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A comprehensive analysis of Delta signaling in pre-gastrular sea urchin embryos

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ABSTRACT

In sea urchin embryos Delta signaling specifies non-skeletogenic mesoderm (NSM). Despite the identification of some direct targets, several aspects of Delta Notch (D/N) signaling remain supported only by circumstantial evidence. To obtain a detailed and more complete image of Delta function we followed a systems biology approach and evaluated the effects of D/N perturbation on expression levels of 205 genes up to gastrulation. This gene set includes virtually all transcription factors that are expressed in a localized fashion by mid-gastrulation, and which thus provide spatial regulatory information to the embryo. Also included are signaling factors and some pigment cell differentiation genes. We show that the number of pregastrular D/N signaling targets among these regulatory genes is small and is almost exclusively restricted to non-skeletogenic mesoderm genes. However, Delta signaling also activates *foxY* in the small micromeres. As is the early NSM, the small micromeres are in direct contact with Delta expressing skeletogenic mesoderm. In contrast, no endoderm regulatory genes are activated by Delta signaling even during the second phase of *delta* expression, when this gene is transcribed in NSM cells adjacent to the endoderm. During this phase Delta provides an ongoing input which continues to activate *foxY* expression in small micromere progeny. Disruption of the second phase of Delta expression specifically abolishes specification of late mesodermal derivatives such as the coelomic pouches to which the small micromeres contribute.

Introduction

The Delta signaling ligand is an important regulator of developmental processes across the animal kingdom. In contrast to many other signaling ligands it is bound to the cell surface of the *delta* expressing cell and not secreted. This limits its effective range to cells that are in direct contact with the source (Wang, 2011). In the receiving cell Delta binds to the Notch receptor causing cleavage of its intracellular domain (N_{ic}). N_{ic} then enters the nucleus where it binds to the transcription factor Suppressor of Hairless (Su(H)) to activate transcription of target genes. In the absence of nuclear N_{ic}, Su(H) is bound to the co-repressor Groucho and becomes a dominant repressor. Thus, as shown in sea urchin embryos as well as in other systems, D/N signaling operates as a toggle switch (Barolo and Posakony, 2002; Ransick and Davidson, 2006).

In sea urchins D/N signaling is required for specification of all non-skeletogenic mesoderm (NSM) cell types, such as pigment cells, blastocoelar cells, coelomic pouch cells and circumesophageal muscle (Sherwood and McClay, 1999; Sweet et al., 2002). The *delta* gene is first expressed between 8 and 9 hours post fertilization (hpf) in the skeletogenic mesoderm at the center of the vegetal plate. Initially it is received by the surrounding ring of veg2 endomesodermal cells. At 7th cleavage, the ring of veg2 cells divides into an inner ring that will develop into NSM and an outer that is specified as endoderm (Peter and Davidson, 2010, 2011b; Ruffins and Ettensohn, 1993). Continued reception of the Delta signal is essential for NSM specification. About the same time that ingression of the skeletogenic micromere descendants begins, *delta* gene expression is extinguished in these cells but is initiated anew in the NSM. Prior studies of D/N signaling have suggested that the first, or skeletogenic, Delta signal is responsible for specification of the earliest NSM cell types, i.e. pigment and blastocoelar cells, whereas NSM Delta functions to specify late mesoderm derivatives such as coelomic pouch cells and muscles (Sweet et al., 2002).

D/N signaling from the NSM to the endoderm has been discussed in previous studies. However, evidence for this remains entirely circumstantial and indirect, inferred from experiments using activated Notch (N^{act}) in which endoderm expands at the expense of ectoderm, and an observation that the endodermal but also mesodermal gene *gataE* (Lee et al., 2007) is apparently affected in D/N perturbations (Davidson et al., 2002; Sweet et al., 2002). Direct evidence for activation of endodermal genes in response to D/N signaling has not been reported. Rather, it has been shown that D/N signaling serves to deactivate endodermal genes in the NSM precursors (Croce and McClay, 2010; Peter and Davidson, 2011b).

In this study we follow a systems biology approach to examine in greater detail the function of both skeletogenic and NSM Delta

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signaling. We analyze the effect of D/N perturbation on transcript levels of 205 genes. This gene set includes the majority of transcription factors that are specifically activated during early development up to mid-gastrulation, including all embryonic transcription factors that are known to be spatially restricted in their expression. We find that by the time *delta* expression in the skeletogenic lineage comes to an end, only 6 NSM transcription factors have been activated, two of which are known direct D/N targets. But no endoderm genes are activated by D/N signaling throughout the time period covered, in either phase of *delta* gene expression. The skeletogenic Delta signal is however received in the small micromeres, where it activates *foxY*, and by specifically perturbing the function of the second, or NSM *delta* expression phase, we show that Delta ligand function is required for maintenance of *foxY* expression into gastrulation.

Materials and methods

Delta/Notch perturbations

Morpholino substituted oligonucleotides (MASOs) were obtained from Gene-tools LLC and injected at 300 μM in 0.12 M KCl. Injection volumes were about 5 pl. Sequences are as follows: Delta — CAAGAAGGCAGTGCGGCCGATCCGT, Notch — CCTGGATGGGTAGTCCGCCTCATCT. The dominant negative (DN) Su(H) contains a mutation in its DNA binding domain that prevent it from binding DNA while leaving its interaction with N_{ic} and other proteins unaffected (Ransick and Davidson, 2006). DN-Su(H) mRNA was injected at 200 ng/μl in 0.12 M KCl. The γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (Hughes et al., 2009) was dissolved in DMSO and added at 3 hpf or 17 hpf to a final concentration of 8 μM. Higher concentrations cause all embryos to exogastrulate, and a concentration >20 μM causes severe, non-specific, defects. Lower inhibitor concentrations result in higher numbers of pigment cells.

Embryo culture and RNA extraction

Sea urchin embryos were cultured at 15 °C and closely monitored for proper development. For lysis, sea water was removed before adding 350 μl RLT buffer from the Qiagen RNeasy Micro Kit (Qiagen, Hilden, Germany). Embryo lysates were immediately stored at −70 °C until use. RNA was extracted according to the manufacturer's instructions; to maximize recovery, RNA was eluted with 50 μl nuclease free water. Samples were ethanol precipitated and resuspended in 11 μl

