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ORIGINAL ARTICLE

Notch-mediated lateral inhibition is an evolutionarily conserved mechanism patterning the ectoderm in echinoids

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Abstract

Notch signaling is a crucial cog in early development of euechinoid sea urchins, specifying both non-skeletogenic mesodermal lineages and serotonergic neurons in the apical neuroectoderm. Here, the spatial distributions and function of *delta*, *gcm*, and *hesc*, three genes critical to these processes in euechinoids, are examined in the distantly related cidaroid sea urchin *Eucidaris tribuloides*. Spatial distribution and experimental perturbation of *delta* and *hesc* suggest that the function of Notch signaling in ectodermal patterning in early development of *E. tr ibuloides* is consistent with canonical lateral inhibition. *Delta* transcripts were observed in t he archenteron, apical ectoderm, and lateral ectoderm in gastrulating e mbryos of *E. tribuloides*. Perturbation of Notch signaling by either *delta* morpholino or treatment of DAPT downregulated *hesc* and upregulated *delta* and *gcm*, resulting in ectopic expression of *delta* and *gcm*. Similarly, *hesc* perturbation mirrored the effects of *delta* perturbation. Interestingly, perturbation of *delta* or *hesc* resulted in more cells expressing *gcm* and supernumerary pigment cells, suggesting that pigment cell proliferation is regulated by Notch in *E. tribuloides*. These results are consistent with an evolutionary scenario whereby, in the echinoid ancestor, Notch signaling was deployed in the ectoderm to specify neurogenic progenitors and controlled pigment cell proliferation in the dorsal ectoderm.

Keywords Notch signaling · HES proteins · Pigment cells · Gene regulatory networks · Sea urchins · Neurogenic ectoderm

Introduction

In multicellular organisms the Notch signaling pathway functions to regulate cell fates by mediating short-range, cell-cell interactions when cells are directly apposed with or in close proximity to one another (Artavanis-Tsakonas et al. 1999). The Notch/LIN-12 protein family comprises single-pass transmembrane receptors that, when tickled by their Delta/Serrate/LAG-2 ligands bound to membranes of neighboring cells, causes release and subsequent intracellular processing of the Notch intracellular domain (NICD) by γ-secretase (Kopan and Ilagan 2009). Once cleaved, the NICD translocates to the nucleus and interacts with members of the Suppressor of Hairless(SuH)/LAG-1 protein family to facilitate DNA binding and alter gene expression. In canonical Notch signaling, members of the Hairy and Enhancer of Split (HES) protein family are present in signal-receiving cells as principal targets of Notch, by which they mediate transcriptional repression of genes expressed in signal-sending, neighboring cells. Notch signaling has been shown to act in diverse metazoan developmental contexts, from the maintenance of neural precursors and neurogenic placodes to the specification of mesenchymal cell lineages, as well as the spacing of sensory organs (Bray 2016).

In early development of euechinoid sea urchins, Notch signaling is deployed as a patterning mechanism in endomesodermal and ectodermal domains. In the endomesoderm of the euechinoid *Lytechinus variegatus* (*Lv*), early cleavage stage embryos exhibit uniform levels of

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Notch protein until early blastula stage, at which time Notch becomes concentrated in a ring of cells near the vegetal plate (Sherwood and McClay 1997). Subsequent experiments revealed that Notch segregates non-skeletogenic mesoderm (NSM) from cells that will become anterior endoderm (Sherwood and McClay 1999). Correspondingly, the absence of Delta ligand, which is expressed specifically in NSM-adjacent skeletogenic mesenchyme (SM), results in complete loss of NSM-precursor lineages that later become pigment cells and other mesenchymal cell types (Sweet et al. 2002). Similarly in *Strongylocentrotus purpuratus* (*Sp*), another camarodont euechinoid, perturbation of Notch signaling results in loss of activation of NSM-specific regulatory genes, e.g., *glial cells missing* (*gcm*), as well as loss of small micromere-specific genes in neighboring small micromeres (Materna et al. 2013a; Materna et al. 2013b; Ransick and Davidson 2006). Downregulation of NSM regulatory genes results in the failure to clear endodermal regulatory genes from mesodermal domains (Croce and McClay 2010; Materna and Davidson 2012; Peter and Davidson 2011). Data from other camarodont euechinoid taxa, viz. *Paracentrotus lividus* (*Pl*) and *Hemicentrotus pulcherrimus* (*Hp*) (Ohguro et al. 2011; Rottinger et al. 2006), as well as an irregular euechinoid, *Astriclypeus manni* (Takata and Kominami 2011), suggest that this early developmental capacity of Notch signaling to specify NSM is a highly conserved feature of euechinoids, dating back at least to the divergence of irregular and non-irregular echinoids more than 210 mya (Smith et al. 2006).

In the ectoderm and later in the endoderm of the euechinoid embryo, Notch signaling functions in a highly conserved role of recruiting neurogenic progenitor cells in the animal pole domain (APD) and ciliary band (CB) (Burke et al. 2014). In *Sp and Pl*, *delta* transcripts are expressed during gastrulation in isolated cells of the apical neuroectoderm (ANE) (Lapraz et al. 2009; Wei et al. 2011; Yaguchi et al. 2012; Yaguchi et al. 2011). Perturbation of Notch results in ectopic patches of serotonergic neurons in those domains, suggesting that Delta/Notch functions to laterally inhibit signal-receiving cells. Indeed, Notch-mediated lateral inhibition recruits neuronal progenitors in APD and in lateral ectoderm (LE), where bilateral postoral neurogenic cells are recruited from CB (Garner et al. 2016; Mellott et al. 2017). Moreover, in the closely related euechinoid *Hp* and the distantly related irregular euechinoid *Scaphechinus mirabilis* (*Sm*), *delta* transcripts are also expressed at mesenchyme blastula in APD, as well as bilaterally in isolated cells in LE, indicating that these echinoids also may possess an ANE domain and bilateral postoral cells (Yamazaki et al. 2010). Hence, in euechinoids, Notch signaling functions as a conserved spatial regulatory mechanism in at least three distinct domains during embryogenesis: in the endomesoderm for cell fate specification and lineage segregation, in the APD to regulate proliferation of neuronal progenitors, and in LE to recruit neuronal progenitors from CB.

