Title: Dorsoventral polarity of the *Nasonia* embryo primarily relies on a BMP gradient formed without input from Toll

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Running Title: Nasonia DV patterning

Highlights:

- BMP signaling is required for global embryonic DV polarity in *Nasonia*
- BMP signaling at the dorsal pole is independent of ventral Toll signaling
- Toll signaling has a limited role in *Nasonia*
- BMP signaling gradient is established by novel means in *Nasonia*

Summary: In *Drosophila*, Toll signaling leads to a gradient of nuclear uptake of Dorsal with a peak at the ventral egg pole, and is the source for dorsal-ventral (DV) patterning and polarity of the embryo [1-3]. In contrast, Toll signaling plays no role in embryonic patterning in most animals [4, 5], while BMP signaling plays the major role [6]. In order to understand the origin of the novelty of the *Drosophila* system, we have examined DV patterning in *Nasonia vitripennis* (Nv) [7], a representative of the Hymenoptera, and thus the most ancient branch points within the Holometabola [8]. We have previously shown that while the expression of several conserved DV patterning genes is almost identical in *Nasonia* and *Drosophila* embryos at the onset of gastrulation, the way these patterns evolve in early embryogenesis are very different from what is seen in *Drosophila* [7] or the beetle *Tribolium* [9]. In contrast to *Drosophila* or *Tribolium*, we find that wasp Toll has a very limited ventral role, BMP is required for almost all DV polarity of the embryo, and that these two signaling systems act independently of each other to generate DV polarity. This result gives insights into how the Toll pathway could have usurped a BMP based DV patterning system in insects. In addition, our work strongly suggests that a novel system for BMP activity gradient formation must be employed in the wasp, since orthologs of crucial components of the fly system are either missing entirely or lack function in the embryo.

Main Text:

BMP and Toll pathways act independently in DV axis formation

Multiplex *in situ* hybridization techniques with *Nv-twist* (*-twi*), *Nv-brinker* (*-brk*), and *Nv-zerknuellt* (*-zen*) allow a simultaneous global view of the DV axis of *Nasonia* blastoderm embryos (nuclear cycles 11-12, which are equivalent to cycles 13-14 in *Drosophila)* (Fig. 1A, see [7]). At cycle 12 *Nv-twi* is expressed in a ventral domain, giving rise to the mesoderm, *Nv-brk* is expressed in ventrolateral domains giving rise to CNS and ventral epidermis and *Nv-zen* is expressed in a narrow dorsal domain, which forms extraembryonic tissue. The association of expression pattern with cell type rests on the observation of gastrulating embryos showing that cells derived from particular regions execute a characteristic morphogenetic program (see [7]). *Drosophila* cycle 14 embryos show similar expression patterns of orthologous genes giving rise to the same or to similar cell types. In *Drosophila* these patterns are established by the consecutive action of the Toll and BMP signaling pathways. Toll signaling acts as a morphogen to directly control the expression of ventral and ventrolateral genes. It also directly regulates components of the BMP pathway, which are in turn responsible for patterning the dorsal half of the embryo [10, 11]. Therefore, in *Drosophila* loss of Toll signaling completely abrogates DV polarity while loss of BMP signaling only affects dorsal and lateral regions. We used parental RNAi (pRNAi) [12] targeting either the *Nasonia* Toll receptor (*Nv-Toll*) or the BMP ligand Decapentaplegic (*Nv-dpp*) to investigate the relative roles of the two pathways in the wasp embryo.

If Toll had a similar role in *Nasonia* as it does in *Drosophila,* we would expect the loss of both *Nv-brk* and *Nv-twi* expression, and the expansion of *Nv-zen* to the ventral side, reflecting the absence of DV polarity. As expected, *Nv-Toll* pRNAi embryos show a complete loss *Nv-twi* expression (Fig. 1B). This indicates a conserved role of Toll in at

least the induction of ventral most cell fates (mesoderm). However, all *Nv-Toll* pRNAi embryos are still highly polarized , with normal *Nv-zen* and dorsal border of *Nv-brk* expression (Fig. 1B). In addition, the ventral expansion *Nv-brk* (Fig. 1B) in *Nv-Toll* pRNAi indicates that *Nv-brk* is activated independently of Toll signaling, which is in contrast to the complete dependence of early *Drosophila brk* on Toll (compare Fig. 1E to 1F) [1, 13].

Together, these data indicate that the patterning role of Nv-Toll is much reduced compared to *Drosophila,* and that a Toll independent patterning system operates on the dorsal side of the *Nasonia* embryo.

In *Drosophila* BMP signaling is required for specification of the amnioserosa and dorsal ectoderm, has minor roles in refining the borders of some laterally expressed genes [14-16], but has no effect on the ventral mesodermal domain (Fig. 1F). In contrast knockdown of the BMP 2/4 like ligand *Nv-dpp* leads to loss of not only the dorsal fates, but also to the expansion of the *Nv-twi* domain to almost completely cover the dorsal surface of the embryo (Fig. 1C). The dorsal-anterior region is consistently resistant to complete ventralization in *Nasonia* BMP knockdowns. This indicates that while BMP signaling is required for most DV polarity in the embryo, there is an additional source of dorsalizing activity centered at the dorsal anterior corner of the embryo.

