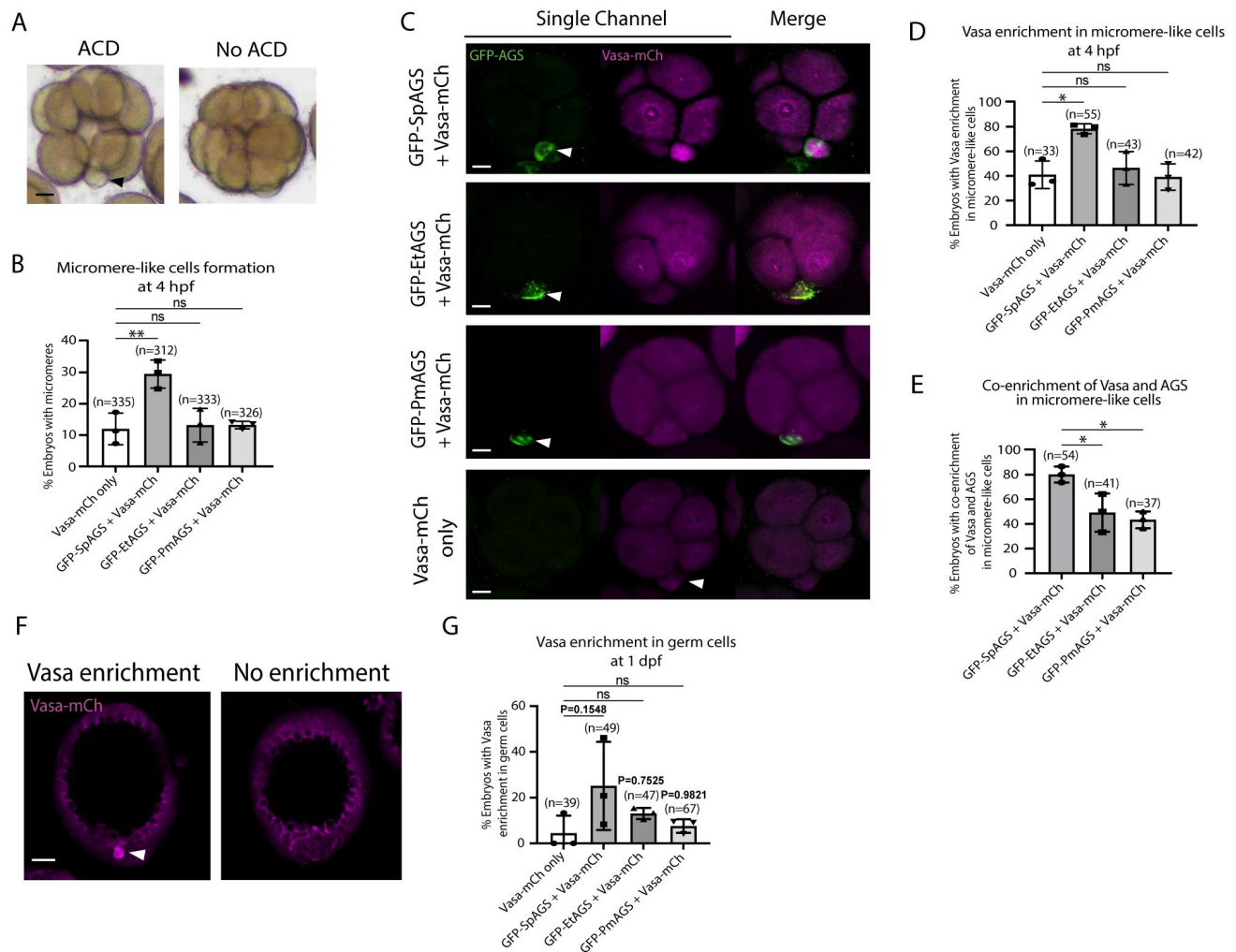


Figure 10.

SpAGS but not EtAGS and PmAGS can induce a functional ACD in pencil urchin (Et) embryos.

A, Representative brightfield images of Et embryos with or without micromere-like cells. Black arrowhead indicates micromere-like cells. **B**, Et embryos were injected with 0.3µg/µl stock of GFP-AGS mRNAs and 1µg/µl stock of Vasa-mCherry mRNA. The number of embryos making micromere-like cells was scored and normalized to that of the Vasa-mCherry only. Statistical analysis was performed against Vasa-mCh only by One-Way ANOVA. **C**, Representative 2D-projection images of the injected Et embryos. Z-stack images were taken at 1µm intervals to cover a layer of the embryo. White arrowheads indicate micromere-like cells. Scale bars=10µm. **D**, The number of embryos showing Vasa enrichment in the micromere-like cells was scored and normalized to that of the Vasa-mCherry only. Only the embryos that formed micromere-like cells were scored. Statistical analysis was performed against Vasa-mCh only by One-Way ANOVA. **E**, % of total embryos showing co-enrichment of Vasa and AGS in the micromere-like cells. Statistical analysis was performed against GFP-SpAGS by One-Way ANOVA. **F-G**, Representative 2D-projection images of Et embryos at 1 dpf. White arrowhead indicates Vasa enrichment in germ cells. Z-stack images were taken at 1µm intervals. % of total embryos showing Vasa enrichment in germ cells at 1 dpf was scored. Et embryos were injected with 0.3µg/µl stock of GFP-AGS mRNAs. Statistical analysis was performed against Vasa-mCh only by One-Way ANOVA. n indicates the total number of embryos scored. * represents p-value < 0.05, and ** p-value < 0.01. Each experiment was performed at least three independent times. Error bars represent standard error. Scale bars=20µm.

et al., 2019 [DOI](#)). This study demonstrates that the GL1 motif of SpAGS is key for its vegetal cortical localization and function in micromere formation.

Importantly, this unique role of the GL1 motif appears to be conserved across organisms. In *Drosophila* Pins and humans LGN, GL1 is free from TPR binding, making it essential for the recruitment of Pins/LGN to the cortex (Nipper et al., 2007 [DOI](#); Takayanagi et al., 2019 [DOI](#)). Thus, the evolutionary introduction of the GL1 motif into SpAGS likely increased recruitment affinity to the vegetal cortex, inducing ACD in the sea urchin embryo.

The GL1 deletion significantly disrupted micromere formation, while its replacement with other GL motifs had no effect. Therefore, the GL1 position rather than the sequence is essential for SpAGS function in ACD regulation. In contrast, GL3 and GL4 sequences are crucial for SpAGS activity, which also appears to be conserved across organisms. In *Drosophila* Pins and human LGN, GL2-3 and GL3-4 sequences, respectively, are essential for their intramolecular interactions with TPR motifs, which control Pins/LGN's autoinhibited conformation (Nipper et al., 2007 [DOI](#); Pan et al., 2013 [DOI](#); Smith & Prehoda, 2011 [DOI](#); Takayanagi et al., 2019 [DOI](#)). In a closed conformation, Pins/LGN are unable to bind to Mud/NuMA. Therefore, Goi binding to GL1 relieves the autoinhibition (Du & Macara, 2004 [DOI](#); Nipper et al., 2007 [DOI](#); Takayanagi et al., 2019 [DOI](#); Pan et al., 2013 [DOI](#)). Indeed, the TPR4-6 motifs are necessary to restrict SpAGS localization to the vegetal cortex, suggesting their interactions with GL motifs to maintain autoinhibition.

While the role of AGS protein in spindle orientation has been established in several model organisms, it was unknown if or how far AGS could regulate the fate determinants to facilitate ACD diversity. In this study, we learned that SpAGS is essential for the recruitment of ACD factors, such as Insc and NuMA, and fate determinants, such as Vasa and β -catenin, to micromeres. Notably, in pencil urchin embryos, SpAGS recruited Vasa protein into micromeres, suggesting SpAGS may be sufficient to recruit necessary fate determinants to create cell lineage segregation in another species. Although such a lineage segregation of micromeres may be mediated solely by ACD, their function as organizers might require additional changes in the developmental program of the entire embryo. For example, sea urchin embryos have a robust hyaline layer to keep blastomeres together, which presumably increases the cell-cell interaction and may also enhance cell signaling during early embryogenesis. In contrast, a hyaline layer is not or little present in sea star or pencil urchin embryos, respectively. At present, we do not know what developmental changes are upstream or downstream of micromere formation during sea urchin diversification. It will be important to identify in the future how far SpAGS impacts the developmental program other than inducing ACD and what other developmental elements play critical roles in establishing micromeres as a new cell lineage and organizers during sea urchin diversification.

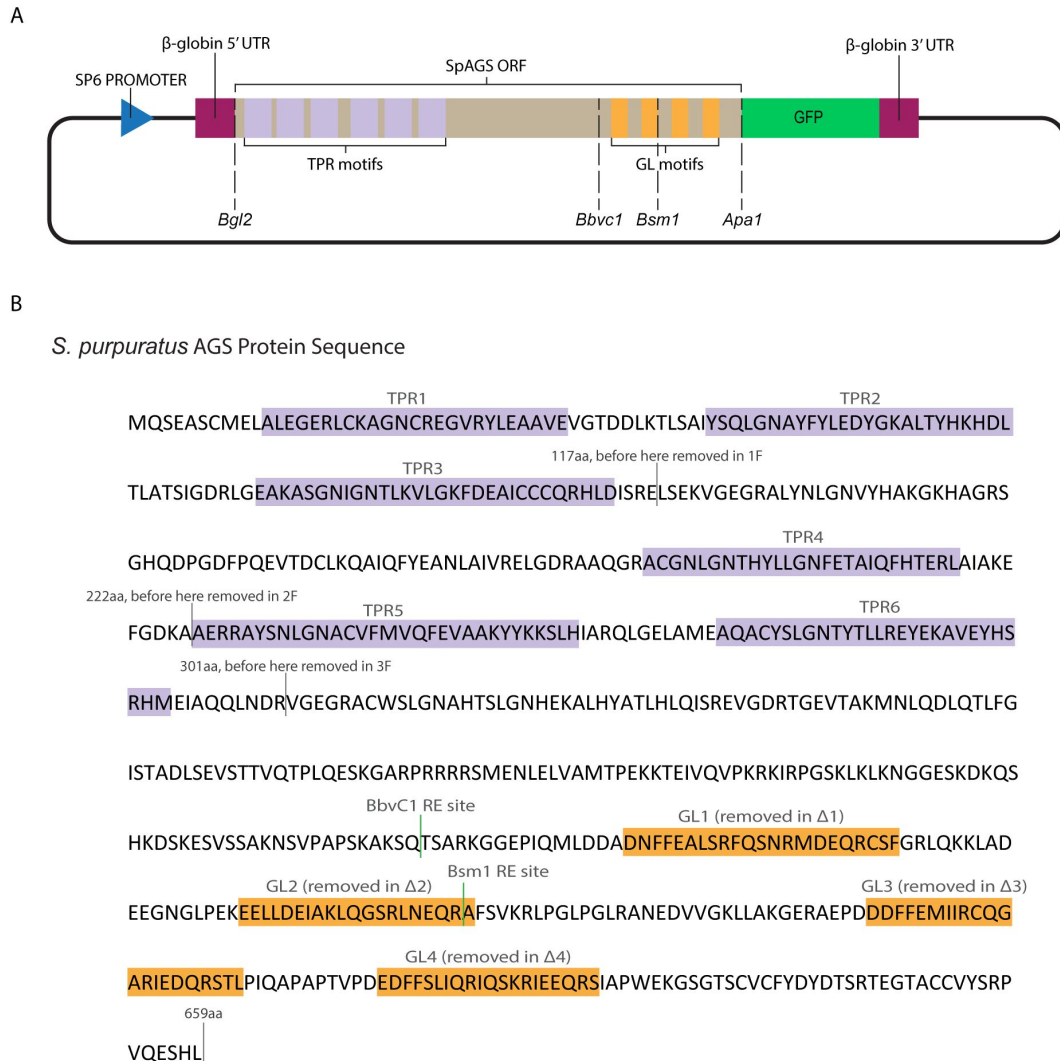
Overall, we conclude that the GL1 motif unique to sea urchin AGS orthologs is critical for SpAGS function in micromere formation. Since the unique role of the GL1 motif appears to be conserved across organisms, including *Drosophila* and humans, it is possible that the GL1 motif was once lost in the echinoderm common ancestor and recovered during sea urchin diversification. The recovery of this GL1 motif also resumed the interaction between SpAGS and other ACD machinery, such as NuMA, Insc, and Dlg, at the cortex in a similar manner to its orthologs Pins and LGN in other phyla, resulting in the controlled ACD and further interactions with fate determinants to form a new cell type in the sea urchin embryo. Therefore, unlike random unequal cell divisions that do not alter cell fates, AGS-mediated cell divisions appear to be highly organized and may be programmed to cause cell fate changes. Considering great variations within the C-terminus of AGS orthologs and their immediate impact on micromere formation, we propose that AGS is a variable factor in facilitating ACD diversity among echinoderm embryos, contributing to developmental diversity within a phylum. Future studies in other taxa are awaited to prove this concept further.