The euechinoid taxa described above are members of one great branch of a two pronged lineage of extant sea urchins—the other lineage being the cidaroid echinoids. These two sister groups, cidaroids and euechinoids, diverged from each other at least 268 mya (Thompson et al. 2015). Studies focusing on the function and spatiotemporal deployment of regulatory genes in early development of cidaroids are beginning to reveal the degree to which developmental gene regulatory networks change in deep time (Bishop et al. 2013; Erkenbrack 2016; Erkenbrack et al. 2016; Erkenbrack and Davidson 2015; Yamazaki et al. 2014). For instance, it is now clear that the endomesodermal role of Notch signaling in early development of the cidaroid echinoids *Eucidaris tribuloides* (*Et*) and *Prionocidaris baculosa (Pb)* exhibits important functional differences when contrasted to euechinoids. In blastular stages of *Et*, *delta* transcripts are present in micromere-descendants, a homologous cell type to euechinoid SM and that also expresses the SM regulatory gene *alx1*. Neighboring signal-receiving cells are comprised of NSM that abut the SM. After the onset of *delta* expression, NSM begin to accumulate transcripts of the HES-1 protein family member *hesc*. When *delta* translation is disrupted, expression of *hesc* is extinguished in SM-neighboring cells, and the SM domain expands into surrounding NSM (Erkenbrack and Davidson 2015). Rather than a cell lineage segregation mechanism as in euechinoids, a canonical Notch lateral inhibition mechanism is deployed whereby *delta* is confined to SM by Notch-mediated HES protein family members. Thus, the endomesodermal role of Notch diverged after the cidaroid-euechinoid split.

To date, no studies have addressed these mechanisms in the distantly related cidaroid lineage. The study by Erkenbrack and Davidson (2015) showed *delta* and *hesc* transcripts in the ectoderm of *Et* at early gastrula stage, hinting that Notch signaling also may play a role during ectodermal patterning in *Et*. Here, I elaborate on that preliminary observation by presenting spatial and perturbation data on three core regulatory genes—*delta*, *hesc*, and *gcm*—involved in euechinoid neurogenic ectodermal progenitor specification and mesenchymal pigment cell specification during gastrulation of *Et*. These results suggest that the predominant mechanism of Notch signaling in *Et* is via canonical lateral inhibition. The spatial confinement of *delta* transcripts is under control of this mechanism in the archenteron, the APD, and in isolated cells in LE. These cells in LE are likely homologous to euechinoid postoral cells in CB. The regulatory mechanisms installing *hesc* transcripts in the ectoderm are complex, consisting of one that is tightly linked to the spatial localization of *delta*, and one that is independent of *delta* expression. Perturbation

of both *delta* and *hesc* results in ectopic expression of *delta*. *Delta* perturbation or disruption of Notch downregulates zygotic *hesc*. Intriguingly, ectodermally confined *gcm* expression was sensitive to perturbation of Notch and *hesc*, increasing the abundance of *gcm* transcripts, *gcm*-positive cells, and pigment cells. These results suggest that the spatial confinement and proliferation of *gcm* in LE during *Et* gastrulation are Notch-dependent. Lastly, employing a comparative developmental framework (Table 1), these data paint a picture of the evolution of Notch signaling in early development of echinoderms.

Materials and methods

Animals, cloning, whole-mount in situ hybridization, qPCR

Animals were obtained from SeaLife, Inc. (KP Aquatics, FL) and were maintained at room temperature. Embryos were cultured in Millipore-filtered seawater (MFSW) in temperature controlled conditions at 22 °C. Full-length or partial coding sequences for *delta*, *hesc*, and *gcm* were obtained by PCR from a cDNA library comprised of mixed developmental time points. Primer sequences for qPCR and for whole-mount in situ hybridization (WMISH) probe construction for *delta*, *hesc*, and *gcm* have been previously described (Erkenbrack 2016; Erkenbrack and Davidson 2015). Full-length or partial PCR products were subsequently cloned into *E. coli* by standard procedures. Plasmids were isolated and sequenced for confirmation of the correct insert. Detailed protocols for WMISH and qPCR were previously described (Erkenbrack 2016; Erkenbrack and Davidson 2015).

Morpholino perturbations

A morpholino microinjection protocol modified from Materna (2017) was carried out as previously described (Erkenbrack and Davidson 2015). Morpholinos targeting *delta* and *hesc* translation start sites were synthesized by GeneTools (Philomath, OR, USA), and their sequences are 5′-ATAACATATAGCAC GCCGAGAAGGC-3′ and 5′-AATCACAAGGTAAG ACGAGGATGGT-3′, respectively. Injection solutions of each morpholino were 1 mM, and approximately 10 pL was injected into each zygote. Embryos were then cultured to the desired time point and processed either for qPCR via RNA isolation (RNeasy; Qiagen) and subsequent cDNA synthesis (iScript cDNA Synthesis; Bio-Rad) or for WMISH by overnight fixation in MOPS-buffered MFSW with 4% paraformaldehyde. WMISH and qPCR analyses were replicated twice in the case of morpholinos and three times in the case of DAPT treatment (Supplementary Table 1).

