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A *caudal* mRNA gradient controls posterior development in the wasp *Nasonia*

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One of the earliest steps of embryonic development is the establishment of polarity along the anteroposterior axis. Extensive studies of *Drosophila* embryonic development have elucidated mechanisms for establishing polarity, while studies with other model systems have found that many of these molecular components are conserved through evolution. One exception is Bicoid, the master organizer of anterior development in *Drosophila* and higher dipterans, which is not conserved. Thus, the study of anteroposterior patterning in insects that lack Bicoid can provide insight into the evolution of the diversity of body plan patterning networks. To this end, we have established the long germ parasitic wasp *Nasonia vitripennis* as a model for comparative studies with *Drosophila*. Here we report that, in *Nasonia*, a gradient of localized *caudal* mRNA directs posterior patterning, whereas, in *Drosophila*, the gradient of maternal Caudal protein is established through translational repression by Bicoid of homogeneous *caudal* mRNA. Loss of *caudal* function in *Nasonia* results in severe segmentation defects. We show that *Nasonia caudal* is an activator of gap gene expression that acts far towards the anterior of the embryo, placing it atop a cascade of early patterning. By contrast, activation of gap genes in flies relies on redundant functions of Bicoid and Caudal, leading to a lack of dramatic action on gap gene expression: *caudal* instead plays a limited role as an activator of pair-rule gene expression. These studies, together with studies in short germ insects, suggest that *caudal* is an ancestral master organizer of patterning, and that its role has been reduced in higher dipterans such as *Drosophila*.

KEY WORDS: *Caudal*, *Nasonia*, Segmentation

INTRODUCTION

Recent progress in genomic and molecular techniques in different insect species has allowed deep insights into the evolution of developmental regulatory gene networks. As *Drosophila* provides an unmatched in-depth description of the regulatory network that directs early development, other systems have emerged to take advantage of this knowledge and compare developmental strategies. Studies in the beetle *Tribolium*, in the cricket *Gryllus*, in the milkweed bug *Oncopeltus* and in the grasshopper *Schistocerca* have highlighted several common and different pathways to pattern the embryo (reviewed by Liu and Kaufman, 2005). We have chosen to study the wasp *Nasonia*, an important model system with which compare early development that is functionally accessible, through both genetics and parental RNAi (Pultz and Leaf, 2003; Lynch and Desplan, 2006).

Much of our understanding of anteroposterior body axis formation has been a result of elegant screens for segmentation defects in *Drosophila*. The syncytial environment of the *Drosophila* embryo allows for the generation of morphogenetic gradients of transcription factors, which are established via mRNA localization, protein diffusion and translational repression. Reciprocal gradients are then interpreted by downstream targets in a concentration-dependent manner to establish a complex anteroposterior patterning system that will eventually form the segmented insect body plan. However, much of development in most other insects takes place in a cellularized environment, and thus not all basic mechanisms and principles used in *Drosophila* patterning can be conserved. Still, many of the genes involved in fly segmentation are well conserved (Tautz, 2004; Liu and Kaufman, 2005).

Although much attention has been focused on anterior patterning in the fly, the major anterior patterning factor *bicoid* (*bcd*) is not found outside the dipteran lineage (Dearden and Akam, 1999; Stauber et al., 1999; Stauber et al., 2000; Lynch and Desplan, 2003), and this has thus led researchers to investigate the patterning networks of other insects, such as *Tribolium* (Tautz, 2004; Liu and Kaufman, 2005). Beetles use an ancestral mode of embryogenesis, termed short-germ embryogenesis, in which the embryo develops in the posterior of the egg and only anterior structures are patterned in a syncytial environment. Later, abdominal and posterior structures are formed in a cellularized environment through a region in the posterior of the germ rudiment termed the 'growth zone'. This is in contrast to the more derived long-germ mode of patterning found in flies, where the embryo occupies the entire egg, is patterned completely within a syncytial environment and, thus, lacks a posterior growth zone (Davis and Patel, 2002). It has been proposed that an anterior patterning center, such as Bcd in the long-germ *Drosophila*, would not function well to pattern the anterior of the embryo in short germ insects (Stauber et al., 1999; Stauber et al., 2000): anteriorly localized factors would not be able to reach the germ rudiment at the posterior of the oocyte and would instead pattern the extra-embryonic membranes, which lie at the anterior (Lall and Patel, 2001; van der Zee et al., 2005). Instead, the ancestral system may have exclusively used a posterior patterning center, allowing for posteriorly localized factors to reach the developing embryo easily.

The posterior patterning homeoprotein Caudal (Cad) is conserved throughout evolution from *C. elegans* to mammals. The *Drosophila caudal* gene (*Dm cad*) is involved in posterior embryonic patterning and hindgut formation (Macdonald and Struhl, 1987; Moreno and

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Morata, 1999; Schulz and Tautz, 1995; Wu and Lengyel, 1998). *Dm cad* zygotic mutant embryos are not viable and exhibit posterior defects: a lack of anal pad, anal tuft structures and anal sense organs. This rather mild phenotype has been attributed to maternal rescue of the loss of zygotic *Dm cad*. Maternally mutant embryos that have been paternally rescued also show mild phenotypes with deletions in abdominal segment 8 (A8) and sometimes A4, but are viable. Embryos lacking both maternal and zygotic *Dm cad*, however, show severe segmentation defects. Although the head and thorax are normal, the body is shortened owing to elimination of all anal structures and disruption of more anterior abdominal segments (Macdonald and Struhl, 1986) (Fig. 3).

The phenotype resulting from loss of *cad* has also been investigated in *Tribolium*, *Gryllus* (cricket) and *Artemia* (brine shrimp) using RNA interference (RNAi) (Copf et al., 2003; Copf et al., 2004; Shinmyo et al., 2005), and also studied in *Sacculina* (barnacle) (Rabet et al., 2001). Strikingly, in each organism examined, loss of *cad* results in embryos where only anterior head structures remain and all thoracic, abdominal and posterior structures fail to form. This phenotype is more severe than the *Dm* $cad^{mat+zyg}$ phenotype, and suggests that *cad* may play a greater role in patterning ancestral insects than in *Drosophila*. In the intermediate germ *Gryllus* embryo, *cad* plays a major role in thoracic and gnathal patterning by activating transcription of the gap genes *hunchback* (*hb*) and *Krüppel* (*Kr*). This role in gap gene activation is played by *bcd* and maternal *hb* in *Drosophila*. It has thus been proposed that, in ancestral insects, *cad* sits at the top of the segmentation cascade and regulates gap gene expression, while *bcd* has usurped this role in higher dipterans (Shinmyo et al., 2005).

The *Dm cad* gene is expressed maternally and zygotically in the embryo. Both transcripts share an identical open reading frame and encode a homeodomain protein of 427 amino acids. Maternal *Dm cad* RNA is first made in the nurse cells and is found evenly distributed throughout the embryo (Mlodzik and Gehring, 1987a) (Fig. 1E). The maternal *Dm* Cad protein product forms a posterior to anterior gradient via translational repression by Bcd in the anterior. Bcd binding to the *cad* mRNA is mediated by the Bicoid response element (BRE) in the 3′UTR of the *cad* transcript (Dubnau and Struhl, 1996; Rivera Pomar et al., 1996). A translationally controlled maternal gradient is also observed in both *Bombyx mori* (silk moth) and *Tribolium*, although it is not understood how it is established in these species (Wolff et al., 1998; Xu et al., 1994). Furthermore, the *C. elegans cad* homolog *pal-1* is maternally expressed and its protein product is restricted to cells of the posterior lineage via translational repression by *mex-3*, a gene that encodes an mRNA-binding protein that shares no homology with Bcd (Hunter and Kenyon, 1996).