Acknowledgements

We would like to thank Mr. Ronit Sethi for providing assistance in identifying the optimal conditions for AGS-MO and OE experiments. N.E. and F.D.M.W. were responsible for the concept, experimental design and undertaking, data analysis, and manuscript construction and editing regarding all bioinformatics analyses; A.F. was responsible for initial conceptualization, experimental design, undertaking, and data analysis; M.Y. was responsible for concepts, experimental design and undertaking, data analysis, manuscript construction, and editing for all sections.

Funding

This work was supported by NSF (IOS-1940975) and NIH (1R01GM126043-01)



Supplemental Figure 1.

Dissection of SpAGS motifs.

A, The construct design of SpAGS-GFP, including the restriction enzyme sites used to prepare SpAGS mutants. **B**, The protein sequence of SpAGS. Predicted domains are labeled based on NCBI blast results, and the sequence portions deleted for each N-terminal construct are marked. The sequences for each GL motif used for deletion or swapping are indicated in orange. The internal restriction enzyme sites for *BbvCI* and *BsmI* are shown in green.

```

OjAGS -----MRANMDMPCMEIALEGERLCKMGCCREGVYFEAAVVTGDDDLKTLSA
SbAGS -----MEASCMELALEGERLCKTGNCREAVRYLEAAVVTGDDDLQTLSA
PbAGS MRLELEGRASTNAFMDASCMELALEGERLCKAGNCKSGVQVFEAAVVTGDDDLRTLSA
PbAGS MRLELEGRASTNAFMDASCMELALEGERLCKAGNCKSGVQVFEAAVVTGDDDLRTLSA
EtAGS -----MQSEASCMELALEGERLCKVGNCREGVYLEAAVVTGDDDLKTLSA
EpAGS -----MQSETSCMELALEGERLCKVGNCREGVYLEAAVVTGDDDLKMLSA
SpAGS -----MQSEASCMELALEGERLCKAGNCREGVRYLEAAVVTGDDDLKTLSA
SgAGS -----MQSEASCMELALEGERLCKAGNCREGVRYLEAAVVTGDDDLKTLSA
LvAGS -----MQSEASCMELALEGERLCKAGNCREGVRYLEAAVVTGDDDLKTLSA
      1 ***** * .,*,*,*,*, *,*,*

OjAGS IYSQLGNAYFYLQYKALTYHHRDITLASTIGDRLGEAKAGNLGNTLKVLGKFDEAVM
SbAGS IYSQLGNAYFYLDYKALTYHHRDITLARSIEDRLGEAKASGNIGMTLKVLGKYDEAIL
PbAGS IYSQLGNAYFYLDYTRALEYHHRDITLACTLGDHIGEAKASGNIGMTLKVLGKFDEAIV
PbAGS IYSQLGNAYFYLDYTRALEYHHRDITLACTLGDHIGEAKASGNIGMTLKVLGKFDEAIV
EtAGS IYSQLGNAYFYLDYKALTYHHRDITLATSIGDRLGEAKASGNIGMTLKVLGKFDEAIV
EpAGS IYSQLGNAYFYLDYKALTYHHRDITLATSIGDRLGEAKASGNIGMTLKVLGKFDEAIV
SpAGS IYSQLGNAYFYLDYKALTYHHRDITLATSIGDRLGEAKASGNIGMTLKVLGKFDEAIV
SgAGS IYSQLGNAYFYLDYKALTYHHRDITLATSIGDRLGEAKASGNIGMTLKVLGKFDEAIV
LvAGS IYSQLGNAYFYLDYKALTYHHRDITLATSIGDRLGEAKASGNIGMTLKVLGKFDEAIV
      *****:*,* :*,* ***** :*,* ***** :*,* ***** :*,*

OjAGS CCQCHLIDISRELGDKMGEARALYNLGNVYHAKGHSNGSHQDGGDFPRDVQKSLTAID
SbAGS CCQKHLIEISKELDVTGEARALYNLGNVYHAKGHSAGKAGHQEPDFTVTSCLKQAIIV
PbAGS CCQCHLIDISRELCDRVGEARALYNLGNVYHAKGHSAGKAGHQDGGDFPEEVIICGLRAKAVE
PbAGS CCQCHLIDISRELCDRVGEARALYNLGNVYHAKGHSAGKAGHQDGGDFPEEVIICGLRAKAVE
EpAGS CCERHNIIVISRELSDKIGEARLYNLGNVYHAKGHSAGKAGHQDGGDFPQEVTECLKQAIQ
SpAGS CCQCHLIDISRELSKIVGEARALYNLGNVYHAKGHSAGKAGHQDGGDFPQEVTECLKQAIQ
SgAGS CCQCHLIDISRELSKIVGEARALYNLGNVYHAKGHSAGKAGHQDGGDFPQEVTECLKQAIQ
LvAGS CCQCHLIDISRELSKIVGEARALYNLGNVYHAKGHSAGKAGHQDGGDFPQEVTECLKQAIQ
      **:*,* *:*,* *:*,* *****:*,* *:*,* *:*,* *****:*,* *:*,*

OjAGS VYEANLKLIVRELGDRAAQGRACGNLGNTHYLLGNFDTAIGFHKERLSIAKEFGDKKAERR
SbAGS VYETNLIQVRELNDRAAQGRACGNLGNTHYLLGNFDTAIGFHKERLSIAKEFGDKKAERR
PbAGS FYEMNLAIIVKSLVDKAAQGRACGNLGNTHYLLGNFDTAIGFHKERLSIAKEFGDKKAERR
PbAGS FYEMNLAIIVKSLVDKAAQGRACGNLGNTHYLLGNFDTAIGFHKERLSIAKEFGDKKAERR
EtAGS FYEANLAIIVRELGDRAAQGRACGNLGNTHYLLGNFDTAIGFHKERLSIAKEFGDKKAERR
EpAGS FYEANLAIIVRELGDRAAQGRACGNLGNTHYLLGNFDTAIGFHKERLSIAKEFGDKKAERR
SpAGS FYEANLAIIVRELGDRAAQGRACGNLGNTHYLLGNFDTAIGFHKERLSIAKEFGDKKAERR
SgAGS FYEANLAIIVRELGDRAAQGRACGNLGNTHYLLGNFDTAIGFHKERLSIAKEFGDKKAERR
LvAGS FYEANLAIIVRELGDRAAQGRACGNLGNTHYLLGNFDTAIGFHKERLSIAKEFGDKKAERR
      :*,* *:*,* *:*,* *****:*,* *:*,* *:*,* *****:*,* *:*,*

OjAGS AYSNLGNHVFKEGFEFAADYKRTLLIARGLADQAIIEAQACYSLGNFTYLLRDYETAIVE
SbAGS AYSNLGNHVFKEGFEFAADYKRTLLIARGLADQAIIEAQACYSLGNFTYLLRDYETAIVE
PbAGS AYSNLGNACVFMGDFETAAADYKRTLLIARGLADQAIIEAQACYSLGNFTYLLRDYETAIVE
PbAGS AYSNLGNACVFMGDFETAAADYKRTLLIARGLADQAIIEAQACYSLGNFTYLLRDYETAIVE
EtAGS AYSNLGNACVFLVKFESAAEYKKAHLIARQLGESAMEAQACYSLGNFTYLLRDYETAIVE
EpAGS AYSNLGNACVFMVFEFAAAYKKAHLIARQLGESAMEAQACYSLGNFTYLLRDYETAIVE
EpAGS AYSNLGNACVFMVFEFAAAYKKAHLIARQLGESAMEAQACYSLGNFTYLLRDYETAIVE
SgAGS AYSNLGNACVFMVFEFAAAYKKAHLIARQLGESAMEAQACYSLGNFTYLLRDYETAIVE
