The Phylogenetic Origin of oskar Coincided with the Origin of Maternally Provisioned Germ Plasm and Pole Cells at the Base of the Holometabola

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Abstract

The establishment of the germline is a critical, yet surprisingly evolutionarily labile, event in the development of sexually reproducing animals. In the fly Drosophila, germ cells acquire their fate early during development through the inheritance of the germ plasm, a specialized maternal cytoplasm localized at the posterior pole of the oocyte. The gene oskar (osk) is both necessary and sufficient for assembling this substance. Both maternal germ plasm and osk are evolutionary novelties within the insects, as the germline is specified by zygotic induction in basally branching insects, and osk has until now only been detected in dipterans. In order to understand the origin of these evolutionary novelties, we used comparative genomics, parental RNAi, and gene expression analyses in multiple insect species. We have found that the origin of osk and its role in specifying the germline coincided with the innovation of maternal germ plasm and pole cells at the base of the holometabolous insects and that losses of osk are correlated with changes in germline determination strategies within the Holometabola. Our results indicate that the invention of the novel gene osk was a key innovation that allowed the transition from the ancestral late zygotic mode of germline induction to a maternally controlled establishment of the germline found in many holometabolous insect species. We propose that the ancestral role of osk was to connect an upstream network ancestrally involved in mRNA localization and translational control to a downstream regulatory network ancestrally involved in executing the germ cell program.

Introduction

Germ cells are essential for the transfer of heritable information and, therefore, the determination of their fate is a critical event in the development and evolution of sexually reproducing organisms. Two general strategies for generating the germline have evolved in animals: cytoplasmic inheritance or zygotic induction. Inheritance requires that determinants of the germ cell fate (mRNAs and proteins that form the pole plasm) are maternally generated and provisioned to the oocyte. In contrast, induction involves the acquisition de novo of the germ cell fate in a subset of cells later during embryonic development [1,2].

Some of the first experiments that proved the existence of a maternally generated substance capable of inducing the germ cell fate were conducted in insects. It had been observed that in many insect species, a distinct region of cytoplasm (called pole plasm, or oosome) is localized to the posterior pole of the oocyte during oogenesis. This pole plasm remains at the posterior during early embryogenesis, until cleavage nuclei reach the embryo cortex. Those nuclei that reach the posterior pole of the embryo interact with the pole plasm, bud from the posterior pole, and become cellularized precociously in comparison to the other blastodermal nuclei [3]. These cells are termed pole cells, and will give rise to the germline [4,5]. Classical embryonic manipulations showed that the pole plasm is both necessary [6], and sufficient [7] to produce the primordial germ cells.

Genetic analyses have identified numerous molecular factors that are required for the proper production of the pole plasm and pole cells in Drosophila. Only one of these, oskar (osk), is both necessary and sufficient to induce the production of polar granules and pole cells [8]. Due to the sufficiency of Osk to induce germ plasm, it must be tightly regulated to prevent ectopic induction of germline fate. To this end, genes upstream of osk are generally required to regulate translation of osk mRNA and to mediate its transport between the time it is transcribed in the nurse cells and the time it is properly posteriorly localized in the oocyte [9]. Genes downstream of osk are generally required to assemble the polar granules or to mediate proper behavior of the pole cells [9], and have highly conserved functions in the germline throughout the Metazoa [10–12].

Current data suggest that the mode of germline determination found in Drosophila is not the ancestral mode among the insects. So
The establishment of the germline during embryogenesis is a critical milestone for sexually reproducing organisms, and one that is surprisingly labile in evolution. For example, in the fly Drosophila, the germline is set aside early in embryogenesis due to the localized synthesis of the germ plasm at the posterior pole of the oocyte, and the gene oskar is both necessary and sufficient for assembly of the germ plasm. However, oskar orthologs have not been found outside of flies and mosquitoes, while the maternal provisioning of germ plasm and the early setting aside of the germline are unique to, but not universal within, the holometabolous insects. In order to understand how the novel mode of germline determination found in Drosophila could have evolved, we have examined this process in the wasp Nasonia. Our results indicate that the phylogenetic origin of the insect mode of maternal germ plasm provision and early establishment of the germline coincided with the origin oskar at the base of the holometabolous insects. Our results further suggest that osk was independently lost in multiple holometabolous insect lineages and that these losses are phylogenetically correlated with changes in germline determination strategies in these species.