As the *Drosophila* embryo develops, the Cad protein gradient becomes steeper and recedes from the anterior, forming a zygotic abdominal expression domain (Macdonald and Struhl, 1987; Mlodzik and Gehring, 1987a) (Fig. 2H,I). Later, the abdominal expression domain disappears and only a posterior stripe remains (Wu and Lengyel, 1998) (Fig. 2J). In *Gryllus*, *cad* is expressed in the early embryo in a posterior-to-anterior gradient, and later is restricted to the posterior growth zone, as in *Tribolium* and *Artemia* (Shinmyo et al., 2005; Schulz et al., 1998; Copf et al., 2003).

In order to study evolution of insect patterning, we have chosen the long-germ hymenopteran *Nasonia vitripennis* (*Nvit*). This parasitic wasp is a model system where a forwards genetic screen and functional parental RNAi studies have been performed (Pultz et al., 1999; Pultz et al., 2000; Pultz et al., 2005; Lynch et al., 2006a; Lynch et al., 2006b). *Nasonia* uses a long-germ mode of embryogenesis similar to that of highly derived *Drosophila*. However, *Nasonia* does not possess a *bcd* homolog and might therefore rely on an ancestral patterning system. Thus, *Nasonia* is an ideal system in which to study the evolution of patterning gene networks in general, as well as to study the specific patterning changes that have occurred during the evolution of long-germ embryogenesis.

In a screen to identify genes involved in embryonic patterning in *Nasonia*, many mutations in segmentation genes were identified, including a large number that resemble mutations in *Drosophila* genes of the gap, pair-rule and Polycomb-group (Pultz et al., 1999). In particular, one mutant, *head only* (*ho*), has a phenotype very similar to *Tc cad* RNAi embryos. It is also reminiscent of *Dm* $cad^{mat+zyg}$ mutant embryos, although more severe, and was thus hypothesized to be due to a lesion in the *Nvit cad* locus (Pultz et al., 1999; Pultz et al., 2000). Here, we use parental RNAi to show that *ho* is most likely to be a zygotic *Nvit cad* mutant. Using *ho* together with parental RNAi, we assessed the role of the maternal and zygotic *Nvit cad* components as compared with that of *Dm cad*. We find that *Nasonia* uses mRNA localization to generate a posterior to anterior *cad* mRNA gradient in the absence of translational regulation of Cad by Bcd. Furthermore, we provide evidence that in *Nasonia*, *cad* acts as a crucial posterior patterning center sitting atop the ancestral patterning hierarchy.

MATERIALS AND METHODS

Fixation and in situ hybridization

Nasonia wild-type and headless stocks were kept at 28°C. *head only* stocks were raised at 18°C. Embryos were collected and fixed as described in Pultz et al. (Pultz et al., 1999). Embryos were hand peeled on double sided sticky tape in 0.1% Tween 20 in 1× PBS. Ovaries were fixed in 4% formaldehyde 0.1% Tween 20 in 1× PBS for 20 minutes, and dehydrated in methanol. Cuticles were mounted in 50% Hoyer's medium and 50% lactic acid. In situ hybridization was performed as previously described (Brent et al., 2003).

RNA interference

Nvit cad parental RNAi was performed as described (Lynch and Desplan, 2006) using forward (5′ **TAATACGACTCACTATAGGGAGACCAC**CAGAACCGCCGAGCTAAAGAC 3′ and reverse (5′ **TAATACGACTCACTATAGGGAGACCAC**TCAGCGGCGAGATCAGTTAAA 3′) primers to generate templates via PCR for transcription of double-stranded RNA. (T7 promoters are in bold.)

Fly lines

cad zygotic mutants were generated by crossing *pr*[1]*cad*[2]P{*ry*[+t7.2]=*neoFRT*}^{40A}/*CyO* virgin females to *b*[1]*pr*[1]*cad*[3]/In(2LR)*Gla*,*wg*[*Gla*-1]/*CyO*. Maternal *cad* mutants were generated by crossing *pr*[1]*cad*[2]P{*ry*[+t7.2]=*neoFRT*}40A/*CyO* virgin females to *P*[ry$^+$;*hs-FLP*]^{12}; *P*[w^+;*Ovo*^{D1}]^{2L} *P*[*hs-neo*;*ry*$^+$*FRT*]^{40A}/*CyO* males. Third instar larvae and virgin progeny were heat shocked at 37°C for 90 minutes. Virgin females were crossed to *b*[1]*pr*[1]*cad*[3]/In(2LR)*Gla*,*wg*[*Gla*-1]/*CyO* males to generate progeny of which half are maternally mutant and half are maternal and zygotic mutants. To generate only maternally mutant progeny, heat-shocked virgin females were crossed to *yw* males. Ventral misexpression lines (sna>Kr; sna>hb; sna>otd; sna>tll) were gifts from Stephen Small. Males carrying both the snail misexpression transgene and a β2-*tubulin-FLP* transgene were crossed to *yw* virgin females to activate ventral misexpression. The resulting progeny were assayed using in situ hybridization.

Degenerate PCR

Nv *caudal* was initially cloned by PCR using degenerate forward (5′ CATGAATTCAARACKCGNACKAARGAYAARTA 3′), and the reverse (5′ TGAGTCGACRTTYTGRAACCADATYTTNAC 3′) primers.

RACE PCR

Total RNA was isolated from pooled embryos collected 0-4 hours or 4-10 hours after egg laying. SMART PCR cDNA synthesis kit (Clontech) was used for first-strand cDNA synthesis. The 5′ RACE primer (5′

GCGGATGGTGATGTACCGGCTAGAGTAG 3′) and 3′ RACE primer (5′ AACTCGCCAGCAGCCTCGCCTTGTC 3′) were used to clone the 5′ and 3′ ends of Nasonia caudal mRNA.

Genomic PCR

PCR was used to characterize Nv caudal genomic sequence. Forward and reverse primer sets included: 5′ CAAGACACGAACGAAGGACAAGTACAG 3′, 5′ ACGGTTAGCACTCGGGTATGAACAACT 3′; and 5′ GTTGTTGAATTCGCCGAGCTAAAGACCGCAAGCAG 3′, 5′ ATTGTTAACGTTGAGCACCGAGTGTTG 3′.

Molecular mapping

To determine whether *head only* was linked with Nv *caudal*, degenerate PCR was used to also clone *caudal* from the sister species *Nasonia giraulti* (Ng) for molecular mapping (Pultz et al., 2005). A polymorphism distinguishing the Nv and Ng *caudal* was detected by Ambion RNAse Mismatch Detect II kit. This allowed testing surviving sons of a Nv *head only* × Ng cross to determine if they had Ng or Nv *caudal*. All surviving sons (45/45) had Ng *caudal*, suggesting that *caudal* is linked to *head only*.