LvAGS AYSNLGNACVFMVFEFAAAYKKAHLIARQLGESAMEAQACYSLGNFTYLLRDYETAIVE
      *****:*,* *:*,* *****:*,* *:*,* *****:*,* *:*,*

OjAGS YHMRHNEIHAQHVNDRVGEGRACWSLGNATHALQNHKEKALHYATLHLQISREVGDRTGEVT
SbAGS YHVHKHIAIQAQLQDRVGEGRACWSLGNATHALQNHKEKALHYATLHLQISREVGDRTGEVT
PbAGS YHERHRIHAQKLDKRVGEGRACWSLGNATHALQNHKEKALHYATLHLQISREVGDRTGEVT
PbAGS YHERHRIHAQKLDKRVGEGRACWSLGNATHALQNHKEKALHYATLHLQISREVGDRTGEVT
EtAGS YHMRHNEIHAQQLQDRVGEGRACWSLGNATHALQNHKEKALHYATLHLQISREVGDRTGEVT
EpAGS YHMRHNEIHAQQLNDRVGEGRACWSLGNATHALQNHKEKALHYATLHLQISREVGDRTGEVT
SpAGS YHMRHNEIHAQQLNDRVGEGRACWSLGNATHALQNHKEKALHYATLHLQISREVGDRTGEVT
SgAGS YHGRHNEIHAQELNDRVGEGRACWSLGNATHALQNHKEKALHYATLHLQISREVGDRTGEVT
LvAGS YHGRHNEIHAQELNDRVGEGRACWSLGNATHALQNHKEKALHYATLHLQISREVGDRTGEVT
      **:*,* *:*,* *****:*,* *:*,* *****:*,* *:*,* *****:*,* *:*,*

OjAGS AQMNLSDLITLVGNQFNERNBERGSSICS-----REDDGKPRRRSMENLELVMTPEKNS
SbAGS AQMNLVDLQTVLGLSKKGFE-----KQGRDARRRKSMENLELVMTPEKNG
PbAGS AQMNLADLRTVLGLDKDPCSDGLLQNASKTKLKEKELNRQRQSMERLELVMTPEKNA
PbAGS AQMNLADLRTVLGLDKDPCSDGLLQNASKTKLKEKELNRQRQSMERLELVMTPEKNA
EtAGS AQMNLADLRTVLFGIGTODLSHDVTAAC-PPAMQPTKGATPRRRSMENLELVMTPEKNT
EpAGS AQMNLSDLQKIPGLSPDINSNAT-----QVPDKATPGARPRRRSMENLELVMTPE-
SgAGS AKMNLQDLQTLFGISTADLSEVSTTV-----QTLQESKAGARPRRRSMENLELVMTPEKKT
SgAGS AKMNLQDLQSLFGISTDLSVSTTV-----QTLQETKAGARPRRRSMENLELVMTPEKKA
LvAGS AKMNLQDLQSLFGISTDLSVSTTV-----QTLQETKAGARPRRRSMENLELVMTPEKKA
      **:*,* *:*,* *:*,* *****:*,* *:*,* *****:*,* *:*,*

OjAGS SEKPNKDQVDGKPKGARVKE---KSSKEKLVHAGSNNISGANKATDPQVTV-----NL
SbAGS ATAIPVAKPLKPV---HMKKKTSSKEKLSRKSGDTSSSSGSAGSNK---AESN---
PbAGS AATANGK-PKQSTGAKFKRK---GSNKEKLSRKSSNTSSTSSSGSAACTRTENVNIQV
PbAGS AATANGK-PKQSTGAKFKRK---GSNKEKLSRKSSNTSSTSSSGSAACTRTENVNIQV
EtAGS DSA-VPNG-KLKPTGSKSKRNGKE---KLSRKSGNTSTGSSNSSSSAPS---QASTQA
EpAGS -----
SpAGS EIVQVPRK-KIRP-GSKLKLKNGGESKDKQSHKSDKESVSSAKNSAPAPS---KAKSQT
SgAGS EIVQVPRK-KIRP-GSKLKLKNGGESKDKQSHKSDKESVSSAKNSAPAPS---KAKTQT
LvAGS EIVQVPRK-KIRP-GSKLKLKNGGESKDKQSHKSDKESVSSAKNSAPAPS---KAKTRT

OjAGS ---KPPRYKSSPDFFDSFFDLGRFQGNRMDEQMSLPSKNSL-----DKCDTSDEGKEE
SbAGS -GVKLNGSLDDEDDQDFDALGRFQEKRINDQVSGFLDD-----TDAANKED
PbAGS SP-QQSSPEFMDDDDSFFDLGRFQGNRMDEQRCFSNRMQDKQRENDERDRTYPMKEE
PbAGS SP-QQSSPEFMDDDDSFFDLGRFQGNRMDEQRCFSNRMQDKQRENDERDRTYPMKEE
EtAGS SSQKGGEPQMLDDADNFFEALSRFQGNRMDEQRCFSGRMQKQLQE---DEANGLEPEEE
EpAGS ---KGGEPTQMLDDADNFFEALSRFQGNRMDEQRCFSGRMQKQLQE---DDEANGLEPEEL
SpAGS SARKGGEPQMLDDADNFFEALSRFQGNRMDEQRCFSGRMQKQLQE---DEANGLEPEEE
SgAGS SARKGGEPQMLDDADNFFEALSRFQGNRMDEQRCFSGRMQKQLQE---DEANGLEPEEE
LvAGS SARKGGEPQMLDDADNFFEALSRFQGNRMDEQRCFSGRMQKQLQE---DEANGLEPEEE
      :*,* *:*,* *****:*,* *:*,* *:*,*

OjAGS LMNSTAKFGQKRMNEQRMHV-----ASLPGLRGKDEELSLKIL---EASSVPDDDFFDMLIR
SbAGS LLDEIAKVQGNRLNEQRANV-----ELLPLGLR-GHREVDRLLAGDGAVPDDDFFDMLMR
PbAGS LMNQIASFORSRLNEQRSSI-----CNPLGLKTNNEVLGQLQKGNQGVPDDDFFDMLMR
PbAGS LMNQIASFORSRLNEQRSSI-----CNPLGLKTNNEVLGQLQKGNQGVPDDDFFDMLMR
EtAGS LLDEIAKLQGSRLNEQRAFSQAQRLPGLPLGLR-ANENVLGLKLAKGDLAVPDDDFFDMLMR
EpAGS LLDEIAKLQGSRLNEQRVSVARLPLGLPLGLR-ANDKVLGTLLAKGERAVPDDDFFDMLMR
SbAGS LLDEIAKLQGSRLNEQRAFSVQRLPGLPLGLR-ANEDVVGKLLAKGERAEVDDDFFDMIIR
SgAGS LLDEIAKLQGSRLNEQRASSAQLRPLGLPLGLR-ANEDVVGKLLAKGERAEVDDDFFDMIIR
LvAGS LLDEIAKLQGSRLNEQRASSAQLRPLGLPLGLR-ANEDVVGKLLAKGERAEVDDDFFDMIIR
      *:*,* *:*,* *:*,* *****:*,* *:*,* *:*,*

OjAGS CQGTRIEDQRTDAPERVRAPTVPDEDFFSLIGSVQSRLEEQRCDLPELSRSDMITDEK*
SbAGS CQGARMEQDQSSRLPSSRPAPTVPDEDFFLIQRIQSKRIEEQRTDAPERVPTKRES*
PbAGS CQGARINDQSEPPVLQAPAPTVPDEDFFLIQRIQSVQSRLEEQRCDKGPDQQT*
PbAGS CQGARINDQSEPPVLQAPAPTVPDEDFFLIQRIQSVQSRLEEQRCDKGPDQQT*
EtAGS CQGARIEDQRTSLPMPPTAPTVPDEDFFLIQRIQSKRIEEQRTIPWEKGPKPSDK*
EpAGS CQGARIEDQRTSLPVPAPAPTVPDEDFFLIQRIQSKRIEEQRTIPWEKGPKPSDK*
SpAGS CQGARIEDQRTSLPIQAPAPTVPDEDFFLIQRIQSKRIEEQRTIPWEKSGTSCVCFY
SgAGS CQGARIEDQRTSLPIQAPAPTVPDEDFFLIQRIQSKRIEEQRTIPWEKSGTSCVCFY
LvAGS CQGARIEDQRTSLPIQAPAPTVPDEDFFLIQRIQSKRIEEQRTIPWEKSGTSCVCFY
      *:*,* *:*,* *:*,* *****:*,* *:*,* *:*,*

OjAGS -----
SbAGS -----
PbAGS -----
EtAGS -----
EpAGS SYESSTHGSACCVYRRPIQQTISI*
SgAGS DYDTSRTETGACCVYIRPVQESH*
SgAGS DYDTSRTETGACCVYIRPVQESH*
LvAGS DYDTSRTETGACCVYIRPVQESH*

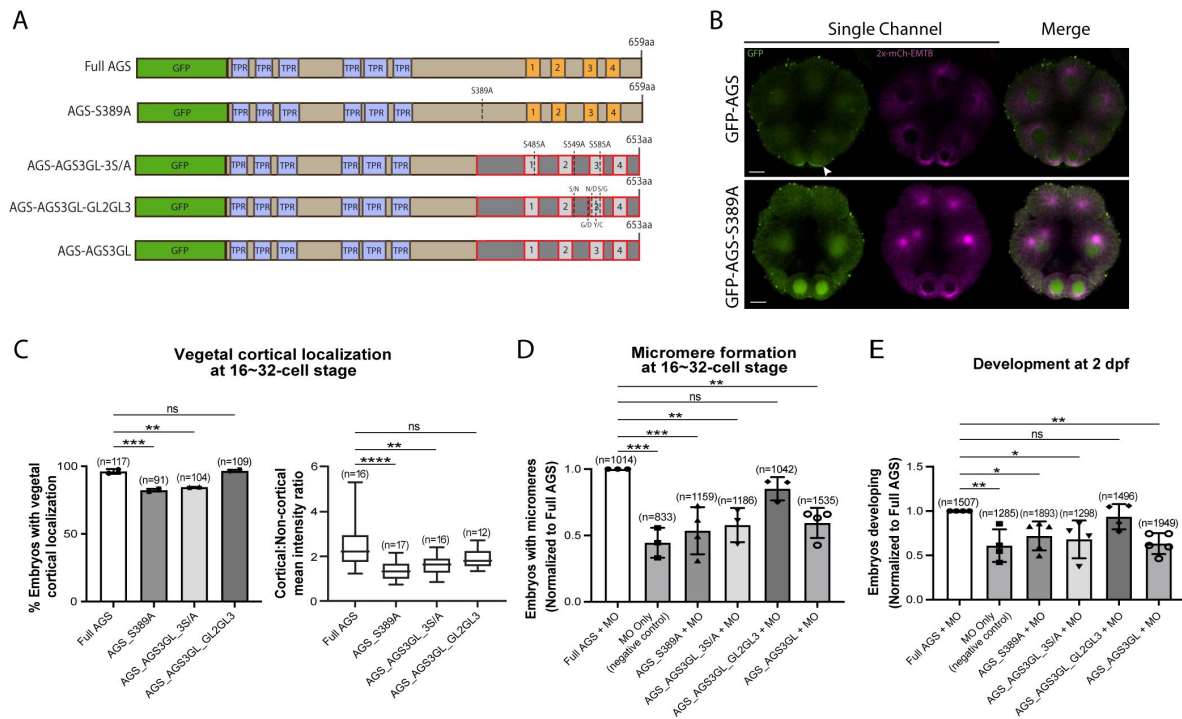
TPR motifs
TPR-like motifs
GL motifs

```

Supplemental Figure 2.

Echinoderm AGS sequence alignment.