Far neither unequivocal maternal germ plasm nor pole cells have been detected in representatives of basally branching hemimetabolous insect orders. Rather, species from these orders instead appear to rely on zygotic induction mechanisms to specify their germline [13–17] (Figure 1). Consistent with absence of cytoplasmic inheritance of germline determinants and the production of pole cells, the processes for which osk is required, orthologs of osk have not been detected in any of the sequenced genomes of the hemimetabolous insects Aedes aegypti [18], Rhodius prolixus (http://genome.wustl.edu/genomes/view/rhodius_prolixus/), and Pediculus humanus http://phumanus.vectorbase.org/SequenceData/Ge nome/ (Figure 1, Table S1).

Among the Holometabola, osk orthologs are also apparently absent from the sequenced genomes of the silk moth Bombyx mori (Lepidoptera) [19], the beetle Tribolium castaneum (Coleoptera) [20], and the honeybee Apis mellifera (Hymenoptera) [21] (Figure 1, Table S1). Consistent with this absence osk, Bombyx, Tribolium, and Apis all also lack maternal germ plasm, do not produce pole cells, and appear to rather use zygotic inductive strategies to generate the germline [22–25] (Figure 1).

These observations led to the idea that osk may have been a novelty that originated within the dipteran lineage [26,27]. However, Drosophila-like modes of germline determination through posteriorly localized maternal germ plasm and pole cells are also found throughout the Holometabola, including most major lineages of the Hymenoptera (e.g., Nasonia vitripennis [28] sawflies [29] and multiple ant species [30,31]), the Coleoptera (e.g., Acanthoscelides obtectus [32], Dermestes frischii [33]), Megaloptera (Sialis misuhashi [34]) and Lepidoptera (Pectinophora gossypiella [35]) (Figure 1). Despite the similarity of the strategies for germline determination in the above species to that employed in Drosophila, osk orthologs have only been identified in the genomes of the dipters Anopheles gambiae, Aedes aegypti, and Culex pipiens [36,37] (Figure 1).

These observations raised the question of evolutionary origin of osk in the insects and whether or not this gene is associated with the evolution of the inheritance mode of germline specification. To answer these fundamental questions, we examined the molecular basis of maternal germ plasm production in the wasp Nasonia vitripennis. We chose Nasonia because its genome was recently sequenced [30], it is amenable to functional manipulation by pRNAi [39], and its key phylogenetic position within the most basally branching holometabolous order, the Hymenoptera [40,41]. We show that the regulatory network underlying the production of maternal germ plasm and pole cells is largely conserved between Nasonia and Drosophila, and argue that these features had a common phylogenetic origin at the base of the Holometabola. In addition, we provide evidence that the possession of an oskar ortholog is a general feature of insects that produce pole cells, and that oskar has likely been lost independently multiple times within the Holometabola in correlation with shifts in strategies for establishing the germline.

Results

Cloning and sequence analysis of Nv-Osk

Attempts to detect a Nasonia ortholog by BLAST [42] searches using the Drosophila Osk sequence as the query failed to return significant hits. However, using Oskar sequences identified in the mosquitoes Culex and Aedes, we identified a Nasonia genomic region that showed significant similarity to the mosquito sequences. Using the predicted peptide sequence in this region, reciprocal BLAST against the mosquito and Drosophila genome databases returned results with significant E-values that corresponded to osk genes in each of these species (Table S1). We thus hypothesized that the region in the wasp genome detected by mosquito Osk BLASTs corresponded to Nasonia osk, and cloned a 1500 base pair fragment representing the full length complementary DNA of Nasonia osk using RACE PCR. This sequence contains an open reading frame that is predicted to generate a protein of 375 amino acids.

The overall Nv-Osk sequence is similar to that of Drosophila Osk (16% identity, 33% similarity, 44% gaps), and many of the residues critical for fly Osk function are conserved in the Nasonia sequence (Figure 2). However, we could identify two regions that appear to be unique to the fly sequence. One is the region that is specific to the Drosophila long-Osk isoform [43] (Figure 2, red text). No similarity to this region appears to be encoded in the Nv-osk mRNA, nor is it present in mosquitos Osk sequences. The other region that is absent in Nv-Osk includes amino acids 290 to 396 in Dm-Osk (Figure 2, blue text), which corresponds to the domain interacting with LASP to regulate Osk anchoring to the actin cytoskeleton [44]. Interestingly, this region is also absent from the mosquito Osk sequences, which appear to be more similar to Nv-Osk in sequence and general structure (Culex/Nasonia: 24% identity, 42% similarity, 22% gaps).