Table 1 Species abbreviations used in this study and their taxonomic relations

Species (class: order)	Abbreviation
Patiria miniata (Asteroidea: Valvatida)	*Pm*
Strongylocentrotus purpuratus (Echinoidea: Camarodonta)	*Sp*
Lytechinus variegatus (Echinoidea: Camarodonta)	*Lv*
Paracentrotus lividus (Echinoidea: Camarodonta)	*Pl*
Hemicentrotus pulcherrimus (Echinoidea: Camarodonta)	*Hp*
Scaphechinus mirabilis (Echinoidea: Irregularia)	*Sm*
Eucidaris tribuloides (Echinoidea: Cidaroida)	*Et*
Prionocidaris baculosa (Echinoidea: Cidaroida)	*Pb*

Treatment of embryos with DAPT

For treatment with *N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethyl ester (DAPT) (cat no. 2634; Tocris Biosciences), embryos developing in MFSW were transferred at 2 h postfertilization (hpf) into an equal volume of MFSW containing 20 μM DAPT, giving a final concentration of 10 μM DAPT. Embryos were allowed to develop to the desired stage and fixed either for qPCR or WMISH as described above.

Results

Spatial distribution of mRNAs encoding *delta*, *gcm*, and *hesc* during *E. tribuloides* gastrulation

As the archenteron extends during early gastrulation in *Et*, and just prior to the ingression of mesenchyme, *delta* transcripts are observed in the archenteron and in isolated cells in LE (Fig. 1a) (Erkenbrack and Davidson 2015). At 36 hpf, as mesenchyme invades the blastocoel, *delta* transcripts are seen in APD, in addition to the archenteron and LE (Fig. 1a). By 50 h, *delta* increases in ectoderm that will become CB. At 28 h, prior to ingression of mesenchyme, *gcm* is detected just below the anterior end of the archenteron, as well as in isolated cells in LE (Fig. 1b) (Erkenbrack 2016; Erkenbrack and Davidson 2015). By mid-gastrula *gcm*-positive cells are in the blastocoel and in LE (Fig. 1b). At 50 h, *gcm* is seen in the future CB and is in a small cluster in the anterior archenteron (Fig. 1b). *Hesc* transcripts are detected in a complex distribution throughout the ectoderm at 28 h, as well as in the archenteron (Fig. 1c). Independent of its expression in the NSM during late blastula stages, *hesc* is installed gradually in the ectoderm beginning from mid-blastula stage to early gastrula, when it is observed in a mottled pattern (Erkenbrack and Davidson 2015). At 28 h, *hesc* is conspicuously absent from particular anterior regions of the embryo and from a ring of 5–6 cell diameters surrounding the blastopore (Fig. 1c). At 36 h, *hesc* transcripts are

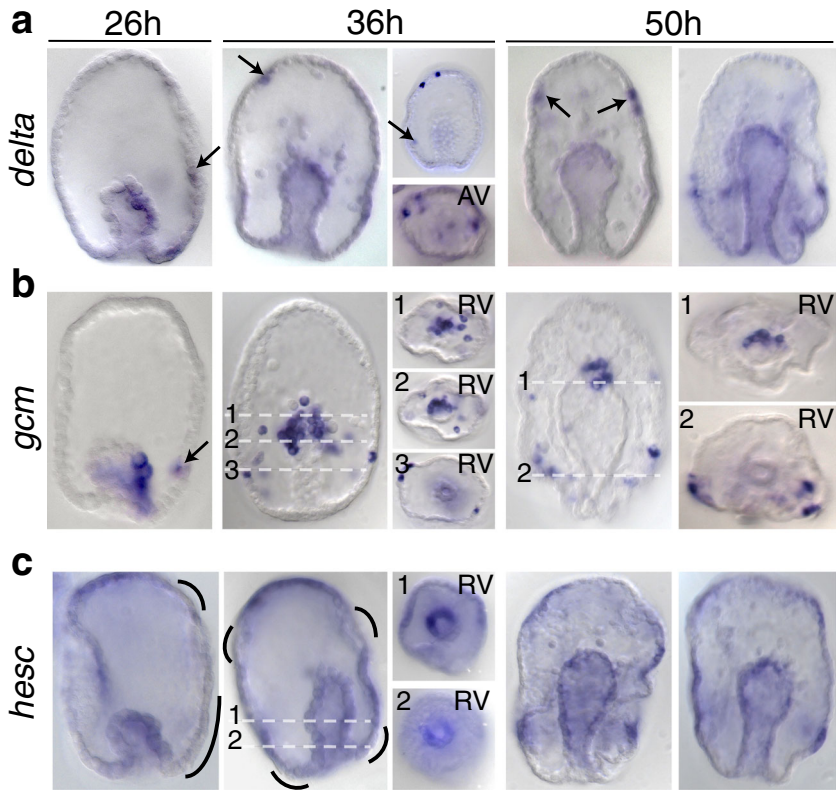

Fig. 1 Spatial distribution of mRNAs encoding *delta*, *gcm*, and *hesc* during *E. tribuloides* gastrulation. **a** At early gastrula, *delta* is expressed at the anterior end of the archenteron and in a few isolated ectodermal cells near the boundary of endoderm and ectoderm. As mesenchyme ingresses at 36 h, *delta* is detected as at 28 h but also in isolated cells at the apical plate. By mid-gastrula at 50 h, *delta* is detected in cells just below the larval equator, as well as the apical plate and archenteron. **b** At early gastrula, *gcm* is detected in a patch of cells just under the anterior end of the archenteron and in isolated cells in the ectoderm. By 36 h, *gcm* is seen also in mesenchymal cells ingressing into the blastocoel. Apical views of embryos at this stage show the restriction of *gcm* to one side of the archenteron and its expression in isolated ectodermal cells more near the blastopore. At mid-gastrula, *gcm* is detected in cells at the tip of the archenteron as well as in scattered ectodermal cells including the larval equator. **c** *Hesc* at early gastrula is detected in the endoderm, ectoderm, and mesoderm. At this stage, *hesc* is spatially distributed throughout much of the ectoderm with isolated patches cleared of its expression. At 36 h, *hesc* is broadly expressed as at 28 h, with some cells in the mesoderm and the region near the blastopore void of its expression. By 50 h, *hesc* is detected in cells near the region where the ciliary band will form in the apical plate and between the larval equator and blastopore, as well as in the archenteron. Arrows show detected staining. Bars show regions of the embryo cleared of transcripts. Dashed lines indicate regions of the larva that were also imaged from the apical view shown immediately to the right and with a corresponding number. AV apical view, RV archenteron view

detected in the archenteron and are more localized in the APD and in LE (Fig. 1c). By 50 h, *hesc* transcripts are seen in the future CB and in the archenteron and are cleared from the most apical region of the APD as well as the area near the blastopore (Fig. 1c). The spatial distribution of *hesc* transcripts in *Et* agrees well with those observed in the closely related, Pacific-dwelling cidaroid *Pb* (Yamazaki et al. 2014).

