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Patterning the dorsal–ventral axis of the wasp *Nasonia vitripennis*

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ABSTRACT

Regulatory networks composed of interacting genes are responsible for pattern formation and cell type specification in a wide variety of developmental contexts. Evolution must act on these regulatory networks in order to change the proportions, distribution, and characteristics of specified cells. Thus, understanding how these networks operate in homologous systems across multiple levels of phylogenetic divergence is critical for understanding the evolution of developmental systems. Among the most thoroughly characterized regulatory networks is the dorsal–ventral patterning system of the fly *Drosophila melanogaster*. Due to the thorough understanding of this system, it is an ideal starting point for comparative analyses. Here we report an analysis of the DV patterning system of the wasp, *Nasonia vitripennis*. This wasp undergoes a mode of long germ embryogenesis that is superficially nearly identical to that of *Drosophila*, but one that was likely independently derived. We have found that while the expression of genes just prior to the onset of gastrulation is almost identical in *Nasonia* and *Drosophila*, both the upstream network responsible for generating this pattern, and the downstream morphogenetic movements that it sets in motion, are significantly diverged. From this we conclude that many network structures are available to evolution to achieve particular developmental ends.

Introduction

All bilaterally symmetric animals face the problem of setting up two orthogonal body axes during embryogenesis. The mechanisms employed in these processes are variable across evolutionary time, with embryological and environmental factors influencing the strategies employed in various lineages. In order to understand how axis determination and patterning processes can change in the course of evolution, a comparative approach that incorporates highly detailed descriptions of homologous developmental processes is required. The establishment and patterning of the dorsal–ventral axis of the fruit fly *Drosophila melanogaster* is one of the most thoroughly described embryonic patterning systems among the bilateria, and thus serves as a valuable point of comparison for studies focused on the evolution of patterning processes.

The chain of events that leads to cell fate determination along the DV axis of the *Drosophila* (and other insects (Lynch et al., 2010)) embryo begins in the ovary (reviewed in (Roth and Schüpbach, 1994)). Here, *gurken* mRNA localized anteriorly and asymmetrically with regard to the short axis of the oocyte, leads to the localized activation of EGF signaling in the overlying follicle cells, and this signal specifies the future dorsal side of the egg (Neuman-Silberberg and Schüpbach, 1993; Roth, 2003). EGF signaling also precisely restricts the expression of the sulfotransferase *pipe* to the ventral follicle cells, which in turn leads to a localized activation of a protease cascade in the perivitelline space (Sen et al., 1998; Cho et al., 2010). The outcome of the activated protease cascade is the graded cleavage, and thus activation, of the Toll ligand Spätzle (Spz) in the ventral half of the perivitelline space (Moussian and Roth, 2005).

In the early embryo, cleaved Spz protein binds the maternally provided Toll receptor present in the plasma membrane. Upon Toll activation by Spz the IκB homolog Cactus (Cact) becomes phosphorylated and degraded, which in turn leads to the release and translocation of the NF-κB transcription factor Dorsal to the nucleus, creating a stable DV gradient of nuclear Dorsal with peak levels at the ventral midline (Moussian and Roth, 2005). Dorsal acts as a morphogen, directly regulating around 50 genes in a concentration dependent manner (Stathopoulos et al., 2002; Hong et al., 2008).

Dorsal target genes contain enhancers that vary in the number, affinity, and arrangement of Dorsal binding sites and determine their sensitivity to nuclear Dorsal concentrations (Stathopoulos and Levine, 2004; Hong et al., 2008). The expression of genes with

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enhancers containing low affinity Dorsal binding sites can only be activated by high levels of nuclear Dorsal, and are thereby restricted to the ventral region of the embryo. Examples are genes like *twist* and *snail*, which are involved in the specification and morphogenesis of mesoderm. Genes such as *ventral neuroblasts defective (vnd)* and *brinker (brk)* that react to moderate and low nuclear Dorsal concentrations are characterized by enhancers containing high affinity affinity Dorsal binding sites in combination with binding sites for bHLH, Supressor of Hairless and/or ETS domain transcription factors. They have lateral, stripe like expression domains, due to repression by Snail ventrally. Finally, genes like *short gastrulation (sog)* and *zerknuellt (zen)*, that react to low levels of Dorsal, are characterized by enhancers containing high affinity Dorsal binding sites with either activating (e.g. *sog* enhancer) or repressing influence (e.g. *zen* enhancer) depending on the presence of closely linked co-activator or co-repressor binding sites, respectively (Rusch and Levine, 1996; Reeves and Stathopoulos, 2009).

Several Dorsal target genes are transcription factors that interact with each other and which further refine and elaborate the expression of downstream target genes. Dorsal not only patterns the ventral and lateral parts of the *Drosophila* embryo, but also plays a major role in regulating the BMP signaling pathway, which patterns the dorsal half of the embryo. By activating the BMP inhibitor *sog* in a broad lateral domain and repressing the BMP2/4-like ligand *decapentaplegic (dpp)* and the metalloprotease *tolloid (tld)* in the ventral half of the embryo, Dorsal facilitates the establishment of a gradient of BMP activation with a sharp peak at the dorsal midline (O'Connor et al., 2006).

Althoughh the *Drosophila* DV patterning system is one of the best understood gene regulatory networks (GRNs), and thus is the gold standard to which other insects will be compared, the fly is not typical of insects in many respects. It shows highly derived features and undergoes a long germ mode of embryogenesis, which is only found among holometabolous insects. In this type of embryogenesis all segments are specified simultaneously within the blastoderm stage embryo (Davis and Patel, 2002). In contrast, all hemimetabolous and some holometabolous insects, such as the beetle *Tribolium castaneum*, undergo a short germ mode of embryogenesis. In this mode of embryogenesis only the head and thoracic segments are specified prior to gastrulation, while the remaining segments are generated and patterned progressively after gastrulation (Davis and Patel, 2002). Thus, short germ patterning requires at least two steps in DV patterning: one acting at the blastoderm stage which partitions the head and thoracic segments, and a second one being active in the post-gastrulation growth zone.

The Dorsal protein of *Tribolium* (Tc-Dorsal), like its *Drosophila* counterpart, forms a gradient during early embryogenesis, and is involved in patterning cell fates along the DV axis of the early embryo (Chen et al., 2000; Nunes da Fonseca et al., 2008). However, the function of Tc-Dorsal differs from that of fly Dorsal in two fundamental ways. First, the Tc-Dorsal gradient is dynamic over developmental time, while the shape of the *Drosophila* gradient is relatively stable (Chen et al., 2000; Kanodia et al., 2009; Liberman et al., 2009). The domain of nuclear Tc-Dorsal is initially weak and shallowly graded, then progressively shrinks to form a steeply graded stripe straddling the ventral midline, before finally disappearing completely just prior to gastrulation. The dynamics of the Tc-Dorsal gradient are a result of a feedback loop of zygotic target genes of Tc-Dorsal, which include both its upstream activating receptor Tc-Toll, and its inhibitor, Tc-Cactus (Nunes da Fonseca et al., 2008). *Tc-Toll*, *Tc-cactus*, and at least one additional zygotic target of Tc-Dorsal (*Tc-twist*) are expressed in dynamic patterns that seem to follow the changes in the Tc-Dorsal gradient (Nunes da Fonseca et al., 2008).

A second difference between the fly and beetle systems is that Tc-Dorsal is only directly involved in specifying cell fates along the DV axis in a fraction of the embryo, since the Tc-Dorsal nuclear gradient is only present prior to gastrulation, and does not operate in the growth zone (Chen et al., 2000). In contrast, fly Dorsal assigns DV cell fates to all segmental primordia prior to gastrulation.

These differences between *Tribolium* and *Drosophila* Dorsal function lead to questions about which characteristics of the *Drosophila* DV patterning system are due to its mode of embryogenesis, and which characteristics of the *Tribolium* system are truly representative of the ancestral mode for insects.

To begin to address these questions, we have initiated an examination of the DV patterning process of the wasp *Nasonia vitripennis*. This wasp has a mode of long germ embryogenesis similar to, but independently derived from, that of the fly, which makes it an ideal model for understanding the patterning requirements for long germ embryogenesis (Lynch et al., 2012). On the other hand, it is a member of the Hymenoptera, the most basally branching order of the Holometabola (fully metamorphosing insects), and *Nasonia* thus represents a key phylogenetic sampling point for reconstructing features of the ancestral DV patterning mechanism within this clade (Lynch et al., 2012).

To fully understand how establishment and patterning of the DV axis come about at a functional level, the process must first be well described observationally. Only after such a thorough description can perturbations of the system be robustly interpreted. Thus, to provide a basis for future functional experiments, and to gain insights into how DV axial patterning comes about in *Nasonia*, we have cloned and analyzed the expression of *Nasonia* orthologs of *Drosophila* DV marker genes covering the entire embryonic axis. We have found that the expression patterns of these genes just prior to gastrulation are highly similar between *Nasonia* and *Drosophila*. However, our results also show that some aspects of the gene regulatory networks both upstream and downstream of this conserved arrangement have diverged significantly between the wasp and the fly. Finally, incorporation of gene expression data from the beetle *Tribolium* into this comparative work has shed light onto features and components of DV patterning that were likely present in the last common ancestor of the Holometabola.

Results

Characterization of early Nasonia embryogenesis

In order to best be able to interpret the dynamics of gene expression, the process of *Nasonia* embryogenesis needed further characterization. We took two approaches to this end. One was timed egg collections and DAPI staining to characterize the stages present at different time points at 29 °C (the temperature at which embryos were laid and incubated) (Fig. S1). The other was to take advantage of the optically clear embryo of *Nasonia* to make time lapse DIC movies of embryogenesis (supplemental movie 1). These two approaches were complementary, and led us to the same conclusions about early *Nasonia* embryogenesis, that were generally consistent with previous work (Bull, 1982). Like *Drosophila*, there are rapid, synchronous syncytial divisions of nuclei before the onset of gastrulation. In *Nasonia*, there are 12 of these divisions, rather than 13 observed in *Drosophila*. The initial pole cell bud occurs after 6 divisions (02:00, supplemental movie 1), one cycle prior to the appearance of the rest of the nuclei on the egg surface (02:20, supplemental movie 1). This is in contrast to *Drosophila*, where the pole cells form simultaneously with the arrival of nuclei to the embryo surface.

Supplementary material related to this article can be found online at doi:10.1016/j.ydbio.2013.05.026.