RESULTS

Nasonia cad mRNA is posteriorly localized in the ovary and early embryo

We first cloned the *Nasonia caudal* (*Nvit cad*) sequence with a degenerate PCR approach using primers directed towards a 120 bp fragment of the conserved homeodomain (see Materials and methods). We next analyzed *Nvit cad* expression in both the ovary and throughout embryogenesis using whole-mount in situ hybridization. *Nvit cad* is first observed during oogenesis within the nurse cells; later, *Nvit cad* mRNA is also found in the oocyte. Unexpectedly, *Nvit cad* mRNA is localized to the posterior pole of the oocyte in ovarian follicles (Fig. 1A,B). In early embryos, maternal *Nvit cad* mRNA is localized to a structure containing the germ plasm known as the oosome. Later, *Nvit cad* mRNA appears to be released and diffuses anteriorly, establishing a graded expression that reaches very far anteriorly (Fig. 1C-F). As this gradient is observed very shortly after embryos are laid, before expression of any of the (zygotic) gap genes, it is unlikely that zygotic transcription has initiated. The resulting posterior to anterior mRNA gradient suggests that a Cad protein gradient exists in *Nasonia*.

Zygotic *Nvit cad* expression resembles that of both short and long germ insects

Two to 3 hours after egg laying (hAEL) at 28°C, during pole cell formation, a zygotic gradient of *Nvit cad* expression forms in the syncytial embryo. Although the exact timing of midblastula transition in *Nasonia* is not known, it is likely that this expression is zygotic as it coincides with the onset of gap gene expression. The initiation of zygotic *Nvit cad* transcription overlaps with the maternal expression, extending anteriorly to cover approximately three-quarters of the embryo (Fig. 1E). The expression pattern is complementary to that of *Nvit otd*, which is expressed at both poles (Lynch et al., 2006a), and *cad* mRNA is indeed excluded from both the anterior and posterior poles (Fig. 1G, Fig. 3C). By 4 hAEL, after nuclei have migrated towards the surface of the embryo, *Nvit cad* is absent from a dorsal strip corresponding to the extra-embryonic

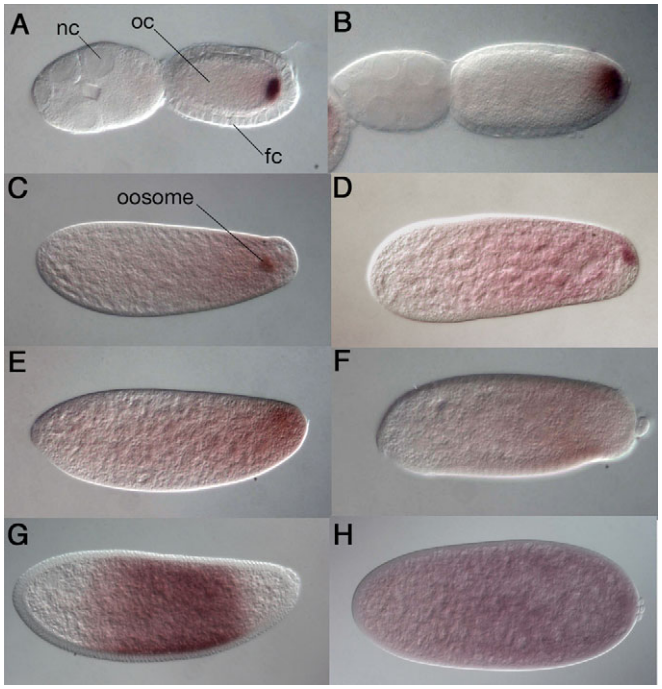

Fig. 1. Posterior localization of *cad* mRNA. (**A**) Nvit cad is posteriorly localized in the ovary and early embryo. (**B**) *Nvit cad* localization in later stage follicles prior to nurse cell degeneration. (**C**,**D**) mRNA localizes to the oosome in freshly laid embryos. (**E**,**F**) A posterior-to-anterior *Nvit cad* mRNA gradient forms. (**G**) This gradient is also present during early zygotic transcription 2-3 hours after egg laying. (**H**) In *Drosophila*, *cad* is found throughout the early embryo. oc, oocyte; fc, follicle cells; nc, nurse cells.

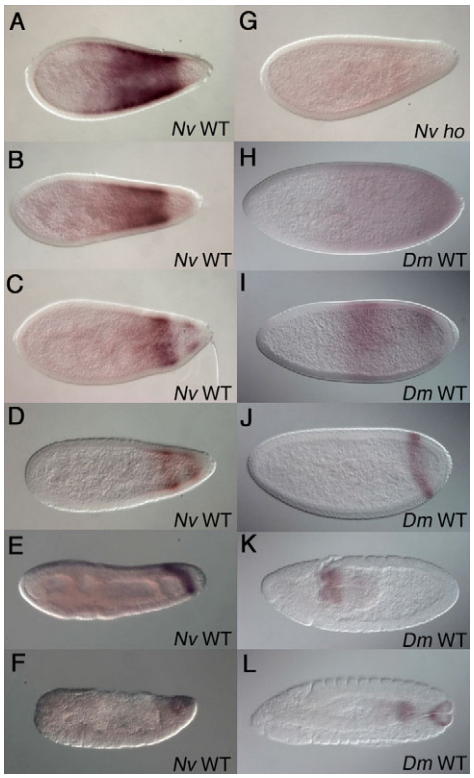

Fig. 2. Zygotic expression of *cad* in both *Nasonia* and *Drosophila*. (**A**,**B**) Zygotic *Nvit cad* is first expressed in the posterior three quarters of the embryo from 0-3 hAEL. (**C-F**) Expression recedes to form two and finally one posterior stripe at 3-5 hAEL. (**G**) *Nvit cad* expression in *ho* mutant embryos. (**H-L**) The *Dm cad* expression is similar to *Nvit cad* expression (compare A-F with H-L).

membranes, which extend along the anteroposterior axis of the embryo (data not shown). *Nvit cad* early zygotic expression extends further anteriorly than that of *Dm cad* (compare Fig. 1E and Fig. 2A with Fig. 2H,I), yet it is very reminiscent of *cad* expression in short-germ insects. At later stages of blastoderm development, *Nvit cad* zygotic expression begins to clear from the anterior to create a strong abdominal expression domain (Fig. 2A,B). By 5-6 hAEL, at the beginning of cellularization, expression recedes further from the anterior, refining into a posterior stripe (Fig. 2C). Later, a second stripe forms directly posterior to the first stripe, which eventually fades (Fig. 2C-E). This later expression of *Nvit cad*, just prior to and during gastrulation, is similar to *Dm cad* expression, where cells expressing this posterior stripe migrate during germband extension and eventually form a cluster of *cad*-expressing cells corresponding to the hindgut and anal plate primordia (Fig. 2E,F,K,L).

Regulation of *Nvit cad* by gap genes

In *Dm*, *hb* regulates the abdominal expression of *Dm cad* in a concentration-dependent manner: High levels of *hb* repress, whereas low levels activate, *Dm cad* transcription (Schulz and Tautz, 1995). Additionally, in *hb* zygotic mutants, the posterior stripe of *cad* is expanded (Mlodzik and Gehring, 1987b).