Notch signaling regulates *delta* and locally regulates *hesc* expression

Previous work indicated that transcription of *delta* increases in pregastrular *Et* embryos injected with *delta* morpholino, suggesting that either *delta* is increasing in the micromere descendants or that its domain of expression is expanding (Erkenbrack and Davidson 2015). As with the SM-specific regulatory gene *alx1*, the domain of *delta* indeed expands into the NSM prior to gastrulation (Fig. S1(a)). Later, at 36 h, *delta* continues to increase in embryos injected with *delta* morpholino (Fig. 2a). Spatially, *delta* morpholino resulted in ectopic *delta* expression in the APD, LE, and archenteron (Figs. 2b, S1(b), and S2(a)). This was also the case for embryos cultured to 50 hpf (Figs. 2c and S1(c)). Moreover, the Notch-signaling responsive regulatory gene *hesc* showed spatially specific downregulation in the archenteron and the ectoderm at 26 h (Figs. 2d and S1(d)). Correspondingly, disruption of NICD cleavage by culturing embryos in the presence of the γ-secretase inhibitor DAPT increased *delta* mRNA and induced ectopic expression of *delta* (Figs. 2e, g and S3(a)). As observed in *delta* MASO background, *hesc* continued to show localized downregulation in the archenteron and depressed expression in ectoderm in DAPT-treated embryos (Figs. 2g and S3(b)). These results suggest (1) that Notch signal-receiving cells express a repressor, likely *hesc*, that functions by lateral inhibition

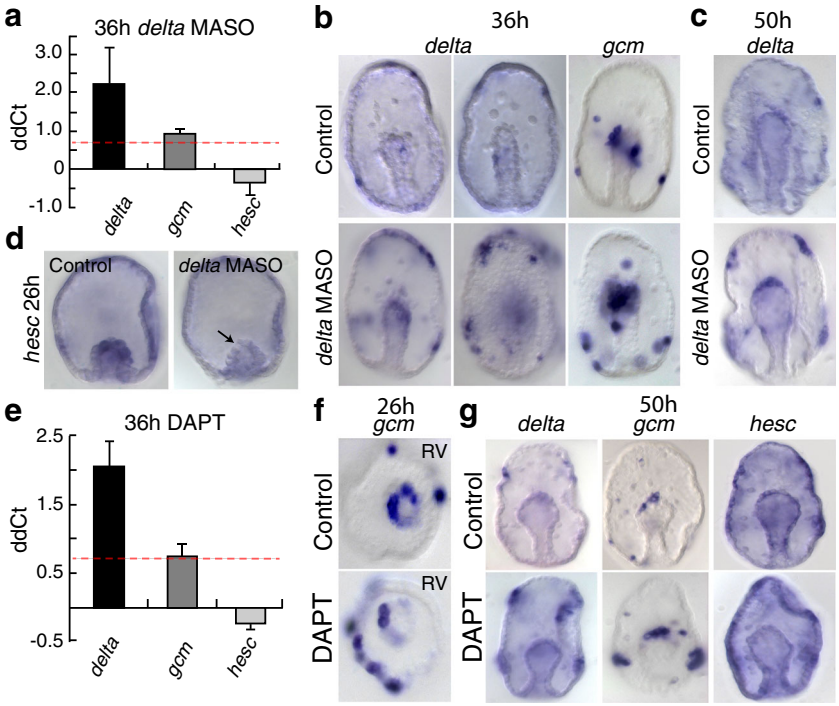

Fig. 2 Perturbation of Notch signaling by blocking translation of Delta ligand or by culturing embryos in the presence of DAPT increases the abundance and results in ectopic expression of *delta* and *gcm*. **a** *Delta* morpholino (MASO) increases RNA abundance of *delta* and *gcm*. **b** Spatial distribution of *delta* and *gcm* at 36 h in *delta* MASO background and uninjected controls. Whereas the number of *delta*-positive cells increases in both the apical ectoderm and the endodermal-ectodermal boundary, the number of gcm-positive cells only increases in the latter region. **c** At 50 h, embryos injected with *delta* MASO show well-defined regions of *delta* expression, expanding domains in the mesoderm, apical ectodermal, and endodermal-ectodermal boundary. **d** Expression of *hesc* is extinguished specifically in the archenteron in the presence of *delta* MASO. **e** Abundance of *delta* and *gcm* RNA increases in embryos cultured in the presence of 10 μM DAPT. **f** At 26 h, the number of ectodermal *gcm*-positive cells increases in the presence of DAPT. **g** At 50 h, DAPT-treated embryos exhibit increases in both *delta*- and *gcm*-positive cells. This treatment mirrors that seen in *delta* MASO. The expression of *hesc* is reduced in the mesoderm at the archenteron. RV archenteron view. Red dashed line in qPCR graphs indicates a difference of 0.7 cycles or 1.6-fold change

to confine *delta* transcripts to Notch signal-sending cells, and (2) that there likely exists a local activator of *delta* in these regions where ectopic expression is occurring.