Once nuclei have arrived at the egg surface, they undergo 5 division cycles of increasing duration. Once the 12th division has been completed, there is a long period before gastrulation (~1 h), during which membrane furrows form between the nuclei, and a clear boundary forms between the nuclei and the yolk sac, completing the process of cellularization (07:00, supplemental movie 1). Once this boundary is completed the embryo begins the process of gastrulation with the mesoderm internalizing ventrally starting at the anterior, and progressing posteriorly, and the posterior gut invaginating posterior-dorsally. As these processes complete, cells on the dorsal side differentiate and migrate ventrally over the ectoderm, forming the serosa (onset at 09:00, supplemental movie 1). All of these morphogenetic movements are distinct from equivalent events occurring in the *Drosophila* embryo (see Discussion).

These observations were made with embryos deriving from both mated and virgin females, which should give rise to mostly female embryos, and all male embryos, respectively. We could not detect any differences between male or female embryos in our time lapse, or in our DAPI analyses. This was somewhat surprising, since in *Drosophila*, haploid embryos undergo an additional, and triploid embryos undergo one less, division cycle prior to gastrulation (Erickson and Quintero, 2007). It may be that since *Nasonia* is obligately haplodiploid, it has developed means to ensure a consistent number of blastoderm nuclei prior to gastrulation between the sexes.

Aside from the morphological movements, the structure of the blastoderm and the arrangement of cell fates prior to gastrulation appear to be almost identical between *Nasonia* and *Drosophila* as described above. Since this similarity is likely due to convergent evolution, it is of interest to compare the molecular patterning processes occurring during this time. We have identified and examined *Nasonia* orthologs of genes known to play a crucial role in DV patterning in other insects, and examined their expression to understand how the mesoderm (ventral region), ectoderm (lateral regions) and extraembryonic membranes (dorsal region) are specified and patterned.

Dynamic gene expression on the ventral side

Two transcription factors, Twist and Snail, are critical factors both in patterning the *Drosophila* DV axis and in specifying the ventral most tissue, the mesoderm (Leptin, 1991). Both of these transcription factors are direct targets of Dorsal, and are expressed in broad overlapping stripes that are initiated weakly and slightly narrower than their broad, largely overlapping final domains (Fig. 1A″–C″).

Orthologs of these genes were cloned, and their expression was observed in the blastoderm embryo of *Nasonia*. Like their fly counterparts, both of these genes are expressed ventrally. However the dynamics of their patterns over time are distinct. Both *Nv-twi* and *sna* are initially detected at cycle 11 in very narrow stripes covering 3–4 ventral nuclei (Fig. 1D″). Over the course of cycle 11, *Nv-twi* and *sna* expression domains expand more or less in concert (Fig. 1E″), until they reach their finally width (16–17 nuclei) and take on their characteristic "slug" shape in cycle 12 (Fig. 1F″). We do not observe any major differences between patterns of *Nv-sna* and *twi* expression during the dynamic or final domain stages, in contrast to the graded *twi* and extremely sharp "on/off" character of *sna* (Fig. 1C and C′) in *Drosophila*. Aside from its expression on the blastoderm, *Nv-sna* is expressed in the yolk nuclei (Fig. 1D″–F″).

Given the apparent dynamic nature of the ventral patterning system in *Nasonia*, it was of interest to compare our wasp results with another system with known dynamic patterning properties: the beetle *T. castaneum*. It is known that the Dorsal gradient and the expression of its early target genes are dynamic in the early *Tribolium* embryo, and it was of interest to determine whether the pattern of simultaneous gene expression in the beetle resembled that of *Nasonia*.

In *Tribolium*, both the temporal and spatial relationship between *Tc-twi* and *Tc-sna* appear to be quite different from that found in either *Nasonia* or *Drosophila*. *Tc-twist* is a very early target of the dynamic Tc-Dorsal gradient, and is first easily detectable as a fairly broad ventral stripe (approximately 9 nuclei wide) expressed over most (Fig. 1G), or the entire length (see Fig. 2G) of the AP axis. This stripe then retracts from the anterior pole, narrows and increases in intensity (Fig. 1H and I). *Tc-sna* expression is not detectable in the early stages of development (Fig. 1G′), and was only seen after *Tc-twi* expression retracts from the anterior pole (Fig. 1H′). *Tc-sna* is not only expressed later, but it is initially expressed in a domain that is significantly narrower than the concurrent *Tc-twi* domain (Fig. 1H″). Prior to gastrulation the *Tc-sna* domain becomes nearly coincident with the slightly broader *Tc-twi* domain (Fig. 1I″).

In the course of our analysis of DV marker genes in *Nasonia*, we unexpectedly observed that the temporal and spatial pattern of *Nv-sim* early expression resembled strongly those of *Nv-sna* and *twi*. The *Nv-sim* stripe starts narrowly, just as the *Nv-sna* and *twi* stripes do (Fig. 2D″, S2E″), and undergoes expansion and refinement as well. The *Nv-sim* domain is consistently wider than both the *Nv-twi* and *Nv-sna* domains, but is largely coexpressed with the other two markers during cycle 11 and a portion of cycle 12 (Fig. 2D″ and E″). In the latter portion of cycle 12 *Nv-sim* begins to clear ventrally (Fig. 2F″, S2F″). The end result of this clearing is that *Nv-sim* is no longer co-expressed at any position with *Nv-twi* or *Nv-sna*, and is found in stripes of 1–2 cells wide flanking the domains of the mesodermal genes (Fig. 2F″, S2H″). After gastrulation, these stripes will constitute the leading edge of the migrating ectoderm, and eventually fuse, forming the ventral midline (Fig. S3A and B).

The early and dynamic expression of *Nv-sim* is in stark contrast to the pattern of *sim* expression in *Drosophila*. Here, *sim* is not detected prior to cycle 14 (Fig. 2A′), and is initially expressed only in a posterior domain (Fig. 2B′). Only after *sna* and *twi* achieve their final domains, is striped *sim* expression observed (Fig. 2C″, S2C″). Theses stripes emerge somewhat gradually in the cells directly adjacent to the lateral edges of the *sna* domain (Fig. 2B′, S2C′).

The *Tribolium* mode of generating *sim* stripes contains elements of both the *Nasonia* and *Drosophila* systems, but also differs from both. Like *Drosophila*, *Tc-sim* expression is initiated well after the first appearance of a ventral *Tc-twi* domain (Fig. 2G″). Like *Nasonia, Tc-sim* appears at a slightly later stage and is expressed in a stripe that is slightly broader than, and completely covering the *Tc-twi* domain (Fig. 2H″). Later, *Tc-sim* is repressed in the most ventral regions, leading to the production of two stripes of 1–4 cells wide flanking the mesoderm (Fig. 2I″). The clearing of *Tc-sim* from the ventral region seems to be correlated with the onset of *Tc-sna* expression (Fig. S2K″–M″), in contrast to *Nasonia*, where *Nv-sim* and *Nv-sna* are initially completely overlapping ventrally (Fig. S2E).

In *Tribolium*, the *cactus* gene was an important marker that gave great insight into how the dynamic, self-regulatory DV patterning system functions in the beetle embryo. It is initially expressed very broadly, then narrows progressively over time, mimicking the pattern of nuclear Tc-Dorsal (Nunes da Fonseca et al., 2008). Given the apparent dynamic nature of ventral patterning in *Nasonia*, as indicated by the behavior of *Nv-twi, Nv-sna,* and *Nv-sim*, it was of interest to determine how the