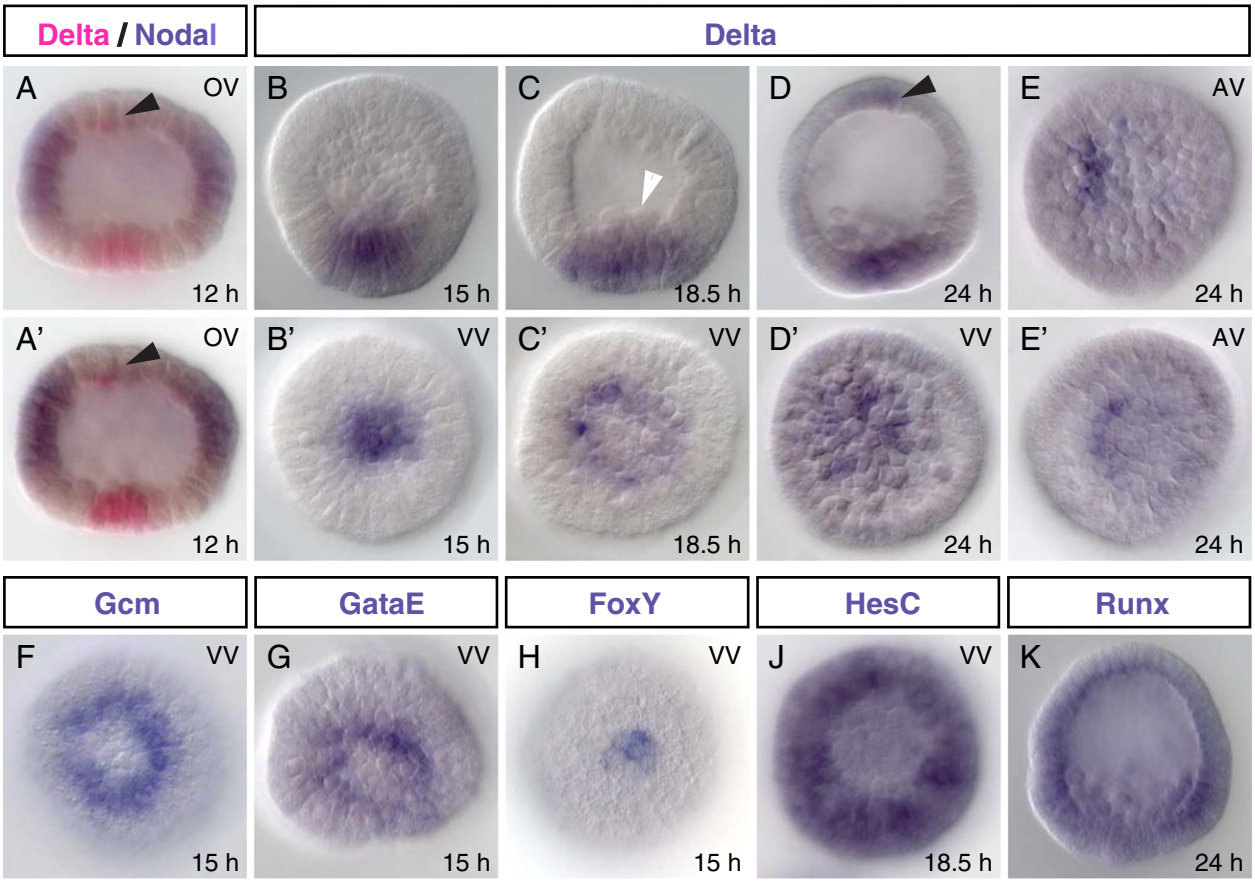

Fig. 1. Expression patterns of the *delta* gene and its early targets. (A, B) *delta* transcripts are localized to the skeletogenic lineage. Transcription is detectable between 8 and 9 hpf by QPCR but is visible by in situ staining only after 10 hpf when it reaches a significant level (Materna et al., 2010; Sweet et al., 2002). *nodal* expression marks the oral ectoderm that at this stage spans about half the embryo (Duboc et al., 2004). (C, D) Between 18 and 19 hpf the skeletogenic cells lose *delta* expression as they ingress into the blastocoel (white arrowhead). (C, C′) At the same time the mesoderm that is adjacent to the skeletogenic cells starts to express *delta* (Sweet et al., 2002). (D, D′) After ingression of the skeletogenic cells is complete the mesoderm occupies the center of the vegetal plate and expresses *delta* throughout (Sweet et al., 2002). In addition, *delta* is expressed in the apical plate. With strong staining *delta* expression can be observed in the apical plate as early as 12 hpf (A, A′, black arrow head), but is more easily detected at 24 hpf (D, black arrowhead). (E, E′) Apical *delta* expression is limited to a few cells that appear to be slightly off center of the apical domain. (F, G) *gcm* and *gataE*, the direct early targets of Delta, are expressed in a ring of mesodermal precursor cells that surround the Delta source (Lee and Davidson, 2004; Ransick et al., 2002); (H) *foxY* is expressed in the small micromeres, which are surrounded by *delta* expressing cells (Ransick et al., 2002). (J) By the time *delta* transcription is activated in the mesoderm, *hesC*, a strong repressor of *delta*, has turned off there (compare to C′) (Smith and Davidson, 2008). (K) The *delta* activator *runx* is expressed ubiquitously throughout the sea urchin embryo (Robertson et al., 2002; Smith and Davidson, 2008). In lateral views apical is at the top. VV — vegetal view; OV — oral view.

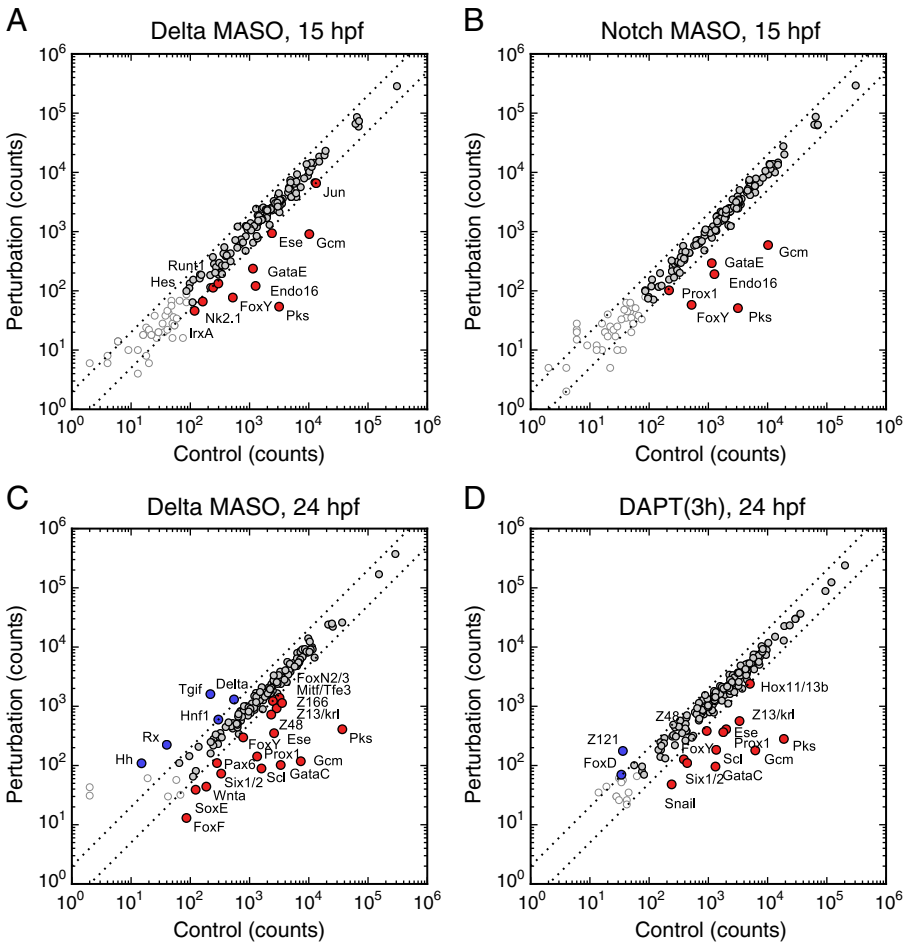

Fig. 2. Quantitative evaluation of Delta/Notch perturbations. (A, B) RNA from Delta MASO or Notch MASO injected, and DAPT treated embryos were extracted and quantified using the NanoString nCounter. The counts obtained for each gene in the codeset in perturbed embryos are plotted against those of control embryos. Perturbation with Delta MASO or Notch MASO produces almost identical results at 15 hpf, except minor differences that are not substantiated in repeat experiments. Only five genes are reproducibly affected indicating that Delta/Notch signaling has a small number of direct targets. (C, D) Application of DAPT, a Notch inhibitor, at 3 hpf produces results equivalent to Delta MASO treatment. At 24 hpf essentially all mesodermal genes included in the NanoString codeset are affected by both perturbations. The dotted lines indicate a threshold of 2-fold change. Transcription levels were estimated from previous quantification data (Materna et al., 2010); genes present with 25 transcripts or less per embryo are marked with an open, gray circle.

nuclease free water. Samples were split and 5 µl was used in NanoString nCounter assays. The leftovers were reverse transcribed.

Transcriptional profiling

For each timepoint and condition, transcript prevalence was measured using the NanoString nCounter. Probe sequences and accession numbers for the genes included in the codeset are given in Supplemental Table 1. Hybridization reactions were performed according to the manufacturer's instructions in 5 µl RNA solution. Care was taken to minimize the time after addition of the capture probe set in order to minimize background due to non-specific interactions between detection probes and capture probes. All hybridization reactions were incubated at 65 °C for a minimum of 18 h. Hybridized probes were recovered with the NanoString Prep Station and immediately evaluated with the NanoString nCounter. For each reaction 1150 fields of view were counted. The resulting counts were normalized using the sum of all counts for all sea urchin genes in the codeset. When injecting mRNA, the counts for the injected transcript were excluded from the normalization. Fold differences were calculated between experiment and control counts.

For quantitative PCR assays leftover RNA was converted to cDNA using the BioRad iScript cDNA synthesis kit (BioRad, Carlsbad, CA). QPCR was performed with the BioRad SYBR Green reagent on an AB 7900 HT instrument (Applied Biosystems, Foster City, CA). Data were evaluated with the dCt method using the average Ct of the polyubiquitin (*ubq*) and *hmg1* genes as reference (Materna and Oliveri, 2008). ddCt values were calculated between experiment and control embryos and converted to fold differences to be comparable with NanoString data. Primer sequences and accession numbers for genes included in the QPCR analysis are provided in Supplemental Table 2.