A search in the Conserved Domain Database indicates that the central portion of the Nv-Osk protein shares similarity with a GDSL/SGNH-hydrolase or lipase-like domain (Figure 2, orange boxes), consistent with similar observations made for C. pipiens and A. aegypti Osk orthologs [36]. This domain is weakly detected in Drosophila Osk and it is not clear whether it is necessary for Osk function in pole plasm assembly.

In addition, the N-terminal region of Nv-Osk shows strong similarity to a domain also present at the N-termini of highly conserved tudor-domain containing proteins. This domain has been independently identified in silico as either the Lotus domain [45], or Tejas domain [46]. This domain is present at the N-terminus of orthologs of tudor-domain-containing-7 and -5 (tdrd7, tdrd5), and related tudor domain containing genes [47], and is detected only weakly in fly Osk. tdrd7 and tdrd5 orthologs are found throughout the Metazoa, including all sequenced insect genomes (JAL, personal observation), and are characterized by the presence of...
of Tudor domains toward the C-terminus of the protein, which are absent in Osk proteins. The N-terminal 100 amino acids of Nv-Osk show strong homology to Tdrd7 orthologs throughout the Metazoa, ranging from 39% identical (BLAST E-value 8e-09) to the Apis ortholog, 31% identical (BLAST E-value 1e-05) to the Hydra ortholog, and 29% identical (BLAST E-value 7e-05) for the

Figure 1. Current understanding of the distribution of maternal germ plasm, pole cells, and oskar orthologs in the insects. Genus names in blue are those in which maternal germ plasm and pole cells have been described. Asterisks indicate a sequenced genome. Green boxes indicate confirmed presence of osk. Red boxes indicate apparent absence of osk in the genome. Dashed green box indicates the hypothesis that species with posteriorly localized maternal germ plasm and pole cells require a factor with Osk-like function and regulation.

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Danio (zebrafish) ortholog. In comparison, the *Apis* and *Danio* Tdrd7 C-termini are 49% identical (BLAST E-value 2e-13), and *Apis* and *Hydra* proteins are 30% identical (BLAST E-value 3e-09) in the N-terminal region.

In zebrafish, *tdrd7* has a role in controlling germ granule morphology and number during embryogenesis [48]. Furthermore, the *Drosophila* *tdrd5* ortholog, *tejas*, has a critical role in germine development, and the N-terminal region of this protein (including the Tejas domain, which is similar to the N-terminus of *Nv-Osk*) has been shown to physically interact with Vas [46]. Finally, a bioinformatic analysis of proteins containing domains similar to those found in Osk and Tdrd7/5 N-termini (termed by

### Figure 2. Sequence features of *Nv*-Osk protein.

CLUSTALW generated alignment of *D. melanogaster* and *N. vitripennis* Osk proteins. Red text is the fly long-Osk specific region. Blue indicates the putative LASP binding domain of fly Osk. Pink text indicates the Lotus/Tejas homology domain. The characterized missense mutations in fly *osk* were mapped on the alignment, and were categorized as follows: green shaded residues are those that are conserved between wasp and fly Osk, but are not conserved in the mosquito sequences (osk2). Red shaded residues are conserved in wasp, mosquito, and fly (osk6B10 and osk 5). Pink shading indicates residues that are conserved between wasp and mosquito Osk, but not in *Drosophila* (osk8). Finally, light blue shaded residues are conserved between mosquitoes and fly, but not in the wasp (osk3 and osk7). Orange boxes delineate the putative hydrolase homology domains in *Drosophila* and *Nasonia* Osk.

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the authors OST-HTH) indicated that these domains may bind double-stranded RNA [49]. These results indicate that Oskar is at least partially related to genes that had ancestral germline and/or RNA binding functions.

**Nv-osk is expressed in the germline and is localized to the posterior of the oocyte and early embryos**

*Nasonia* oogenesis occurs in ovarioles of the polytrophic-meristic type, where each oocyte is associated with its own population of nurse cells, and has been described in detail previously [50]. *Nv-osk* mRNA is detected quite early in oogenesis, just after the time that the nurse cells become distinguishable from the oocyte (Figure 3A, 3A'). As the egg chambers mature (Figure 3B), *Nv-osk* is expressed at very high levels in only the posterior nurse cells nearest to the oocyte. Within these cells, *Nv-osk* mRNA is incorporated into particles (Figure 3B), a pattern similar to that of *Nv-odd1* [51]. From the very early stages of oogenesis, *Nv-osk* is transported from the nurse cells to the oocyte, where it is localized to the posterior pole in a pattern similar to that of *Nv-nos* (Figure 3A', 3B, 3C). During late oogenesis, *Nv-osk* mRNA levels go from high to barely detectable in the nurse cells of adjacent egg chambers (Figure 3C). This likely indicates the onset of nurse cell dumping, as from this point on the nurse cells will become progressively smaller and eventually disappear. This pattern of rapid transfer of mRNA is similar to what is seen for *Nv-odd1* during late oogenesis, except that *Nv-odd1* mRNA accumulates at the anterior pole of the oocyte at this stage [51].