Ectopic expression of *gcm* is observed in the lateral ectoderm in *delta* morpholino background

In *Et*, lineage tracing studies and WMISH have indicated that SM is the first mesenchyme to ingress, and that this only occurs well after the archenteron has extended into the blastocoel at early gastrula (Erkenbrack and Davidson 2015; Urben et al. 1988; Wray and McClay 1988). These lines of evidence make the previously observed presence of *gcm*--positive cells in LE all the more interesting (Erkenbrack 2016), since in euechinoids *gcm*-positive cells come solely from Notch signal-receiving cells in the endomesoderm (Ransick and Davidson 2006). However, since we do not know the source of *gcm*-positive cells in *Et*, we cannot definitively say whether these cells in LE are of mesodermal origin or ectodermal origin. *Delta* transcripts are also present in LE at this time and show ectopic expression upon perturbation of Notch signaling (Figs. 1a and 2b). Similar to *delta*, *gcm* expression was increased in *delta* MASO background (Fig. 2a) and exhibited ectopic expression in LE at 36 h (Figs. 2b and S2(b)). Embryos cultured in DAPT also showed increased *gcm* abundance and ectopic expression at three different time points (Figs. 2e–g and S3(c,d)). Importantly, these data do not indicate whether the spatial expression of *gcm* is also changing in the archenteron.

Ectopic expression of *delta* and *gcm* upon perturbation of Hesc

The data described thus far suggest that Notch signal-receiving cells come to express the obligate repressor Hesc, which in turn confines *delta* and *gcm* transcripts in different regions of the embryo to isolated ectodermal cells. Therefore, *delta* and *gcm* should respond in a similar fashion to *hesc* morpholino. At 36 h, *hesc*, *gcm*, and *hesc* transcripts increased in *Et* embryos injected with *hesc* morpholino (Fig. 3a). At 36 and 50 h, embryos injected with *hesc* MASO induced ectopic expression of *delta* transcripts in APD as well as in LE (Figs. 3b, c and S4(a)). Similarly *gcm* transcripts exhibited ectopic expression in LE (Figs. 3b, d and S4(b,c)). These results suggest that Notch signal-receiving cells

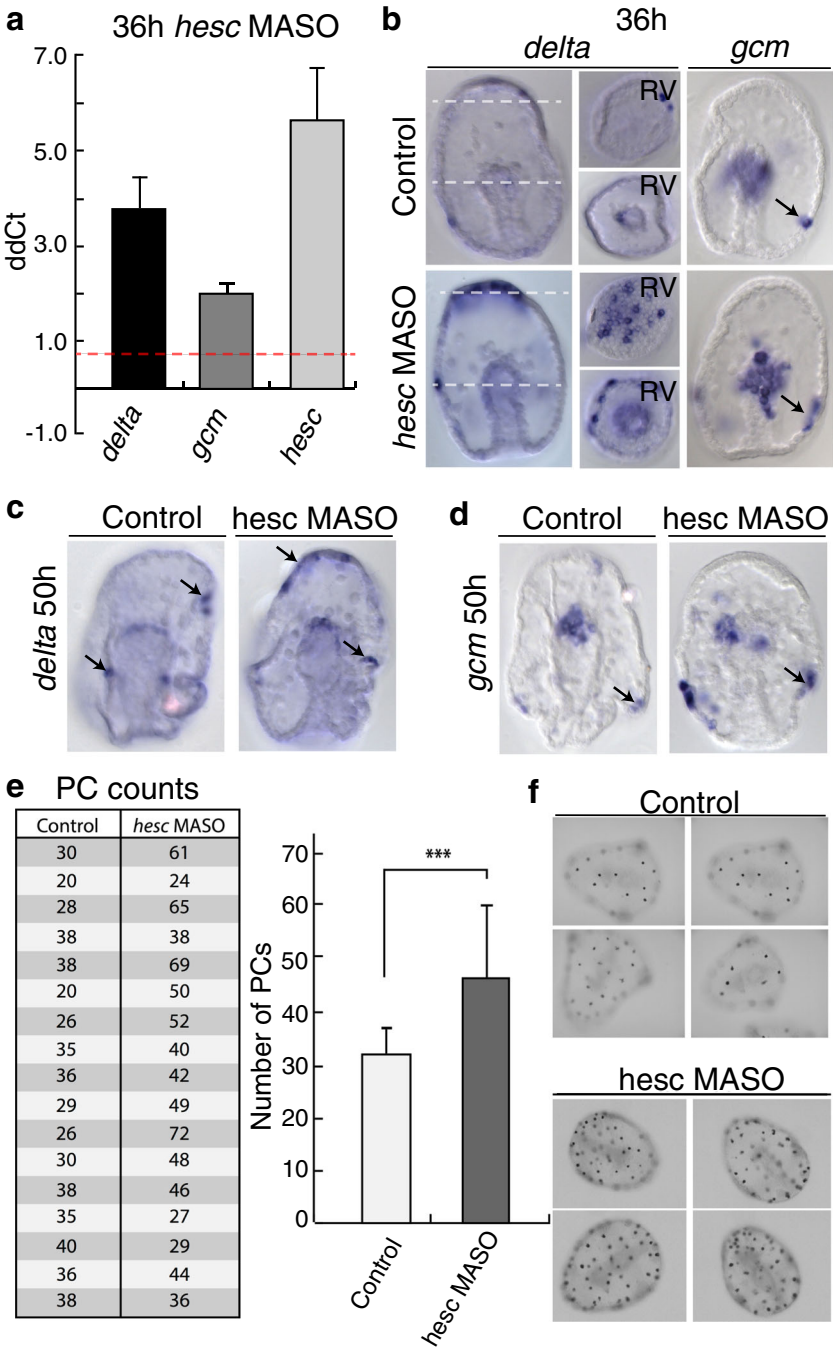

Fig. 3 Perturbation of *hesc* results in increased abundance of *delta* and *gcm* transcripts, as well as supernumerary pigment cell formation. **a** Microinjection of *hesc* MASO increases *delta*, *gcm* and *hesc* RNA transcripts at 36 h. **b** Embryos injected with *hesc* MASO exhibit ectopic *delta*-positive cells in the apical ectoderm as well as near the endodermal-ectodermal boundary. An increase in *gcm*-positive cells is observed near the vegetal lateral clusters in the ectoderm at 36 h. **c**, **d** At 50 h, the spatial domains of *delta* and *gcm* expand in the apical ectoderm and near the vegetal lateral clusters, respectively, in *hesc* MASO background. **e** Microinjection of *hesc* MASO resulted in embryos with increased pigment cell (PC) numbers, on average, relative to uninjected control. ***$p < 0.01$, Mann-Whitney *U* test. **f** Example embryos in uninjected control and *hesc* MASO background showing pigment cells at 120 h