Double knockdown of *Nv-dpp* and *Nv-Toll* results in embryos expressing *Nv-brk* ubiquitously, while *Nv-twi* and *Nv-zen* are absent (Fig. 1D). This shows that the ventralization of *Nv-dpp* pRNAi embryos requires at least initial induction of *Nv-twi* by Nv-Toll. This also shows that *Nv-brk* is activated independently of both BMP and Toll signaling in the wasp.

Altogether, these phenotypes indicate that Nv-Toll signaling has a relatively limited ventral role, while BMP signaling appears to have a broad patterning role encompassing most of the embryo. Thus, *Nasonia* uses novel strategies for patterning its DV axis, and we hoped to gain insights into the molecular bases of the differences by looking in more detail how these phenotypes come about.

BMP signaling opposes expansion of ventral fates

Our previous analyses showed [7] that ventral genes such as *Nv-twi* (Fig 2A), -*sim*, and –*sna*, start to be expressed from very narrow early domains (division cycles 10 & 11) and expand laterally until the end of cycle 12, where they achieve domains of characteristic width and shape (Fig 2B). However, *Nv-cactus (Nv-cact)* is an exception to this pattern. Cactus keeps NF-κB/Dorsal, the canonical transcription factor downstream of Toll signaling, sequestered in the cytoplasm [17], effectively repressing the pathway. In other holometabolous insects and in vertebrates *cactus* is a direct transcriptional target of Toll signaling, and establishes a negative feedback loop, which terminates signaling [9, 18, 19]. A highly significant cluster of Dorsal binding sites found just upstream of the *Nv-cact* coding sequence (Fig. S2) supports the idea that at least the remnants of a similar loop exist in *Nasonia*. Unfortunately, a direct test of *Nv-cact* function in *Nasonia* has so far not been possible.

Here, we use the zygotic expression of *Nv-cactus* (see [7]) as a marker gene indicating high levels of Toll signaling. Interestingly, while *Nv-cact* is initially expressed in a very narrow domain similar to *Nv-twi* (Fig 2A') and other ventrally expressed genes,

it does not expand significantly in later stages (Fig 2B'), but rather disappears just prior to gastrulation (see [7]). This indicates that Toll signaling remains local and is eventually extinguished, while some of its ventral targets are expanding.

This idea is supported by the dynamics of gene expression after *Nv-dpp* pRNAi. **All** early *Nv-dpp* pRNAi embryos show narrow *Nv-twi* and *Nv-cact* domains that are indistinguishable from wild-type (Fig. 2C-C'', S1E-F). However, later *Nv-twi* is massively expanded, while *Nv-cact* remains narrow, then disappears, which is indistinguishable from wild-type (Fig. 2D-D'', see also Fig S1 A, C-E). This result, in combination with that in Fig. 2B'' shows that the *Nv-dpp* pRNAi phenotypes seen at cycle 12 are the result of a dynamic process, as *Nv-twi* expression must expand massively from its initially narrow domain to reach the dorsal pole of the embryo. The simplest explanation for these results is that the massive expansion of ventral gene expression domains after *Nv-dpp* pRNAi is driven by the same mechanism as the smaller expansion observed normally in wild type embryos.

Thus, a major role of BMP signaling in *Nasonia* could be to provide negative inputs into a dynamic system operating on the ventral side of the embryo (See Fig. 4). The observation that *Nv-cact* does not expand in *Nv-dpp* pRNAi embryos further supports the idea that the expansion of *Nv-twi* in both *Nv-dpp* pRNAi and wt embryos is not driven by expansion of Toll signaling, and that BMP signaling is not a negative regulator of Nv-Toll signaling.

It is interesting to compare the *Nv-dpp* RNAi phenotype with that of the *Nasonia EGF receptor* (*Nv-egfr*). In *Nasonia, Tribolium* and *Drosophila,* a source EGF signaling associated with the oocyte nucleus leads to asymmetric activation of the EGFR in the overlying follicle cells [20]. This results in the confinement of eggshell cues required for activation of Toll signaling to the ventral side. Thus, a loss of EGF signaling should expand the region in which Toll signaling is activated. In agreement with this notion *Nv-cact* expression expands and becomes less regular along the AP axis, indicating a broader peak level region of Toll signaling. While *Nv-twi* expression is also broader after *Nv-egfr* pRNAi (Fig 2E-E''), it never expands as dramatically as after *Nv-dpp* pRNAi, and we never observe the *Nv-twi* domain to expand beyond ~50% embryo circumference after EGF signaling knockdown [20].

The expansion of *Nv-cact* in *Nv-egfr* pRNAi is in stark contrast to *Nv-dpp* pRNAi, where no matter how far the *Nv-twi* domain expands, *Nv-cact* remains narrow (Fig. 2D''). We interpret this result as confirming that Toll signaling occurs in a broader domain after knockdown of EGF signaling, and that the expansion of ventral genes in both wild-type and *Nv-dpp* pRNAi is not due to a dynamically expanding region of Toll signaling (since *Nv-cact* does not expand in these cases). The above results also indicate that there is not significant cross talk between BMP and EGF signaling in regulating Toll activity, since the knockdown phenotypes, while both ventralizing in nature, are clearly distinct from one another.