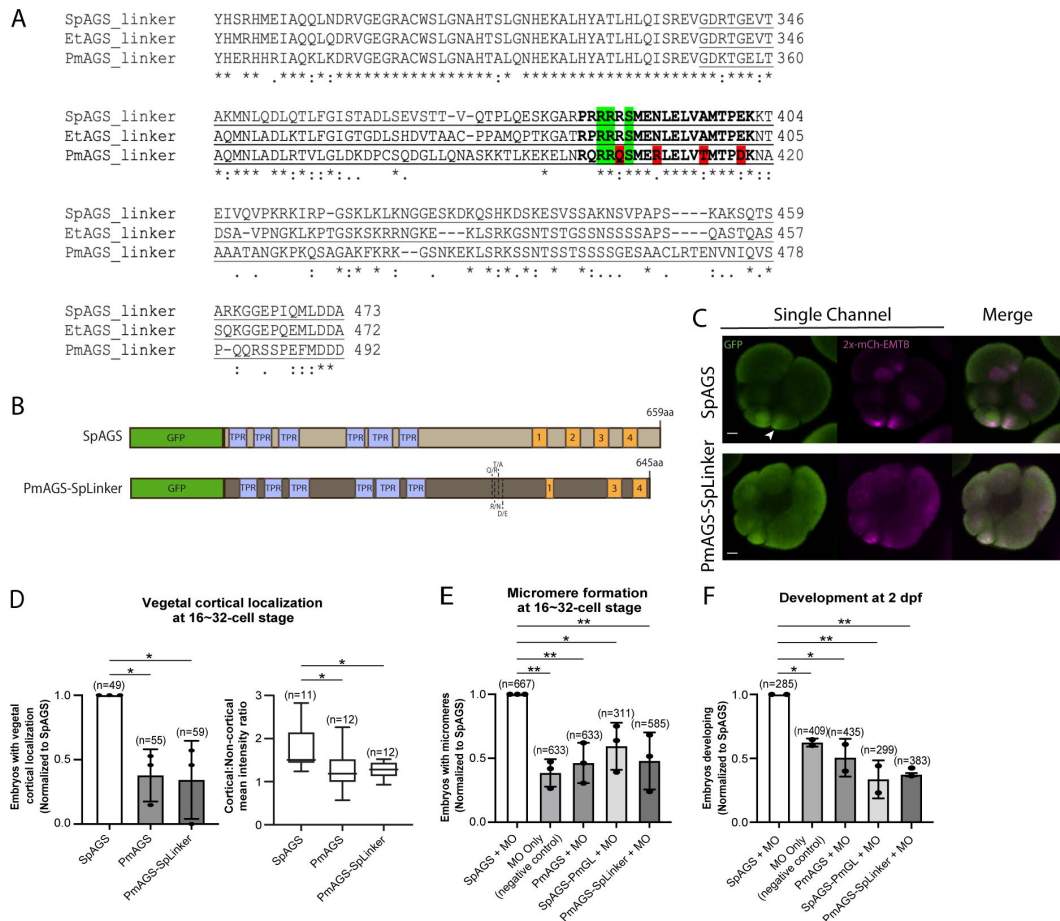
All AGS are similar in the N-terminus with the predicted conserved TPR motifs (blue) but are highly variable in the C-terminus with the predicted GL motifs (yellow).



Supplemental Figure 3.

The linker domain and GL2-GL3 region are important for AGS localization and function.

A, Design of GFP-AGS mutant constructs tested in this study. TPR motifs are marked in blue, and GL motifs are in orange. The brown section shows the SpAGS portion and the red and grey boxes show the non-sea urchin (non-SpAGS) C-terminal sequence introduced. The dotted lines represent single amino acid mutations. **B**, Single Z-slice confocal images of sea urchin embryos at 8~16-cell stage showing localization of GFP-AGS-S389A mutant. Embryos were injected with 0.3µg/µl stock of GFP-AGS mRNA and 0.25µg/µl stock of 2x-mCherry-EMTB mRNA. The white arrowhead indicates vegetal cortical localization of GFP-AGS. **C**, % of the embryos with vegetal cortical localization of GFP-AGS mutants (left) and the ratio of the cortical-to-non-cortical mean intensity (right) in 16~32-cell embryos. Statistical analysis was performed against Full AGS by One-Way ANOVA. **D-E**, Embryos were injected with 0.15µg/µl stock of GFP-AGS mRNAs and 0.75mM SpAGS MO. The number of embryos making micromeres (D) and developing to gastrula or pluteus stage (E) were scored and normalized to that of the Full AGS. Statistical analysis was performed by One-Way ANOVA. n indicates the total number of embryos scored. * represents p-value<0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001. Each experiment was performed at least two independent times. Error bars represent standard error. Scale bars=10µm.



Supplemental Figure 4.

The Dlg recognition sequence in the linker domain is important for the AGS function.

A, Alignment of linker domain between echinoderms including sea urchin (SpAGS_linker), pencil urchin (EtAGS_linker), and sea star (PmAGS_linker). Bold letters represent the conserved core of the linker domain. The green highlight indicates conserved amino acids in the consensus kinase recognition motif. The red highlight represents amino acid mutated to match SpAGS residues in the PmAGS-SpLinker mutant. **B**, Design of GFP-AGS constructs tested in this study from *S. purpuratus* (Sp) and *P. miniata* (Pm). TPR motifs are marked in blue, and GL motifs are in orange. The dotted lines represent single amino acid mutations. **C** Single Z-slice confocal images of sea urchin embryos at 8~16-cell stage showing localization of GFP-AGS. Embryos were injected with 0.3µg/µl stock of GFP-AGS mRNAs and 0.25µg/µl stock of 2x-mCherry-EMTB mRNA. The white arrowhead indicates vegetal cortical localization of GFP-AGS. **D**, The number of embryos with vegetal cortical localization of GFP-AGS mutants in 16~32-cell embryos was scored and normalized to that of the SpAGS (left graph). Right graph shows the ratio of the cortical-to-non-cortical mean intensity. Statistical analysis was performed against SpAGS by One-Way ANOVA. **E-F**, Embryos were injected with 0.3µg/µl stock of GFP-AGS mRNAs and 0.75mM SpAGS MO. The number of embryos making micromeres (E) and developing to gastrula or pluteus stage (F) were scored and normalized to that of the SpAGS. Statistical analysis was performed by One-Way ANOVA. n indicates the total number of embryos scored. * represents p-value<0.05, and ** p-value < 0.01. Each experiment was performed at least two independent times. Error bars represent standard error. Scale bars=10µm.


```

SpAGS_GL 461 RKGGEPIQMLDDADNFFEALSRFQSNRMDEQRCSEFRLQKKLA-----
DmPins_GL 455 EKLRQAATRKAKDDFFEMLSRSQSKRMDDQRCSEIKVNPAGAPAVATGATRKPL----
PmAGS_GL 479 PQRSSPEFMDDDDSFDFVLSRFQSKRMDEQRCSEFNRMQDKQERE-----
EtAGS_GL 459 QKGGEPEMLDDADNFFEALSRFQSNRMDEQRCSEFGRMQRKLE-----
HsLGN_GL 468 NSQRKISADTIGDEGFFDLSRFQSNRMDDQRCCLQEKNCHTASTTTSSTPPKMMLKT
HsAGS3_GL 460 PRTSIPRAPSSDEECFFDLLTKFQSESRMDDQRCPLDDGQAGAAEATAAPTLEDRI--A
      : ** : ** : *..***:*** :

SpAGS_GL DEENGLEPEKEELLDEIAKLQGSRLNEQRAFSVKRLPGLPGLR-ANE---DVVGK---LL
DmPins_GL -----VQONSIFVDPNTLPGLKSPSSANPSAIGHGPLAR
PmAGS_GL NDEDRDYPKKEELMNQIASFQRSRLNEQRSS----ICNLPLKTNNE---EVLGQ---LL
EtAGS_GL DEEANGLEPEKEELLDEIAKLQGSRLNEQRAFSQRLPGLPGLR-ANE---NVLGK---LL
HsLGN_GL SSVPVVSPNTDEFLLASQSRRLDDQRAE---FNLPLGLRLTQNSQ-SVLS---HL
HsAGS3_GL QPSMTASPQTEEFFDLIASQSRRLDDQRAE---VGLPLGLRITHSNA-GHLR---G-
      * : .****: . :

SpAGS_GL AKGERAEPDDDFFEMIIRCOGARIEDQRTLP-I-----
DmPins_GL SATTTQQPDDDFLDMLMRCQGSRLQSELPFPNVMTDAEAEAPPSVPEAAVPGAPRG
PmAGS_GL QKGNQGVDPDDFFDMLMRCQGSRLNDQSEPP-V-----
EtAGS_GL AKGDLAVPDDDFFEMLMRCQGARIEDQRTLP-M-----
HsLGN_GL MTNDNKEADEDDFFDILVKCQGSRLDDQRCAPPPA-----
HsAGS3_GL -HGEPEPEDDFFMLIKQSRIDDQRCPPPDV-----
      .***:***: *.*:***: *

SpAGS_GL -QAPATVPDEDDFFSLIQRIQSKRIEQRSIAPWEKSGT-----SCVCFDYDTSR
DmPins_GL QTGRGATVPDEDDFFSLIMKVQSGRMEDQRAEIPFRNANNNNNSRS-----NNNG
PmAGS_GL -LQAPATVPDEDDFFALIQRVQSKRMDAQRSKGPQEQTKT-----SD-----
EtAGS_GL -PTAPATVPDEDDFTLIQRIQSRRIEQRTIPWEKGPKE-----SDK-----
HsLGN_GL -TTKGPTVPDEDDFFSLILRSQKRMDEQRLVLLQRDQNRDQDFGLKDFLQNNALLEFKNSG
HsAGS3_GL -LPRGPTMPDEDDFFSLIQRVQAKRMDEQRVLDAGGPEQGAGGPPEPQQQC-----QPG
      . *.*:***:*** : *.*:***

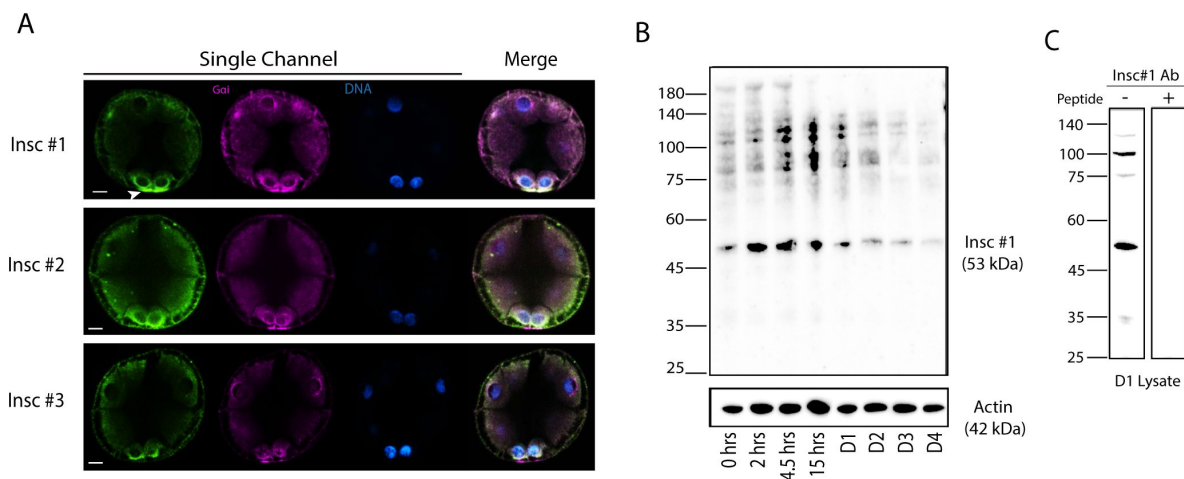
SpAGS_GL TEGTACCVYSRPVQESHL
DmPins_GL SAGGAGK-----
PmAGS_GL -----
EtAGS_GL -----
HsLGN_GL KKSADH-----
HsAGS3_GL AS-----

```

Supplemental Figure 5.

Alignment of C-terminus GoLoco domain sequences used for chimeric mutants.