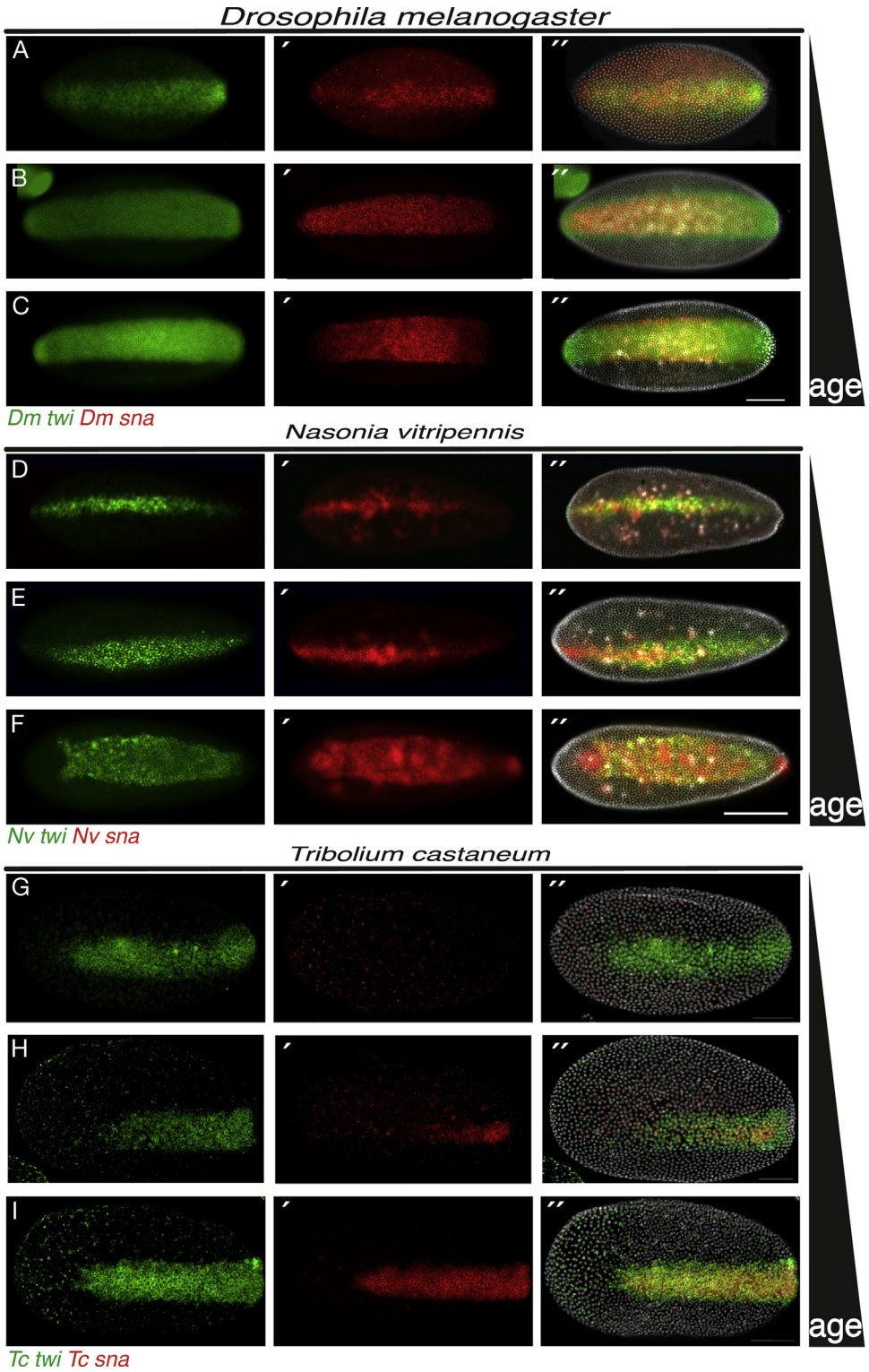

Fig. 1. Simultaneous detection of *twist* and *snail* in *Nasonia*, *Drosophila* and *Tribolium* embryos. (A)–(C″) Ventral views of *Drosophila* embryos in cycle 13 (A)–(A″) and cycle 14 (B)–(C″) comparing *Dm-twi* (A)–(C) and *Dm-sna* (A′)–(C′). Overlays with DAPI (white) (A″)–(C″). (D)–(F″) Ventral views of *Nasonia* embryos in cycle 11 (D)–(E″) and cycle 12 (F)–(F″) comparing *Nv-twi* (D)–(F) with *Nv-sna* (D′)–(F′). Overlays with DAPI (white) (D″)–(F″). (G)–(I″) Ventral views of *Tribolium* embryos in undifferentiated blastoderm (G)–(G″), early differentiated blastoderm (H)–(H″) and late differentiated blastoderm (I)–(I″) comparing *Tc-twi* (G)–(I) and *Tc-sna* (G′)–(I′) expression. Overlays with DAPI (white) (G″)–(I″). Embryos in panels (A″), (D″), (E″), and (G″) are at an equivalent developmental stage (penultimate nuclear division before gastrulation). Embryos in (B″), (C″), (F″), (H″), and (I″) are also at an equivalent stage in the last division directly preceding gastrulation. Scale bar 100 μm. Anterior is left.

Nasonia cactus gene behaves in the early embryo, as it could provide insight into the potential for feedback regulation in the *Nasonia* embryo.

In *Nasonia* there are three *cact* paralogs tandemly arrayed in the genome. Only one of these (XM_001602977) is differentially expressed along the DV axis. In the early stages, (cycle 11) this gene is expressed in a narrow ventral stripe (Fig. 3A′), similar to the early narrow stripes of *Nv-twi, sna*, and *sim* (Fig. 1D–D′, Fig. 2D–D′). In contrast to the other *Nasonia* ventral genes, the *Nv-cact1* expression domain does not expand over the course of development

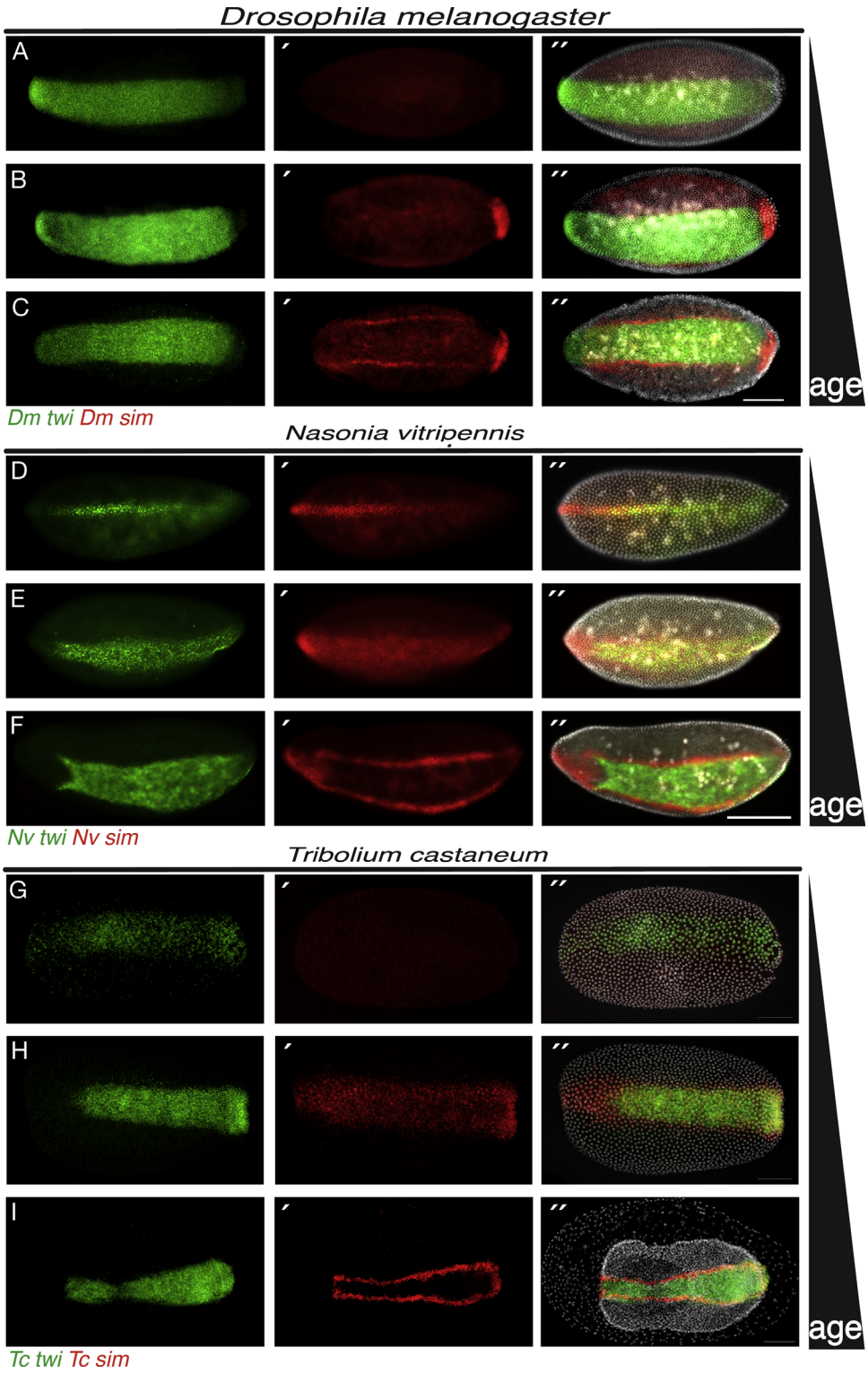

Fig. 2. Simultaneous detection of *twist* and *single-minded* in *Nasonia*, *Drosophila* and *Tribolium* embryos. (A)–(C″) Ventral views of *Drosophila* embryos in cycle 14 (A)–(A″) and (B)–(B″) and at initiation of gastrulation (C)–(C″) comparing *Dm-twi* (A)–(C) and *Dm-sim* (A′)–(C′). Overlays with DAPI (white) (A″)–(C″). (D)–(F″) Ventral views of *Nasonia* embryos in cycle 10 (D)–(D″), cycle 11 (E)–(E″) and cycle 12 (F)–(F″) comparing *Nv-twi* (D)–(F) and *Nv-sim* (D′)–(F′). Overlays with DAPI (white) (D″)–(F″). (G)–(I″) Ventral views of *Tribolium* embryos in undifferentiated blastoderm (G)–(G″), start of primitive pit formation (H)–(H″) and before serosal window closes (I)–(I″) comparing *Tc-twi* (G)–(I) and *Tc-sim* (G′)–(I′). Overlays with DAPI (white) (G″)–(I″). Embryos in (A″), (B″), (F″), (H″) are at equivalent stages, while (D″), (E″), and (G″) are at earlier stages, and (C″) and (I″) are at later (post-gastrulation) stages. Scale bar 100 μm. Anterior is left.

(Fig. 3B′). Rather, the narrow stripe disappears, leaving two terminal spots at each of the AP poles, during cycle 12, prior to gastrulation (Fig. 3C′). Thus unlike *Tc-cact*, *Nv-cact* is not dynamic in early development, which may have implications for the nature of the Dorsal gradient behavior in *Nasonia*.

Lateral markers

The specification and pattern of the neurogenic ectoderm is another function of DV pattering in insect embryos. In *Drosophila*, genes involved in this process are typically expressed in symmetric

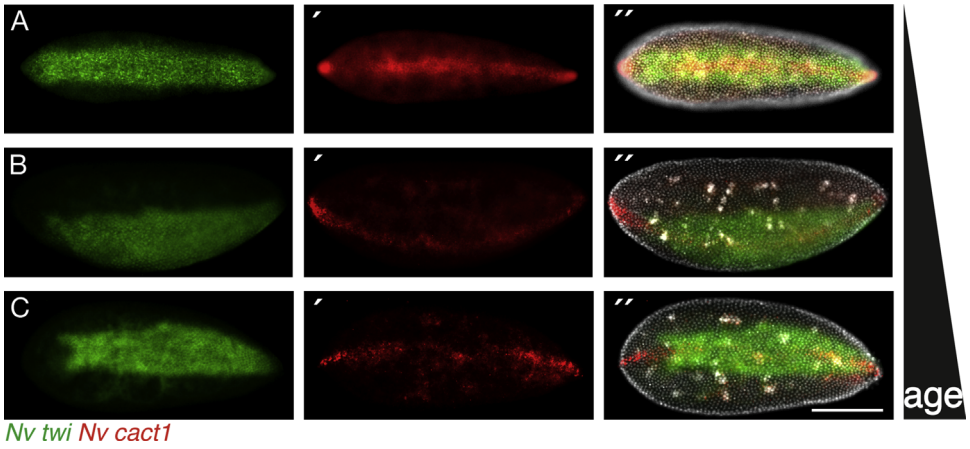

Fig. 3. Characterization of the expression and dynamics of *cact* in *Nasonia*. (A)–(A″) Ventral view of a *Nasonia* embryo in cycle 11 comparing *Nv-twi* (green) and *Nv-cact1* (red) and DAPI (white). (B)–(B″) Ventro-lateral view of a *Nasonia* embryo in cycle 12 comparing *Nv-twi* (green) and *Nv-cact1* (red) and DAPI (white). (C)–(C″) Ventral view of a *Nasonia* embryo in late cycle 12 comparing *Nv-twi* (green) and *Nv-cact1* (red) and DAPI (white). Scale bar 100 μm. Anterior is left. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