In the early embryo, *Nv-osk* mRNA remains localized to the posterior pole, and most of the mRNA is associated with the oosome, a large, discreet structure associated with the posterior pole. The oosome migrates within the embryo during the early cleavages (Figure 3D), before returning to the posterior pole just before the formation of pole cells (Figure 3E, see [51] for details). At this stage, a population of *Nv-osk* mRNA not contained within the oosome is observed in a gradient at the posterior pole, a pattern which is typical for oosome associated mRNAs (e.g., *odd1* and *namos* in *Nasonia* [52]). *Nv-osk* mRNA still associated with the oosome is then incorporated into the pole cells (Figure 3F), while the cytoplasmic population remains in the embryo proper (not shown, but see [51] for expression of *Nv-nos* mRNA, which shows identical behavior at these stages). Both populations of mRNA are finally degraded as the cellular blastoderm begins to form (Figure 3G).

**Nv-osk is required for oosome assembly and pole cell formation**

We used parental RNA interference (pRNAi) to analyze the function of *Nv-osk* during *Nasonia* development. We obtained specific phenotypes that vary in terms of intensity allowing us to infer a number of potential functions for *Nv-osk* during oogenesis and early embryogenesis.

In ovarioles showing the strongest *Nv-osk* pRNAi effect, only a few egg chambers are produced (Figure 4B, compare to 4A) indicating that *Nv-osk* has an early role in promoting oogenesis. This may be related to a similar phenotype produced by mRNA null mutations in *fly osk* [53].

The *Nasonia* ovariole normally consists of a linear array of egg chambers, with the oocytes always lying directly posterior to their sister nurse cells and directly anterior to the next older egg chamber (Figure 4A). In the milder phenotypes of *Nv-osk* pRNAi, this linear arrangement is disrupted, and egg chambers are arranged perpendicularly to the long axis of the ovariole (arrows in Figure 4C and 4D), or with reversed polarity (arrowhead Figure 4C) are observed. Egg chamber polarity defects are also observed after pRNAi against *Nv-nos* (not shown) and *Nv-tud* (see below), indicating that there is a novel role for germ plasm components in establishing polarity of egg chambers within the ovarioles of *Nasonia*. Due to the variability in the final morphology of ovarioles after pRNAi for *Nv-nos, -osk*, and -tud, it is not clear whether these phenotypes are all the result of the disruption of a single developmental process.

Within the oocytes, *Nv-nos* and *odd1* mRNAs are sometimes localized more loosely than normal (asterisk and arrowhead Figure 4C) or mislocalized in relation to the AP axis of the oocyte (asterisk Figure 4D) after *Nv-osk* pRNAi. These phenotypes may represent a disruption of the internal polarity of the oocytes and/or proper anchoring of localized mRNAs. A more detailed understanding of oocyte cytoskeletal polarity and mRNA anchoring mechanisms in *Nasonia* will be required to resolve this uncertainty. In any case, these results indicate that *Nv-osk* is required for germline development, for establishing the polarity of the egg chambers, and for the proper localization of the pole plasm to the posterior pole.

In *Drosophila*, the recruitment of Vas protein to the posterior pole of the oocyte by Osk is a critical step in polar granule assembly. To test whether *Nv-Osk* functions in a similar way, we examined the distribution of *Nv-Vas* using a *Nasonia* specific Vasa antiserum in wild type and *Nv-osk* pRNAi ovaries. During early oogenesis, *Nv-Vas* protein is detected primarily on the surface of the nuclei of the most anterior nurse cells (Figure 4E'). This is consistent with the strong transcription of *Nv-Vas* detected in these cells (Figure 3A). Localized *Nv-Vas* protein is not seen in early oocytes (Figure 4E'), even though *Nv-osk* is already localized at high levels at the posterior (Figure 4E). Localized *Nv-Vas* becomes visible in the oocyte relatively late in oogenesis, when the oocyte is of the same size as the nurse cell cluster (Figure 4F, 4F'). This accumulation of *Nv-Vas* at the posterior pole is abolished after *Nv-osk* pRNAi (Figure 4G), while *Nv-Vas* production in anterior nurse cells appears unaffected (Figure 4G'). Thus, the role of Osk in recruiting germ plasm components to the posterior pole is conserved between *Drosophila* and *Nasonia*.