In summary, the initial, narrow, domains of ventral genes such as *Nv-cact* and *Nv-twi* are dependent on Toll signaling (which in turn is regulated by maternal EGF signaling), but are completely **independent** of BMP signaling in the early embryo (cycle 10-11, meaning that the narrowness is not due to repression by BMP at this stage). Conversely, the expansion of these domains in cycle 12 appears to be independent of further Toll input, and is negatively regulated by BMP. We propose that a positive

feedback loop involving one or more early targets of Toll signaling drives expansion of some ventral gene expression domains, and that robust ventral patterning in *Nasonia* relies on a balance between this positive feedback loop and the negative influence of BMP signaling emanating from the dorsal side (Fig. 4).

Neither Toll nor EGF signaling have significant inputs into BMP signaling in Nasonia *embryogenesis*

Effects of changes in Toll signaling on BMP signaling were also examined. Expanding ventral Toll signaling by *Nv-egfr* RNAi leaves *Nv-zen* expression unchanged (Fig. 2F-F'', compare *Nv-zen* domain to Fig. 1A and [7]), while *Nv-twi* is significantly expanded. This is in contrast to *Nv-dpp* pRNAi, where genes such as *Nv-zen* and *Nv-doc* are lost in concert with the expansion of *Nv-twi* (Fig. S1F-H). This indicates that maternal EGFR signaling is not involved in regulating or initiating the BMP pathway in the *Nasonia* embryo, either through Toll or by any other means. As a caveat, since EGF signaling is required for oogenesis in *Nasonia*, all embryos produced by *Nv-egfr* pRNAi mothers are escapers, and likely experienced some residual EGF signaling throughout oogenesis. It is thus possible that if the oocyte to follicle cell EGF signal were completely and specifically knocked out, an effect on BMP signaling and dorsal patterning would be observed. However, given that *Nv-zen* expression is unchanged despite the dramatic changes in ventral patterning and the sometimes severe perturbations in embryonic morphology after EGF knockdown, any effect of EGF signaling on the pattern of BMP activation is likely to be small.

Similarly, loss of ventral Toll signaling (*Nv-Toll* pRNAi) has no noticeable effects on the dorsal half of the embryo. Even the broadly expressed gene *Nv-ara* [7], which should be most sensitive to changes on the ventral side, is expressed in a domain within the normal range of variability in the stage just before gastrulation (Figure 2G-H''). Together, these two results strongly suggest that the BMP based patterning system on the dorsal side of the embryo is completely independent from the ventral Toll signaling system.

Nasonia *BMP activity gradient formation and regulation is distinct from* Drosophila

The independence of *Nasonia* BMP-based dorsal patterning from Toll signaling is distinct from both *Drosophila* and *Tribolium*, where crucial components of the BMP pathway are directly regulated by Toll, and whose expression is required for the formation of BMP activity gradients [21].

In *Drosophila* three genes directly regulated by Toll are required for proper BMP activity gradient formation: the ligand *dpp*, the inhibitor *short gastrulation* (*sog*), and the metalloprotease *tolloid* (*tld*) [22]. *dpp,* and *tld* are expressed in broad dorsal domains and are repressed by Dorsal, while *sog* is activated by Dorsal laterally. Sog protein binds Dpp, inhibits its signaling, and facilitates its diffusion. Tld cleaves Sog, leading to its destruction, and frees Dpp to signal. The interactions among these and other BMP components are critical for the proper formation of the BMP signaling gradient, particularly the peak levels at the dorsal midline [22]. The formation of this peak is dynamic, and takes place completely within nuclear cycle 14. The gradient of BMP

activity starts relatively broad and flat, and then refines and intensifies, generating a high peak at the dorsal midline [23].

We used cross-reactive phosphorylated MAD antiserum to monitor the pattern of BMP activity in *Nasonia* [23, 24]. pMAD is found in a broad, shallowly graded pattern in cycle 10 embryos, significantly earlier than in *Drosophila* (Fig. 3A). Over the next division cycle this gradient intensifies and becomes more dorsally restricted, and more obviously graded (Fig. 3B). By cycle 12 it is found in an intense stripe over the dorsal midline (Fig. 3C). Eventually, the stripe narrows further and begins to disappear just prior to gastrulation (Fig. 3D). Thus, while the end result of a narrow intense peak of BMP activity just prior to gastrulation is nearly identical between *Drosophila* and *Nasonia,* the way this pattern evolves is distinct. Interestingly, the pattern of change over time of pMAD in *Nasonia* is very similar to that seen for *Nv-zen* [7], indicating that *Nv-zen* is regulated by BMP signaling throughout early embryogenesis.

Nv-dpp is expressed maternally, and does not show spatial regulation until after gastrulation (Özüak et al., submitted). In addition, no *sog* ortholog has been detected in any genome or transcriptome data set in *Nasonia* ([7, 25] Supplemental Text) or in the genome of the closely related wasp *Trichogramma* (JAL personal observation). The very restricted anterior expression (Fig. S3) and lack of patterning function of *Nv-tld* (tested by pRNAi, not shown) are also consistent with the absence of a Sog based transport system.