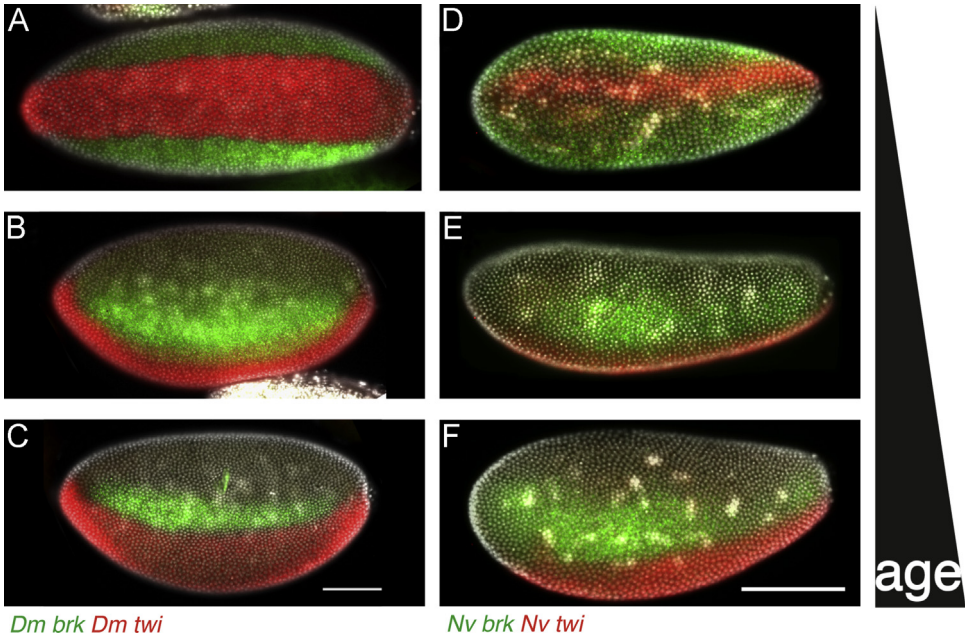

Fig. 4. Comparison of *brinker* expression dynamics in *Drosophila* and *Nasonia*. (A) Ventral, and (B) and (C) lateral views of *Drosophila* embryos comparing *Dm-brk* (green) mRNA and *Dm-twi* (red) expression with DAPI (white) in *Drosophila* during cycle 14. (D) Ventral, and (E) and (F) lateral views of *Nv-brk* mRNA expression with DAPI (white) in cycle 10 (D), cycle 11 (E) and cycle 12 (F) in *Nasonia*. Scale bar 100 μm. Embryos arranged from youngest to oldest from top to bottom in each species panel. Anterior is left. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lateral stripes, reflecting their activation by low to moderate levels of nuclear Dorsal, as well as repression on the ventral side by Snail. Here three markers of differential fates within the neuroectoderm were examined in *Nasonia*: *ventral neuroblasts defective (vnd)*, *intermediate neuroblasts defective (ind)* and *muscle segment homeobox (msh)* (Von Ohlen and Doe, 2000). In addition, an important specifier of general neuroectoderm fate, *brinker (brk)* was also examined in the wasp.

In *Drosophila*, *brk* (Jaźwińska et al., 1999) is expressed in two lateral stripes with sharp ventral borders adjacent to the mesoderm and with relatively fuzzy dorsal borders extending into the region of the dorsal neurogenic ectoderm (Fig. 4A and B). This pattern is somewhat dynamic, being initially broad (Fig. 4A and B), narrowing early in cycle 14 (Fig. 4C), then expanding again just prior to gastrulation (not shown, (Jaźwińska et al., 1999)).

In *Nasonia*, *Nv-brk* is the only neurogenic gene examined that shows expression at the time when the ventral markers (e.g., *Nv-twi*) are expressed in early, very narrow stripes (Fig. 4D). At this stage *Nv-brk* is expressed in broad ventro-lateral domains with relatively fuzzy borders on both the ventral and dorsal edges, and is excluded from the domain of the ventral markers (Fig. 4D). As the embryo ages and the expression domain of *Nv-twi* expands, *Nv-brk* expression is correspondingly cleared from the ventral side (Fig. 4E). During this time, the dorsal border of the *Nv-brk* domain becomes more defined, until the domains of *Nv-twi* and *Nv-brk* are very similar to their counterparts in *Drosophila* (Fig. 4C and F).

The neurogenic ectoderm in *Drosophila* is subdivided by a set of factors known as the columnar genes (*vnd*, *ind*, and *msh*, listed in order of expression from ventral to dorsal). *vnd* is a direct target of the Dorsal gradient, is expressed in stripes of 7–8 nuclei (Jiménez et al., 1995) that lie just dorsally to the stripes of sim expression, and its expression is stable at the blastoderm stage. Expression can be detected early in cycle 14 in stripes that correspond well with the AP and DV extent of the final domain prior to gastrulation

(Fig. 5A). These stripes intensify and become more refined in the time approaching gastrulation (Fig. 5B and C).

ind is expressed just dorsal of *vnd*, in a stripe of approximately the same width, and with limited dynamics (Fig. 5B and C). Like *vnd*, *ind* expression is initiated in a stripe that is complete along the AP axis (Fig. 5B). *ind* initiation occurs later than *vnd*, as we have observed multiple embryos with *vnd* stripes lacking the *ind* domain (Fig. 5A).

In *Nasonia*, the final domain of *Nv-vnd* is also in the form of two broad stripes directly dorsal to the *Nv-sim* domain (not shown). Unlike *Drosophila vnd* and *Nv-brk*, the *Nv-vnd* domain appears relatively late in embryogenesis, just prior to gastrulation. In addition, *Nv-vnd* expression is dynamic with respect to the AP axis. It is first observed at the anterior in stripes extending to approximately 40% egg length (Fig. 5D). These stripes progressively lengthen over time, finally extending to the posterior pole of the embryo just prior to the initiation of gastrulation (Fig. 5E and F, S3C). During gastrulation, *Nv-vnd* marks most of the ectoderm that migrates over the mesoderm (Fig. S3D). Once gastrulation is completed, *Nv-vnd* is expressed in two stripes flanking the ventral midline (S3E).

Nv-ind is initiated later than *Nv-vnd*, and is first detected after the *Nv-vnd* stripe has extended most of the way toward the posterior pole (Fig. 5E). Like *Nv-vnd*, *Nv-ind* expression is also

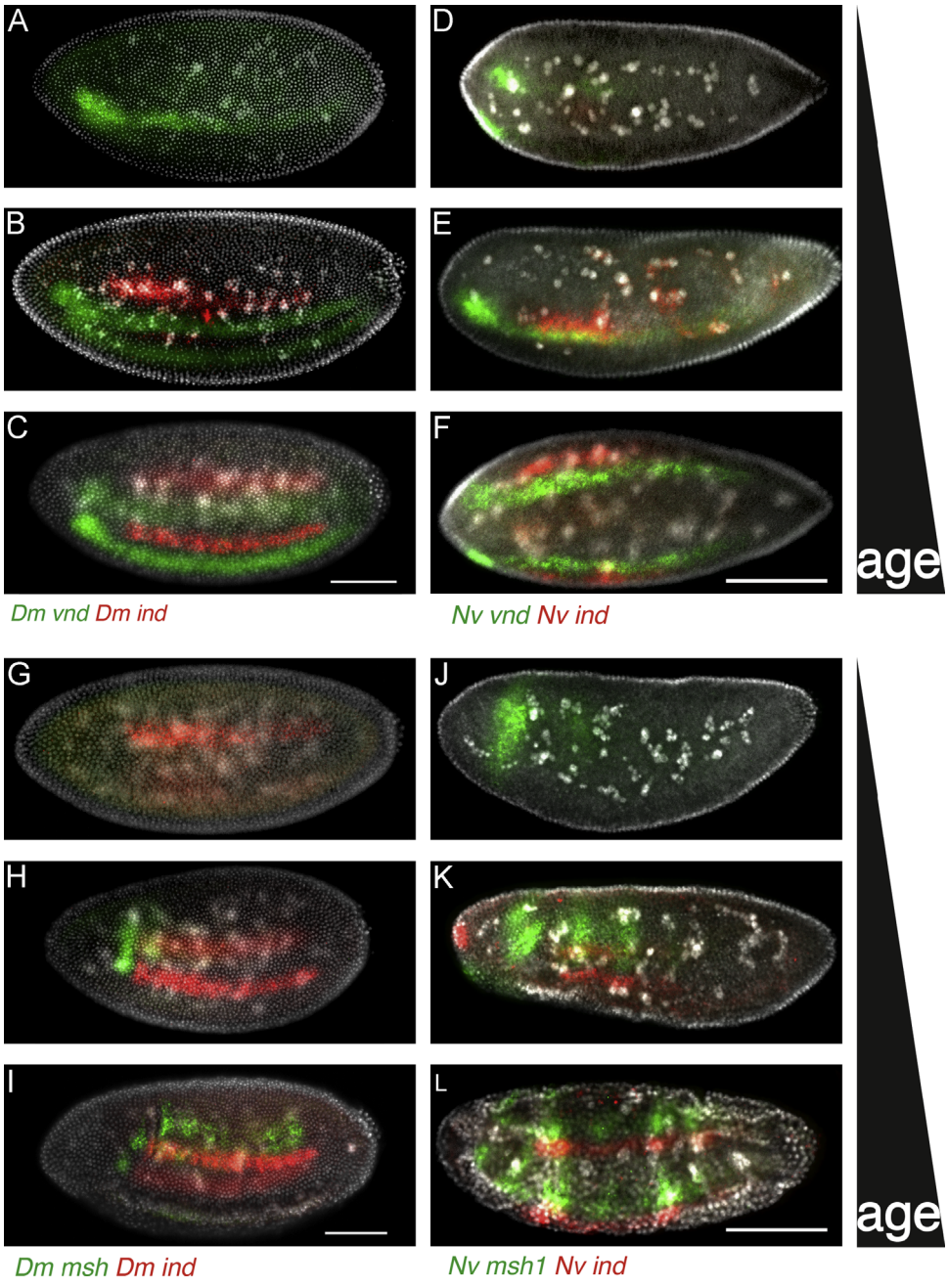

Fig. 5. Comparsion of columnar gene dynamics in *Drosophila* and *Nasonia*. (A)–(C) Lateral views of *Drosophila* embryos in cycle 14 comparing *Dm-vnd* (green) and *Dm-ind* (red) mRNA expression with DAPI (white) in *Drosophila*. (D) and (E) Lateral and (F) ventral views of *Nasonia* embryos in cycle 11–12 comparing *Nv-vnd* (green) and *Nv-ind* (red) mRNA expression with DAPI (white) in *Nasonia*. (G)–(I) Lateral views of *Drosophila* embryos in cycle 14 (G) and (H) and early gastrulating embryo (I) comparing *Dm-msh* (green) and *Dm-ind* (red) mRNA expression with DAPI (white). (J)–(L) Lateral views of *Nasonia* embryos in cycle 11–12 (J), early gastrulation (K) and ongoing gastrulation (L) comparing *Nv-msh1* (green) and *Nv-ind* (red) mRNA expression with DAPI (white). Scale bar 100 μm. Embryos arranged from youngest to oldest from top to bottom in each species panel. Anterior is left. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

initially restricted to the anterior portion of the embryo, and then extends over time toward the posterior pole (Fig. 5E, F, K and L). This process of extension is not completed until after gastrulation has been initiated at the anterior of the embryo.