A table with all perturbation data obtained in NanoString nCounter and QPCR assays is available as Supplemental Material.

Whole mount in situ hybridization

Probe templates were amplified from cDNA by PCR. DIG labeled antisense probes were transcribed with Roche Sp6 or T7 RNA polymerase. Embryos were fixed in 2.5% glutaraldehyde, 32.5% sea water, 32.5 mM MOPS (pH 7) and 162.5 mM NaCl on ice overnight. Embryos were treated with Proteinase K for 5 min at room temperature (25 ng/µl in TBST) followed by a 30 min fixation step in 4% paraformaldehyde, 32.5% sea water, 32.5 mM MOPS (pH 7) and 162.5 mM NaCl at room temperature. Hybridizations were performed using a standard protocol (Ransick, 2004). Probes were hybridized overnight at 65 °C using a concentration of 1 ng/µl hybridization buffer. Probes were detected using anti-DIG Fab fragments conjugated to alkaline phosphatase (1/1000 dilution) and NBT/BCIP. Probe sequences used to amplify the probe template or source of the template are provided in Supplemental Table 3.

Results

delta gene expression in the early sea urchin embryo

The *Strongylocentrotus purpuratus delta* gene is first expressed between 8 and 9 hpf in the cells of the skeletogenic micromere lineage that lie at the center of the vegetal plate (Materna et al., 2010;

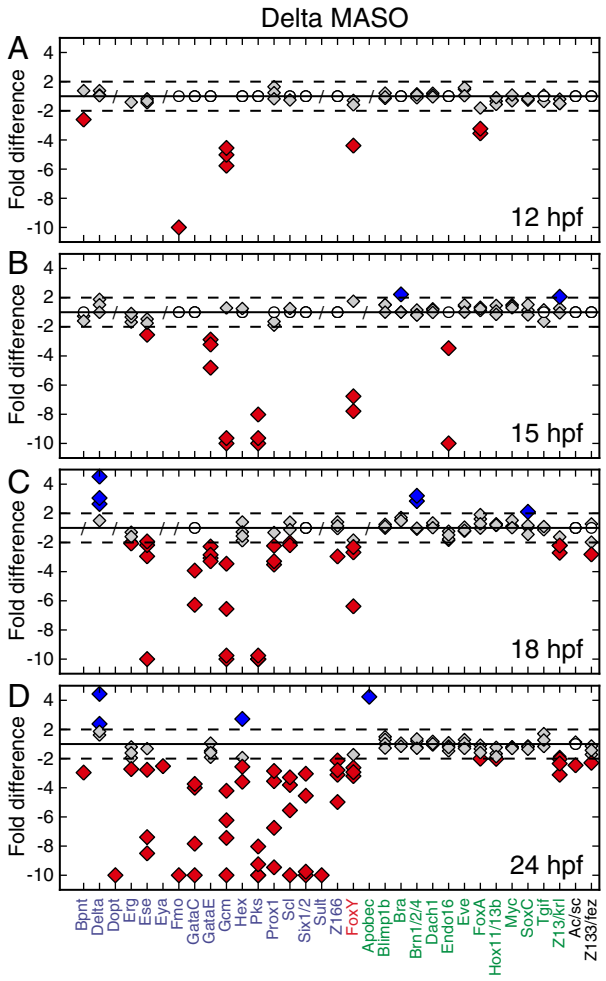

Fig. 3. Effect of Delta MASO treatment on transcript levels. Fold differences were calculated using the quantitative data obtained with the NanoString nCounter and supplemented with QPCR data for genes not included in the codeset. Each diamond represents a single experiment. (A) The earliest gene affected by the perturbation is *gcm*, a known, direct target in the mesoderm. The expression level of *foxY*, a small micromere gene, is also affected at 12 hpf. The early Delta/Notch input into *foxA* (12 hpf) is only transitory (see also Figs. 4A and 5A). (B) The transcription factor *gataE* and pigment cell differentiation gene *pks*, which are both confirmed direct targets of D/N, have reduced expression levels at 15 hpf. *endo16* is strongly activated in endoderm at about 15 hpf but at this time its expression level is significant in only some experiments. The large fold change that is observed in a few experiments is thus due to the relatively big difference between small numbers of transcripts. As soon as it reaches a higher expression level, *endo16* is unaffected by D/N perturbations. (C) At 18 hpf three (oral) mesodermal genes (*ese*, *gatac*, *prox1*) have reduced expression levels in perturbed embryos, but whether they are direct Delta/Notch targets is unknown. (D) At 24 hpf essentially all mesodermal genes (purple labels) have strongly reduced transcript levels. In contrast, expression of endodermal genes (green labels) is impacted only minimally if at all. Apical genes (black labels) are only weakly perturbed. Delta MASO treatment causes increased *delta* expression levels at 18 and 24 hpf. Perturbations affecting the reception of the Delta signal do not cause a similar effect (compare to Figs. 4D, 5D, 7A). Thus, Delta protein itself must contribute to the regulation of Delta transcript, presumably in the SM cells as these are the cells from which Delta is cleared at this time. Dashed lines indicate a significance threshold of 2-fold difference. Genes that are expressed at about 50 molecules or less per embryo are considered insignificant and marked with an open circle. Genes that were not evaluated are marked with a slash (/). For presentation purposes fold differences bigger than 10 fold are shown as 10 fold. A table with all perturbation data is provided as Supplemental Material.

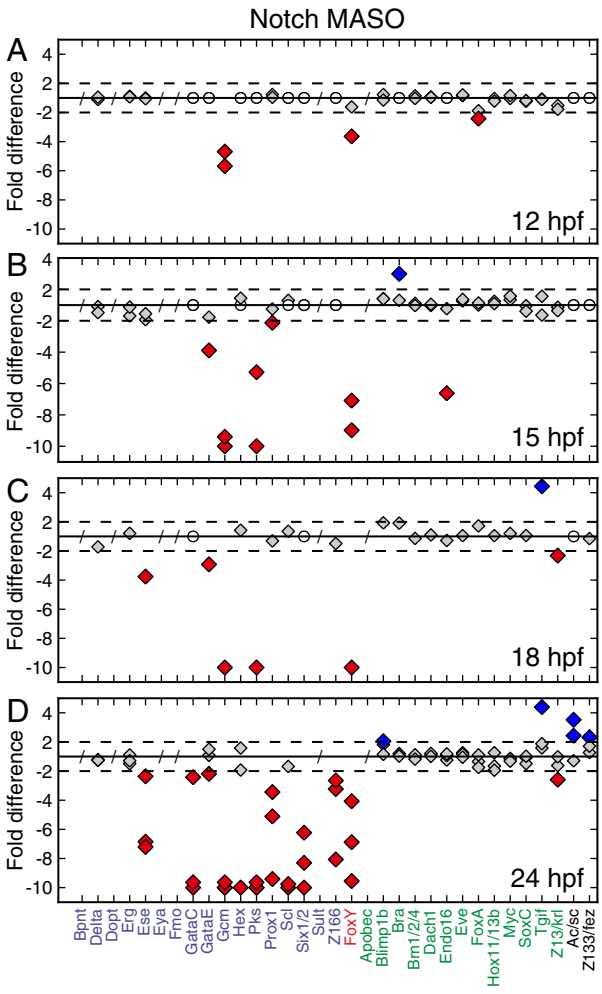

Fig. 4. Effect of Notch MASO treatment on transcript levels. The results are essentially identical to Delta MASO treatment (see Fig. 3): (A–D) Mesodermal genes, and *foxY* in the small micromeres, are strongly affected by Notch MASO injection while endodermal genes are not affected. (C, D) In contrast to Delta MASO injection, which causes an upregulation of *delta* transcripts at 18 hpf and 24 hpf (see Figs. 3C,D), Notch MASO has no effect on *delta* expression. (D) At 24 hpf the apical genes *ac/sc* and *z133/fez* exhibit increased abundance. This effect is stronger in Notch MASO injected embryos (and DN-Su(H) expressing embryos; see Fig. 5D) as compared to Delta MASO injected embryos, where these transcripts are slightly down-regulated (Fig. 3D). The exact spatial relationship between *ac/sc*, *z133/fez*, and *delta* expression is currently unknown just as their connection to other regulatory genes in the apical domain. *z133/fez* has recently been shown to antagonize Bmp signaling in the apical domain (Yaguchi et al., 2011). Data were acquired and analyzed as for Delta MASO. Thresholds and symbols are as in Fig. 3.