Other components are required for BMP signaling in *Drosophila*. One is *screw,* a highly diverged BMP 5/7 paralog related to *glass bottom boat* (*gbb)* [26], which is required along with *dpp* for signaling prior to gastrulation [23]. In *Nasonia,* a BMP 5/7-like ligand (*Nv-gbb*) is weakly, maternally, and ubiquitously expressed throughout early embryogenesis (Özüak et al., submitted) and gives an identical pRNAi phenotype to *Nv-dpp* (Fig. 3F). Therefore, BMP signaling requires both *Nv-dpp* and *Nv-gbb* together to induce BMP signaling in *Nasonia,* similarly to *Drosophila.*

The BMP binding protein Twisted gastrulation (Tsg), is required for peak levels of BMP signaling in the embryo by both interacting with Sog to transport BMP ligand complexes to the dorsal side, and by having an independent positive influence on signaling [27]. pRNAi against *Nv-tsg* gives identical phenotypes to *Nv-dpp* and *Nv-gbb*, indicating that *Nv-tsg* is also required for BMP signaling in *Nasonia* (Fig. 3G). An exclusively pro-BMP, *sog*-independent role for *tsg* was also observed in *Tribolium* [28]. Finally, the receptors *thickveins* (*tkv*) and *saxophone* (*sax*) are required for peak BMP signaling in *Drosophila*, but do act independently in other signaling situations [29] [26]. We find that both receptors are required for normal patterning in *Nasonia*, but the lack of either one alone does not completely abrogate the signal (Fig. 3H, only *Nv-tkv* pRNAi result shown), although we cannot exclude coincidental inefficient knockdown for both of these genes.

Thus, while the dynamics of BMP signaling (pMAD) are similar between wasp and fly, we have shown that the molecular mechanisms producing the dynamics must be highly diverged, due primarily to the absence of Sog and Tld function in *Nasonia*. This shows that not only are the downstream regulatory networks targets of evolutionary change, but that the upstream signaling modules are also prone to significant alterations in the course of evolution.

Conclusions

We have shown here the first example of an insect that relies almost completely on BMP signaling for DV axis patterning (Fig. 4, right side). This BMP system is independent of Toll signaling, which is in stark contrast to the *Drosophila* paradigm, where crucial BMP components are regulated by Toll signaling (Fig. 4). We have also shown that Toll signaling induces only a narrow ventral expression domain of genes whose final pattern depends on a combination of dynamic expansion and BMP mediated repression (Fig. 4), rather than on a stable gradient.

Our results also show that *Nasonia* uses a mode of BMP gradient formation and refinement that is distinct from what is known in *Drosophila*, and may be novel among animals. A major part of the novelty has to do with the absence of *sog* from the *Nasonia* genome. *sog/chordin* orthologs are present in most animals (except the two Pteromalid wasp species with sequenced genomes) and are important for shaping BMP gradients in diverse developmental contexts. At present we understand very little about how the BMP signaling dynamics are regulated in the absence of the Sog transport system in *Nasonia*. One missing piece is the source of initial asymmetry, which in *Drosophila* is provided by Toll regulation of *sog, dpp,* and *tld* expression. Since BMP signaling shows complete independence from Toll in *Nasonia*, another mode of symmetry breaking must be employed.

Another major missing piece is the factor that regulates the dynamic refinement of BMP signaling in *Nasonia*. An interesting possibility is that the refinement could depend on intracellular processes (such as BMP dependent transcription) that can lead to the transformation of a broad, shallow activity gradient in to a narrow, steep one, as has been shown recently in *Drosophila* [30]. In general, the wealth of knowledge of BMP patterning systems will guide future efforts to unravel the *Nasonia* DV patterning system.

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Figure Legends:

Fig. 1. Effects of *Toll*, *dpp,* and *Toll/dpp* double pRNAi.
A-D, *Nv-zen* (purple), -*brk* (green) and -*twi* (red) expression in wild-type (**A**) *Nv-Toll* (**B**), *Nv-dpp* (**C**) and *Nv-Toll*/*dpp* (**D**) RNAi embryos. In all panels white is DAPI, views are lateral, anterior is to the left and dorsal up, and embryos are in late nuclear cycle 12. Embryos shown are representative of the strongest knockdown phenotypes the frequencies of which are: *Nv-Toll* pRNAi (B) 21/27, *Nv-dpp* pRNAi (C) 6/26, and *Nv-Toll+dpp* pRNAi (D) 21/40. See Methods for more details.
E,**F,** Schematic mid-body cross sections of *Nasonia* (**E**) and *Drosophila* (**F**) embryos corresponding to the four different conditions shown in (**A**-**D**) (Red=Mesodern, Green=Neurogenic ectoderm, Grey=Dorsal ectoderm, and Magenta=extraembryonic. Tissue types in *Nasonia* are extrapolated from conserved function of marker genes expressed in areas where mesoderm is internalized (*Nv-twi*), cells that remain on the exterior surface of the embryo after gastrulation (*Nv-brk*), and cells on the dorsal midline that migrate over the ectoderm and form the extraembryonic serosa (*Nv-zen*). Although not indicated for simplicity's sake, scattered extraembryonic cells are seen in *Drosophila* embryos with abrogated Toll signaling (**F**, second column).