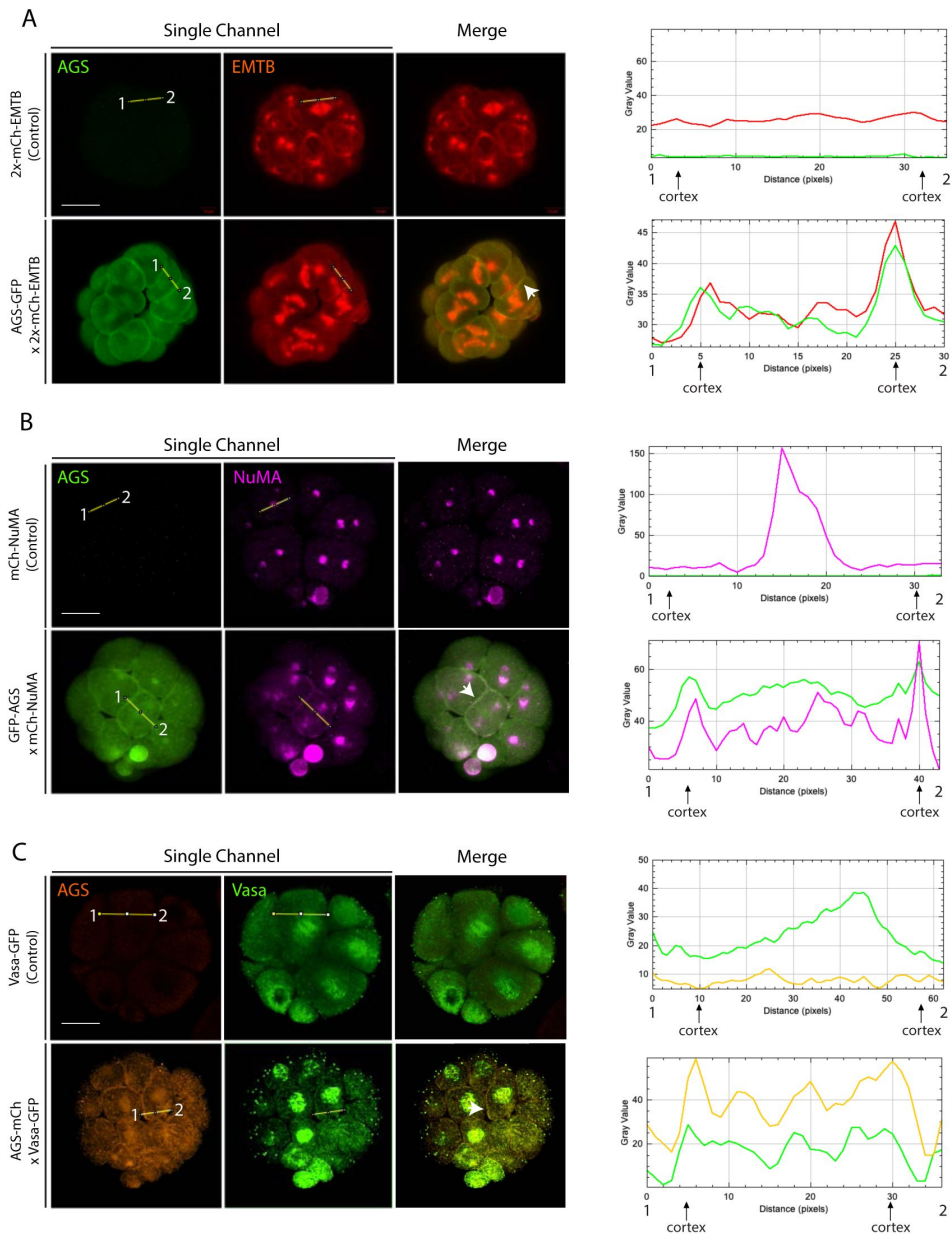
Sea urchin *S. purpuratus* (SpAGS_GL), *Drosophila* (DmPins_GL), sea star *P. miniata* (PmAGS_GL), pencil urchin *E. tribuloides* (EtAGS_GL), human *H. sapiens* LGN (HsLGN_GL) and human *H. sapiens* AGS3 (HsAGS3_GL). Bold letters indicate GoLoco motif sequences. The green highlight indicates additional serine amino acid present uniquely in HsAGS3_GL and mutated to Alanine in AGS_AG3GL_3S/A mutant. The highlighted amino acids between GL2 and GL2 and within GL3 are those mutated to match HsLGN_GL in AGS_AG3GL_GL2GL3 mutant.



Supplemental Figure 6.

Insc protein expression during embryonic development.

A, Endogenous Insc protein localization by immunofluorescence. Embryos were stained with three Insc antibodies (green) designed for different Insc amino acid sequence sections. Embryos were stained with Gai antibody (magenta) and Hoechst dye (blue). During the 16-cell stage, all antibodies show signal enriched at the vegetal pole. With #2 and #3 antibodies, some non-specific cortex signals were also observed around the entire embryo. **B**, Insc immunoblot analysis. Embryos were collected at 0, 2, 4.5, 15, 24, 48, 72, and 96 hours post fertilization and subjected to immunoblot with Insc #1 antibody. Actin (42kDa) was used as a loading control. The expected size of Insc is 53kDa. **C**, Peptide competition assay with Insc #1 antibody. The 24-hour lysate was used. Each experiment was performed at least two independent times. Error bars represent standard error. Scale bars=10µm.



Supplemental Figure 7.

SpAGS colocalizes with micromere-specific fate determinants.

A, Embryos were co-injected with 2x-mCherry-EMTB (0.5µg/µl stock) mRNA with or without GFP-AGS (0.5µg/µl stock) mRNA. **B**, Embryos were co-injected with mCherry-NuMA (0.15µg/ul) mRNA with or without GFP-AGS (0.5µg/µl stock). **C**, Embryos were co-injected with Vasa-GFP (1µg/µl stock) mRNA with or without AGS-mCherry (0.5µg/µl stock) mRNA. The intensity of each signal, from one cortex to the other, was measured and plotted from point 1 to 2 on the corresponding graph (right) using *ImageJ*. White arrows indicate the cortical colocalization of each construct. All images represent over 50% of the embryos observed (n=30 or larger) per group. Scale bars=20µm.

SpDlg	MNNIEEWEYEDIIILNRGGAGLGFSIAGGVDPNPHVPDDPSIFITKLI PGGAAAVGGRLLRN	0
PmDlg		60
SpDlg	-----MVLRRWERNPSTGKTLSIELIKSGKGLGFS	30
PmDlg	DVIIRVNE TEIHNV PHQNAVDALKKTGSIVNLVV RRRRQRRTGD VTRIKL VKGT KGLGFS :: * *: **: . : *:*:*:*****	120
SpDlg	IAGQGNGHVPGDNIGFVTKIIDGGAAEQDRLQVGDKITAVGDNNLVEVTHEDAVAVLK	90
PmDlg	IAGCGGNQHVGADNGIFVTKIIDGGAAQMDGNLQIGDKIIAVGNDKLEEVTHENAVAVLK **** *****: *:*.:** **:.: * *****:	180
SpDlg	ATSNRVLLTVIKNATLLAGTQMAYPSQNDENAQPPDVSVNVDMSAANFVYAT-----	141
PmDlg	ATSEVLLTVIKGAVISYPSPASI-----TSQPDVSTMQPSSPPPPYPEAIVSSVAGV ***: *****,.: : * .. ***.: *: *	234
SpDlg	--MQPTLMQEPRSDQLQELDGAFGTNAVFGTNVANANGANGSNGEPSPYNRVNTNSQPP	198
PmDlg	SLQSEPAL--QRPTTPHQEMSNMMNPPLYTHQPPQDTAYTGM-----KKMHVFPSP *: * *: .. : : : : :	284
SpDlg	NAFNNTPAKFIQNDGIPREPRQVVLNKGATGLGFNI VGGEDGE GEFISFILAGGVAD	258
PmDlg	TANYEGNGVKPYQNDDFPREARTVVLNKGATGLGFNI VGGEDGE GEFISFILAGGVAD .* : . * :.***:*** * *****	344
SpDlg	LSGALRGDQILAVNSKDVLNATHEDAALALKAGQVVTIDAQYKP EYNCFEAKIQSLR	318
PmDlg	LSGELKRGDQILSVNKKDLRGATHEDAALALKGTGQTVL IEAQYKEE YNRF EAKIQSLR *** * ,*****:*,** * ,*****:*,* * ,***** *****	404
SpDlg	EEMMNQSVS--PTGSLRTSAKRTLFVRTLFYDKSRDSGLPSQGLSFDFGDIHVTNASD	376
PmDlg	EEMNNSVSSTTTGSLRTSAKRSLYVRALFDYDKTKDSGLPSQGLSFNYGDI LHV TNASD ***:,* *****:*,*:*****:;*****:;*,*:*****	464
SpDlg	DEWMQARHILENGEEGEIGIIPSKRRVERKEKARLR SVKFSGNKDRNNIQEPAGTVNSKG	436
PmDlg	DEWMQAQKVLPTEEGEGMVIPSKRRVERKEREARLKS VKFSGRGGS---LESKGS MNEKR *****:;*,* *****:*,*****:;*,*:*****. * *:;*,*	521
SpDlg	KKGFF-RKFKNRSDSQETS DPEFTSNASDES SYRNEEVL S YEAVVQQEIKYTRFVI	495
PmDlg	KKGFFSRKFKKGRESLDLTSDAEHITSNASDES SYRNDEMILSYEGVVQQEVKYTRFVI ***** *****:*,* :*** * :*****:*,*:*****.***:*****	581
SpDlg	ILGP GKDRINDDLISEMPDEF GSCVPHT TRARPHEV DGRDYHFVESRQMEKDI QNHLE	555
PmDlg	ILSGSKDRVNDLISEMPEKFGSCVPHTTRARPHEV DKRDYHFVESRQMEKDIQDHLE *** *****:***** ***** *****:***	641
SpDlg	IEAGQYNNENLYGTSVASVKEVSEK GKHC ILDVD SGNAIKRLQVAQLPIAFLFKPSVESI	615
PmDlg	IEAGQYNNENLYGTSVASVREVA DGKGC ILDVD SGNAIRRLQVANLHPAIFIRPKPSVESV *****:*,* :*****:*****:*****:*,*:*****:*****:	701
SpDlg	VEMNRVSEDQARKTYERALKLEQEFLEYFTAIL LGDTIDDIYQQSKEILHQSGETIWI	675
PmDlg	VEMNRMRSEDAEKTYERA TKLEIEGFEFFTATISGDTLDEIVYCKEVIHDQSGSTIWV :*****:*,* **,*****:*** * * ***** *:*,* :*,*:*** **:	761
SpDlg	PAKEKL 681	
PmDlg	PAKDKL 767 ***:,**	

34 of 46

Supplemental Figure 9 Sea urchin SpInsc, pencil urchin EtInsc and sea star PmInsc sequence alignment