Posteriorly localized mRNAs (e.g., *Nv-nos, -odd1*, and *Nv-osk*) are incorporated into the oosome in early *Nasonia* embryos (Figure 5A). After *Nv-osk* pRNAi, these mRNAs remain in a homogenous cap at the posterior pole of the embryo, and the oosome is not formed (100% penetrance, *N* = 60) (Figure 5B, 5C). In addition, the anterior localization of *Nv-odd1* mRNA is disrupted. Rather than being tightly localized at the anterior pole, *Nv-odd1* mRNA is often seen in particles distributed throughout the anterior half of the embryo (Figure 5B). This part of the phenotype may be related to the polarity defects observed in *Nv-osk* pRNAi oocytes.

pRNAi against *Nv-osk* also results in the completely penetrant (N = 57) loss of pole cells (Compare wild type in Figure 5D to 5E). In the absence of the protective environment of the pole cells, all *Nv-nos* mRNA is lost from the embryo by the late blastoderm stage (Figure 5F). A similar phenomenon is seen after *Nv-nos* pRNAi [51]. *Nv-osk* pRNAi also causes embryonic patterning phenotypes that result in larval lethality (42%, *N* = 75). Only a portion (13%) showed phenotypes similar to *Nv-nos* pRNAi [51], while the remainder of affected cuticles showed defects in head patterning, or more severe patterning disruptions of unclear origin. This range of phenotype was also seen for *Nv-tud* [51], and these observations indicate that the roles of *Nasonia* germ plasm assembly factors in embryonic patterning are much more complicated than they are in the fly, where *nos* mRNA translation is the main embryonic patterning output of germ plasm assembly [54].
**Figure 3. Expression of Nv-osk during oogenesis and embryogenesis.** During oogenesis (A–C) and embryogenesis (D–G). A, A’: Expression of Nv-osk (green) and Nv-nos (red) in early oogenesis. Arrows mark oocyte. B: Later stage of oogenesis, after completion of encapsulation of the oocyte by follicle cells. nc = nurse cells. C: Toward the end of oogenesis, most Nv-osk mRNA is rapidly dumped from the nurse cells into the oocyte (compare lower egg chamber to the upper). D: Embryo in division cycle 2–3 stained for Nv-osk. E: Embryo just before syncytial blasoderm formation. F: Embryo in early syncytial blastoderm stage. G: Embryo just before cellularization of the blastoderm. Scale bars = 100 micrometers.

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*Nv-osk* function is upstream of *Nv-vas* and *Nv-tud*

In *D. erecta*, Oskar acts through two main downstream proteins to produce polar granules: Vas and Tud [9]. As shown above, Nv-Osk functions upstream of Nv-Vas recruitment to the posterior during oogenesis (Figure 4G). However, the functional relationship between Nv-Osk and Nv-Vas in the ovary may not be strictly hierarchical, as Nv-Vas knockdown (Figure 6A) leads to defects in the proper anchoring and tight localization of Nv-osk mRNA to the posterior pole of the oocyte (Figure 6A). In the embryo, Nv-vasa pRNAi results in the completely penetrant loss of the oosome (Figure 6E) and pole cells (Figure 6F), similar to the effects of Nv-ask pRNAi.

In contrast to Nv-ask and Nv-vas pRNAi, knockdown of Nv-tud, which is expressed weakly and ubiquitously in the nurse cells and oocyte (Figure S1B), has only a minor effect on posterior accumulation of Nv-Vas protein in the oocyte, even when strong polarity defects within the ovariole are observed (Figure 6B, 6B'). In the embryo, the oosome is still formed, but is significantly reduced in size (Compare Figure 6G to 6C). In line with these apparently
weaker effects, Nv-tud pRNAi leads to a reduction in the number of pole cells, and those that do form are smaller, less spherical, and less segregated from the somatic nuclei at the posterior pole which may indicate that they are not completely differentiated as primordial germ cells (Compare Figure 6H to 6D). These results indicate that, similar to fly tud [8,55], Nv-tud function is downstream of Nv-vas and