The most dorsally expressed of the *Drosophila* columnar genes is *msh*, which is initiated still later than *ind* (Fig. 5G). The first evidence of *msh* expression is in the form of a stripe in the head region of the embryo, that extends ventrally in nuclei just anterior to the *ind* domain (Fig. 5H). Subsequently to this, a segmentally modulated stripe of *msh* appears dorsally to the *ind* domain (Fig. 5I). Again this stripe appears all at once, with no detectable AP progression.

As is the case in *Tribolium*, there are two msh-like genes in the *Nasonia* genome (Wheeler et al., 2005; Nunes da Fonseca et al., 2008). Only one of these genes is expressed in a columnar pattern in *Nasonia*, and we refer to this gene as *Nv-msh1*. *Nv-msh1* expression shares characteristics with both fly msh expression, and with the other *Nasonia* columnar genes, but also exhibits some differences. The first evidence of *Nv-msh1* is a stripe covering the dorsal part of the embryonic head anlage, quite similar to that seen in the fly (Fig. 5J). At the time of the appearance of this stripe, a second stripe can be seen arising toward the posterior. This indicates that the initiation of the neurogenic ectoderm domain of *Nv-msh1* occurs prior to the onset of *Nv-ind*, which is in contrast to the pattern observed for both *Drosophila* and *Tribolium ind* and *msh* (Fig. 5K) (Von Ohlen and Doe, 2000; Wheeler et al., 2005). Over time, the full *Nv-msh1* expression domain appears progressively, again, from anterior to posterior, in segmental blocks (Fig. 5K). The full extent of the *Nv-msh1* domain is not completed until well after the initiation of gastrulation (Fig. 5L).

Complexity of dorsal ectodermal and extraembryonic patterning

In *Drosophila* a set of genes is expressed in the dorsal part of the embryo that is used to pattern and differentiate the dorsal ectoderm and amnioserosa (the single extraembryonic tissue in the fly embryo) (Ashe et al., 2000). The novelty of the higher dipteran amnioserosa (Schmidt-Ott, 2000) makes a comparison to *Nasonia* dorsal genes significant, as this wasp which exhibits an independently derived mode of long germ embryogenesis, but has been proposed to posses the ancestral complement of extraembryonic tissues (amnion+serosa) (Bull, 1982; Fleig and Sander, 1988). These two tissues have been described to arise from a narrow dorsal domain that is more similar to the fly amnioserosal anlage than to the typical pattern for amnion and serosa specification found in short and intermediate germ insects, such as *Tribolium* (Van der Zee et al., 2005).

The homeodomain transcription factor *zerknuellt* (*zen*) is a highly conserved marker of extraembryonic fate throughout the insects so far examined (Falciani et al., 1996; Dearden et al., 2000; Panfilio et al., 2006). In *Drosophila* it is initially expressed in a very broad domain covering most of the dorsal side of the embryo (Fig. S4A′). This broad domain later resolves into a narrow stripe that corresponds to the future amnioserosal cells (Fig. S4B′ and C′) (Rushlow et al., 1987).

Drosophila zen is part of a BMP signaling dependent gene regulatory network that produces different threshold outputs of gene expression on the dorsal half of the embryo, which leads to the patterning and subdivision of the amnioserosa and dorsal ectoderm. Genes such as *pannier* (*pnr*) are expressed in broad domains covering most of the dorsal surface of the embryo, and respond to the lowest threshold levels of BMP signaling+zen

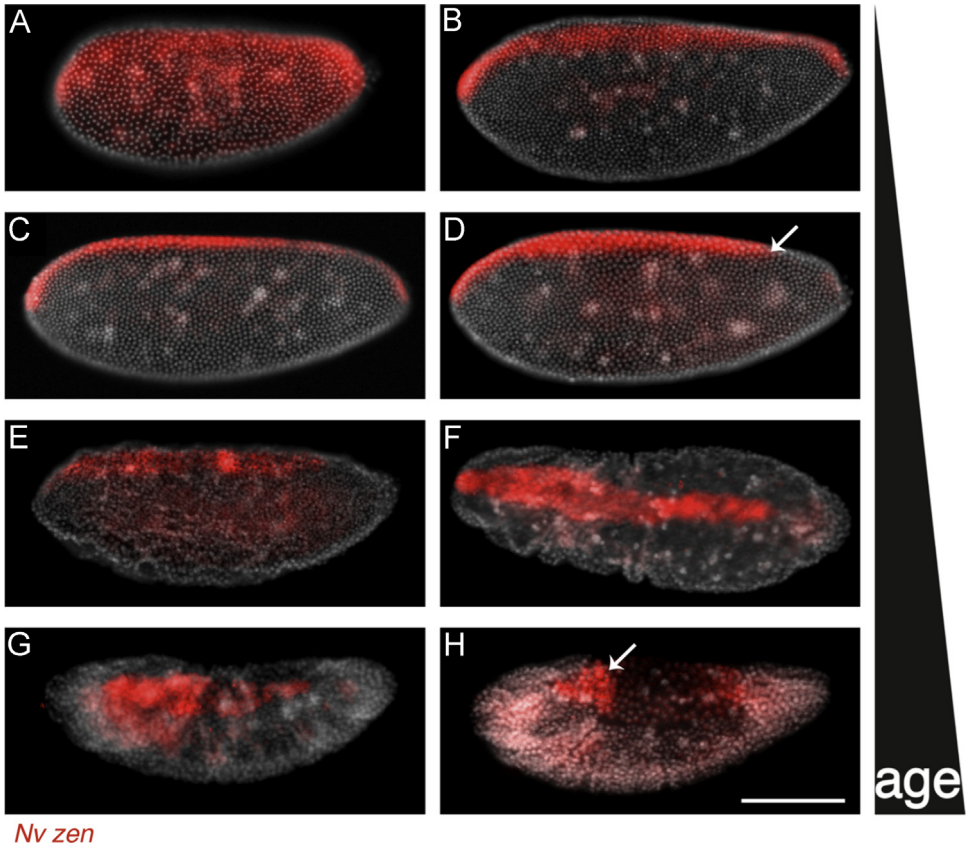

Fig. 6. Dynamics of *Nv-zen* expression in *Nasonia* embryos. (A)–(E) Lateral and (F)–(H) dorso-lateral views of *Nasonia* embryos expressing *Nv-zen* with DAPI (white) in cycle 9 (A), cycle 12 (B)–(D), initiation of gastrulation (E), continuation of gastrulation (F), onset of germ band extension (G) and completion of germ band extension (H). Arrow in D indicates retraction of *Nv-zen* on the posterior pole. Arrow in H indicates *Nv-zen* expression in anterior portion of the serosa. Scale bar 100 μm. Anterior is left.

(Fig. S5A′–C′). Genes such as *tail-up* (*tup*) and *dorsocross* (*doc*) respond to moderate to high levels of BMP+Zen (Fig. S6B and C). Peak levels of BMP signaling are required to activate a third class of genes that includes genes such as *RACE* and *hindsight* (*hnt*) (Fig. S4A–C). In other insects, many of these have conserved roles in the amnion and/or serosa (Van der Zee et al., 2006; Goltsev et al., 2007; Rafiqi et al., 2010). We have cloned and analyzed orthologs of the above mentioned genes from *Nasonia* in order to understand the patterning and tissue specification of the extraembryonic membranes and dorsal ectoderm of the wasp.

Nasonia zen, like its *Drosophila* ortholog is expressed in a narrow stripe over the dorsal midline in the last nuclear cycle before gastrulation. *Nv-zen* is one of the earliest detectable dorsal genes in the embryo. We have detected it in cycle 9 where it is weakly expressed in a rather broad domain (Fig. 6A). In the next cycle, the *Nv-zen* domain is found in a narrow stripe with fuzzy borders that extends from the extreme anterior to posterior pole (Fig. 6B). After the 12th nuclear division, the *Nv-zen* stripe further refines, gains sharp borders and retracts from the posterior pole (Fig. 6C and D). After gastrulation, *Nv-zen* expression is initially strong, then progressively reduced in the presumptive serosa, with strong staining remaining only in the anterior portion of the tissue (Fig. 6E–H).

RACE has a strong requirement both for peak levels of BMP signaling, and for the late phase of narrow zen expression to be fully expressed in the *Drosophila* embryo (Ashe et al., 2000). To determine whether a similar gene regulatory network may be operating in the *Nasonia* embryo, we cloned the *Nasonia* ortholog of this gene, and examined its expression. *Nv-RACE* appears late relative to *Nv-zen*, just before gastrulation, in a stripe that mostly overlaps the *Nv-zen* domain (Fig. 7A″). After gastrulation, *Nv-RACE* expressing cells of the serosa break their epithelial continuity with ectodermal cells, and spread over the surface of the embryo (Fig. S7). The function of RACE in the fly amnioserosa is not clear, but our results indicate that this factor was included in the extraembryonic GRN in the common ancestor of the holometabolous insects.

Another high level target of the BMP+Zen GRN in *Drosophila* is hnt. This gene is expressed in a narrow stripe similar in width to the zen domain, but restricted to the posterior half of the embryo (Fig. S4C″). *Nv-hnt* is unique among the DV genes examined so far. It is initially detected ubiquitously in embryos at cycle 11 (Fig. 7B) and earlier stages (not shown). In cycle 12, most of the transcript disappears leaving a continuous stripe covering both the ventral and dorsal midlines (Fig. 7C). Eventually, the ventral half of this ring disappears, and what remains is a dorsal narrow stripe that corresponds exactly with the *Nv-zen* domain (Fig. 7D″). Again, this gene remains expressed in the presumptive serosa after gastrulation (not shown).

We examined two *Nasonia* orthologs of genes that respond to moderate levels of BMP+*Zen*, *Nv-tup* and *Nv-doc*. *tup* is expressed in a moderately broad stripe in *Drosophila*, similar to that of *doc*. In the wasp, however, the *Nv-tup* expression is quite dynamic, and rather different from *Nv-doc*. *Nv-tup* is first detected in spot-like domains just anterior and posterior to the *Nv-zen* expression stripe, just prior to gastrulation (Fig. 8A). As gastrulation proceeds, and the presumptive serosa begins to expand, *Nv-tup* expression becomes localized to the lateral margins of the serosal region, which may represent the Nasonia amnion (Fig. 8B and C).