Revilla-i-Domingo et al., 2004). At the peak of expression (12 hpf) there are only a few hundred transcripts per embryo (Materna et al., 2010). In addition Delta transcript can also be detected as early as 12 hpf in the apical domain, albeit weakly (Figs. 1A, A′). Delta expression ceases in the skeletogenic lineage as these cells prepare for ingression between 18 and 19 hpf. At about the same time, *delta* transcripts appear in the entire NSM (Sweet et al., 2002). Initially, the NSM forms a ring around the skeletogenic micromere descendants (Figs. 1C, C′), but when ingression is complete it has replaced the latter at the center of the vegetal plate (Fig. 1D′). At this stage (24 hpf) strong *delta* expression is also visible in the apical domain (Fig. 1D). There, it is expressed in only a few cells that align in a row or in a small cluster that appears to be off center relative to the middle of the apical domain (Figs. 1E, E′).

Delta expression is activated by ubiquitously expressed transcription factors, most notably Runx (Fig. 1K) (Smith and Davidson, 2008), but its spatial expression is tightly regulated by the widely expressed

repressor HesC (Revilla-i-Domingo et al., 2007; Smith and Davidson, 2008). In the skeletogenic micromeres *hesC* transcription is repressed by Pmar1, while in the NSM and later skeletogenic lineage hesC is repressed by Blimp1. We show here that *hesC* mRNA has already disappeared from the NSM even before ingression of SM cells is complete (Fig. 1J). But *hesC* transcript does not clear from the neighboring endodermal cells and this limits expression of the *delta* gene to the NSM (Revilla-i-Domingo et al., 2007).

Effects on NSM regulatory gene expression of Delta signaling from the skeletogenic lineage

To obtain a comprehensive picture of the effects of Delta expression on regulatory gene transcription in pregastrular sea urchin development, we assessed the effects of several kinds of D/N signaling perturbation by use of NanoString technology. This method affords simultaneous quantitative measurement of hundreds of mRNA transcripts (Geiss et al., 2008; Materna et al., 2010) under normal or perturbed conditions. We collected perturbed embryos at four timepoints in short succession (12, 15, 18, 24 hpf) to identify genes that are activated by D/N signaling in a time resolved manner. RNA was extracted from these embryos and quantified using the NanoString nCounter. The probe sets used for this study uniquely identified 182 gene transcripts, including genes encoding almost all transcription factors expressed in a localized fashion by 36 hpf, according to published sources (Howard-Ashby et al., 2006a,b; Materna et al., 2006; Rizzo et al., 2006; Tu et al., 2006) plus extensive additional unpublished data from this laboratory. The NanoString data were supplemented with a number of genes, usually pigment cell specific markers, that are not covered by the codeset. The transcript abundances of these genes were determined by QPCR using cDNA generated from the same batch of RNA as used in NanoString runs. QPCR evaluation was usually limited to timepoints at which the genes examined were known to be expressed in unperturbed embryos. All perturbation experiments were carried out at least in duplicate. A table containing all perturbation data is available as Supplemental Material.

D/N signaling was perturbed for these experiments by several different means, all of which gave similar results. These were injection of morpholino-substituted antisense oligonucleotides (MASO) to block translation of the Delta ligand; injection of MASO targeting the Notch receptor; expression of a dominant negative form of the Suppressor of Hairless (Su(H)) that contains a mutation in its DNA binding domain (Ransick and Davidson, 2006); treatment with the γ-secretase inhibitor DAPT, which inhibits cleavage of the Notch intracellular domain following signal reception (Hughes et al., 2009). Any of these perturbations cause embryos to fail to specify NSM, and consequently to lack pigment and blastocoelar cells (or develop only a few), and to fail to form coelomic pouches and circumesophageal muscle, phenotypic effects earlier observed to result from interference with D/N signaling (Sherwood and McClay, 1999; Sweet et al., 2002). A significant fraction of embryos usually exogastrulates if the perturbation agent is injected at fertilization.

Plotting the RNA counts for perturbed embryos against those of control embryos revealed that the prevalence of the vast majority of regulatory gene transcripts is not affected by the perturbations (Fig. 2). This is consistent with the crude phenotypic assessment that, aside from some specific mesodermal defects, development proceeds normally when D/N signaling is blocked. Differences between types of perturbation are negligible compared to biological variation: Genes that show more than two-fold change are robustly affected regardless of the kind of perturbation applied, thus demonstrating the equivalence of Delta and Notch MASOs (after 15 hpf), the expression of DN-Su(H), and treatment with DAPT (Fig. 2). A very few subtle differences in results of these treatments are noted in captions.

By 15 hpf D/N signaling has been active for more than six hours. When D/N signaling is blocked from the beginning, by 12 and 15 hpf the levels of only a small set of regulatory gene transcripts are strongly and specifically reduced. In our data set, *gcm*, *gataE*, *foxA*, and *foxY* are significantly affected in their expression level in repeat experiments (Figs. 3A, B; 4A, B; 5A, B). Given that we queried the regulome comprehensively with regard to spatially restricted gene regulatory factors, this result strongly suggests that the number of direct targets of D/N signaling among regulatory genes is low. *gcm* was previously shown to be a direct *cis*-regulatory target of the N_{ic}/Su(H) complex (Ransick and Davidson, 2006), and is the first gene to become activated by D/N signaling. It is turned on in the veg2 tier of endomesodermal cells that surround the skeletogenic cells when the *delta* gene is first activated in these cells (Fig. 1F). Following the next cleavage, which creates an inner and outer tier of veg2 cells, *gcm* expression is restricted to the inner tier. Only these cells are contiguous to the Delta source on which *gcm* expression is dependent. The spatial expression pattern of *gataE* is in this period similar to *gcm* (Fig. 1G) but it is activated only about three hours after *gcm* (Lee and Davidson, 2004; Materna et al., 2010). The *cis*-regulatory module controlling *gataE* expression also contains functional Su(H) sites, thus proving that, like *gcm*, *gataE* is a direct target of D/N signaling (Lee, 2007; Lee et al., 2007). However, the delay between *gcm*

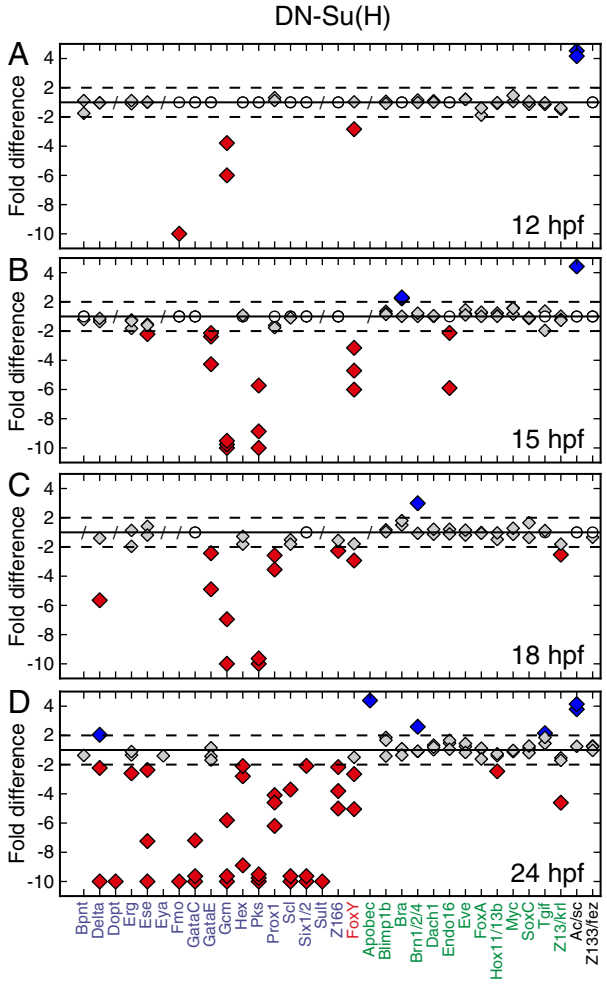

Fig. 5. Effect of dominant negative (DN) Su(H) expression on transcript levels. Overall the effects are essentially identical to Delta MASO treatment (see Fig. 3). DN-Su(H) treatment causes an upregulation of the apical gene *ac/sc* starting at 12 hpf, which is earlier than the effects of the Delta MASO and Notch MASO on this gene. Thresholds and symbols as in Fig. 3.

expression and activation of *gataE* indicates that other inputs are necessary, and in fact as we show elsewhere, Gcm itself is an activator required, in a feed forward relationship with respect to the D/N input, for *gataE* expression to occur in the NSM.