Figure 4. Effects of Nv-osk pRNAi during oogenesis. A: Wild type Nasonia ovariole stained with Nv-otd1 (green) Nv-nos (red), and DAPI (blue). B: Strong Nv-osk pRNAi knockdown, very few mature egg chambers are formed. C,D: In weaker Nv-osk pRNAi knockdowns the linear arrangement of egg-chambers is severely disrupted. Egg chambers in reverse orientation (arrowhead) or perpendicular to the AP axis of the ovariole (arrows) are observed. Within the oocytes, axial polarity (asterisks) and mRNA localization (arrowhead in C) defects occur. E, E': In wild type, Nv-Vas protein is not localized in young oocytes (E') even though high levels of Nv-osk mRNA are localized at the posterior pole (E). Nv-Vas protein appears to be concentrated on the surface of the most anterior nurse cell nuclei. F, F': Nv-osk mRNA (F) and Nv-Vas (F') accumulation late in oogenesis. G, G': Expression of Nv-osk (G) and Nv-Vas (G') after Nv-osk pRNAi. doi:10.1371/journal.pgen.1002029.g004
Nv-osk in the production of the germ plasm. However, due to the incompleteness and variability of pRNAi efficiency, we cannot exclude the possibility that the weaker defects are the result of general weaker knockdown of Nv-tud with pRNAi.

Regulation of Nv-osk function

In Drosophila, the localization and regulation of osk translation is tightly regulated in order to prevent ectopic pole plasm and disruptions in segmental patterning. A critical factor in ensuring proper control of osk translation is the RNA binding protein Bruno, which binds the UTRs of osk mRNA and represses its translation. This repression is relieved under normal circumstances only upon localization of osk mRNA to the posterior pole of the oocyte [56]. We analyzed the function of Nasonia bruno to test whether a similar mechanism of translational repression operates in Nasonia to prevent the ectopic assembly of the oosome.

In wild-type egg chambers, Nv-osk and otd1 mRNAs are co-expressed in the posterior nurse cells and localized at the posterior pole of the oocyte, while Nv-otd1 is additionally localized to the anterior pole (Figure 7A, 7A'). The distribution of these mRNAs is dramatically altered after Nv-bruno RNAi: both Nv-osk and Nv-otd1 (and Nv-nos, data not shown) mRNAs are concentrated in large, dense, spheroid particles in the posterior-most nurse cells (Figure 7B, 7B'). These large particles seem to originate at the nuclear envelope, and smaller particles are observed on the surface of the nurse cell nuclei membranes in some egg-chambers (Figure 7C, 7C'). The morphology (density, large size, spheroidal shape) and molecular composition of the ectopic particles seen after Nv-bruno RNAi are similar to the corresponding features of the oosome, indicating that this structure is being ectopically produced in the nurse cells.

If the role of Nv-bruno is similar to that of its Drosophila ortholog, the production of these oosome-like structures in the nurse cells could be due to the ectopic translation of Nv-osk in the nurse cells in the absence of Nv-bruno. In support of this conclusion, the large particles are only produced in the most posterior nurse cells nearest to the oocyte, to which Nv-osk is restricted (Figure 3), while Nv-bruno is expressed in nurse cells located more anteriorly (Figure 8C). However, we cannot exclude that the restriction of large oosome-like particles to the posterior nurse cells is a result of higher levels of Nv-Bruno protein in these cells. In addition, in late Nv-bruno pRNAi egg chambers, Nv-Vas protein is associated with the dense accumulation of Nv-osk mRNA (Figure 7D), further indicating that oosome formation is being completed ectopically within the nurse cells. Conclusive evidence for a direct role of Nv-Bruno in repressing Nv-osk translation will come only with the availability of an antibody against Nv-Osk protein.

Another Drosophila RNA binding protein, Hrp48, is critical for both silencing of unlocalized osk mRNA translation, and for the proper initiation of its translation once the mRNA is localized to the posterior [57,58]. Nv-hrp48 is expressed strongly throughout the nurse cells in the wasp ovary (Figure S1D), and when its function is knocked down, ectopic oosome-like structures are not seen in the nurse cells (Figure 7E, 7F), in contrast to what is seen after Nv-bruno pRNAi. In most egg chambers, both Nv-osk and Nv-otd1 mRNAs are expressed normally in the nurse cells, and are transported to the oocyte (Figure 7E). Once in the oocyte, however, these mRNAs do not become localized normally. The extent of mislocalization varies from oocytes that show a looser localization of posterior mRNAs (Figure 7E) to those where Nv-osk and Nv-otd1 mRNAs fail to localize to a distinct cortical location, and are diffusely expressed throughout the smaller than usual oocytes (Figure 7F, arrow). In more weakly affected egg chambers, which have established normal polarity, the pattern of Nv-Vas accumulation appears to be only weakly affected, with the protein appearing at slightly lower levels, and loosely organized, likely reflecting a mild disruption in the proper assembly of the oosome during late oogenesis (Figure 7G, 7G').