Nv-doc also has a rather complex expression pattern in *Nasonia* (Fig. 8D and F). Dorsally, the *Nv-doc* stripe is variable in width. It is broader than the *Nv-zen* domain anteriorly (Fig. 8E″). Starting at about 50% egg length, the *Nv-doc* domain gets narrower than the *Nv-zen* domain. Then, close to the posterior pole, it is wider again. In addition, its domain extends over the extreme anterior pole of the embryo and continues on the ventral side in the presumptive head region (Fig. 8E), unlike most of the dorsally expressed genes that we have examined. This stripe terminates in a broad domain on the ventral side (Fig. 8F).

We next describe the expression of *Nv-pnr*, whose *Drosophila* ortholog is expressed broadly, covering most of the dorsal surface of the embryo (Fig. S5A′). Just before gastrulation, *Nv-pnr* is expressed in a very broad domain straddling the dorsal midline, and extending several cell rows wider than *Nv-zen* (Fig. 9A″ and B″).

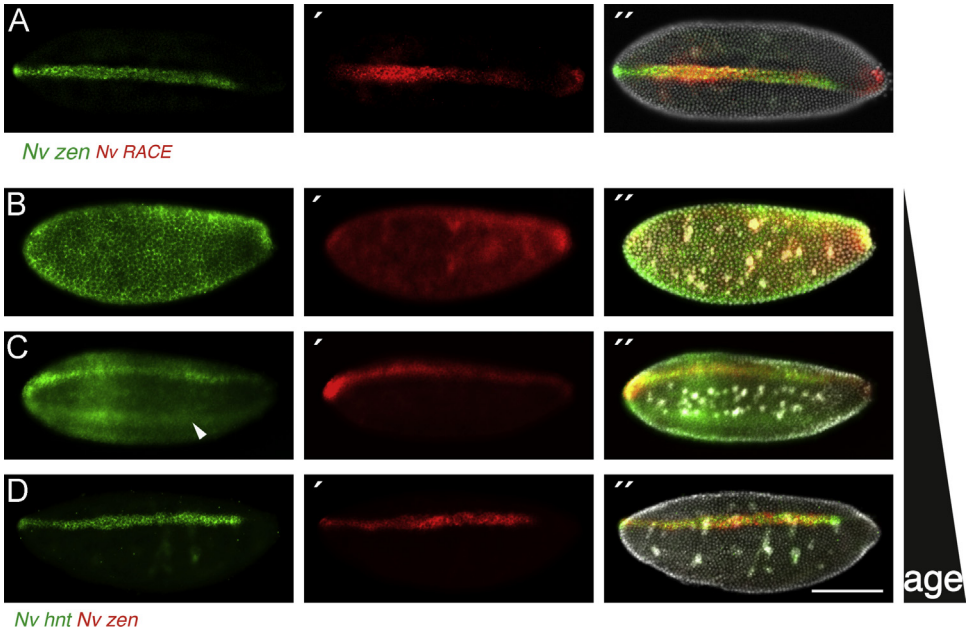

Fig. 7. Dynamics of *Nv-RACE* and *Nv-hnt* in relation to those of *Nv-zen*. (A)–(A″) Dorsal view of a *Nasonia* embryo between cycle 11 and 12 comparing *Nv-zen* (green) and *Nv-RACE* (red) expression with DAPI (white). (B)–(B″) Lateral view of a *Nasonia* embryo in cycle 11–12 comparing *Nv-hnt* (green) and *Nv-zen* (red) expression with DAPI (white). (C)–(C″) Dorso-lateral view of a *Nasonia* embryo in cycle 11 comparing *Nv-hnt* (green) and *Nv-zen* (red) expression with DAPI (white). Note the ventral stripe of *Nv-hnt* is still discernable (arrowhead in C), despite being out of focus. Dorsal view of a *Nasonia* embryo in cycle 12 comparing *Nv-hnt* (green) and *Nv-zen* (red) expression with DAPI (white). Scale bar 100 μm. Anterior is left. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

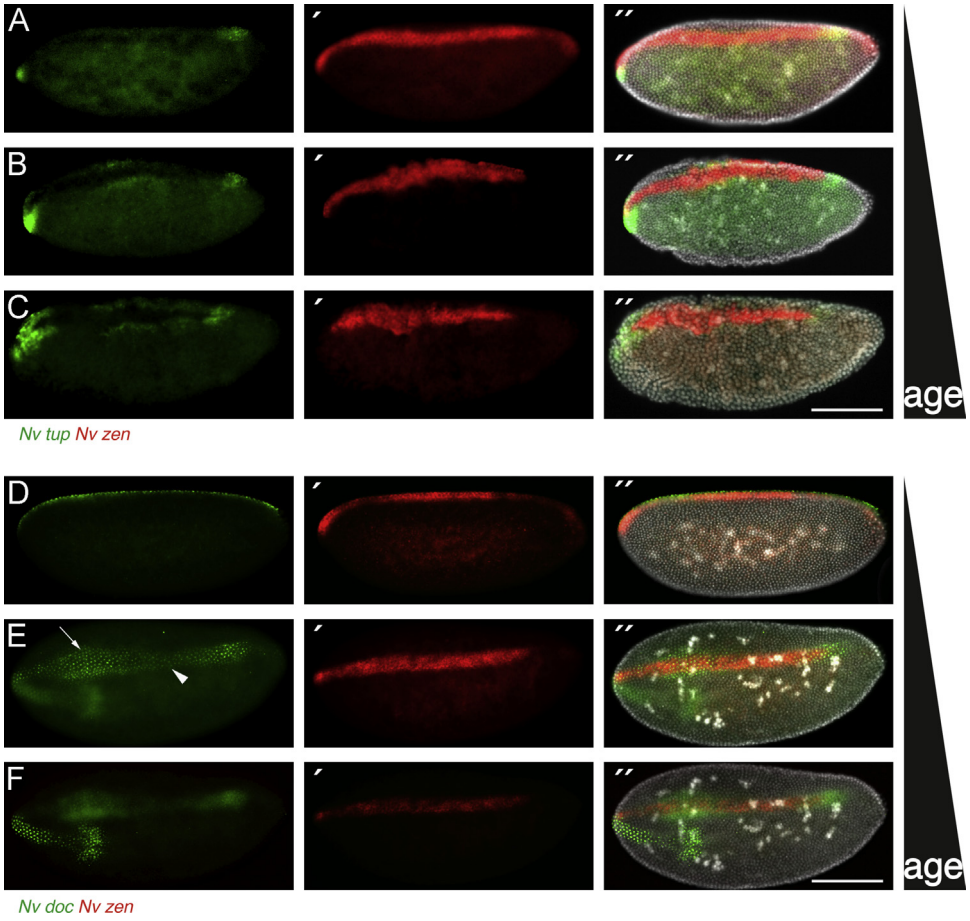

Fig. 8. Dynamics of *Nv-tup* and *Nv-doc* in relation to those of *Nv-zen*. (A″)–(C″) Lateral views of *Nasonia* embryos in cycle 11 (A″), at initiation of gastrulation (B″) and completion of gastrulation (C″) comparing *Nv-tup* (A)–(C) and *Nv-zen* (A′)–(C′) with DAPI (white). (D)–(D″) Lateral view of a *Nasonia* embryo in cycle 11 comparing *Nv-doc* (green) and *Nv-zen* (red) with DAPI (white). (E″)–(F″) Ventro-lateral views of a *Nasonia* embryo in cycle 11–12 comparing *Nv-doc* (green) and *Nv-zen* (red) with DAPI (white) focusing on the dorsal side (E)–(E″) and ventral side (F)–(F″). Arrow in E indicates broadened area of *Nv-doc* expression, arrowhead in E indicates narrowed area. Scale bar 100 μm. Anterior is left. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As development proceeds, *Nv-pnr* is cleared from the presumptive serosal region, and flanks the expanding *Nv-zen* domain in a very similar way to *Nv-tup* (Fig. 9C″).

Finally, we examined the expression of the single *Nasonia* ortholog of the *Drosophila iro-c* genes *araucan/caupolican* (referred to here as *Nv-ara*). In the fly, these genes are expressed late in the dorsal ectodermal region, but the single *Tribolium* ortholog (referred to as *Tc-iroquois*) has served as a useful marker for the amnion (Nunes da Fonseca et al., 2010). *Nv-ara* expression is first detected in two broad patches toward the posterior end of the embryo that flank the domain of *Nv-zen* (Fig. 9D″). As the embryo nears gastrulation, the stripe-like expression extends anteriorly, until the *Nv-ara* domain completely flanks the presumptive serosa, with the stripes being narrow at the anterior, and becoming quite broad at the posterior (Fig. 9E″). After gastrulation, *Nv-ara* refines to two broad, even stripes flanking the serosa (Fig. 9F″).

Discussion

With our analyses of time lapse movies and DAPI stainings in *Nasonia* embryos, we have shown that in general the cellular processes leading up to gastrulation are quite similar, with some interesting differences, between *Nasonia* and *Drosophila*. In our gene expression analyses, we have uncovered points of strong convergence between these embryos, and also cases of major divergence between them in the three main DV domains (ventral (mesoderm), lateral (ectoderm), dorsal (extraembryonic)).

Ventral side

One of our striking results is the observation that the expression of *Nv-cact1*, *twi*, *sna*, and *sim* all start out as very narrow stripes at the ventral midline. The latter three expand over time until they reach their full domains, while *Nv-cact* remains the same width until it is cleared from most of the ventral side. This result gives some insights into the patterning system operating in the *Nasonia* blastoderm embryo. First, it appears that there is a very restricted peak of initial gene activation in early (cycle 11) embryos, covering only a few nuclei at the ventral midline. The sharp edges of these early stripes indicate that the activation is either very steeply graded with a sharp peak at the ventral side, or that these target genes are exquisitely sensitive to specific threshold levels of activation that are only present in this region (or a combination of both). This differs from *Drosophila*, where the early DV regulated domains of *twi* and *sna* are only slightly narrower, weaker, and more graded than their mature domains, likely reflecting the increasing amplitude of the broad, stably shaped nuclear Dorsal gradient (Liberman et al., 2009).