PmInsc	-----MNGFNES-ALAGWLADDVIEMHMTDEDSVVRWLQDLRTMTTECECMCI LQG	49
SpInsc	-----MSDN---KNTESFP-EKKLMQRCDVDSVQQWLIDIRLMPEQECTTVMET	45
EtInsc	MTCLYGVVRTMYGPQAPVICEEKLPTSTSRFQLTEVRSVQQWLSDLQGMMECECN-ILET	59
	* . : . : : ** : ** * : * * * * : : :	
PmInsc	KPIRTSAHDMAKVTSSARDNIYILRQRASSISADFAKLYYKLDSGSWDQVH-WLCLSLTI	108
SpInsc	KPIHNSPLNLENLAATVKESVYRLQSIKDGLFSDLQIYMCIDLSDQ-----	92
EtInsc	KPIHNSPLNLENVAATKENIYRLRNRAENLARELQQLYHKLTSGTYSQRLRPVLAITL	119
	***. * : : : : : : * * . . : : : * * .	
PmInsc	RLRTFLHECTSYVPTMPLHLFQRQQTVLEECGRLVKHAESFEELNGTLPPALPTLNVLS	168
SpInsc	-----SVK--LFIQSLTT	103
EtInsc	HVRSCLEECNEYCPDF-LHMKEKQQVVIDAMATLVKNFEMYDNSKGCELD--SLIHSITS	176
	: : * : :	
PmInsc	LGKAFNDLVHMQGILIQTVVRSIHESASEYNIKIAINS IHCLSEDDSEIRLLAKEGAI	228
SpInsc	VAKAIKAYLEMVQGALVRKLVDMVQGAATELNVKFAVLTVHSLAQDGVWLRRLIQEDTM	163
EtInsc	LGNALNSIRQTVQSALVRKLVMMIQDSASELNVKFAVITAHSLSQDSIWLRLRIQENAM	236
	: : * : : . ** . * : : * : : * * * : : * . * : : . * : : * : : :	
PmInsc	QALLQLCQQASL--NAMKPMALKTVANLCNVAEGRQELERASGVEYLSEILSSPTATESV	286
SpInsc	RALLAICRITTFIRSIEPTALEIVSILCTEVEGRAELEKVGVGVALSEILASPTILEAV	223
EtInsc	GALFAVCRIITLSIPSIVTTALQAISTLCAEPEGRVELEKIGGLGFLGEILSSPSTSEAV	296
	* : : * : : : : * : : * * * * * : * : * * * * * : * : *	
PmInsc	KAEAA SVIAQITSPNIDNYHLLGFIEYMEDLLKDLTALSGSASSSVFVIATSAIANIT	346
SpInsc	KKEAASVLAEITSPDADIFHRMLCFIEHMEDLLRNLTVLCDQTSSEPGVFLVATTAIANIT	283
EtInsc	KLEAASVLAEITSPDINSYHRMLCFIEHMEDLLKNLTALCDQTSNPLVFLVSASAIANIT	356
	* * * * * : * * * * : : * : * * * * * : * * * . * : : : * * * * *	
PmInsc	FMDSMACDCLASFHTARALVKGCCNNGMANTLYCKDQVATILANMAVTDQCRKEIMVSGGT	406
SpInsc	FMDAMASDYLAEFQTAEVLIQGYLKGKAPSIYSKHQVITIIANISCSKSCREQIVSSGGM	343
EtInsc	FMDTMSSDFLAMENTATILIDGYTSGKAQNMYSKDQVVTIIANMSGSEKCREQIIKAGGM	416
	*** : . * * * * * * : . * . * * . : * . * * * * : : . * * : : * : *	
PmInsc	ELLVDFLHTRALVHPSSAK---KGLKGSGLSGGCSSGGKQDVVMSSSCEKVHQKAAIALA	463
SpInsc	NILAE LLQGVPTSRPTSLSRKLSFDGN-----VSLGQMGEESAYEDIYQKAATALG	395
EtInsc	KVLVELLHTRAPFTTVKTNGVN---QRA-----DSKAACGMASCTCEHVHQSAIALG	465
	: * : : * : : : : . . . * : * : : * * * * *	
PmInsc	RLARDQDSAEILELDAIPRLVELCCNRRENDSEFVLVACLAALRKLNLLGPSVFKSS	523
SpInsc	RLCQDYETCKMAVDLKVVSRLADLCKHARERNHRSDVLVACLAALRRIHSHIGSSPLKEN	455
EtInsc	RLCQDHD TAMMAVDLGVIPRLVDMCRHRRERNDSEFVLVACLAALRKLSVVGASKFEPK	525
	* * . * : . : : * . : * . : * * . * * * * * : * . * * : .	
PmInsc	DVDQLIKPRLIDSYLMCSATK--ESFV	548
SpInsc	DVEQLIQPRLMESFLHCSTTVRRETFV	482
EtInsc	ALQQLIRPRLIESFVTCSSIGSTETFV	552
	: * * * : * * * : * : * : * * : * * *	

LBD (LGN-binding domain)

Supplemental Figure 9.

Sea urchin (SpInsc), pencil urchin (EtInsc), and sea star (PmInsc) sequence alignment.

The blue highlight indicates the LBD domain.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-SpAGS	Voronina & Wessel, 2006	N/A
Anti-Gai	Santa Cruz Biotech	# sc-56536
Anti- β -catenin	Yazaki et al., 2015	N/A
Anti-SpInsc	This article	N/A
Anti-SpNuMA	Poon et al., 2019	N/A
Anti- β -actin (8H10D10)	Cell Signaling Technology	# 3700S
Anti-Flag	Millipore-Sigma	# F1804
Alexa 488-conjugated goat anti-mouse IgG	Cell Signaling Technology	# 4412
Alexa 555-conjugated goat anti-mouse IgG	Cell Signaling Technology	# 4409
HRP-conjugated anti-Protein A antibody	Abcam	# ab7245
HRP-conjugated goat anti-mouse IgG	Cell Signaling Technology	# 7076
Anti-Digoxigenin-AP, Fab fragments	Roche	# 11093274910
Chemicals		
Hoechst 33342	Thermo Fisher Scientific	# 62249
DTT	Millipore-Sigma	# 10197777001
Tris buffered saline, with tween (TBST)	Millipore-Sigma	# T9039
Tris-MOPS-SDS Running Buffer	Genscript	# M00138
Transfer buffer powder	Genscript	# M00139
DIG RNA labeling mix	Roche	#11277073910
Critical Commercial Assays		
mMESSAGE mMACHINE SP6 Transcription Kit	Thermo Fisher Scientific	# AM1340
MEGAscript SP6 Transcription kit	Thermo Fisher Scientific	# AM1330
MEGAscript T7 Transcription kit	Thermo Fisher Scientific	# AM1333
In-Fusion HD Cloning	Clontech	# 639648

Supplementary Table 1.

A key resource table

Duolink [®] In Situ Red Starter Kit Mouse/Rabbit	Millipore-Sigma	# DUO92101
Recombinant DNA		
Plasmid: SpAGS-GFP	Poon et al., 2019	N/A
Plasmids: SpAGS-dC-term-GFP, SpAGS-dGL1/2/3/4-GFP, SpAGS1111/2222/2134/4234-GFP	This article	N/A
Plasmids: SpAGS-dN-term-GFP	This article	N/A
Plasmid: SpAGS-mCherry	Poon et al., 2019	N/A
Plasmid: GFP-SpAGS, GFP-EtAGS, GFP-PmAGS	This article	N/A
Plasmid: GFP-SpAGS4444/GL1GL2/LGNGL/DmGL/EtGL/AGS3GL/PmGL/S389A/AGS3GL-3S/A/AGS3GL-GL2GL3	This article	N/A
Plasmid: GFP-PmAGS-SpLinker	This article	N/A
Plasmid: GFP-SpDlg/PmDlg	This article	N/A
Plasmid: GFP-SpInsc/EtInsc/PmInsc	This article	N/A
Plasmid: GFP-NuMA	This article	N/A
Plasmid: mCherry-NuMA	This article	N/A
Plasmid: GFP-Par3	This article	N/A
Plasmid: Vasa-GFP	Yajima & Wessel, 2011	N/A
Plasmid: Vasa-mCherry	Uchida & Yajima, 2018	N/A
Plasmid : 3xFlag-GFP-SpAGS/SpDlg/SpNuMA, 3xFlag-Vasa-GFP	This article	N/A
Plasmid: 2x-mCherry-EMTB	Addgene	# 26742
Software and Algorithms		
Echinoderm protein sequences	EchinoBase	http://www.echinobase.org/Echinobase/
Protein motif search	NCBI blast	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Protein sequence alignment	Clustal Omega	https://www.ebi.ac.uk/Tools/msa/clustalo/
Quantitative analysis	Image J	https://imagej.nih.gov/ij/
Statistical analysis	Graphpad PRISM 8	https://www.graphpad.com/scientific-software/prism/
Morpholino antisense oligos (Gene Tools: http://www.gene-tools.com/)		
NCBI reference sequence	Name	Sequence
NM_001040405.1	SpAGS	GGCCCGTTTCACAAAGCCTTTGTTT
Primers for ISH probes		
NCBI reference sequence / Source	Name	Sequence

Supplementary Table 1. (continued)

XM_011663478.2	SpAlx1	F: GGATATTTTCTCGACCGGGATC R: CGAGTAACCGTTCATCATCCCC
NM_214574.3	SpBlimp1b	F: ATGGGGTGCAACGACAACGCCGTG R: CTATGATTTGTTTCGTACGATTGAG
NM_214519.1	SpEndo16	F: GCAGAGTTCAACAGAATCGAC R: GCCAGTAGACGTAGCAGAAG
XM_030976919.1	SpEts1	F: TCAATCATGGCGTCTATGCACTG R: ACAGCTGCAGGGATAACAGG
NM_001079542.1	SpFoxA	F: ATGGCCAATAGTGCCATGATCTCG R: TCACATTGCATGGTTTGCTTG
XM_003731512.3	SpFoxQ2	F: ATGACTTTATTTCAGCATTGACAAC R: TAGCAGGATCCTACAGAAGACCAG
NM_214610.3	SpSm50	F: ATGAAGGGAGTTTGTATTATTGTGG CTAGTC R: GTTATGCCAACGCGTCTGCCTCTTG AAGC
XM_786173.5	SpTbr1	F: CCACCGCTGCACCAGACGAC R: CTGCCGGCTGGCGCCAATTGCG
NM_214667.1	SpWnt8	F: ATGGATGTTTTTACGGAATTTGTTTCG R: CTACAGCCTCGATCCAACGGGCTG

Supplementary Table 1. (continued)

References

1. Bate C. M., Jacobson M (1978) **Development of Sensory Systems in Arthropods** *Handbook of Sensory Physiology* :1–53 https://doi.org/10.1007/978-3-642-66880-7_1
2. Bernard M. L., Peterson Y. K., Chung P., Jourdan J., Lanier S. M (2001) **Selective interaction of AGS3 with G-proteins and the influence of AGS3 on the activation state of G-proteins** *J Biol Chem* **276**:1585–1593 <https://doi.org/10.1074/jbc.M005291200>
3. Bowman S. K., Neumuller R. A., Novatchkova M., Du Q., Knoblich J. A (2006) **The Drosophila NuMA Homolog Mud regulates spindle orientation in asymmetric cell division** *Dev Cell* **10**:731–742 <https://doi.org/10.1016/j.devcel.2006.05.005>
4. Chenn A., McConnell S. K (1995) **Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis** *Cell* **82**:631–641 [https://doi.org/10.1016/0092-8674\(95\)90035-7](https://doi.org/10.1016/0092-8674(95)90035-7)
5. Culurgioni S., Alfieri A., Pendolino V., Laddomada F., Mapelli M (2011) **Inscuteable and NuMA proteins bind competitively to Leu-Gly-Asn repeat-enriched protein (LGN) during asymmetric cell divisions** *Proc Natl Acad Sci U S A* **108**:20998–21003 <https://doi.org/10.1073/pnas.1113077108>
6. Culurgioni S., Mari S., Bonetti P., Gallini S., Bonetto G., Brennich M., Round A., Nicassio F., Mapelli M (2018) **Insc:LGN tetramers promote asymmetric divisions of mammary stem cells** *Nat Commun* **9** <https://doi.org/10.1038/s41467-018-03343-4>
7. di Pietro F., Echard A., Morin X. (2016) **Regulation of mitotic spindle orientation: an integrated view** *EMBO Rep* **17**:1106–1130 <https://doi.org/10.15252/embr.201642292>
8. Doe C. Q (2008) **Neural stem cells: balancing self-renewal with differentiation** *Development* **135**:1575–1587 <https://doi.org/10.1242/dev.014977>
9. Doe C. Q., Hiromi Y., Gehring W. J., Goodman C. S (1988) **Expression and function of the segmentation gene fushi tarazu during Drosophila neurogenesis** *Science* **239**:170–175 <https://doi.org/10.1126/science.2892267>
10. Du Q., Macara I. G (2004) **Mammalian Pins is a conformational switch that links NuMA to heterotrimeric G proteins** *Cell* **119**:503–516 <https://doi.org/10.1016/j.cell.2004.10.028>
11. Erkenbrack E. M (2016) **Divergence of ectodermal and mesodermal gene regulatory network linkages in early development of sea urchins** *Proc Natl Acad Sci U S A* **113**:E7202–E7211 <https://doi.org/10.1073/pnas.1612820113>
12. Erkenbrack E. M., Davidson E. H (2015) **Evolutionary rewiring of gene regulatory network linkages at divergence of the echinoid subclasses** *Proc Natl Acad Sci U S A* **112**:E4075–4084 <https://doi.org/10.1073/pnas.1509845112>
13. Erkenbrack E. M., Davidson E. H., Peter I. S (2018) **Conserved regulatory state expression controlled by divergent developmental gene regulatory networks in echinoids** *Development* **145** <https://doi.org/10.1242/dev.167288>