Thus, Nv-hrp48 appears to have a conserved role in the assembly of the germ plasm in Nasonia, and by extension may have a conserved function in regulating the translation of Nv-osk. Our results indicate that the primary role of this factor is to promote oosome assembly (and thus, by analogy to Drosophila, Nv-osk function). However, we cannot completely exclude a second role, such as that seen in Drosophila, for Nv-hrp48 in Nasonia in repressing the translation of unlocalized Nv-osk in the oocyte [57,58].

osk is present in a close relative of Apis, and likely in a close relative of Tribolium.

Our results show that a regulatory network of protein interaction centered on Nv-Osk is required for the maternal
production of germ plasm, and that this network is highly similar to that found in *Drosophila*. This suggests that, given the basally branching phylogenetic position of the Hymenoptera among the Holometabola, this regulatory network arose in a common ancestor of all Holometabola, and that transitions to the zygotic induction mode of germ cell specification are associated with secondary disruptions of this network. To test this hypothesis, we sought to determine if *osk*, as the central component of this network, is conserved in other species that produce maternal germ plasm and pole cells.

Multiple ant species have been shown to specify their pole cells through the assembly of a posterior pole plasm that is incorporated...
into pole cells during early embryogenesis [30,31]. Consistent with our hypothesis, we successfully cloned an osk ortholog in the ant *Messor pergandei*, whose protein sequence shows 46.4% similarity to that of *Nv-Osk*. Moreover, *Messor osk* (*Mp-osk*) mRNA is localized to the posterior pole of the oocyte during oogenesis (Figure 8A), and embryogenesis (Figure 8B). This pattern of *Mp-osk* mRNA accumulation is similar to that of insects that specify germ cell through cytoplasmic inheritance (e.g., *Nasonia* and *Drosophila*), and suggests that its function in germ cell specification is conserved in ants. In addition, the localization of *Mp-osk* corresponds well to the previously observed localization of Vasa protein and *nanos* mRNA in the oocyte and embryo at equivalent stages in *Messor* and other closely related ant species [30,31]. *Messor* is a much closer relative of *Apis* than is *Nasonia* [59], and the discovery of osk in this ant species strongly indicates that the absence of osk in the bee genome is a derived state.

We also analyzed the molecular basis of maternal germ plasm formation in the beetle *Acanthoscelides obtectus*, which, like *Nasonia*, but unlike *Tribolium*, produces an oosome and pole cells [32]. Like *Tribolium* and many other beetle species, *Acanthoscelides* possesses teletrophic ovarioles. In this type of oogenesis, a common pool of nurse cells is located at the anterior of the ovariole, which is connected with progressively maturing oocytes toward the posterior by actin and microtubule-rich structures called trophic cords [60]. In early oogenesis, Vasa protein is highly enriched around the surface of the oocyte nucleuses (Figure 8C). The presence of Vasa protein is also detected in the nurse cells and trophic cords. In more mature oocytes, Vasa protein is strongly enriched at the posterior pole, where the oosome will be formed (Figure 8D). This indicates that, despite employing a mode of oogenesis quite divergent from that seen in *Nasonia* and *Drosophila*, this beetle possesses similar capabilities for directing the localization and assembly of the germ plasm components to the posterior pole.

This is in contrast to *Tribolium*, where Vasa protein is never found in a localized pattern in later oocytes (Figure 8F) despite its presence in the cytoplasm of early oocytes and in the trophic cords (Figure 8E), correlating well with the absence of pole cells and maternal germ plasm in this species. Based on the similarity of the pattern of Vasa protein accumulation in *Acanthoscelides* to the osk
dependent Vas localization patterns in Nasonia and Drosophila, we predict that an osk ortholog is present in the genome of Acanthoscelides, and that it functions in recruiting Vas protein to the posterior pole of the oocyte and in assembling the oosome similar to its orthologs in Nasonia and Drosophila. Attempts to clone osk from the beetle by degenerate PCR have so far failed, and transcriptome or genome sequencing may be required to resolve this question.