The expansion of the *Nv-twi*, *sim*, and *sna* domains from their initial very narrow domains to their broad, final shape indicates that the ventral patterning GRN has strong dynamic, and possibly self-regulatory, properties. In this respect, the *Nasonia* system has

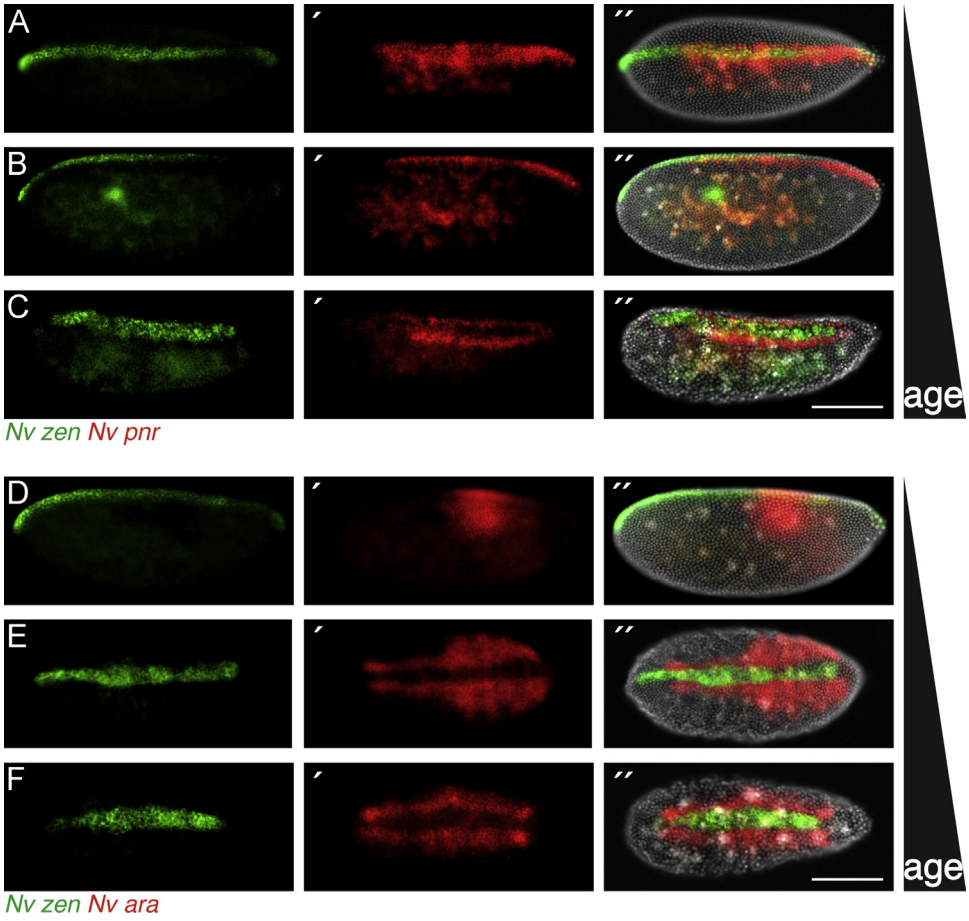

Fig. 9. Dynamics of *Nv-pnr* and *Nv-ara* expression in relation to those of *Nv-zen*. (A)–(A″) Dorsal view of a *Nasonia* embryo in cycle 11 comparing *Nv-zen* (green) and *Nv-pnr* (red) with DAPI (white). (B)–(B″) Lateral view of a *Nasonia* embryo in cycle 12 comparing *Nv-zen* (green) and *Nv-pnr* (red) with DAPI (white). (C)–(C″) Dorso-lateral view of a *Nasonia* embryo in continuation of gastrulation comparing *Nv-zen* (green) and *Nv-pnr* (red) with DAPI (white). (D)–(D″) Lateral view of a *Nasonia* embryo in cycle 11 comparing *Nv-zen* (green) and *Nv-ara* (red) with DAPI (white). (E)–(E″ and F)–(F″) Dorsal view of *Nasonia* embryos in continuation of gastrulation comparing *Nv-zen* (green) and *Nv-ara* (red) with DAPI (white). Scale bar 100 μm. Embryos arranged from youngest to oldest from top to bottom in each species panel. Anterior is left. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

more in common with that of *Tribolium* than with *Drosophila*. In *Tribolium*, a self-regulatory loop composed of components and direct targets of the Toll signaling pathway leads to a dynamic source of DV patterning information in the form of a gradient of nuclear Dorsal that progressively narrows over the course of embryonic development (Nunes da Fonseca et al., 2008). In contrast, while there is quite some crosstalk among Dorsal target genes in the process of refinement of their domains, the *Drosophila* system does not appear to have significant dynamic self-regulatory properties downstream of Toll activation.

While the apparent use of a highly dynamic, and self-regulatory system for patterning the ventral side appears to be conserved between *Tribolium* and *Nasonia*, it is clear that the regulatory mechanism responsible for these features are not the same. In *Tribolium*, the changes in dynamic ventral genes such as *Tc-twist* are due to the shrinking and refinement of the Dorsal nuclear gradient toward the ventral side. In *Nasonia*, the changes in gene expression domains proceeds in the opposite direction, from narrow to broad.

At present it is not clear at which level the dynamic behavior of ventral genes in the *Nasonia* embryo is generated. Preliminary evidence indicates that Toll signaling is required for the activation of ventral genes in *Nasonia* (in preparation), so one explanation could be that the Dorsal gradient is itself dynamic in *Nasonia*. Other possibilities include the presence of feed-forward or feed-back interactions among ventrally expressed genes. A more complete characterization of all of the genes expressed in the ventral region, the distribution of protein products, and the functional connections among genes expressed in this region, are needed to differentiate among these possibilities.

Our analyses also provide insights into the evolution of *sim* regulation among the insects. In *Drosophila*, the sim stripes appear very late in embryogenesis, and seem to arise de novo in a single row of cells flanking the presumptive mesoderm (Fig. 2A′–C′). The pattern is very different in *Nasonia*, where *Nv-sim* appears simultaneously with *Nv-twi* and *sna* in a stripe that is narrow, but noticeably broader than the expression of *Nv-twi* and *sna*. The *Nv-sim* stripe remains broader than *Nv-sna* and *twi* and is largely coexpressed with them throughout most of blastodermal development. Only toward the end of cycle 12 is *Nv-sim* finally cleared from the ventral side of the embryo, leading to the production of two stripes that in the end cover a single row of nuclei flanking the mesoderm (Fig. 2D″–F″, S2E″–H″).

A similar pattern is seen in *Tribolium*, where *Tc-sim* is a relatively early target of the Tc-Dorsal gradient, appearing later than *Tc-twist* but before *Tc-snail* (Fig. 2G″, S2I′–J″). The initial domain of *Tc-sim* is similar to that of its *Nasonia* counterpart, in that it initially covers the ventral side of the embryo in an unbroken domain, and persists in being coexpressed with *Tc-sna* for a significant period of time, before being cleared from the ventral side at the onset of gastrulation (Fig. S2I″–M″). Thus, an early broad stripe of *sim* expression is likely ancestral for at least the holometabolous insects.

Lateral genes

Nv-brk is the first of the lateral genes to be expressed, and appears at about the same time as the early, narrow stripes of *Nv-twi*, sim and *sna* (Fig. 4D). This indicates that *Nv-brk* might have an important role in the early interpretation and further refinement of positional information in the *Nasonia* embryo.

Our results also raise an interesting question about the origin of the use of *brinker* in insect embryonic patterning, since *brinker* is not expressed in the embryo of *Tribolium*. (R.N. da Fonseca, personal communication). Thus, either embryonic *brinker* was present in the common ancestor of the Holometabola, and was lost in the beetle lineage, or it was independently recruited for DV patterning in the wasp and fly lineages.

In contrast to the early expression of *Nv-brk*, genes involved in the partitioning of the neurogenic ectoderm are expressed relatively late in embryogenesis, at about the time just preceding gastrulation. The first of these to appear is *Nv-vnd*, which is the ventral-most of the columnar genes. Unlike the fly *vnd* domain, the *Nv-vnd* domain is initially incomplete along the AP axis, and is only present in presumptive thoracic regions (Fig. 5D). As development progresses, this stripe extends to the posterior end of the embryo, just as the morphogenetic movements of gastrulation are beginning (Fig. 5E and F). *Nv-ind* and *msh1* columnar expression is initiated later, and again in an anteriorly restricted pattern, at a stage where *Nv-vnd* is extended most of the way to the posterior pole (Fig. 5F and K). Thus, there appear to be two temporal gradients of gene activation that affect columnar gene expression: one along the DV axis and one along the AP axis.

Another potentially useful marker for ectoderm fate would have been *short-gastrulation (sog)*, which is a BMP inhibitor, and responds to the lowest levels of nuclear Dorsal in the fly embryo (Rusch and Levine, 1996; Reeves and Stathopoulos, 2009). To date we have found no evidence that this gene exists in *Nasonia*. It is absent from all three *Nasonia* genome sequences and was not detected in any of the numerous EST and next generation sequencing projects deposited in Genbank. In addition, we have sequenced the transcriptome of the *Nasonia* embryo between blastoderm formation and gastrulation, and were not able to detect any *sog* expression. Together, these data indicate that *sog* has been lost from the *Nasonia* genome. This may not be so surprising, as *sog* appears to have no role in establishing early polarity in honeybee (Wilson and Dearden, 2011). In addition the other known function of *Drosophila sog* is in patterning the wing veins. These structures are strongly reduced in *Nasonia*, a trait that is diagnostic for the Chalcid family of wasps (Grissell and Schauff, 1990), to which *Nasonia* belongs. Thus, the combination of a loss of a role in embryonic patterning early in hymenopteran evolution (prior to the divergence of *Nasonia* and *Apis* lineages), and the lineage specific reduction in wing veins, may have allowed the loss of *sog* somewhere along the lineage leading to *Nasonia*.

Dorsal side

One of the characteristic features of the insect embryo is the presence of extra-embryonic membranes, which are critical for the protection of the embryo from the environment, and for morphogenetic movements taking place after gastrulation. Insects vary widely in regard to the proportion of the egg surface which is dedicated to the production of these membranes, as well as where these structures are specified within the coordinates of the egg axes. *Drosophila* is one extreme, where the extraembryonic membranes are reduced to a single tissue type (the amnioserosa), which is restricted to the extreme dorsal pole of the embryo. On the other hand, most other insects generate two distinct extraembryonic tissues, the amnion and the serosa. In short germ insects, these tissues typically derive from both anterior and dorsal egg regions (Panfilio, 2008).

At first glance the *Nasonia* embryo looks very much like that of *Drosophila* in terms of its arrangement of, and egg surface area commitment to, extraembryonic membranes. The best known *Drosophila* marker for this tissue, *zen*, is expressed initially in a very broad pattern, which then refines to a narrow stripe at the dorsal midline (Fig. S4A′–C′). A similar pattern is observed for *Nv-z*en, which in a very early division cycle (10) is expressed in a fairly broad domain restricted to the dorsal side of the embryo (Fig. 6A), and very quickly refines to a narrow stripe of about 4 nuclei wide covering the dorsal midline in the next nuclear division cycle (Fig. 6B and C). It is not yet clear whether the same molecular mechanisms for generating the two phases of *zen* expression in the fly (ubiquitous activation+repression by Dorsal, followed by refinement and amplification of BMP signaling) are also employed in *Nasonia*.