At 18 hfp – only three hours later – our perturbations reveal several additional mesodermal genes that are dependent on D/N signaling (Figs. 3C, 4C, 5C). These genes encode the transcription factors Prox1, GataC, and Ese and are activated at around 16 hpf (Materna et al., 2010). The *prox1*, *gataC*, and *ese* genes are of particular interest because they are specifically expressed in the oral mesoderm (Fig. 6) (Poustka et al., 2007; Rizzo et al., 2006). Expression of the oral mesoderm gene *scl* is also lost. The sharp reduction in transcript levels of these genes when D/N signaling is blocked indicates that oral mesoderm regulatory genes also directly or indirectly require this signal input. We have already seen that the aboral mesoderm genes *gcm* and *gatae* are direct Su(H) targets; the later datasets shown in Figs. 3D, 4D, and 5D indicate that expression of other aboral mesoderm regulatory genes, i.e., the zinc finger gene *z166*, the *six1/2* gene, and the gene encoding its co-factor Eya, also fails in the absence of D/N signaling. In addition expression of the entire battery of downstream pigment cell differentiation genes is lost, i.e., those encoding Bpnt, Dopt, Fmo, Papps, Pks, and Sult (Calestani et al., 2003). In other words, by 24 hpf, if D/N signaling is disrupted by any of several different means, expression of all known mesodermal regulatory genes, both oral and aboral, is either entirely missing or strongly reduced.

Genes that are expressed both in the NSM and elsewhere in the embryo specifically lose expression in the NSM when D/N signaling is blocked, but not in other territories. For example, the *shr2* regulatory gene is expressed in both the aboral ectoderm and the oral NSM; while oral NSM expression is lost when D/N function is perturbed, expression in the aboral ectoderm is unchanged (Figs. 6M, R). Similarly, the expression of *delta* in the NSM is abolished while its apical expression is unaffected (Figs. 6B, G). Thus, the NSM phase of *delta* transcription is dependent on D/N signaling from the skeletogenic mesoderm, just as is the expression of all other mesodermal genes.

Endodermal genes are not targets of NSM Delta

In direct contrast to mesodermal genes, endodermal regulatory genes are only minimally affected or not at all affected by D/N perturbations. As an example of a minor effect which is fully understood, the expression of *foxA* is reduced early, but Notch-dependence is strictly transitory: At 12 hpf the *foxA* gene is expressed in the single veg2

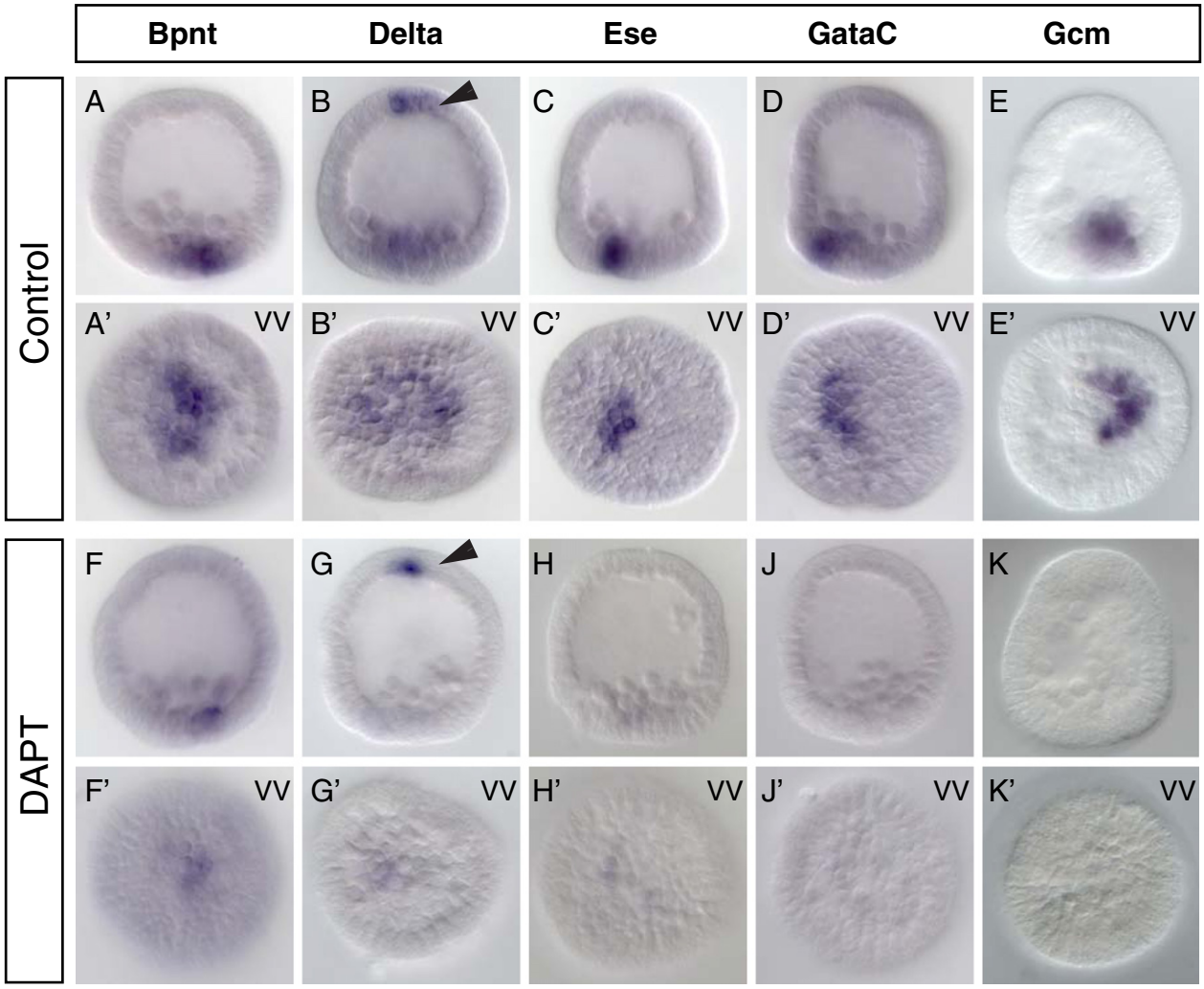

Fig. 6. Spatial effects of Delta/Notch perturbation with DAPT at 24 hpf. (A–N, Q–S) WMISH confirms quantitative results: Following DAPT treatment, mesodermal genes show either severely reduced or no staining by WMISH (*bpnt*, A/F; *ese*, D/H; *gataC*, D/J; *gcm*, E/K; *prox1*, L/Q; *six1/2*, N/S). *delta* and *shr2* are expressed in additional territories, but transcripts are specifically lost in the mesodermal domain (B/G: arrowheads indicate apical expression of *delta*; M/R: asterisk marks mesodermal expression of *shr2*). (O, P, T, U) The endodermal genes *apobec* and *foxA* do not clear from the cells that would normally be specified as mesoderm (compare vegetal view in O′/T′ and P′/U′), but the signal intensity remains the same. In lateral views apical is at the top. VV — vegetal view.