We find that, in addition to an expansion of the posterior *Dm cad* stripe, hb^{zyg} mutants show ectopic dorsal expression of *Dm cad* in the anterior of the embryo (Fig. 3H). Similarly, in the zygotic *Nvit* hb^{hl} mutant (Pultz et al., 2005), the posterior *Nvit cad* stripe is duplicated at the anterior of the embryo. Additionally, faint *Nvit cad* staining spans the region between the wild-type posterior *Nvit cad* stripe and the ectopic anterior *Nvit cad* stripe (Fig. 3D-F). This suggests that *Nvit hb* prevents *Nvit cad* expression in the anterior of the embryo. Furthermore, the ectopic anterior *Dm* and *Nvit cad* stripes are reminiscent of the duplication of the posterior *Dm cad* stripe at the anterior of bcd^{-} mutant embryos (Mlodzik and Gehring, 1987b).

As *Nvit otd-1* to a large extent plays a role similar to that of *Dm bcd* (Lynch et al., 2006a), we examined *Nvit cad* expression in *otd-1* RNAi embryos. Zygotic *Nvit cad* becomes derepressed from both poles, resulting in expression throughout the embryo. The area of *Nvit cad* derepression corresponds to the *otd-1* expression domains (Fig. 3A-C).

To assess in more detail the function of *hb* and *otd* in flies, and to address the function of *Kr* and *tll* in regulating *cad*, we next used ventral misexpression in *Drosophila* to examine the effect of the *Dm* gap genes *tll*, *Kr*, *otd* and *hb* on *Dm cad* expression. We used the *snail* (*sna*) promoter to drive ectopic expression in a ventral stripe (Andrioli et al., 2002). In *sna>tll* embryos, *cad* is activated in the ventral region of the embryo (Fig. 3I). Interestingly, *Dm cad* is not activated in the anterior ventral region of the embryo, suggesting that it is strongly repressed there. This activation by *tll* agrees with previous studies that showed a loss of the posterior *Dm cad* stripe in *tll* mutant embryos (Mlodzik and Gehring, 1987b). Ventral misexpression of the other gap genes does not affect *Dm cad* expression (data not shown). In the case of *hb*, one would have expected ventral repression of the posterior *Dm cad* stripe as it has been shown that high levels of *hb* repress *Dm cad* (Schulz and Tautz, 1995). The lack of repression might be due to insufficient levels of ventral *hb*. The lack of effect of *Kr* misexpression on *Dm cad* is consistent with the wild-type expression of *Dm cad* in *Kr* mutants (data not shown). These results suggest that the role of *hb* and other gap genes in regulating *cad* expression may have changed in the *Drosophila* gene network, when compared with more ancestral patterning networks.

Nvit cad parental RNAi produces severe defects in posterior development

To examine the function of *Nvit cad*, we made use of parental RNAi to downregulate *Nvit cad* function (Lynch and Desplan, 2006). Female pupae were injected with dsRNA and allowed to develop. Embryos from these adult mothers were aged for ~28 hours at 28°C; cuticles were subsequently examined. Interestingly, high concentrations of dsRNA targeting *Nvit cad* results in few embryos being laid: embryos derived from these mothers cease developing and do not reach the cuticular stage, suggesting that *Nvit cad* might play a role in oogenesis that is separate from its role in the early

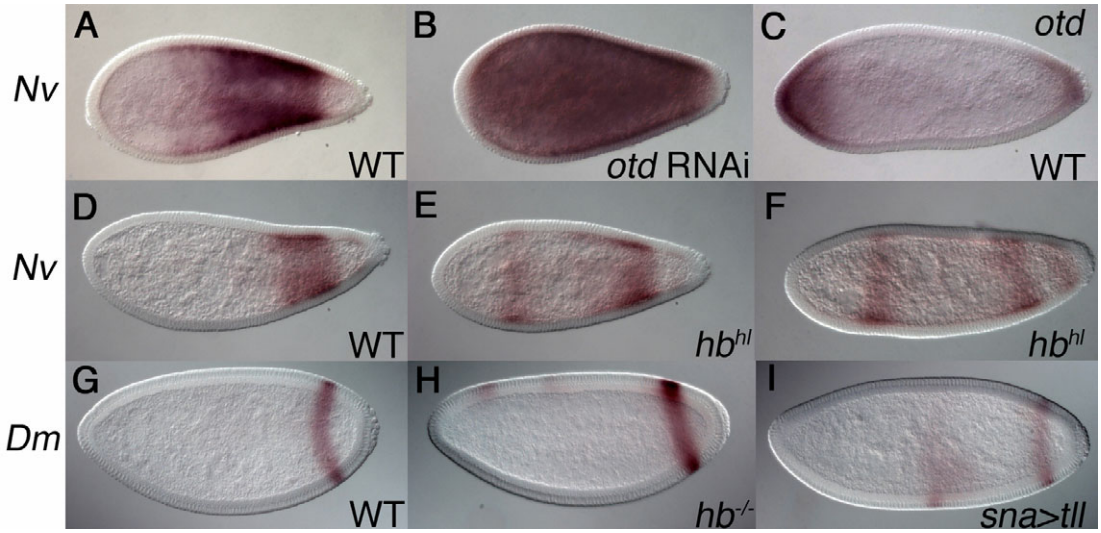

Fig. 3. Regulation of zygotic *Nvit cad* expression. Wild-type zygotic *Nvit cad* expression (**A**,**D**). *Nvit cad* expression is de-repressed throughout embryo in *Nvit otd* RNAi embryos (**B**). *Nvit cad* is expressed in a complimentary pattern to *Nvit otd* (**C**). *Nvit cad* is a expressed with a duplicated stripe at the anterior on hb^{hl} embryos (**E**,**F**). Wild-type expression of *Dm cad* (**G**). *Dm* $hb^{-/-}$ zygotic mutant embryos show a partial dorsal anterior ectopic stripe of *Dm cad* expression (**H**). *Dm cad* is ectopically activated in the presence of ventrally misexpressed *Dm tll* (**I**).

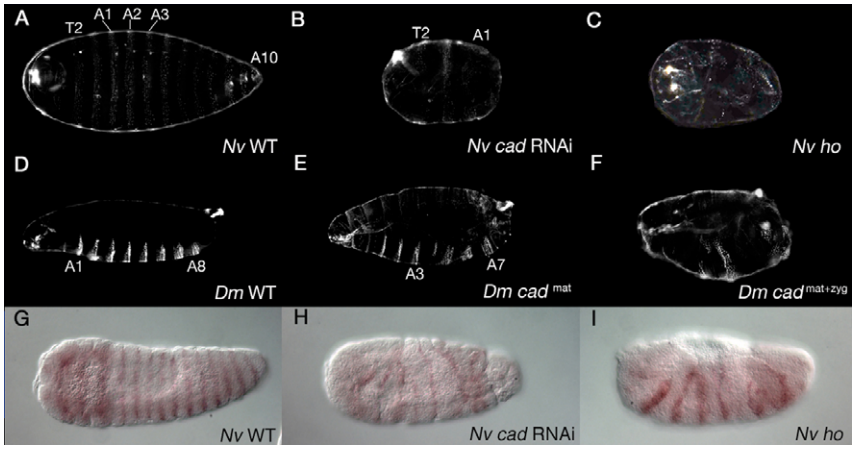

Fig. 4. Loss of *Nvit cad* causes a 'head only' phenotype. (**A**) The wild-type *Nvit* cuticle consists of three thoracic denticle belts and 10 abdominal denticle belts. (**B**) Thoracic segment 2, as well as A1-A3 abdominal denticle belts, show spiracles. *Nvit cad* RNAi results in loss of most abdominal denticle belts. (**C**) *ho* also shows loss of abdominal segments. (**D**) The *Dm* wild-type cuticle consists of eight abdominal denticle belts. (**E**) *Dm cad*^{mat} mutants show loss of A4 and A8. (**F**) *Dm cad*$^{mat+zyg}$ show loss of many abdominal segments. (**G-I**) *engrailed* expression in *Nvit* wild-type (G), *Nvit cad* RNAi (H) and *ho* (I) embryos.