Fig. 2. **Differing mechanisms of BMP and EGF signaling in repressing ventral fates**
A-B", *Nv-twi* (red) and *Nv-cact* (green) expression in early (cycle 11, A-A", ventral view) and late (cycle 12, B-B", ventral view) wild-type embryos.
C-D" *Nv-twi* (red), *Nv-cact* (green) in early (cycle 11, C-C", ventral view, representing 12/12 at this stage), and late (cycle 12, D-D", ventro-lateral view, representing 4/15 embryos at this stage) *Nv-dpp* pRNAi embryos.
E-E", *Nv-twi* (red, E), *Nv-cact* (green, E') expression in an *Nv-egfr* pRNAi embryo, ventral view, cycle 12. Note the expanded and less straight shape of the *Nv-cact* domain. This is representative of 15/15 embryos showing noticeably expanded *Nv-twi* domains.
F-F", *Nv-twi* expression (F) in cycle 12 *Nv-egfr* pRNAi embryo (ventral view); *Nv-zen* expression (F') in same *Nv-egfr* pRNAi embryo as in F, showing an expression pattern indistinguishable from wild-type (dorsal view); and overlay with DAPI (F"), (dorsal view, ventral domain out of focus). This is representative of 21/21 embryos showing significantly expanded *Nv-twi* domains.
G-G", *Nv-twi* (red, G, ventro-lateral view), and *Nv-ara* **(**a marker gene covering a broad dorsal area, green, G', dorsolateral view) expression in the same wt cycle 12 embryo. G" overlay of both with DAPI, (dorsolateral view, ventro-lateral domain out of focus).
H-H", *Nv-twi* (red, G, ventro-lateral view), and *Nv-ara* **(**green, G', dorsolateral view) expression in the same cycle 12 *Nv-Toll* RNAi embryo. G" overlay of both with DAPI, dorsolateral view. This is representative of 10/10 embryos where *Nv-twi* is absent

Fig. 3. BMP signaling in the *Nasonia* embryo.
A,B,C,D, Distribution of pMAD (green), arranged by age from cycle 10, (**A**, lateral view), cycle 11 (**B**, lateral view), early cycle 12 (**C**, dorsolateral view) to late cycle 12, (**D**, dorsal view) "nc"=nuclear cycle.
E-H, *Nv-twi* (red)*, Nv-vnd* (green) expression in wt (**E**, ventrolateral view), *Nv-gbb* (**F**, lateral view), *Nv-tsg* (**G**, lateral view) and *Nv-tkv* (**H,** lateral view) pRNAi embryos. All embryos are in late cycle 12, with H just beginning gastrulation. Images are representative of the strongest phenotypes observed, but were not quantified.

Fig. 4. Illustration of the difference between *Nasonia* and *Drosophila* embryonic patterning.
Scheme on left represents early cycle 14 *Drosophila* embryo (left half) and cycle 11 *Nasonia* embryo (right half). Scheme on right shows late cycle 14 *Drosophila* embryo (left half) and late cycle 12 *Nasonia* embryo (right half). Orange cycling arrows at left represent the interactions among BMP components regulated by Toll signaling that produce peak levels of BMP signaling (magenta). Orange cycling arrows on right represent the unknown mechanism by which BMP signaling refines in *Nasonia*. Red cycling arrows at bottom right indicate an unknown positive feedback mechanism that leads to the expansion of the mesodermal markers. Thickened orange arrows indicate peak levels of BMP signaling. For clarity, not all possible interactions are indicated.

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Supplemental Data:

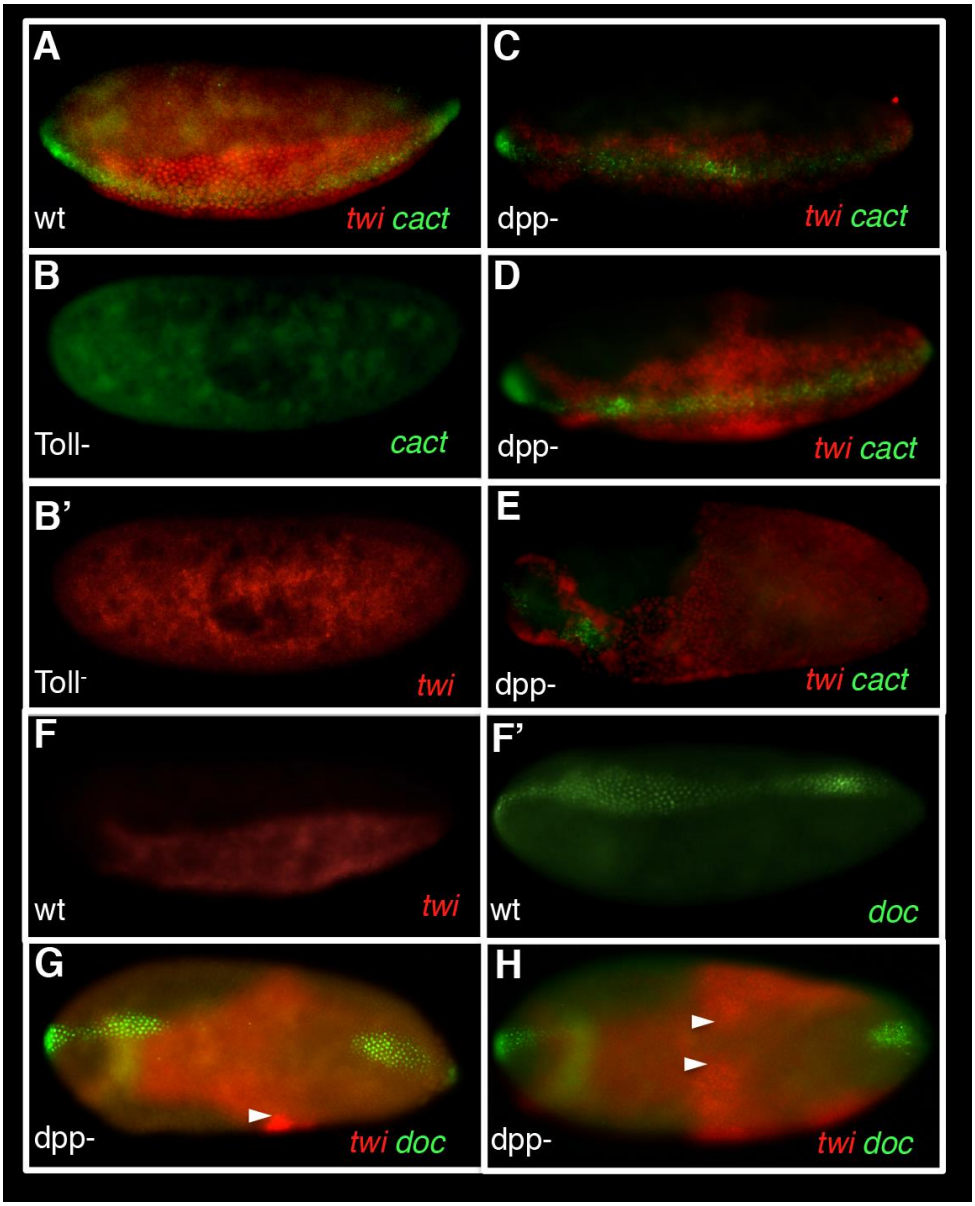

Figure S1. Detailed effects of *dpp* and Toll pRNAi during early embryogenesis in *N. vitripennis* (Related to Figure 1)

A-E, *Nv-cact* (green) and *Nv-twi* (red) expression in wildtype (A), *Nv-Toll* (B,B'), and (C-E) *Nv-dpp* RNAi embryos of increasing phenotypic strength and/or age. A, C-E are ventrolateral views while orientation of B,B' is ambiguous. In B and B' signal intensity was increased to give a visible image, but what is seen is only background staining primarily in the yolk.

F,F' expression of *Nv-twi* (red, F, ventrolateral view) and *Nv-dorsocross* (*Nv-doc*, F', green, dorsolateral view) in the same wt embryo.

G-H *Nv-twi* and *Nv-doc* in *Nv-dpp* RNAi (Both dorsal views). White arrowheads show the most dorsal of the *Nv-twi* domain.

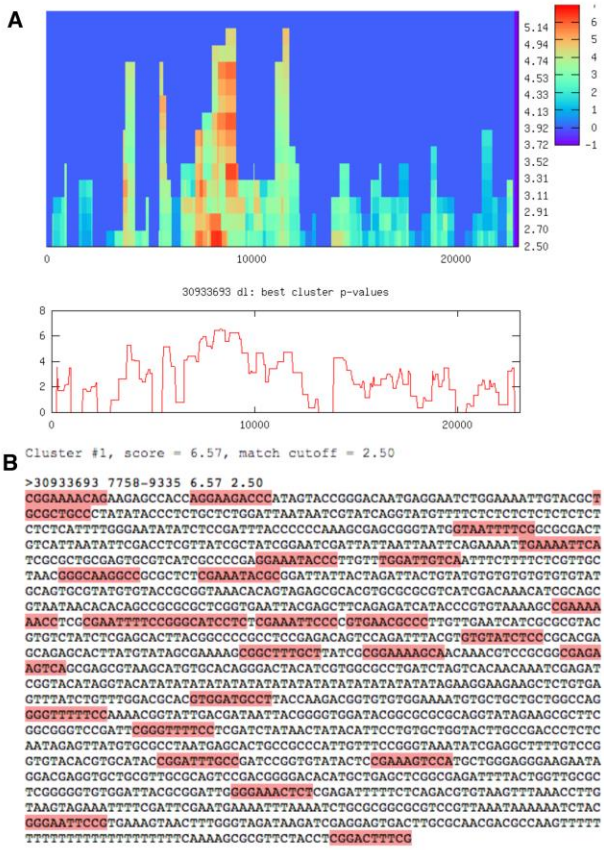

Figure S2. **Illustration of clustered Dorsal binding sites in the *Nv-cact* genomic region (Related to Figure 2).** A genomic fragment including the *Nv-cact1* locus plus 10kb upstream and downstream was submitted to the Clusterdraw web server [S1] using default parameters. Output of the highest scoring cluster is shown. **A).** 2D plot of cluster E-values showing regions with clusters of high scoring Dorsal binding sites. Note that the strongest cluster is located just before the 10kb mark, which is the start of the *Nv-cact* coding locus. **B)** Distribution of Dorsal binding sites in best scoring cluster. **C).** Position and score (higher score~higher affinity) of Dorsal sites in best scoring cluster.