restrict the spatial distribution of *delta* and *gcm* to isolated cells in the archenteron and ectoderm. In the case of *gcm*, ectopic expression is induced, minimally, in LE; whether or not a similar effect occurs where *gcm* is expressed in the archenteron will require additional experimental data.

Induction of supernumerary pigment cells in *hesc* perturbation background

In euechinoids, Delta presentation in SM directly regulates specification of NSM via Notch-mediated regulatory control of *gcm* and other NSM regulatory genes (Materna and Davidson 2012; Ransick and Davidson 2006; Ransick and Davidson 2012; Sherwood and McClay 1999; Sweet et al. 2002). Perturbation of either Notch signaling or *gcm* translation produces albino embryos (Ransick and Davidson 2006; Sherwood and McClay 1999). As seen above, in *Et* the early transcription of *gcm* does not depend on Notch for its activation, rather its spatial confinement depends on Notch. Lastly, I sought to determine if changes in *gcm* abundance affected pigment cell specification. Intriguingly, embryos cultured in the presence of *hesc* morpholino exhibited supernumerary

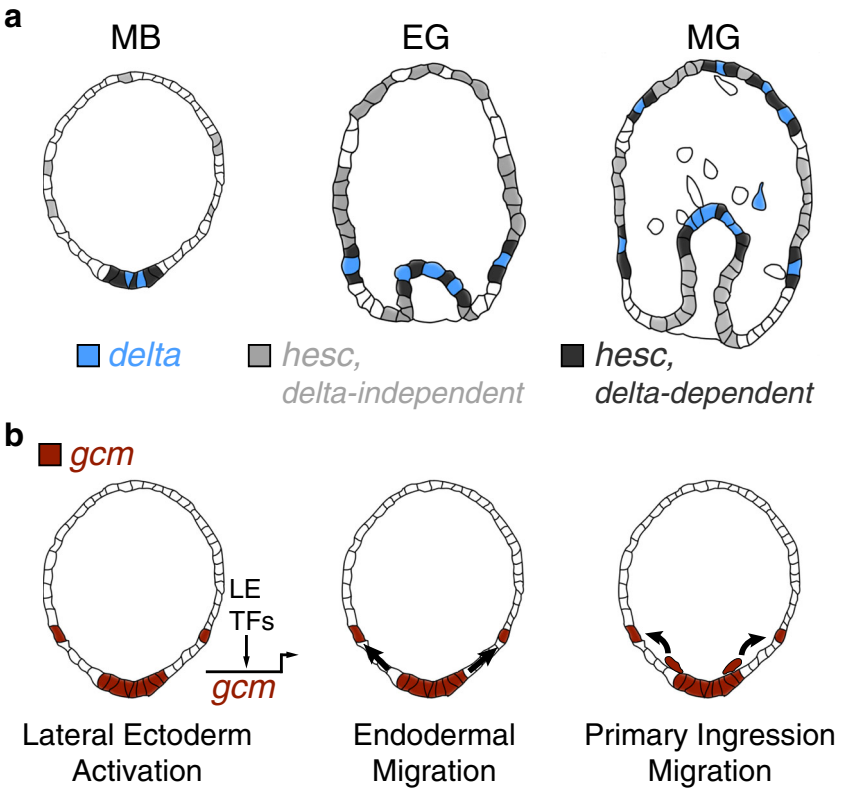

Fig. 4 Models and hypothetical scenarios for the installation and function of Notch signaling components and *gcm* in early development of *Eucidaris tribuloides.* **a** Installation of zygotic *hesc* transcripts comes in two forms in *E. tribuloides* from mid-blastula to mid-gastrula. *Expression of hesc* (dark gray) abutting *delta*-positive cells (light blue) is sensitive to Notch perturbation, whereas most *hesc* expression in the ectoderm are insensitive to Notch perturbation (light gray) and is installed independent of Notch. **b** Ontogenetic source of *gcm*-positive cells in the lateral ectoderm of *E. tribuloides.* Three scenarios are shown: in the first, transcription factors (TFs) in the lateral ectoderm activate *gcm* expression independently of its mesodermal activity; in the second, mesodermally derived *gcm*-positive cells migrate directly through the endoderm to arrive at LE; in the third, a pregastrular ingression of mesodermally derived *gcm*-positive cells migrates through the blastocoel to LE

pigment cells (Fig. 3e, f), suggesting that *hesc* perturbation increased the number of *gcm*-positive cells. These data do not indicate whether supernumerary pigment cells are coming from *gcm*-positive cells in LE or the archenteron (Fig. 4). On average, there were 15–30 additional pigment cells in embryos injected with *hesc* MASO (Fig. 3e), which is consistent with the hypothesis that a small population of cells in the embryo are transfated to become pigment cells.