embryo. Females injected with control *gfp* dsRNA show no cuticular defects and no difference in egg laying from wild-type females (data not shown). Lowering the concentration of *Nvit cad* dsRNA, however, results in a range of *cad* phenotypes. The wild-type *Nasonia* cuticle is composed of mouth hooks at the anterior, three thoracic and ten abdominal denticle belts. The second thoracic, as well as the first three abdominal denticle belts are easily identified by the presence of spiracles (Fig. 4A). Weak *Nvit cad* RNAi phenotypes show fusion of denticle belts throughout the abdomen but most commonly between segments A2 and A3 (data not shown). Stronger RNAi phenotypes exhibit a combination of fused or missing denticle belts, with progressive loss of segments starting from the posterior. Although, the number of denticle belts missing ranges from 0 to 13, most embryos retain six or seven denticle belts. Embryos exhibiting strong phenotypes, however, typically retain only three or four denticle belts, with T3 or A1 being the most posterior denticle belt remaining (Fig. 4B). These phenotypes are reminiscent of, but more severe than, the *Dm cad*$^{mat+zyg}$ phenotype, which also typically shows few abdominal denticle belts and often exhibits fusion of belts. Rarely do *Dm cad*$^{mat+zyg}$ phenotypes show loss of denticle belts as far anteriorly as A2 (Fig. 4F).

head only: a zygotic mutation in Nvit cad?

It has been proposed that the *head only* (*ho*) phenotype (Pultz et al., 1999) results from a mutation in *Nvit cad*. *ho* cuticles exhibit a loss of posterior denticle belts (compare Fig. 4B with 4C). This phenotype is exacerbated with decreasing temperature. We carried out a meiotic mapping experiment to determine whether the *ho* mutation is linked to *Nvit cad* (see Materials and methods). We tested 50 individuals and recovered no crossovers, placing *ho* within 2 cM of *Nvit cad*. Consistent with this hypothesis, the *Nvit cad* RNAi phenotypic series closely phenocopies the range of *ho* phenotypes, and affects all of the structures affected by *ho*, strongly suggesting that *ho* is a mutation in *cad*. We also compared the pattern of *engrailed* (*en*) mRNA staining in *ho* and *cad* RNAi embryos. The wild-type staining pattern for *en* consists of five head stripes and 12 trunk stripes (Pultz et al., 1999) (Fig. 4G). *en* staining in both *ho* and *cad* RNAi mutant embryos display variability that reflects their cuticular phenotypes. However, *en* stripes in the head always form normally. The most typical class of severely affected embryos displays all five normal head stripes but lacks 6 or seven trunk stripes. *en* trunk stripes also often display fusion, as is seen in the cuticles of both *ho* and *cad* RNAi embryos (Fig. 3H,I). These results are consistent with those previously reported for the *ho* mutant (Pultz et al., 1999). We examined *Nvit cad* expression in *ho* embryos using a probe directed against the region encoding the homeodomain and 3′UTR. *Nvit cad* expression is somewhat reduced at 28°C in *ho* mutants. However, when females are allowed to lay at 18°C (when the *ho* phenotype is strongest), *Nvit cad* zygotic expression is almost completely absent (Fig. 2G).

Taken together, the linkage analysis, paternal RNAi phenotypic series, as well as loss of *Nvit cad* expression in *ho* strongly suggest that *ho* is due to a lesion in the *Nvit cad* locus. Therefore, *ho* mutant embryos will be used here to examine the effects of zygotic lack of *Nvit cad* expression, in contrast to parental *Nvit cad* RNAi, which knocks down both maternal and zygotic *Nvt cad* expression.

Nvit cad regulates *hb* expression through *Kr*

To test whether maternal *Nvit cad* contributes to embryonic patterning and to decipher its place in the *Nasonia* patterning hierarchy, we examined the effects on gap gene expression of knocking down maternal and zygotic *Nvit cad* and compared our results with those obtained in *ho* mutants. We also compared the function of *cad* in *Nasonia* with its role in *Drosophil*a.

Nvit hunchback (*Nvit hb*) is expressed maternally and zygotically in the *Nasonia* embryo (Pultz et al., 2005; Lynch et al., 2006a) (Fig. 5A). Maternal *Nvit hb* is first distributed throughout the embryo and remains unaffected in *ho*, as well as in *Nvit cad* RNAi embryos. Later zygotic *Nvit hb* appears as an anterior cap and as a broad stripe in the posterior of the embryo (Pultz et al., 2005). In *ho* mutants, the anterior expression domain of *Nvit hb* expands toward the posterior. The same effect is seen in embryos derived from females injected with *Nvit cad* dsRNA, suggesting that zygotic, but not maternal *Nvit cad*, positions the posterior boundary of the *Nvit hb* anterior zygotic expression domain (Fig. 5B,C). As *cad* is generally thought of as a transcriptional activator, we examined whether *Nvit cad* might activate a repressor of *hb*. A candidate for this repressor is *Krüppel* (*Kr*).

Nvit Kr is expressed in a broad stripe in the center of the *Nasonia* embryo resembling *Dm Kr* expression (Fig. 5G). In *ho* embryos, there is a clear reduction in *Nvit Kr* transcription, leaving only a thin stripe of expression (Fig. 5I). In *Nvit cad* RNAi embryos, the central broad expression domain of *Nvit Kr* is absent (Fig. 5H). Therefore, both maternal and zygotic *Nvit cad* components are required to activate *Nvit Kr* expression. Anterior *Nvit hb* expression might therefore expand towards the posterior in *Nvit cad* mutants owing to the absence of *Nvit Kr* expression (Fig. 5G-I). Indeed, in the absence of *Nvit Kr*, *Nvit hb* shows a similar posterior expansion (A.E.B. and C.D., unpublished). We next examined whether loss of *Dm cad* also affects *Dm Kr* expression. In sharp contrast to the role of *Nvit cad* in