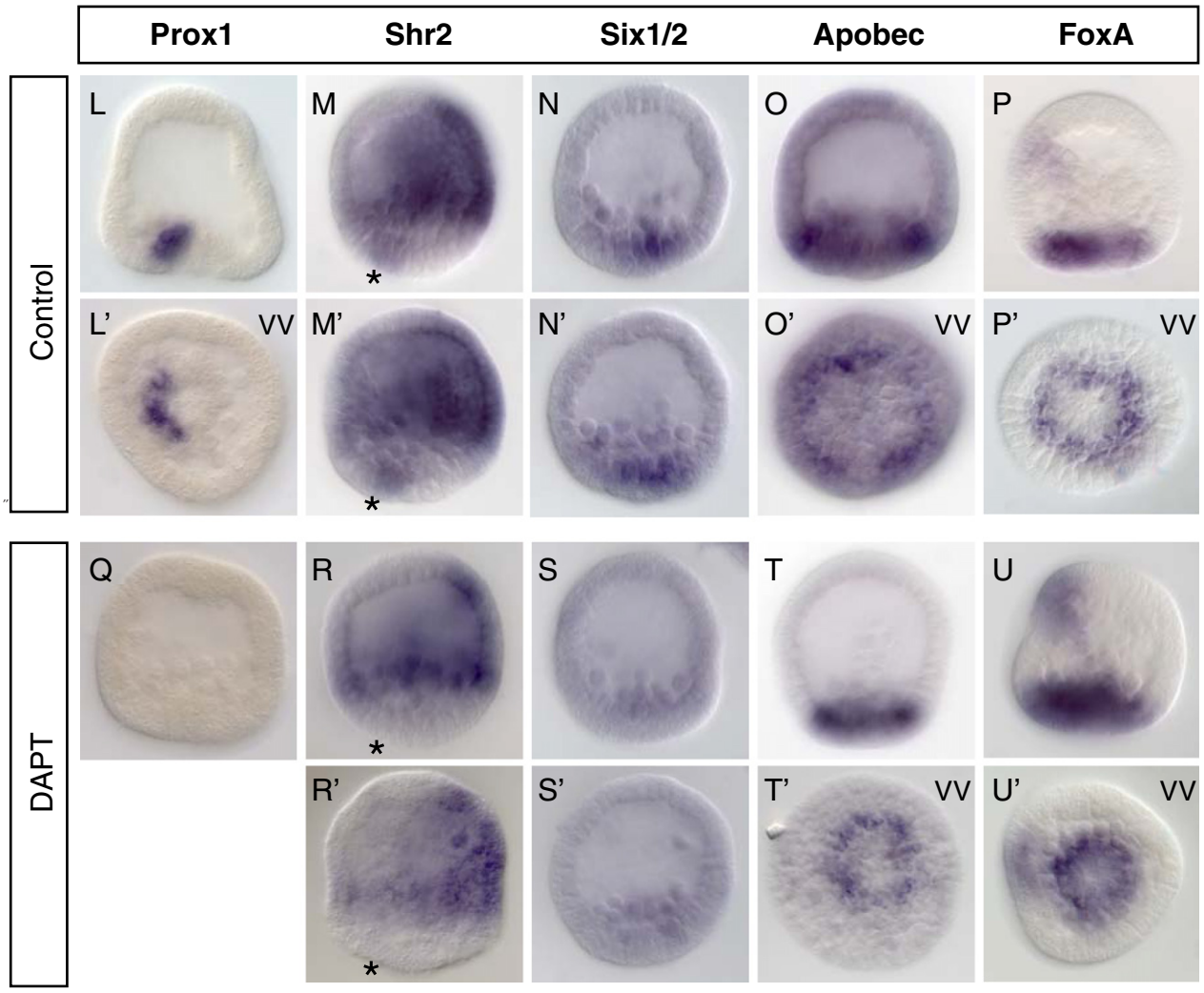

Fig. 6 (*continued*).

tier of cells directly adjacent to the Delta source, and is a direct target of D/N signaling due to a *cis*-regulatory module that contains functional Su(H) sites (de-Leon and Davidson, 2010). However, when these cells undergo radial cleavage *foxA* is also expressed in what is now the outer, or endodermal, ring of veg2 cells which is not in contact with the skeletogenic cells and cannot receive the Delta input. *foxA* expression is predominantly due to activation by β-catenin/TCF, the main early driver of the endoderm gene regulatory network (de-Leon and Davidson, 2010; Peter and Davidson, 2010, 2011b). After 16 hpf, expression of *foxA* and all other endodermal genes normally clears from the mesodermal tier as a consequence of D/N signaling. D/N perturbation interferes with this clearance, because β-catenin is not removed from the NSM nuclei, as discussed elsewhere (Peter and Davidson, 2011b; Sherwood and McClay, 2001). This results in the continued expression of endodermal genes in cells that would normally become NSM. As a further example, we show here that the rings of expression of *foxA* and *apobec*, an endoderm differentiation gene, are substantially smaller in DAPT treated embryos (Figs. 6O, T, P, U). Overall, the absolute prevalence of endodermal transcripts is not significantly altered by D/N perturbation, aside from some very minor effects which are to be expected given the spatial rearrangements caused by the perturbation. The main conclusion, seen explicitly in Figs. 2–5, is that none of the many endoderm regulatory genes included in the NanoString codeset is quantitatively dependent on D/N signaling, so none can be a direct target. This in turn means that the function of *delta* expression in its second, NSM phase is something other than to signal to the adjacent endoderm.

A small micromere target of the skeletogenic Delta signal

An unexpected finding in our NanoString data was the observation that D/N signaling provides a required activating input to the *foxY* gene, which was significantly affected even at the earliest sampling time (12 hpf, Figs. 3A, 4A, 5A). This is surprising because *foxY* is expressed only in the small micromeres and not the NSM (Ransick et al., 2002) (Fig. 1H). The four small micromeres are the product of the unequal 5th cleavage of the micromeres and are generally thought of as 'set aside' in early development for incorporation, after one further round of division, in the coelomic pouches. Their progeny ultimately contribute to the adult rudiment. These cells have generated a unique regulatory state by the early cleavage stages, of which *foxY* expression is one component. The small micromeres also express a set of genes associated with conserved pluripotency and germ line functions, including *vasa*, *nanos*, and *piwi* genes (Juliano et al., 2010; Voronina et al., 2008). After their birth the small micromeres remain located on top of their sister cells the skeletogenic micromere lineage in the center of the vegetal plate. Once

Delta is activated in the skeletogenic cells, the small micromeres are encircled by cells that present the Delta ligand on their surface, which accounts for the availability of the D/N signaling required for their activation of *foxY*.

Function of NSM Delta signaling

In order to block only NSM *delta* expression so as to determine its role separately from the prior skeletogenic phase of *delta* expression, we added DAPT at 17 hpf, a time just prior to the normal handoff of Delta expression from the skeletogenic lineage to the NSM. By 17 h skeletogenic D/N signaling has basically run its course. As shown in Figs. 2 and 7A, B, when introduced at the beginning of development (3hpf) the effects of DAPT are essentially identical to those of delta MASO, dnSu(H), and N MASO.

The effects of DAPT treatment starting at 17 hpf were evaluated by NanoString on embryos collected at 24 and 30 hpf. Remarkably, out of all genes tested, the only gene activated in response to NSM Delta is the small micromere gene *foxY* (Figs. 7C, D). No other gene, neither NSM, nor endoderm, is affected by this perturbation. When the skeletogenic cells ingress, the small micromeres stay behind and come to lie on top of the NSM cells toward the center of the vegetal plate (Fig. 1H). Since skeletogenic cell ingression coincides with the start of *delta* transcription in the NSM, the Delta ligand is continuously presented to the small micromeres, first from the skeletogenic cells and then from the adjacent NSM cells. Thus, D/N signaling functions continuously to maintain *foxY* expression. Phenotypic evaluation of embryos in which only the NSM Delta function has been perturbed produces a consistent result: late treatment with DAPT does not affect pigment cell formation (Figs. 8D–F), but the embryos fail to develop coelomic pouches (Figs. 8D′–F′). Evidently absence of *foxY* expression precludes normal functions in small micromere descendants required for their role in building the coelomic pouches, which are normally composed of about 50% small micromere descendants (Cameron et al., 1991).

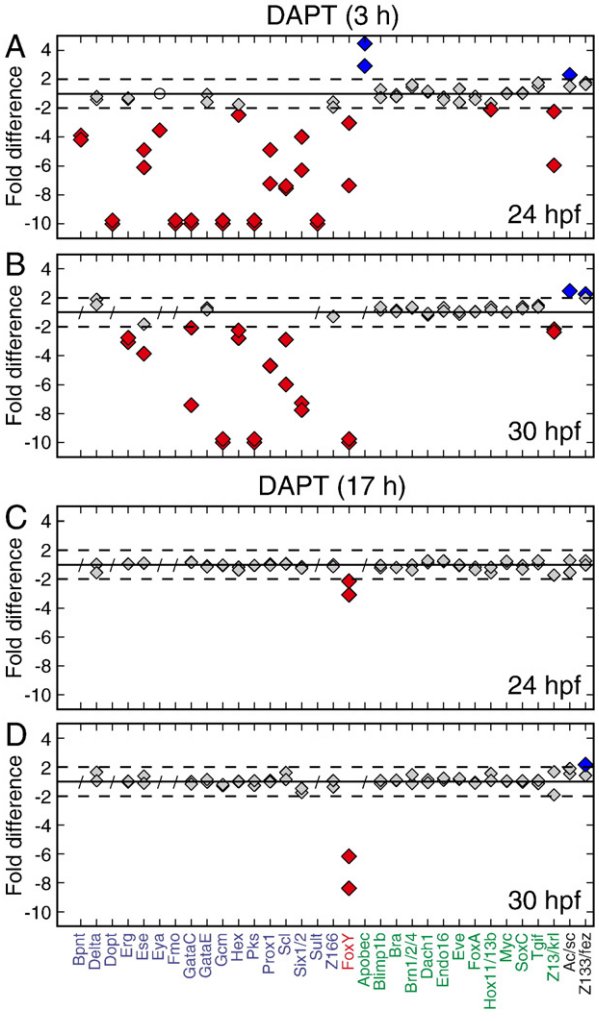

Fig. 7. Effect of mesodermal Delta function perturbation on transcript levels. (A, B) Early addition of DAPT (at 3 hpf) prevents the activation of mesodermal genes while endodermal genes are not affected. (C, D) Addition of DAPT at 17 hpf perturbs the function of late, i.e. mesodermal, Delta. The only gene affected by this perturbation is *foxY* indicating that it requires a continuing activating input from Delta/Notch signaling for its expression. No mesodermal or endodermal genes are affected by loss of mesodermal Delta.