Discussion

Spatial installation of hesc in *E. tribuloides* and Hesc regulation of *delta*

In euechinoids, Hesc functions globally throughout the early embryo to repress SM specific genes, e.g., *delta*, until another repressor, Pmar1, relieves that repression specifically in the large micromeres, whose ontogenetic descendants are SM (Oliveri et al. 2008; Revilla-i-Domingo et al. 2007). This double-negative gate circuitry is a highly conserved feature of euechinoid sea urchins (Minokawa 2017; Thompson et al. 2017). Perturbation of Hesc activity results in global expression of *delta* transcripts (Revilla-i-Domingo et al. 2007). In contrast, it was previously reported that Hesc perturbation in *Et* does not result in global expression of the SM-specific gene *alx1* (Erkenbrack and Davidson 2015). Here, we see *delta* responding similarly to Hesc perturbation as did *alx1* in previous reports, suggesting that there is no global driver of SM gene expression in *Et*. Furthermore, these data argue for two distinct modes of spatial regulation of *hesc* transcription: a Notch-mediated mode in the endomesoderm, the APD, and LE, and a second Notch-independent mode of regulation that is installed in the ectoderm from late blastula and beyond (Fig. 4a). Given Hesc is a repressor of *delta* transcription and since *hesc* transcripts are present throughout most of the ectoderm, it is still not clear how *delta* transcription is activated if Hesc is uniformly expressed. Thus, in order for *delta* expression to be turned on in isolated APD and LE cells, *hesc* must either be kept transcriptionally silent in particular regions of the ectoderm or, if *hesc* is globally expressed, regions of the ectoderm must be cleared of Hesc. Unfortunately, the data

presented here do not allow for resolution of this issue, and the only thing that is clear is that Hesc perturbation does not expand the domain of *delta* and *alx1* throughout the embryo. One possible solution is that Hesc protein is regulated locally by posttranslational modifications, as phosphorylation of HES proteins is known to interfere with their DNA-binding activity (Popovic et al. 2014; Strom et al. 1997). Thus, even if *hesc* transcripts are expressed throughout the ectoderm, their mere presence does not allow us to rule out posttranslational regulation of Hesc protein.

A conserved role for Hesc in restricting *delta* and *gcm* during ectoderm development

One scenario suggested by these data is that *delta* and *gcm* are co-expressed in LE. This would not be the first instance of this in the echinoid clade. The simultaneous transcriptional activation of *delta* and *gcm* has been previously observed during early development of the irregular echinoid *Sm* (Yamazaki et al. 2010; Yamazaki and Minokawa 2016), as well as the likely co-expression of *delta* and *gcm* in the micromeres of the spatangoid echinoid *Echinocardium cordatum* (Yamazaki and Minokawa 2015). Indeed, it has been reported that Hesc also functions upstream of *delta* and *gcm* during NSM and SM specification in *Sm* and *Hp* (Yamazaki and Minokawa 2016). These studies showed that Hesc perturbation resulted in expanding domains of *delta* and *gcm*, as well as an increase in the number of pigment cells in these echinoids. Here, in the cidaroid *Et*, we also see an increase in pigment cells as well as expanding domains of spatial expression of *delta* and *gcm*. From an evolutionary perspective, these data argue for conserved gene regulatory network (GRN) circuitry linking *delta*, *hesc*, and *gcm* in both SM and NSM specification since the divergence of cidaroids and euechinoids. Furthermore, similarities in the regulatory connectivity of these three genes in three distantly related taxa suggest that canonical Notch lateral inhibition likely plays a widespread role in echinoid NSM specification.

Gcm in pigment cell specification GRN cicruitry in echinoids

The transcriptional regulation of *gcm* is one of the most thoroughly dissected *cis*-regulatory loci in the *Sp* developmental GRN (Ransick and Davidson 2006; Ransick and Davidson 2012). Gcm is a crucial regulator of pigment cell specification, the regulatory state of which is locked down by an intergenic feedback circuit involving Gcm, Gatae, and Six1/2 (Ransick and Davidson 2012). During NSM specification in euechinoids, *gcm* activation and transcription depend entirely upon Delta presentation in SM and subsequent NICD nuclearization (Ransick and Davidson 2006). Data reported here and elsewhere (Erkenbrack 2016) show *gcm*-positive cells near the endodermal-ectodermal boundary prior to mesenchymal ingression in *Et*. One question remaining is whether or not the *gcm*-positive cells in the LE are truly pigment cells. This question can likely be addressed by surveying the regulatory state of those cells for *gatae* and *six1/2*. Unfortunately, no data are published for *six1/2* in *Et.* However, there are data for *gatae* (Erkenbrack 2016). At the time of invagination, *gatae* is expressed in endodermal and mesodermal domains and expression is not observed near the ectoderm until after mesenchyme begins to ingress into the blastocoel. Therefore, if we assume that the intergenic stabilization loop Gcm-Gatae-Six1/2 is a marker for pigment cell specification, then it is plausible that the *gcm* activity we are seeing in the ectoderm is not related to a pigment cell lineage in *Et.* Indeed, the data from Erkenbrack (2016) show that *gcm* and *gatae* may be expressed in an overlapping domain at the tip of the archenteron in *Et*, which may correlate with its mesenchymal-derived pigment cell lineage. Double in situ hybridization combined with perturbation of Gcm, Gatae, and Six1/2 are needed in *Et* to resolve this interesting question.

The ontogenetic origin of *gcm*-positive cells in the *E. tribuloides* ectoderm

The ontogenetic source of *gcm*-positive cells populating LE is unclear. In camarodont euechinoids, *gcm*-positive pigment cell precursors arise from blastocoelar ingression of NSM and their subsequent migration from the archenteron to ectoderm at mid-gastrula stage (Gibson and Burke 1985). The most likely scenario in *Et* is that *gcm*- and *gatae*-positive cells ingressing at the tip of the archenteron are the source of all of the pigment cells in *Et.* If that is the case, then perhaps *gcm* serves a distinct regulatory function in LE of *Et*, as well as in LE of the closely related cidaroid *Pb* (Yamazaki et al. 2014). Still, we would like to know whether these *gcm*-positive cells in LE are mesodermally or ectodermally derived. Unfortunately, the scope of this study could not include experiments that specifically addressed this question. However, there are a few scenarios that can be framed around it based on what is seen in non-camarodont euechinoids (Fig. 4b). The presence of a single population of *gcm*-positive cells around the time of gastrulation in *Et* (Erkenbrack 2016) suggests that either *gcm*-positive cells begin to migrate to LE shortly after gastrulation begins or *gcm* transcription begins in LE independently of its NSM activation. As previously mentioned, lineage tracing studies make it clear that mesenchymal ingression in *Et* does not occur until the archenteron has passed two thirds of the way into the blastocoel (Urben et al. 1988; Wray and McClay 1988; Wray and McClay 1989); hence, while it is unlikely that *gcm*-positive cells are ingressing before skeletogenic mesenchyme in *Et*, it is still a possibility. This hypothesis could be called the furtive ingression hypothesis and, importantly, cannot be ruled out. Another scenario is