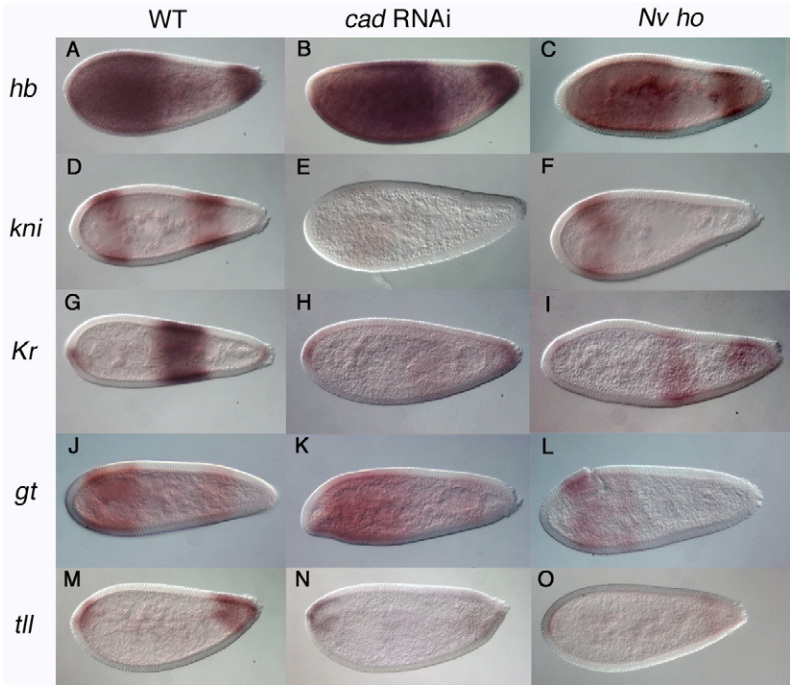

Fig. 5. *Nvit cad* regulates gap gene expression. Expression of *Nvit hb* (**A-C**), *Nvit kni* (**D-F**), *Nvit Kr* (**G-I**), *Nvit gt* (**J-L**) and *Nvit tll* (**M-O**) in wild-type, (A,D,G,J,M), in *Nvit cad* RNAi (B,E,H,K,N) or in *ho* embryos (C,F,I,L,O). *Nvit cad* regulates the expression of these gap genes in the abdomen and thorax.

Nvit Kr activation, we find that *Dm Kr* is not affected in zygotic, maternal or maternal + zygotic *Dm cad* mutant embryos (Fig. 6I,J). Furthermore, there is no effect on *Dm hb* expression in the same mutant genotypes (Fig. 6K,L).

cad has been shown to activate *hb* expression in *Gryllus* embryos where *cad* parental RNAi appears to cause a posterior shift in *Gb hb* expression, suggesting that it not only activates, but also sets the position of *Gb hb* (Shinmyo et al., 2005). This observation suggests that *cad* plays an ancestral role in activating *hb* expression. Our results, however, suggest that *Nvit cad* represses anterior *Nvit hb* through *Nvit Kr*, whereas it is *Nvit otd* that activates posterior *Nvit hb* (Lynch et al., 2006a). Surprisingly, the posterior *Nvit hb* stripe remains unaffected in *Nvit cad* RNAi (Fig. 5A-C).

Nvit cad activates both *Nvit kni* and *Nvit gt*

Dm cad acts as a transcriptional activator of *Dm giant* (*gt*) and *Dm knirps* (*kni*) (Rivera-Pomar et al., 1995; Schulz and Tautz, 1995). We verified that removing either zygotic or maternal *Dm cad* alone shows no effect on *Dm gt* and *Dm kni* expression (data not shown). However, removing both maternal and zygotic *Dm cad* causes a reduction in the expression of the posterior stripe of *Dm gt* (Fig. 6A-D), while the posterior stripe of *Dm kni* is reduced in intensity and expanded posteriorly (Fig. 6E-H). This expansion is probably due to a reduction in *Dm gt*, which acts as a repressor of *Dm kni* (Rivera-Pomar et al., 1995). Nevertheless, these phenotypes are fairly mild.

Nvit kni and *Nvit gt* are expressed zygotically in a similar pattern to their fly counterparts (Fig. 5D,J; Fig. 6A,C,E,G). Zygotic *Nvit cad* appears to be necessary to activate the posterior stripes of both *Nvit gt* and *Nvit kni*, since they are missing in *ho* mutants (Fig. 5F,L). In *Nvit cad* RNAi embryos, the same effect is observed at the posterior, while the anterior expression domains of *Nvit kni* and *Nvit gt* are also affected. Although positioned properly, the anterior *Nvit kni* domain is dramatically reduced (Fig. 5E), while the anterior domain of *Nvit gt* is expanded posteriorly (Fig. 5K). This expansion is not only due to loss of *Kr* as there is no dramatic posterior expansion of *Nvit gt* in *Nvit Kr* RNAi embryos (A.E.B. and C.D., unpublished). Therefore maternal *Nvit cad* probably represses anterior *Nvit gt* directly or activates another repressor of *Nvit gt*, thereby establishing its posterior border of expression.

Nvit cad activates *tll* but not *otd* transcription

Nvit otd has recently been shown to act as a morphogen involved in anterior patterning. Moreover, *Nvit otd* is involved in posterior patterning and its posterior cuticular phenotype partly overlaps with that of *Nvit cad* (Lynch et al., 2006a). We therefore examined whether *Nvit cad* regulates expression of posterior *Nvit otd* and *Nvit tailless* (*Nvit tll*) (Lynch et al., 2006b), which is involved in terminal patterning. *Nvit otd* is expressed maternally at both poles in the early embryo. Zygotic expression later forms caps at both poles of the embryo (Lynch et al., 2006a). *ho* and *Nvit cad* RNAi embryos show normal maternal and zygotic expression of *Nvit otd*, consistent with the model that *Nvit otd* is a maternal morphogen that regulates its own expression (data not shown) (Lynch et al., 2006a).

Nvit tll is expressed zygotically and resembles *Dm tll* expression (compare Fig. 4M with Fig. 5M) (Lynch et al., 2006b). *Nvit tll* is affected identically in both *ho* and *Nvit cad* RNAi embryos, where both the anterior and the posterior expression domains of *Nvit tll* are reduced. Later, however, the anterior expression domain is restored, while the posterior domain remains absent. Zygotic *Nvit cad* therefore activates both *Nvit tll* expression domains, but is not necessary for later activation of anterior *Nvit tll* expression (Fig. 5M-O). In *Drosophila*, *otd* (data not shown) and *tll* remain unaffected in the three different classes of *Dm cad* mutants (Fig. 6M,N).

Dm cad regulates pair rule gene expression

Our results indicate that *Nvit cad* plays a major role in gap gene regulation. As we see a much weaker regulatory contribution of *Dm cad* at the level of the gap genes, it is likely that this role has been taken over by *bcd* in *Drosophila* or become redundant with other patterning factors. However, although *Dm cad* might have become obsolete at the level of gap gene regulation, the *Dm cad*$^{mat+zyg}$ phenotype does show severe segmentation defects. We therefore

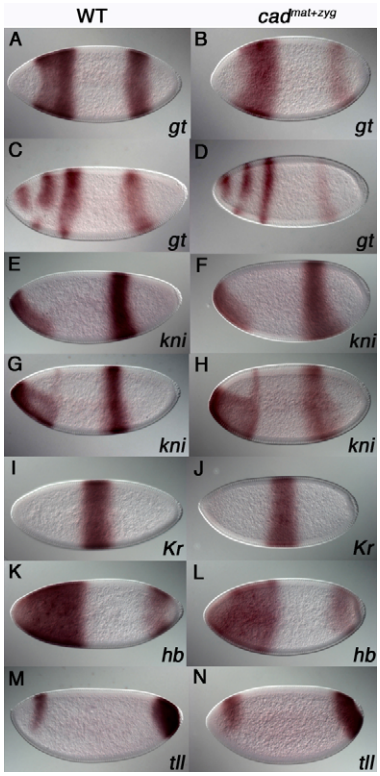

Fig. 6. *Dm cad* is a weak activator of *Dm kni* and *Dm gt*. *Dm gt* expression in wild-type (**A**,**C**) and *Dm cad*$^{mat+zyg}$ embryos (**B**,**D**). *Dm kni* expression in wild-type (**E**,**G**) and *Dm cad*$^{mat+zyg}$ embryos (**F**,**H**). *Dm Kr* expression (**I**,**J**). *Dm hb* expression (**K**,**L**). *Dm tll* expression (**M**,**N**). Wild-type embryos (I,K,M). *Dm cad*$^{mat+zyg}$ embryos (J,L,N).

examined the role of *Dm cad* in regulating pair-rule gene expression as a possible explanation for the severe cuticular phenotype resulting from the complete loss of *Dm cad*.