The expression patterns of two of our dorsal marker genes give an intriguing insight into the possible mechanisms used to generate DV polarity in the wasp. Both *Nv-hnt* and *Nv-doc* show expression in a stripe that covers the dorsal midline and continues over one or both poles onto the ventral side of the embryo (Fig. 7C, Fig. 8E–F). *Nv-hnt* is particularly striking, since it has a transient stripe completely covering the embryo circumference, before disappearing from the ventral side (Fig. 7C and D). The above observations indicate that there is a shared characteristic of both the dorsal and ventral midlines of *Nasonia* that allows the initial circumpolar expression of *Nv-hnt* and *Nv-doc*, and that breaking of DV asymmetry allows differential expression at the two midlines.

BMP signaling in *Drosophila* has three threshold outputs on the dorsal side of the embryo, with genes such as *RACE* and *hnt* expressed in narrow dorsal domains, genes such as *tup* and *doc* expressed in somewhat broader stripes, while genes such as *pnr* are expressed quite broadly, and can be activated in the presence of low levels of BMP activity (Ashe et al., 2000). We have also found that there are genes expressed very narrowly (*Nv-RACE, Nv-hnt*), moderately more broadly (*Nv-tup, Nv-doc*), and significantly more broadly (*Nv-ara*) along the dorsal surface of the *Nasonia*. This indicates that there might be a gradient of positional information on the dorsal side of the *Nasonia* embryo with threshold outputs similar in nature to the one found in *Drosophila.* It is clear that while the location and width of the *Nv-zen* stripe is quite similar to that of its fly counterpart, the behavior of the tissue in which it is expressed is quite different. The fly amnioserosa maintains a border with the surrounding ectoderm throughout development until the end of dorsal closure. In contrast, *Nasonia* serosal cells eventually migrate and surround the entire embryo. This latter behavior is typical of the serosal coverings present in most insects. However the *Nasonia* serosal behavior differs from that of most insects, in that its movement involves a severing of this tissue from the flanking epithelium, and the migration of a free edge over the surface of the ectoderm. This behavior is similar to that observed in the honeybee (Fleig and Sander, 1988), indicating that this type of migration is an ancestral character of the higher hymenoptera. Interestingly, this mode of serosal migration is also found in some fly species, such as the scuttlefly *Megaselia* (Rafiqi et al., 2008). This may be an additional case of convergent evolution between hymenoptera and dipterans.

Many of the dorsal/extraembryonic markers analyzed here have also been examined in other insect species, and have been used as indicators of serosa or amnion fate. *zen* orthologs are generally reliable markers of the serosa, but also have roles in the amnion in *Tribolium* and *Megaselia* (Van der Zee et al., 2005; Rafiqi et al., 2010). *hnt* orthologs seem to vary in their expression, with a narrow, *zen*-like, serosal expression in *Nasonia,* a broader domain encompassing both the amnion and serosa in *Megaselia* (Rafiqi

et al., 2012), and a domain flanking the presumptive serosa in the mosquito *Anopheles* (Goltsev et al., 2007). Expression of *hnt* has so far not been reported in *Tribolium*. In both *Megaselia* and *Anopheles*, *tup* and *doc* are expressed in domains flanking the serosa, which will give rise to the amnion (Goltsev et al., 2007; Rafiqi et al., 2010). While *Nasonia tup* matches this pattern, and likely also is an amnion marker in the wasp, *Nv-doc* differs in that it is only slightly broader than the serosal primordium, and is not repressed at the dorsal midline. *Tribolium doc* is also exceptional, as it is expressed in the dorsal serosa (Van der Zee et al., 2006). *Tribolium pnr* and *ara* are both specific amnion markers, and the expression of *Nv-pnr* and *ara* are also consistent with amniotic roles (Van der Zee et al., 2006; Nunes da Fonseca et al., 2008), with *Nv-ara* likely also having a significant role in the dorsal ectoderm.

Thus it is clear that there is much plasticity in gene regulatory systems in the insect extraembryonic and dorsal ectoderm, but that there also may be a conserved core of factors that are crucial for the specification of amnion, serosa, and dorsal embryonic ectoderm across insects.

Conclusion

In this work we have shown that the arrangement of cell fates, and the expression of genes that mark these fates, are strongly convergent along the DV axis of the long germ embryo of *Nasonia* just prior to gastrulation when compared to those of *Drosophila* at an equivalent stage (Fig. 10). Given the large evolutionary distances between the wasp and the fly this may represent a developmental constraint impinging on the evolution of rapid long germ embryonic patterning among the holometabolous insects. The stringency and true nature of this constraint can be explored by further sampling additional cases of independent evolution of the long germ mode of embryogenesis in other insect orders. Only in this way can lineage specific traits (which are in themselves interesting) and mere coincidence be distinguished from a true signal of constraint.

In contrast to the similarity seen at the stage just prior to gastrulation, we have found that the dynamics of the generation and interpretation of positional information before this stage is quite diverged (Fig. 10). In addition, the behaviors of cells and tissues during and after gastrulation are also quite different between *Drosophila* and *Nasonia* (Fig. S3, Fig. 10). Together these observations indicate that the GRN governing DV patterning and cell type specification in *Nasonia* is structured rather differently and could have novel components that are not found in the *Drosophila* DV GRN. For these reasons, a comprehensive characterization of the *Nasonia* DV patterning system, at the level of its components, their interactions, and the output of the preceding in regard to cell fate and behavior, will give major insights into how convergent traits can evolve.

Methods

Embryo collection and fixation

All *N. vitripennis* embryos were collected using the Waspinator (Fig. S8). This modified petri dish harbors 19 hosts, which can be accessed by up to 120 *Nasonia* females at the same time only from the anterior side. To collect the embryos, parasitized hosts were cracked open at the anterior side and dipped into the fixing solution (5 mL heptane, 2 mL 10% methanol-free formaldehyde, 2 mL 1xPBS, in a 15 mL scintillation vial). Fixation and subsequent hand devitellinization were done as described in Lynch and Desplan (2006).

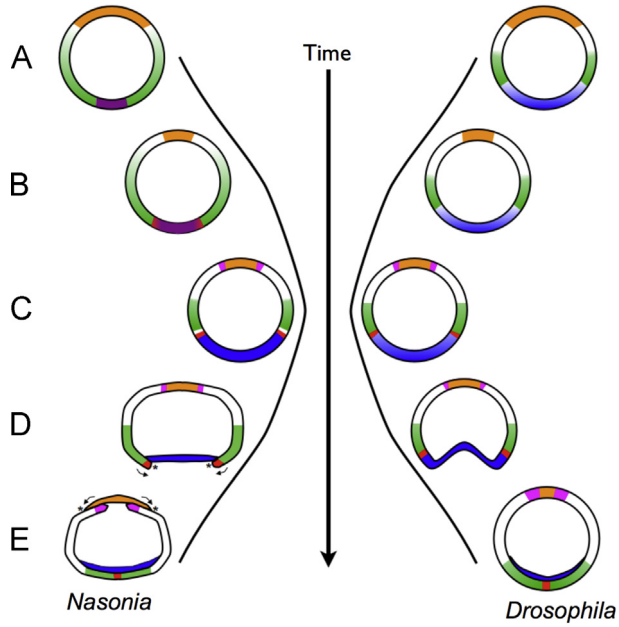

Fig. 10. Summary of convergences and divergences between *Nasonia* and *Drosophila* embryos. Schematic representations of embryogenesis of *Nasonia* (left) and *Drosophila* (right) from mid-blastoderm stage (A) until after the completion of gastrulation (E). Colored regions correspond to different exemplar gene expression patterns: blue=*twi*, red=*sim*, green=*brk*, orange=*zen*, magenta=*tup*, purple=blue(*twi*)+red(*sim*). (A) The *Nasonia* embryo diverges from *Drosophila* in that *Nv-twi* and *sim* are expressed in a very narrow, overlapping (red+blue=purple) domain, while in *Drosophila* twi expression is broad and sim is not yet detected. *Nv-brk* is found quite ventrally, while *Dm-brk* is restricted to the lateral sides. (B) As development proceeds the *Nv-twi+sim* domain dynamically expands, while the *Drosophila* pattern remains for the most part static. In both species, zen expression retracts to a very narrow dorsal stripe. (C) *Dm-sim* becomes expressed in two lateral stripes and *Nv-sim* expression is cleared from the *Nv-twi* expression domain resulting in two lateral stripes of *Nv-sim* expression, too. Hence the arrangement of markers of tissue fates are basically identical between *Nasonia* and *Drosophila* just before gastrulation. (D) *Nasonia* and *Drosophila* embryos diverge again at the onset of gastrulation. *Drosophila* mesoderm is internalized through a ventral furrow, whereas the embryonic epithelium remains intact. In contrast, *Nasonia* mesoderm is internalized by a rupturing of the epithelium at the border between *Nv-sim* and *Nv-twi* expressing cells, leading to free edges (*) that migrate over the mesoderm, and eventually fuse at the midline (see E). (E) After the completion of gastrulation, morphogenetic movements differ on the dorsal side between *Nasonia* and *Drosophila*. In *Nasonia*, the border between serosal cells and putative amnion is ruptured, leading again to free edges (*) that move over the ectodermal surface. In contrast, the fly amnioserosa gradually shrinks as the embryonic flanks expand dorsally. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In situ hybridization

Single in Situ hybridizations for *Nasonia* and *Drosophila* were performed as previously described (Brent et al., 2003).

Double fluorescent in situ hybridizations in *Nasonia* were performed as described in Lynch et al. (2010). In brief, digoxigenin and biotin labeled probes were hybridized simultaneously. They were detected by anti-dig::POD (Roche, 1:100), and anti-Biotin::AP (Roche, 1:2000) antibodies respectively. Fluorescence was generated with the AlexaFluor 488 TSA kit (Invitrogen), and a modification of the HNPP/Fast Red (Roche) protocol, respectively.

Double fluorescent in situ hybridizations in *Tribolium* were performed using a modified version of the protocol described in Lynch and Desplan (2010). Digoxigen-labelled and dinitrophenyl (DNP)-labeled probes were added simultaneously and were detected using anti-digoxigenin::AP antibody (1:2500, Roche) coupled with HNPP Fluorescent detection (Roche), and 1st anti-DNP-rabbit antibody (1:400, Molecular Probes) plus 2nd anti-rabbit-HRP antibody (1:100) combined with AlexaFluor 488 TSA

kit (Invitrogen), respectively. A step by step protocol is available at: http://www.uni-koeln.de/math-nat-fak/ebio/Research/Roth/Protocols/protocols.html.

Probes were produced as in Lynch et al. (2010), using the gene specific primers listed in the Supplementary Table.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.05.026.

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