Discussion

Transcription of *delta* in the skeletogenic micromere lineage occurs immediately downstream of the double negative gate that unlocks regulatory specification of these cells soon after they are born (Oliveri et al., 2008; Revilla-i-Domingo et al., 2007). The expression of the Delta signaling ligand is required for pregastrular specification of all mesoderm derivatives in the adjacent NSM. Here we have attempted to determine the complexity of D/N signaling targets among NSM regulatory genes. In addition we sought to provide an unbiased identification of D/N targets at 3-hr time resolution between 12 and 18 hpf, and at 24 hpf, anywhere else in the pregastrular embryo. The method of this analysis depends on an accurate quantitative measure of differences in mRNA levels of >200 regulatory genes in control embryos as compared to embryos in which D/N signaling has been blocked by any of four different methods all of which give essentially identical results. The inclusiveness or completeness of the codeset used to identify regulatory gene transcripts is of course crucial. This codeset recognizes every known regulatory gene expressed in a spatially restricted way up to 36 hpf in the *S. purpuratus* embryo. This includes not only genes identified in earlier genome wide screens (Howard-Ashby et al., 2006a,b; Materna et al., 2006; Rizzo et al., 2006; Tu et al., 2006) but also regulatory genes identified in an extensive transcriptome analysis and in numerous additional WMISH studies (unpublished data). It includes every gene so far incorporated in our GRN analyses for the whole of the endomesoderm, and for the oral and aboral ectoderm as well, and also many genes reported by others to be expressed in the apical neurogenic domain (a complete list of the genes included in our study and a table of all perturbation data is available as Supplemental Material). With respect to the endomesoderm GRN, the D/N targets of which are our particular interest, we have reason to believe that this GRN is approaching completion with respect to its regulatory components, as will be reported elsewhere. A limitation of this analysis should be noted, which is that quantification of perturbation effects may miss genes that have particularly complex expression patterns extending to more than one domain of the embryo at any given time. An example pointed out above is the *shr2* gene: loss of NSM but not ectodermal expression of this gene when D/N signaling is blocked can only be detected by spatial evaluation.

NSM regulatory gene targets of D/N signaling

We now see that only a few genes can be direct targets of D/N signaling in the NSM, and it is their downstream linkages in the aboral and oral mesoderm GRNs that expand the effects of interference with D/N signaling to virtually all mesodermal specification functions. By the time the expression of Delta in the skeletogenic cell lineage terminates, only *gcm*, *gataE*, *prox1*, *ese*, and *gataC* have been activated in the NSM (Fig. 9). Of these, *gcm* and *gataE* are confirmed direct *cis*-regulatory targets of the Notch signal transduction pathway

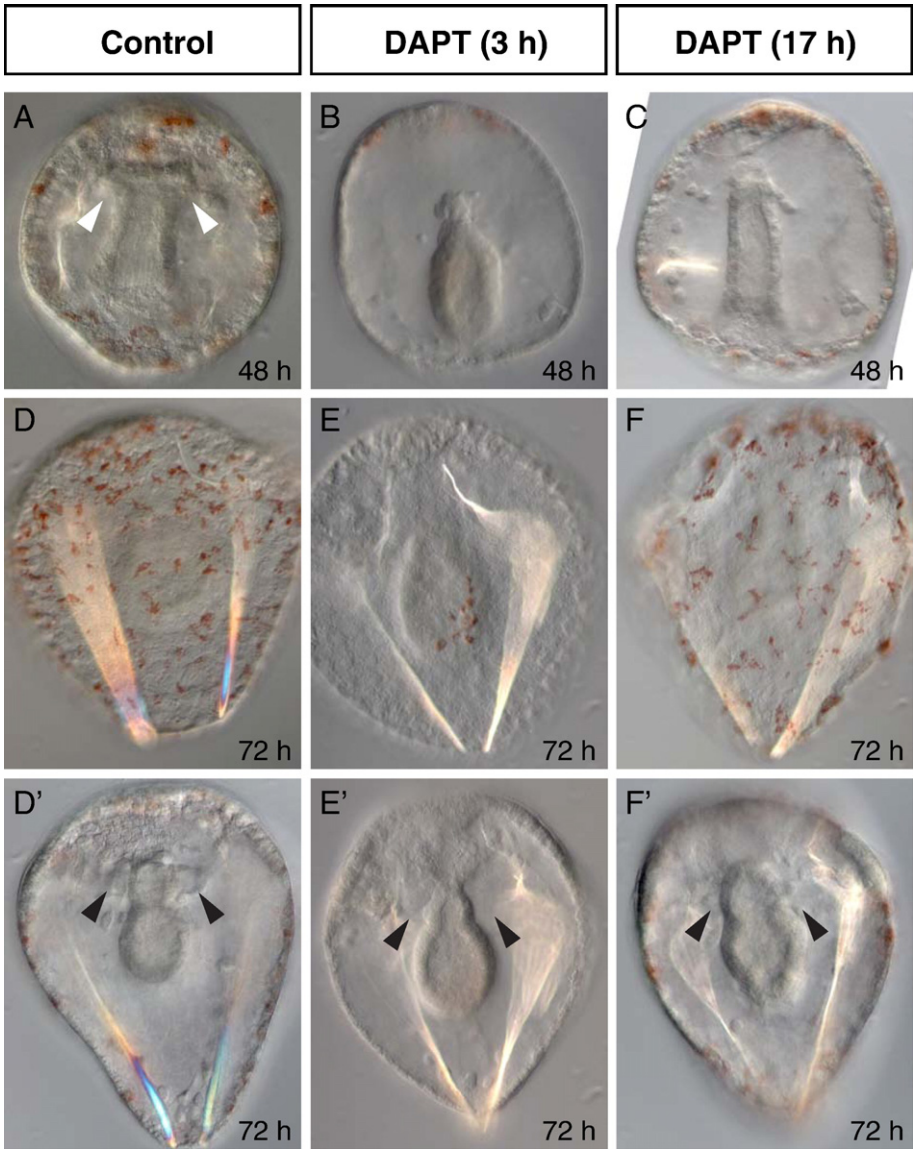

Fig. 8. Phenotype of embryos with perturbed skeletogenic or mesodermal Delta function. (A, D) Control embryos form pigment cells and start developing coelomic pouches at about 48 hpf (A, white arrowheads; D′, black arrowheads). (B, E) Perturbation of skeletogenic Delta (DAPT added at 3 hpf) causes loss of all mesoderm and produces embryos with few, if any, pigments cells. At the DAPT concentration used, about half of the embryos exogastrulate, but of those proceeding normally through gastrulation none form coelomic pouches (black arrowheads in E′). (C, F) In contrast, addition of DAPT at 17 hpf does not interfere with specification of pigment cells. But virtually all embryos lack coelomic pouches (black arrowheads in F′).

(Lee, 2007; Ransick and Davidson, 2006). One or more of *prox1*, *ese*, and *gataC* could potentially be directly activated by D/N signaling as well. However, as we discuss elsewhere, the expression of these particular genes also depends on additional regulatory inputs downstream of Nodal signaling (Duboc et al., 2010), and consequently the onset of their expression is delayed by several hours (Materna et al., 2010).

Expression of pigment cell differentiation genes, such as *pks*, which is expressed precociously in the NSM, is driven by its upstream regulators, the direct D/N targets *gcm* and *gataE* (Calestani and Rogers, 2010). These differentiation genes are thus indirectly affected by D/N signaling. Many additional (regulatory and differentiation) genes are turned on specifically in the NSM after skeletogenic expression of *delta* has stopped (Materna et al., 2010). These genes are affected by D/N perturbations as well and thus indirect targets. In many cases the GRN linkages that relate them to the upstream direct targets are now known (Materna and Davidson, unpublished data). As the addition of DAPT at 17 hpf shows (Fig. 7), NSM genes receive no input from the late phase of *delta* expression in the NSM itself.