Dm cad activates the pair rule gene *fushi tarazu* (*ftz*) through direct binding to the 'zebra stripe' promoter element (Dearolf et al., 1989). Maternal *Dm cad* mutant embryos show an expansion of stripes 2, 4 and 7, while stripes 3, 5 and 6 are narrower than in wild-type embryos (Macdonald and Struhl, 1986). *Dm cad*$^{mat+zyg}$ mutant embryos have loss of up to four posterior *ftz* stripes (MacDonald and Struhl, 1986) (Fig. 7D).

As *Dm cad*-binding sites have been identified in the 3+7 and 4+6 enhancer elements of the pair-rule gene *Drosophila even skipped* (*eve*) (Hader et al., 1998; Schroeder et al., 2004), we looked at the expression of *Dm eve* in the different *Dm cad* mutant backgrounds. In a small number of *Dm cad*^{zyg} mutant embryos, the posterior stripes of *Dm eve* are weakly reduced. In *Dm cad*^{mat} mutants, *Dm eve* stripes 4-7 are expressed weakly with stripes 5 and 6 not well resolved in some cases. In *Dm cad*$^{mat+zyg}$ mutants, however, there is a loss of stripes 4, 6 and 7, a posterior expansion of stripe 5, as well as a reduction in stripe 3 expression (Fig. 7B). These results validate the presence of Cad-binding sites in the stripes 3/7 and 4/6 enhancer elements and further support the idea that the severity of the *Dm cad*$^{mat+zyg}$ phenotype is a result of aberrant pair-rule gene expression rather than of defects in gap gene expression.

We find that the expression of *Nvit eve* and *Nvit ftz* is also severely affected in *ho* mutants, with few stripes remaining. In *Nvit cad* RNAi-treated embryos, both *Nvit ftz* and *Nvit eve* are more severely affected, often with only one or two pair-rule stripes

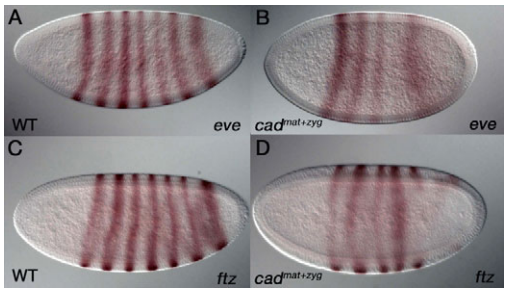

Fig. 7. *Dm cad* regulates pair rule gene expression. (**A**) Wild-type *Dm eve* expression. (**B**) *Dm eve* expression in *Dm cad*$^{mat+zyg}$ embryos. (**C**) Wild-type *Dm ftz* expression. (**D**) *Dm ftz* expression in *Dm cad*$^{mat+zyg}$ embryos.

remaining (data not shown). These results may simply reflect the effect on gap genes in the *ho* and RNAi treated embryos. Alternatively, as in *Drosophila*, *Nvit cad* may also directly regulate pair rule gene expression.

DISCUSSION

Much of our understanding of body plan formation comes from studies in *Drosophila* where Bcd, a factor that is not present outside the dipteran lineage, is a major organizer of the anteroposterior axis and is required for all anterior fates. However, as Bcd is not a conserved feature of anterior patterning, developmental biologists have sought out comparative analyses of anteroposterior development in insects such as *Tribolium* and *Gryllus* that lack Bcd (reviewed by Liu and Kaufman, 2005). However, making direct comparisons in short and intermediate germ insects with the long-germ insect *Drosophila* is complicated by the fact that they represent different modes of embryonic development. Here, we investigate the role of *caudal* in posterior patterning in *Nasonia*, a long germ insect that lacks *bcd*. We find that: (1) a maternal gradient of *Nvit cad* is achieved through mRNA localization rather than through translational repression by Bcd as in *Drosophila*; (2) *Nvit cad* plays a greater role in patterning the embryo than does *Dm cad*, and this role expands far anteriorly; and (3) *Nvit cad* is an activator of gap gene expression, in contrast to its role as a pair-rule gene activator in *Drosophila.*

Extensive function of *Nvit cad*

We have investigated the function of *Nvit cad* in early embryogenesis using both parental RNAi and the *ho* mutation, which probably results from the loss of zygotic *Nvit cad*. This allowed us to distinguish maternal and zygotic functions for a gene in a species other than *Drosophila*. The fact that the *Nvit cad* RNAi phenotype is much more severe than total loss of *Dm cad* is not surprising given the fact that both maternal and zygotic expression patterns of *Nvit cad* reach much further towards the anterior of the embryo than *Dm cad.* Similarly, in *Gryllus*, the *Gb cad* RNAi phenotype includes a complete loss of thoracic, abdominal and posterior structures. This is reflected in the wild-type expression of *Gb cad*, which is expressed in the presumptive gnathal and thoracic regions, as well as in the posterior growth zone (Shinmyo et al., 2005).

The fact that the severe *cad* phenotype is conserved in arthropods suggests that ancestrally, *cad* played a greater role in embryonic development but has lost some of its importance in *Drosophila*. We discuss these roles of *cad*, and what function it has retained in *Drosophila* for pair-rule gene regulation.

Maternal *Nvit cad* mRNA is localized

We have shown that *Nasonia* establishes a maternal mRNA gradient in the early embryo using mRNA localization and diffusion. Maternal *Nvit cad* mRNA is tightly localized to the posterior of the oocyte. After the embryo is laid, however, the mRNA diffuses far towards the anterior creating an mRNA gradient. *Nasonia* has thus devised a new mechanism for establishing a posterior-to-anterior gradient of *cad* mRNA, which probably forms a similar gradient at the protein level. In *Drosophila*, *cad* maternal transcripts are homogenously distributed throughout the early embryo and the Cad protein gradient is produced later through translational repression by Bcd (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996) (Fig. 1D). A redundant translational repression system may exist in *Nasonia* to ensure that no Cad is produced at the anterior.