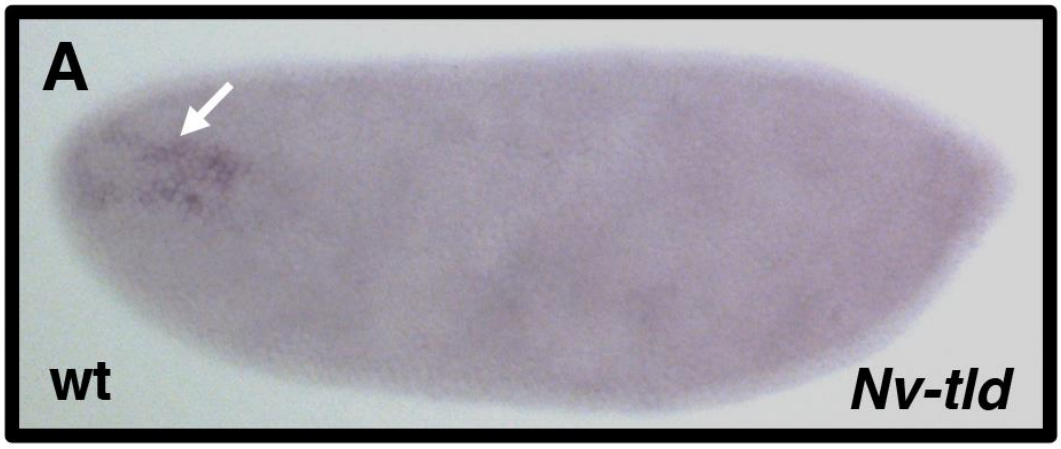

Figure S3. *Nv-tolloid* expression in the embryo. (Related to figure 3)
Arrow marks the small dorso-anterior domain of *Nv-tld* expression on the blastoderm of the embryo.

Supplemental Results: Absence of *short gastrulation* from *Nasonia (Related to Figure 3 and S3)*.

Sog/chordin is highly conserved among all animals, and within insects. For example BLAST using honeybee Sog protein as a query finds *Drosophila* Sog with an E-value of 0.0, and the two sequences are 47% identical along their length. We would expect *Nasonia* Sog to be even more similar to *Apis* Sog.

The *Nasonia vitripennis* genome was sequenced to 7x coverage, with two closely related species sequenced at 1x. No significant hits for Sog from ants, bees, mosquito, fly, or bug were found in any genome assembly or annotation using BLAST searches. The Sog queries did uncover genes of similar size and domain structure-called *crossveinless-2*. There are 4 paralogs of *cv-2* in the *Nasonia* genome (Özüak et al, submitted) and they are all clearly related to *cv2* and not orthologs of Sog. Expression and functional analyses of these genes revealed no Sog-like role (Özüak et al, submitted). Similar results were found when searching a database of over 100,000 ests, and finally our assembly (using Trinity software[S2]) of ~180 million paired end 100 bp reads (36 billion nucleotides, or more than 10x worth of genome coverage) (JAL in preparation). All components of BMP signaling, including the large mRNAs of the structurally similar *cv2* genes were recovered in the RNAseq assembly, so it should be of high enough quality to find Sog if it were present. An identical approach using a beetle (*Callosobruchus maculatus*) embryonic transcriptome did identify a clear Sog ortholog (JAL in preparation). All of this together with the fact that Tld is not expressed dorsally, and seems to have no patterning function, and that there is no input of Toll into BMP refinement, make it all the more plausible that Sog is absent from the *Nasonia* genome.

Supplemental Experimental Procedures:

Embryo collection

All *N. vitripennis* embryos were collected using the *Waspinator* and fixed as described in [S3].

Identification of *Nasonia* orthologs of patterning molecules and markers.

Reciprocal best BLAST hits of *Drosophila* genes of interest to the *Nasonia* genome and transcriptome assemblies [S4] was the criterion to identify likely orthologs. For most of the genes described here, clear 1:1 orthologs were found. In some cases multiple *Nasonia* genes returned the fly gene of interest as the best hit, indicating that gene duplication took place in the *Nasonia* lineage. For *Toll*, four *Nasonia* genes returned *Toll* as their best hit. Of these, only two were expressed maternally, and only one of these two gave an embryonic patterning phenotype. Four potential *Nasonia* Dorsal orthologs were found, and none gave clear pRNAi phenotypes, indicating that there is some redundancy among these genes, all of which are expressed maternally. Three likely *cactus* orthologs were identified, and only the one described here showed zygotic ventral expression. Two *Nasonia gbb* hits were identified, and only the one described here gave a phenotype with pRNAi.