direct migration from NSM through the endoderm without ingressing into the blastocoel (Fig. 4b). Two observations make this scenario plausible: (1) it is known that pigment cell precursors in *Hp* invade the ectoderm at the apical plate and then begin their migration through the aboral ectoderm without ingressing (Kominami et al. 2001) and (2) in the euechinoids *Toxopneustes pileolus* and *Anthocidaris crassispina*, populations of pigment cells in euechinoids migrate directly from the vegetal plate in the direction of the apical plate through the aboral ectoderm without ingressing (Takata and Kominami 2004). However, it is unclear how a few cells of a large population of *gcm*-positive cells would become primed to migrate to LE. The simplest interpretation is that *gcm* turns on independently in LE where the neuronal progenitors arise in the future CB domain, and that the spatial localization is due to convergence of numerous signaling pathways near the endodermal-ectodermal boundary (Range 2014).

Notch signaling as a conserved mechanism specifying neuronal progenitors in echinoids

Notch-mediated neurogenesis in the APD of euechinoids has become an important model contributing to our understanding of the evolution of neurogenesis in metazoans (Burke et al. 2014). Numerous studies have revealed mechanisms underlying the specification of euechinoid larval neurons (Burke et al. 2014; Garner et al. 2016; Lapraz et al. 2009; Mellott et al. 2017; Wei et al. 2011; Yaguchi et al. 2012; Yaguchi et al. 2011). This study, along with another previous study (Bishop et al. 2013), contributes to the evolutionary story of Notch-mediated ectodermal patterning in cidaroids. In euechinoids, serotonergic neurons develop on the aboral side of the APD, and Notch functions to laterally inhibit adjacent, signal-receiving cells from differentiating into neurons (Yaguchi et al. 2012). Perturbation of Notch in euechinoids increases the number of serotonergic neurons and expands the domains of neural markers in the APD and in postoral cells positioned in CB (Mellott et al. 2017; Yaguchi et al. 2012; Yaguchi et al. 2011). Notably, in *Patiria miniata*, *delta* knockdown expands the domain of the neural marker *soxc*, suggesting a lateral inhibition mechanism is also at play in asteroids (Yankura et al. 2013). In *Et*, it was previously shown that serotonergic neural markers are known to be bilaterally expressed in the APD and in CB; later in development, these cells develop into clusters of four or five neurons each (Bishop et al. 2013). Here, I show that Notch-mediated lateral inhibition is active precisely in the locations as those previously shown to exhibit neuronal markers. Furthermore, in the case of the *delta*-positive cells in LE, these cells are positioned in spatially homologous locations in embryos of cidaroids and euechinoids, precisely where CB will later form. These cells are very likely to be components of the CB ganglionic nervous system and are likely to be a homologous cell type to those previously described as neurogenic postoral cells in euechinoids (Burke et al. 2014). Similarly, as in the euechinoids *Sp*, *Pl*, and *Hp* (Materna et al. 2013a; Rottinger et al. 2006; Yaguchi et al. 2012), *delta* is expressed in *Et* in clustered cells within APD (Fig. 4a). Upon perturbation of Notch, *delta* expands, indicating a canonical lateral inhibition mechanism of Notch signaling in *Et* apical neuroectoderm, which is also the case in euechinoids. Integrating these previous lines of evidence with the results presented here makes a very strong case for conserved ontogenetic deployment and function of Notch signaling in both APD and postoral cells in LE of echinoids.

Notch signaling as a model GRN plugin in early development

Intercellular signals have been described as GRN plugins that can be deployed as switches in distinct spatial embryonic addresses by altering their regulatory control during development (Davidson and Erwin 2006; Erwin and Davidson 2009). Affording support for this notion is the partial survey provided in the above description of Notch signaling in early development of echinoids as well as data from echinoderms more broadly. Comparative analyses of developmental GRNs between cidaroids and euechinoids are revealing how plugins and other GRN subcircuits are rewired and altered over vast geological timeframes (Thompson et al. 2017; Thompson et al. 2015), how the regulatory systems that install them evolve (Erkenbrack 2016; Gao et al. 2015), and how embryonic developmental programs likely functioned in the most recent common ancestors of extant sea urchins (Erkenbrack et al. 2016; Erkenbrack and Petsios 2017). Evidence collected from crown group echinoderms makes an illustrative case study showing how regulatory circuitry coupling Notch signaling and *gcm* appears to have been shuffled around in this manner during the evolution of eleutherozoan echinoderm lineages over the last 500 million years. Notch signaling components have been deployed in some aspect of specification of all spatial domains in echinoderms—endoderm (Hinman and Davidson 2007), mesodermal SM restriction (Erkenbrack and Davidson 2015), mesodermal NSM specification (Sherwood and McClay 1999), and ectodermal and endodermal specification of serotonergic neurons and neuronal progenitors (Burke et al. 2014; Garner et al. 2016; Mellott et al. 2017; Wei et al. 2011; Yaguchi et al. 2012). This report adds further evidence to the pliant nature of Notch signaling in development of echinoid neuronal and mesodermal cell lineages, showcasing both the conserved hierarchical nature of developmental GRNs as well as their marked plasticity.

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