The mechanism that establishes the Cad gradient in *Nasonia* is of particular importance as *bcd* is a new addition to the developmental network and is found only in higher dipterans. Consequently, Bcd cannot be responsible for establishing the Cad gradient in more ancestral species (Lynch and Desplan, 2003). Nonetheless the Cad gradient is conserved among insects. In *Tribolium*, Cad protein is first expressed homogenously throughout the embryo. Later, however, a posterior to anterior protein gradient forms but nothing is known about the mechanisms leading to the formation of this gradient. Interestingly, however, when a transgene encoding the *Tc cad* mRNA is placed in *Drosophila*, it leads to the formation of a translational gradient that is dependent on *bcd* (Wolff et al., 1998). This argues that a common underlying mechanism may be responsible for establishing the protein gradient in *Tribolium* and in *Drosophila.* It is likely that *bcd* took over the function of a translational repressor present in ancestral insects, perhaps including *Nasonia*. The mRNA gradient might therefore be specific to the wasp. Interestingly, *Nvit otd* mRNA is also localized to both the anterior and posterior poles of the embryo, which has not been reported in any other species (Lynch et al., 2006a). This suggests that *Nasonia* may extensively use RNA localization mechanisms for setting up the anteroposterior axes in the embryo. Moreover, maternal mRNA localization may be a common feature of long germ development. Studies performed in other Hymenopterans, which undergo extremely diverse modes of embryogenesis, ranging from long-germ embryogenesis in *Apis mellifera* (Davis and Patel, 2002) to the polyembryonic development of *Copidosoma floridanum* (Grbic, 2003) will aid in identifying the conserved mechanisms among these diverse modes of embryogenesis. In *Copidosoma*, up to 2000 embryos may be produced clonally from a single egg, showing that maternal determinants cannot play similar axial patterning roles in this insect as seen in long and short germ insects (Zhurov et al., 2004). However, work in the long germ *Apis mellifera* might elucidate whether maternal mRNA localization is a common feature of long-germ embryogenesis.

cad is the ancestral activator of gap genes

In *Nasonia*, *cad* functions as an activator of gap gene expression, placing it at the top of the segmentation network similar to *bcd* in *Drosophila*. However, we find no evidence that Cad acts as a morphogenetic gradient. Gap genes are primary interpreters of anteroposterior cues and serve to divide the early embryo into broad expression domains. Among gap genes, *Dm Kr* is a particularly important player that acts as a potent repressor of other gap and pair rule genes. Positioning the *Kr* domain is therefore crucial and Bcd is involved in *Dm Kr* regulation in addition to activating a large number of anterior patterning genes such as *Dm hb*. *bcd* is therefore considered a master patterning gene (Hoch et al., 1991). In *Gryllus*, *Gb Kr* and *Gr hb* are activated by *cad* and it was hypothesized that this represents the ancestral function of *cad*, placing it at the top of the segmentation hierarchy (Shinmyo et al., 2005). Cad-binding sites have been identified in *Dm Kr* regulatory region, which may be vestiges that had once functioned in an ancestral patterning system (Schroeder et al., 2004). Our results in *Nasonia* confirm that the role of *cad* to activate *Kr* is conserved and supports the notion that this role has been usurped by *bcd* in *Drosophila*. We also find that *Nvit cad* activates *tll* expression. This role is not conserved in the fly, despite the presence of *cad*-binding sites in the regulatory region of *Dm tll* (Schroeder et al., 2004).

kni and *gt*, which are only weakly affected in *Drosophila cad* mutants, absolutely require *cad* in *Nasonia*. It is likely that *kni* and *gt* rely instead on *bcd* for activation in *Drosophila.* It should be noted that the anterior patterning gene *bcd* is involved in activating gap gene expression in the posterior of the embryo. This is also true of the posterior-most stripe of the pair-rule gene *hairy*, which relies on the combined activity of Bcd and Cad for activation (La Rosee et al., 1997). Similarly, although *cad* is typically thought to regulate expression in the posterior of the embryo, maternal *Nvit cad* is involved in regulating the anterior expression domains of both *kni* and *gt.*

Although the role of *cad* in activating the gap genes seems to have been taken over by *bcd* in *Drosophila*, complete loss of *Dm cad* does result in severe segmentation defects. We have shown that *Dm cad* acts at the level of pair-rule genes instead and that it is a strong transcriptional regulator of *Dm eve* expression.

Nvit otd and *Nvit cad* work together in patterning posterior segments

bcd is believed to have evolved as a duplication of *zen* (Dearden and Akam, 1999; Stauber et al., 1999; Stauber et al., 2000) that later acquired a K_{50} residue within its homeodomain, giving it the same binding specificity as Otd (Treisman et al., 1989). It has thus been proposed that *otd* is a major ancestral anterior patterning gene, the role of which has been taken over by *bcd* (reviewed by Lynch and Desplan, 2003). Interestingly, in *Nasonia*, *otd* is expressed maternally and zygotically at both poles. Loss of *Nvit otd* results in the loss of both anterior and posterior structures (Lynch et al., 2006a). This phenotype is somewhat overlapping in the posterior with *Nvit cad*. Thus, it is likely that *Nvit cad* and *Nvit otd* work in concert to regulate posterior genes, as seen in fly with *bcd* and *cad* activating posterior *hairy* and *kni* stripes. The presence of Bcd (K_{50}) binding sites in promoter elements of genes expressed in the posterior of the *Drosophila* embryo may thus reflect an ancestral role of *otd* in activating posterior genes, although *Nasonia* remains the sole example so far of posterior *otd* expression.

Nvit otd is necessary to repress *Nvit cad* from both poles of the embryo. Additionally, *Nvit hb* represses later *Nvit cad* expression in the anterior of the embryo. This suggests that zygotic *Nvit cad* is first activated throughout the embryo, and that a strong repression system is required to prevent *Nvit cad* from specifying posterior fates in the anterior. In *Drosophila*, the absence of Bcd leads to the expansion of maternal Cad to the anterior of the egg. This results in the duplication at the anterior of the embryo of a telson, a structure that requires *cad*. However, the mere presence of Cad at the anterior is not sufficient to induce the formation of a telson. In embryos where Bcd is present but unable to bind the *cad* 3′UTR, Cad is expanded anteriorly, yet only head involution defects are seen but no telson forms at the anterior (Mlodzik et al., 1990; Neissing et al., 1999; Neissing et al., 2002). This is probably due to the presence of *bcd*-dependent Hb at the anterior, which might inhibit Cad protein

function. Like *bcd*, *Nvit otd* also acts, probably in concert with *Nvit hb*, in repressing posterior development in the anteriormost region of the embryo by repressing *Nvit cad*. However, *Nvit otd* controls *Nvit cad* at the transcriptional level, whereas *bcd* represses *Dm cad* at the translational level.

Conclusion

We propose that *Nvit cad* and *Nvit otd* function together in patterning the posteriormost segments (Lynch et al., 2006a). *Nvit cad* acts as the ancestral posterior patterning center responsible for activating the gap genes in the thoracic, abdominal and posterior regions of the long-germ wasp embryo, but it is *Nvit otd* that functions as a morphogen by setting the positions of the gap genes.

In conclusion, the posterior-to-anterior gradient of *Nasonia* maternal *cad* is established in a novel way through the formation of an mRNA gradient. Moreover, maternal *Nvit cad* plays a distinct role from its zygotic counterpart. Together, maternal and zygotic *Nvit cad* regulate gap gene expression in a non-redundant manner, placing *cad* at the top of the segmentation network. In *Drosophila*, it seems that *cad* has lost, to *bcd*, its ability to activate gap genes and instead its role in the patterning network is to regulate pair rule genes. The combinatorial activation of posterior *kni* by *cad* and *bcd* in *Drosophila* may be a remnant of the ancestral role of *cad* as the key transcriptional activator of gap genes. We thus propose that *cad* is the ancestral patterning center in short-germ embryogenesis and that this role is retained in *Nasonia* long-germ development but largely lost in *Drosophila*.

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