The expression of *delta* in the NSM is dependent on D/N signaling from the SM. However, this is likely indirect: *delta* transcription is spatially controlled by the dominant repressor HesC, and activated by the widespread Runx factor (Smith and Davidson, 2008). Loss of *delta* expression is probably due to failure of clearance of *hesC* expression from the NSM as a downstream consequence of the abrogation of the mesodermal GRNs in the absence of the skeletogenic Delta signal or of Notch signal transduction; the exact linkage to *hesc* expression awaits the completion of the mesodermal GRNs. While it lasts, skeletogenic Delta signaling apparently re-enforces *hesc* expression in the NSM by means of a positively acting *cis*-regulatory Su(H) site in the *hesC* gene (Smith and Davidson, 2008), but this mechanism cannot be relevant to the persistence of *hesC* expression in the absence of D/N signaling.

The developmental role of NSM delta expression

Effective Delta signaling is limited to the contiguous cellular neighbors of the Delta source. After 7th cleavage only the veg2 ring

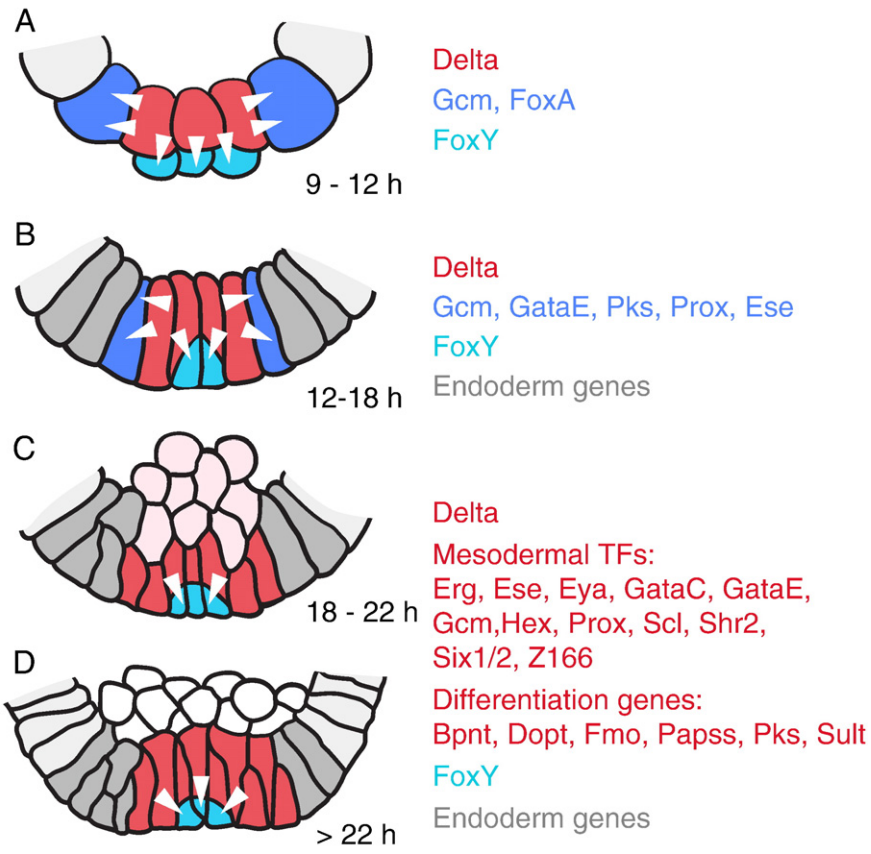

Fig. 9. Schematic representation of effective Delta signaling in the pre-gastrula sea urchin. (A) The Delta ligand is present in the cells of the skeletogenic lineage starting at 9 hpf. It is received in the neighboring cells and turns on *gcm* in the endomesoderm. (B) After the veg2 tier of cells divides into an inner and outer tier, the Delta/Notch signal is only received in the inner tier adjacent to the skeletogenic cells, i.e. the mesodermal precursors. Here, Delta/Notch activates *gataE*, and thereafter the transcription factors *prox1*, *ese* and *gataC* in the oral mesoderm. (C) Concurrent with ingression of skeletogenic cells, *delta* ceases to be expressed in the ingressing cells and instead turns on in the mesoderm. The genes expressed in the mesoderm now run autonomously and no longer require the Delta/Notch signal as an activating input. (D) As skeletogenic cells ingress, the small micromeres remain in the same position at the center of the vegetal plate and come into contact with the mesoderm. Mesodermal Delta is a continuing activating input into *foxY*, which is required to maintain its expression in the small micromeres.

of NSM precursors and the small micromeres are in the position to receive the signal from the skeletogenic micromere descendants. As we see in Figs. 3–5, by 12 hpf *foxY* expression in the small micromeres (Ransick et al., 2002) depends on D/N signaling, and it continues to depend on it to a greater and greater extent throughout the pregastrular period. The 8 small micromeres of the blastula stage embryo remain in contact with the NSM after the *delta* gene begins to be expressed in the NSM. And the experiment of Fig. 7 in which DAPT was added at 17 hpf reveals *foxY* in the small micromeres to be the sole regulatory gene target. No gene in the NSM is affected. Later these embryos exhibit defects in coelomic pouch formation, as was earlier reported for experiments with chimeric embryos (Sweet et al., 2002). It will be interesting to address the function of *foxY* in coelomic pouch development.

It can be predicted that the *foxY* control system holds the key to the mystery of why this gene is not expressed in NSM when Notch signaling is occurring there; the NSM GRN is likely to include a repressive exclusion function (Davidson, 2010; Oliveri and Davidson, 2007) which targets *foxY*. Conversely, *gcm* is expressed only in the NSM and not in the small micromeres, and when the small micromere GRN is solved it will be predicted to include a specific repressor for *gcm* as well. Similarly, *gcm* (indirectly) represses the skeletogenic regulator *alx1* (Damle and Davidson, 2012) and *alx1* reciprocally prevents *gcm* expression in skeletogenic cells (Oliveri et al., 2006).

As *delta* is activated in the NSM, endoderm cells come in direct contact with the Delta source. Yet endoderm genes still show no change in expression level. Endoderm cells express Notch receptor and other genes that are essential for Notch signaling to occur, including *fringe* and *numb*, (Peterson and McClay, 2005; Range et al., 2008; Walton et al., 2006). However, the evidence in Figs. 3–5 shows clearly that no endoderm regulatory gene requires the late NSM Delta signal for expression, though genes such as *foxa* are capable of responding to D/N signaling (de-Leon and Davidson, 2010). Thus the D/N signal appears not to be properly received and/or processed by the endoderm.

Flexibility of D/N signaling function in echinoderm evolution

Despite the fundamentally important role of D/N signaling for NSM specification in the sea urchin embryo, this developmental signaling pathway is used in just the opposite manner in the sea star embryo. These organisms diverged from a common ancestor almost half a billion years ago, and yet they are both indirectly developing echinoderms. In the sea star there is no direct equivalent of the echinoid skeletogenic lineage to serve as a source of Delta ligand. But the equivalent of the NSM – the vegetal disc of mesodermal precursors – does express the *delta* gene in the blastula stage, and this could be regarded as a pleisiomorphic function of vegetal plate mesoderm in the indirectly developing echinoderm embryo. But remarkably from the sea urchin vantage point, *delta* expression is required for regulatory gene expression in the adjacent endoderm of the sea star embryo, not for regulatory gene expression in the mesoderm (Hinman and Davidson, 2007). Interference with D/N signaling in the sea star causes loss of endoderm regulatory specification, and increased mesoderm specification at the expense of presumptive endoderm, in direct contrast with the result in the sea urchin embryo. There, as we have

seen, interference with D/N signaling causes loss of mesoderm and increased endoderm specification at the expense of presumptive mesoderm because of the failure of clearance of endoderm gene expression from the inner ring of veg2 cells (Croce and McClay, 2010; Peter and Davidson, 2011b). As we have pointed out elsewhere (Peter and Davidson, 2011a), the linkages determining deployment of signal systems are commonly among the more evolutionarily flexible aspects of developmental GRN structure, but this is certainly among the most dramatic examples in the literature on comparative GRN evolution.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2012.01.017.

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