Primers used (linker sequences in Bold):

Toll	(*Toll*)	Fw	**GGCCGCGG**GAACTCGCCAAACTGGTCTC
		Rev	**CCCGGGGC**AACGGATTGTTCGTCAGCTC
decapentaplegic	(*dpp*)	Fw	**GGCCGCGG**GTGGTGGGCGAGGCGGTAAA
		Rev	**CCCGGGGC**CACGACCTTGTTCTCCTCGT
glass bottom boat	(*gbb*)	Fw	**GGCCGCGG**ATCCTGCTGCAGTTCGACTT
		Rev	**CCCGGGGC**CCCTGATGATCATGTTGTGG
cactus1	(*cact*)	Fw	**GGCCGCGG**ACTTGGATATGGGCAAGTCG
		Rev	**CCCGGGGC**CTACTGTCGCTGCTGCTGTC
twist	(*twi*)	Fw	**GGCCGCGG**GCTTCTCGCCCAGTAACAAC
		Rev	**CCCGGGGC**ACGTTAGCCATGACCCTCTG
ventral nervous system defective	(*vnd*)	Fw	**GGCCGCGG**GTCGGACTGCTCAACAACTG
		Rev	**CCCGGGGC**AGGTTCCAGGAGCTTCGACT
brinker	(*brk*)	Fw	**GGCCGCGG**ATCCAAAAGTGGCTCCAGTG
		Rev	**CCCGGGGC**CTGAAAGTCGTGCTCCTCGT
zerknüllt	(*zen*)	Fw	**GGCCGCGG**AAGTCGGCTCCTATTCAGCA

		Rev	**CCCGGGGC**TAGGAATAGTGGCCCGAAGA
araucan	(*ara*)	Fw	**GGCCGCGG**CACCACCACCTTCTCATCCT
		Rev	**CCCGGGGC**GCCCTTGTAGAACGGTTTGA
dorsocross	(*doc*)	Fw	**GGCCGCGG**ATAAAATCGGCACCGAGATG
		Rev	**CCCGGGGC**AAATAGGCAGCTGCAATGCT
T7 Universal F			GAGAATTCTAATACGACTCACTATAGGGCCGCGG
T7 Universal R			AGGGATCCTAATACGACTCACTATAGGGCCCGGGGC

RNAi

Young *N. vitripennis* pupae were injected as in [S5]. Briefly young (yellow) pupae were injected with dsRNA 1-2 microgram/microliter for all dsRNAs. These injected pupae were allowed to eclose and the resulting eggs were collected and processed for in situ hybridization. In all cases it is likely that both maternal and zygotic mRNAs are being disrupted, and for all of the genes tested here, the maternal transcript is likely to be the one of import, as all of these genes show weak zygotic staining with in situ hybridization. For all genes tested here, penetrance of pRNAi was 100% at the level of larval lethality. Accurate quantification of the BMP phenotypes is difficult due to the dynamic nature of the patterning system and phenotype. Since all of the expansion of ventral gene expression (e.g. *Nv-twi*) takes place during cycle 12, we cannot confidently distinguish between weaker phenotypes or slightly earlier embryos. However once the expansion begins, BMP knockdown embryos are identifiable by changes in the shape of the *Nv-twi* domain. For quantification in Figure 1, phenotypes where the *Nv-twi* domain expanded to continuously cover the embryo on the dorsal side were counted. *Nv-egfr* pRNAi leads to in most cases defects in oogenesis that prevent viable egg production (similar to *torpedo* or *gurken* phenotypes in *Drosophila*). Therefore those eggs resulting from *Nv-egfr* pRNAi are unlikely to result from complete knockdown. However, all eggs were abnormal in terms of DV patterning

***in situ* Hybridisation (ISH) and Immunohistochemistry (IHC)**

Two-color ISH was performed as described in [S3]

Three-color ISH is a modified version of the two-color ISH and was performed in 4 days as described below.

First day: Probe incubation using biotin-, fluorescein-, and dig labeled probes.

Second day: Antibodies were incubated using α-fluorescein::AP antibodies (1:2500), α-digoxigen::POD antibodies (1:100) and Mouse-α-biotin antibodies (1:100).

Third day: Staining reaction with Fast Red and HNPP (Roche) as substrates for α-fluorescein::AP antibodies to give a red fluorescent signal with AlexaFluor 488 tyramide (Invitrogen) as a substrate α-digoxigen::POD antibodies as described in [S3].

After staining reaction POD was destroyed by incubating embryos for 30 min in a 1% H_2O_2 solution diluted in PBT. Antibody incubation using α-mouse::POD (1:100) antibodies overnight.

Fourth day: Staining reaction with AlexaFluor 647 conjugated tyramide (Invitrogen) as substrate for α-mouse::POD.

Immunohistochemistry was performed as described in [S6]. Rabbit monoclonal antibody 41D10, raised against phospho-Smad 1/5, was obtained from Cell Signaling Sciences, and was used at 1:100. Secondary anti-rabbit::Alexa 568 was used at a concentration of 1:500

Supplemental References

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