14. Fernandez-Nicolas A., Uchida A., Poon J., Yajima M (2022) **Vasa nucleates asymmetric translation along the mitotic spindle during unequal cell divisions** *Nat Commun* **13** <https://doi.org/10.1038/s41467-022-29855-8>
15. Gonczy P (2008) **Mechanisms of asymmetric cell division: flies and worms pave the way** *Nat Rev Mol Cell Biol* **9**:355–366 <https://doi.org/10.1038/nrm2388>
16. Hartenstein V., Campos-Ortega J. A (1984) **Early neurogenesis in wild-type *Drosophila melanogaster*** *Wilehm Roux Arch Dev Biol* **193**:308–325 <https://doi.org/10.1007/bf00848159>
17. Haydar T. F., Ang E., Rakic P (2003) **Mitotic spindle rotation and mode of cell division in the developing telencephalon** *Proc Natl Acad Sci U S A* **100**:2890–2895 <https://doi.org/10.1073/pnas.0437969100>
18. Horstadius S (1928) **Über die determination des keimes bei echinodermen** *Acta Zoologica* **9**:1–191 <https://doi.org/10.1111/j.1463-6395.1928.tb01165.x>
19. Izumi Y., Ohta N., Hisata K., Raabe T., Matsuzaki F (2006) ***Drosophila* Pins-binding protein Mud regulates spindle-polarity coupling and centrosome organization** *Nat Cell Biol* **8**:586–593 <https://doi.org/10.1038/ncb1409>
20. Johnston C. A., Hirono K., Prehoda K. E., Doe C. Q (2009) **Identification of an Aurora-A/PinsLINKER/Dlg spindle orientation pathway using induced cell polarity in S2 cells** *Cell* **138**:1150–1163 <https://doi.org/10.1016/j.cell.2009.07.041>
21. Juliano C. E., Wessel G. M (2009) **An evolutionary transition of Vasa regulation in echinoderms** *Evol Dev* **11**:560–573 <https://doi.org/10.1111/j.1525-142X.2009.00362.x>
22. Konno D., Shioi G., Shitamukai A., Mori A., Kiyonari H., Miyata T., Matsuzaki F (2008) **Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis** *Nat Cell Biol* **10**:93–101 <https://doi.org/10.1038/ncb1673>
23. Kotak S (2019) **Mechanisms of Spindle Positioning: Lessons from Worms and Mammalian Cells** *Biomolecules* **9** <https://doi.org/10.3390/biom9020080>
24. Logan C. Y., Miller J. R., Ferkowicz M. J., McClay D. R (1999) **Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo** *Development* **126**:345–357
25. Miller A. L., Bement W. M (2009) **Regulation of cytokinesis by Rho GTPase flux** *Nat Cell Biol* **11**:71–77 <https://doi.org/10.1038/ncb1814>
26. Minokawa T., Rast J. P., Arenas-Mena C., Franco C. B., Davidson E. H (2004) **Expression patterns of four different regulatory genes that function during sea urchin development** *Gene Expr Patterns* **4**:449–456 <https://doi.org/10.1016/j.modgep.2004.01.009>
27. Nipper R. W., Siller K. H., Smith N. R., Doe C. Q., Prehoda K. E (2007) **Galpai generates multiple Pins activation states to link cortical polarity and spindle orientation in *Drosophila* neuroblasts** *Proc Natl Acad Sci U S A* **104**:14306–14311 <https://doi.org/10.1073/pnas.0701812104>
28. Noctor S. C., Martinez-Cerdeno V., Ivic L., Kriegstein A. R (2004) **Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases** *Nat Neurosci* **7**:136–144 <https://doi.org/10.1038/nn1172>

29. Okazaki K (1975) **Spicule formation by isolated micromeres of the sea urchin embryo** *Amer Zool* **15**:567–581
30. Pan Z., Zhu J., Shang Y., Wei Z., Jia M., Xia C., Wen W., Wang W., Zhang M (2013) **An autoinhibited conformation of LGN reveals a distinct interaction mode between GoLoco motifs and TPR motifs** *Structure* **21**:1007–1017 <https://doi.org/10.1016/j.str.2013.04.005>
31. Parmentier M. L., Woods D., Greig S., Phan P. G., Radovic A., Bryant P., O’Kane C. J. (2000) **Rapsynoid/partner of inscuteable controls asymmetric division of larval neuroblasts in Drosophila** *J Neurosci* **20** <https://doi.org/10.1523/JNEUROSCI.20-14-j0003.2000>
32. Perillo M., Paganos P., Spurrell M., Arnone M. I., Wessel G. M (2021) **Methodology for Whole Mount and Fluorescent RNA In Situ Hybridization in Echinoderms: Single, Double, and Beyond** *Methods Mol Biol* **2219**:195–216 https://doi.org/10.1007/978-1-0716-0974-3_12
33. Poon J., Fries A., Wessel G. M., Yajima M (2019) **Evolutionary modification of AGS protein contributes to formation of micromeres in sea urchins** *Nat Commun* **10** <https://doi.org/10.1038/s41467-019-11560-8>
34. Ransick A., Davidson E. H (1993) **A complete second gut induced by transplanted micromeres in the sea urchin embryo** *Science* **259**:1134–1138 <https://doi.org/10.1126/science.8438164>
35. Rose L., Gönczy P (2014) **Polarity establishment, asymmetric division and segregation of fate determinants in early C. elegans embryos** *WormBook* :1–43 <https://doi.org/10.1895/wormbook.1.30.2>
36. Saadaoui M., Konno D., Loulier K., Goïame R., Jadhav V., Mapelli M., Matsuzaki F., Morin X (2017) **Loss of the canonical spindle orientation function in the Pins/LGN homolog AGS3** *EMBO Rep* **18**:1509–1520 <https://doi.org/10.15252/embr.201643048>
37. Schaefer M., Petronczki M., Dorner D., Forte M., Knoblich J. A (2001) **Heterotrimeric G proteins direct two modes of asymmetric cell division in the Drosophila nervous system** *Cell* **107**:183–194 [https://doi.org/10.1016/s0092-8674\(01\)00521-9](https://doi.org/10.1016/s0092-8674(01)00521-9)
38. Schaefer M., Shevchenko A., Knoblich J. A (2000) **A protein complex containing Inscuteable and the Galpha-binding protein Pins orients asymmetric cell divisions in Drosophila** *Curr Biol* **10**:353–362 [https://doi.org/10.1016/s0960-9822\(00\)00401-2](https://doi.org/10.1016/s0960-9822(00)00401-2)
39. Schnabel R., Weigner C., Hutter H., Feichtinger R., Schnabel H (1996) **mex-1 and the general partitioning of cell fate in the early C. elegans embryo** *Mech Dev* **54**:133–147 [https://doi.org/10.1016/0925-4773\(95\)00466-1](https://doi.org/10.1016/0925-4773(95)00466-1)
40. Schober M., Schaefer M., Knoblich J. A (1999) **Bazooka recruits Inscuteable to orient asymmetric cell divisions in Drosophila neuroblasts** *Nature* **402**:548–551 <https://doi.org/10.1038/990135>
41. Siegrist S. E., Doe C. Q (2005) **Microtubule-induced Pins/Galphai cortical polarity in Drosophila neuroblasts** *Cell* **123**:1323–1335 <https://doi.org/10.1016/j.cell.2005.09.043>

42. Siller K. H., Cabernard C., Doe C. Q (2006) **The NuMA-related Mud protein binds Pins and regulates spindle orientation in Drosophila neuroblasts** *Nat Cell Biol* **8**:594–600 <https://doi.org/10.1038/ncb1412>
43. Siller K. H., Doe C. Q (2009) **Spindle orientation during asymmetric cell division** *Nat Cell Biol* **11**:365–374 <https://doi.org/10.1038/ncb0409-365>
44. Smith N. R., Prehoda K. E (2011) **Robust spindle alignment in Drosophila neuroblasts by ultrasensitive activation of pins** *Mol Cell* **43**:540–549 <https://doi.org/10.1016/j.molcel.2011.06.030>
45. Sulston J. E., Schierenberg E., White J. G., Thomson J. N (1983) **The embryonic cell lineage of the nematode *Caenorhabditis elegans*** *Dev Biol* **100**:64–119 [https://doi.org/10.1016/0012-1606\(83\)90201-4](https://doi.org/10.1016/0012-1606(83)90201-4)
46. Takayanagi H., Hayase J., Kamakura S., Miyano K., Chishiki K., Yuzawa S., Sumimoto H (2019) **Intramolecular interaction in LGN, an adaptor protein that regulates mitotic spindle orientation** *J Biol Chem* **294**:19655–19666 <https://doi.org/10.1074/jbc.RA119.011457>
47. Uchida A., Yajima M (2018) **An optogenetic approach to control protein localization during embryogenesis of the sea urchin** *Dev Biol* **441**:19–30 <https://doi.org/10.1016/j.ydbio.2018.06.015>
48. Voronina E., Wessel G. M (2006) **Activator of G-protein signaling in asymmetric cell divisions of the sea urchin embryo** *Dev Growth Differ* **48**:549–557 <https://doi.org/10.1111/j.1440-169X.2006.00895.x>
49. Voronina E., Lopez M., Juliano C. E., Gustafson E., Song J. L., Extavour C., George S., Oliveri P., McClay D., Wessel G (2008) **Vasa protein expression is restricted to the small micromeres of the sea urchin, but is inducible in other lineages early in development** *Dev Biol* **314**:276–286 <https://doi.org/10.1016/j.ydbio.2007.11.039>
50. Wang C. *et al.* (2011) **An ana2/ctp/mud complex regulates spindle orientation in Drosophila neuroblasts** *Dev Cell* **21**:520–533 <https://doi.org/10.1016/j.devcel.2011.08.002>
51. Watts J. L., Etemad-Moghadam B., Guo S., Boyd L., Draper B. W., Mello C. C., Priess J. R., Kemphues K. J (1996) **par-6, a gene involved in the establishment of asymmetry in early C. elegans embryos, mediates the asymmetric localization of PAR-3** *Development* **122**:3133–3140
52. Wavreil F. D. M., Yajima M (2020) **Diversity of activator of G-protein signaling (AGS)-family proteins and their impact on asymmetric cell division across taxa** *Dev Biol* **465**:89–99 <https://doi.org/10.1016/j.ydbio.2020.07.004>
53. Willard F. S., Kimple R. J., Siderovski D. P (2004) **Return of the GDI: the GoLoco motif in cell division** *Annu Rev Biochem* **73**:925–951 <https://doi.org/10.1146/annurev.biochem.73.011303.073756>
54. Williams S. E., Ratliff L. A., Postiglione M. P., Knoblich J. A., Fuchs E. (2014) **Par3-mInsc and Galphai3 cooperate to promote oriented epidermal cell divisions through LGN** *Nat Cell Biol* **16**:758–769 <https://doi.org/10.1038/ncb3001>

55. Wodarz A., Ramrath A., Grimm A., Knust E (2000) **Drosophila atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts** *J Cell Biol* **150**:1361–1374 <https://doi.org/10.1083/jcb.150.6.1361>
56. Yajima M., Wessel G. M (2011) **Small micromeres contribute to the germline in the sea urchin** *Development* **138**:237–243 <https://doi.org/10.1242/dev.054940>
57. Yajima M., Wessel G. M (2015) **Essential elements for translation: the germline factor Vasa functions broadly in somatic cells** *Development* **142**:1960–1970 <https://doi.org/10.1242/dev.118448>
58. Yu F., Kuo C. T., Jan Y. N (2006) **Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology** *Neuron* **51**:13–20 <https://doi.org/10.1016/j.neuron.2006.06.016>
59. Yu F., Morin X., Cai Y., Yang X., Chia W (2000) **Analysis of partner of inscuteable, a novel player of Drosophila asymmetric divisions, reveals two distinct steps in inscuteable apical localization** *Cell* **100**:399–409 [https://doi.org/10.1016/S0092-8674\(00\)80676-5](https://doi.org/10.1016/S0092-8674(00)80676-5)
60. Yu F., Morin X., Kaushik R., Bahri S., Yang X., Chia W (2003) **A mouse homologue of Drosophila pins can asymmetrically localize and substitute for pins function in Drosophila neuroblasts** *J Cell Sci* **116**:887–896 <https://doi.org/10.1242/jcs.00297>
61. Yuzawa S., Kamakura S., Iwakiri Y., Hayase J., Sumimoto H (2011) **Structural basis for interaction between the conserved cell polarity proteins Inscuteable and Leu-Gly-Asn repeat-enriched protein (LGN)** *Proc Natl Acad Sci U S A* **108**:19210–19215 <https://doi.org/10.1073/pnas.1110951108>
62. Zhu J., Wen W., Zheng Z., Shang Y., Wei Z., Xiao Z., Pan Z., Du Q., Wang W., Zhang M (2011) **LGN/mInsc and LGN/NuMA complex structures suggest distinct functions in asymmetric cell division for the Par3/mInsc/LGN and Galphai/LGN/NuMA pathways** *Mol Cell* **43**:418–431 <https://doi.org/10.1016/j.molcel.2011.07.011>
63. Zhu J., Wen W., Zheng Z., Shang Y., Wei Z., Xiao Z., Pan Z., Du Q., Wang W., Zhang M (2011) **LGN/mInsc and LGN/NuMA complex structures suggest distinct functions in asymmetric cell division for the Par3/mInsc/LGN and Gai/LGN/NuMA pathways** *Mol Cell* **43**:418–431 <https://doi.org/10.1016/j.molcel.2011.07.011>

Editors

Reviewing Editor

Sarah Russell

Peter MacCallum Cancer Centre, East Melbourne, Australia

Senior Editor

Claude Desplan

New York University, New York, United States of America

Reviewer #1 (Public Review):

Summary:

Previous work has shown that the evolutionarily-conserved division-orienting protein LGN/Pins (vertebrates/fly) participates in division orientation across a variety of cell types, perhaps most importantly those that undergo asymmetric divisions. Micromere formation in echinoids relies on asymmetric cell division at the 16-cell stage, and these authors previously demonstrated a role for the LGN/Pins homolog AGS in that ACD process. Here they extend that work by investigating and exploiting the question of why echinoids but not other echinoderms form micromeres. Starting with a phylogenetics approach, they determine that much of the difference in ACD and micromere formation in echinoids can be attributed to differences in the AGS C-terminus, in particular a GoLoco domain (GL1) that is missing in most other echinoderms.

Strengths:

There is a lot to like about this paper. It represents a superlative match of the problem with the model system and the findings it reports are a valuable addition to the literature. It is also an impressively thorough study; the authors should be commended for using a combination of experimental approaches (and consequently generating a mountain of data).

Weaknesses:

There is an intriguing finding described in Figure 1. AGS in sea cucumbers looks identical to AGS in the pencil urchin, at least at the C terminus (including the GL1 domain). Nevertheless, there are no micromeres in sea cucumbers. Therefore another mechanism besides GL motif organization has arisen to support micromere formation. It is a consequential finding and an important consideration in interpreting the data, but I could not find any mention of it in the text. That is a missed opportunity and should be remedied, ideally not only through discussion but also experimentation. Specifically: does sea cucumber AGS (SbAGS) ever localize to the vegetal cortex in sea cucumbers? Can it do so in echinoids? Will that support micromere formation?

The authors point out that AGS-PmGL demonstrates enrichment at the vegetal cortex (arrow in 5G, quantifications in 5H), unlike PmAGS. AGS-PmGL does not however support ACD. They interpret this result to indicate "that other elements of SpAGS outside of its C-terminus can drive its vegetal cortical localization but not function." This is a critical finding and deserves more attention. Put succinctly: Vegetal cortical localization of AGS is insufficient to promote ACD, even in echinoids. Why should this be?

The authors did perform experiments to address this problem, hypothesizing that the difference might be explained by the linker region, which includes a conserved phosphorylation site that mediates binding to Dlg. They write "To test if this serine is essential for SpAGS localization, we mutated it to alanine (AGS-S389A in Fig. S3A). Compared to the Full AGS control, the mutant AGS-S389A showed reduced vegetal cortical localization (Fig. S3B-C) and function (Fig. S3D-E). Furthermore, we replaced the linker region of PmAGS with that of SpAGS (PmAGS-SpLinker in Fig. S4A-B). However, this mutant did not show any cortical localization nor proper function in ACD (Fig. S4C-F). Therefore, the SpAGS C-terminus is the primary element that drives ACD, while the linker region serves as the secondary element to help cortical localization of AGS."

The experiments performed only make sense if the AGS-PmGL chimeric protein used in Figure 5 starts the PmGL sequence only after the Sp linker, or at least after the Sp phosphorylation site. I can't tell from the paper (Figure S3 indicates that it does, whereas S5 suggests otherwise), but it's a critical piece of information for the argument. Another piece of missing information is whether the PmAGS can be phosphorylated at its own conserved phosphorylation site. The authors don't test this, which they could at least try using a phosphosite prediction algorithm, but they do show that the candidate phosphorylation site has a slightly different sequence in Pm than in Et and Sp (Fig. S4A). With impressive rigor, the

authors go on to mutate the PmAGS phosphorylation site to make it identical to Sp. Nothing happens. Vegetal cortical localization does not increase over AGS-PmGL alone. Micromere formation is unrescued.

There is therefore a logic problem in the text, or at least in the way the text is written. The paragraph begins "Additionally, AGS-PmGL unexpectedly showed cortical localization (Figure 5G), while PmAGS showed no cortical localization (Figure 5B)." We want to understand why this is true, but the explanation provided in the remainder of the paragraph doesn't match the question: according to quite a bit of their own data, the phosphorylation site in the linker does not explain the difference. It might explain why AGS-PmGL fails to promote micromere formation, but only if the AGS-PmGL chimeric protein uses the Pm linker domain (see above).

Another concern that is potentially related is the measurement of cortical signal. For example, in the control panel of Figure 5C, there is certainly a substantial amount of "non-cortical" signal that I believe is nuclear. I did not see a discussion of this signal or its implications. My impression of the pictures generally is that the nuclear signal and cortical signal are inversely correlated, which makes sense if they are derived from the same pool of total protein at different points of the cell cycle. If that's the case (and it might not be) I would expect some quantifications to be impacted. For example, the authors show in Figure S3B that AGS-S389A mutant does not localize to the cortex. However, this mutant shows a radically different localization pattern to the accompanying control picture (AGS), namely strong enrichment in what I assume to be the nucleus. Is the S389 mutant preventing AGS from making it to the cortex? Or are these pictures instead temporally distinct, meaning that AGS hasn't yet made it out of the nucleus? Notably, the work of Johnston et al. (Cell 2009), cited in the text, does not show or claim that the linker domain impacts Pins localization. Their model is rather that Pins is anchored at the cortex by Gai, not Dlg, and that is the same model described in this manuscript. In agreement with that model and the results of Johnston et al., a later study (Neville et al. EMBO Reports 2023) failed to find a role for Dlg or the conserved phosphorylation site in Pins localization.

<https://doi.org/10.7554/eLife.100086.1.sa1>

Reviewer #2 (Public Review):

This study from Dr. Emura and colleagues addresses the relevance of AGS3 mutations in the execution of asymmetric cell divisions promoting the formation of the micromere during sea-searching development. To this aim, the authors use quantitative imaging approaches to evaluate the localisation of AGS3 mutants truncated at the N-terminal region or at the C-terminal region, and correlate these distributions with the formation of micromere and correct development of embryos to the pluteus stage. The authors also analyse the capacity of these mutated proteins to rescue developmental defects observed upon AGS3 depletion by morpholino antisense nucleotides (MO). Collectively these experiments revealed that the C-terminus of AGS3, coding for four GoLoco motifs binding to cortical Gaphai proteins, is the molecular determinant for cortical localisation of AGS3 at the micromeres and correct pluteus development. Further genetic dissections and expression of chimeric AGS3 mutants carrying shuffled copies of the GoLoco motifs or four copies of the same motifs revealed that the position of GoLoco1 is essential for AGS3 functioning. To understand whether the AGS3-GoLoco1 evolved specifically to promote asymmetric cell divisions, the authors analyse chimeric AGS3 variants in which they replaced the sea urchin GoLoco region with orthologs from other echinoids that do not form micromeres, or from *Drosophila* Pins or human LGN. These analyses corroborate the notion that the GoLoco1 position is crucial for asymmetric AGS3 functions. In the last part of the manuscript, the authors explore whether SpAGS3 interacts with the molecular machinery described to promote asymmetric cell division in eukaryotes, including Insc, NuMA, Par3, and Gaphai, and show that all these proteins

colocalize at the nascent micromere, together with the fate determinant Vasa. Collectively this evidence highlighted how evolutionarily selected AGS3 modifications are essential to sustain asymmetric divisions and specific developmental programs associated with them.

<https://doi.org/10.7554/eLife.100086.1.sa0>