Discussion

The origin of germ plasm and pole cells in holometabolous insects

Taken together, our results reveal a new picture for the origin and evolution of oskar, maternally provisioned germ plasm, and pole cells. We propose that the origins of these features represent evolutionary novelties of the Holometabola in relation to the rest of the insects, and that the appearance of the latter two features is strongly correlated with the presence of osk (Figure 9). Our conclusions are based on: (1) the presence of osk orthologs in the genomes of Nasonia and Messor, two distantly related hymenopteran species that also both have maternal germ plasm and pole cells; (2) the molecular and developmental similarity of the germ plasm of Acanthoscelides to that of Drosophila and Nasonia, which is consistent with the presence of an osk ortholog in this beetle; (3) the conserved interactions of Nv-Osk with upstream regulators (such as Nv-Bruno and Nv-Hrp48) and downstream partners (such as Nv-Vas and Nv-Tud), which indicate that a protein interaction network centered on Osk for generating maternal germ plasm and pole cells was present at the latest in the most recent common ancestor of the Hymenoptera and Diptera (which, based on current phylogenies would also be the common ancestor of all Holometabola) (Figure 9); and finally (4) the absence of maternal germ plasm, pole cells and osk in hemimetabolous insects, suggesting that the absence of these features is ancestral for the insects (Figure 9), and that these features likely arose after the divergence of the Holometabola from its sister group the Paraneoptera (true bugs, lice, and thrips).

The mapping of our findings on the insect phylogeny also indicates that Apis, Tribolium, and Bombyx may have lost these characters through independent evolutionary events (Figure 9). In addition, the correlation of the loss of maternal germ plasm and pole cells with the absence of oskar in these species (Figure 9), indicate that osk is a key factor in the evolution of germline determination mechanisms in the Holometabola.

Since production of the germline is a critical event in development and evolution, it is surprising that dramatic changes in how this cell fate is established have occurred several times in insect evolution. Such transitions could have been facilitated if redundant mechanisms for generating the germline existed in the ancestors of lineages that eventually lost the ability to maternally specify the germline.

In Drosophila there appears to be no remaining inductive capability; if pole cells are not produced, or are destroyed before reaching the gonad, the resulting fly is sterile. However, this is not
Figure 9. Phylogenetic pattern of losses and gains of maternal germ plasm, pole cells, and oskar among the insects. Genus names in blue are those in which maternal germ plasm and pole cells have been described. Asterisks indicate a sequenced genome. Green boxes indicate confirmed presence of osk. Red boxes indicate apparent absence of osk in the genome. Orange arrow indicates the ancestral use of zygotic induction of germline fate among insects. Green circles and squares indicate the proposed evolutionary origin of osk and maternally synthesized germ plasm, while red circles and squares indicate the proposed loss of these features, respectively. Tree was drawn based on the phylogenetic relationships described in [41,59,75].

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the case in all insects. Destruction or removal of the ososome from the embryo of the wasp *Pimpla turionellae* resulted in the complete absence of pole cells, consistent with the role of the ososome in generating these cells. In spite of this, when embryos subject to these manipulations were examined later, a majority appeared to have germ cells populating the late embryonic gonads [61]. As *Pimpla* is a close relative of ants and bees, it is possible that both maternally provisioned germ plasm and the ability to zygotically induce germ line fate coexisted in an ancestor of *Apis*, and the loss of the former capability thus may not have had dire consequences for the fecundity of species within the lineage leading to *Apis*. Once the presence of pole cells and maternal germ plasm was no longer selected for, it may have been relatively easy to lose osk, as long as another strategy for either localizing posterior nanos, or another mechanism for patterning the posterior is present.

The question of why an insect would lose the capacity to produce pole cells is also difficult to address directly. The likelihood that maternal provisioning of germ line determinants evolved independently multiple times among animals [1,2] implies that this strategy for germ line determination has, at least under certain circumstances, selective benefits. Reciprocally, the multiple independent losses of this strategy indicate that, in other circumstances, zygotic induction may be favored. Broader sampling of germ line specification strategies among the animals could shed light on the possible ecological or embryological traits correlated with the retention of or transition away from maternal synthesis of germ line determinants and early segregation of the germ cell fate.

The origin of oskar

Our finding that Oskar was a critical innovation for the transition to the maternal inheritance mode of germ line determination in insects leads to the question of how such a novel protein could have been invented.

The strong similarity of the N-terminus of *Nv*-Osk to the N-terminus of *Tdrd-7* orthologs found throughout the Metazoa, indicates that the origin of *osk* involved the duplication and divergence of this locus in an ancestor of the Holometabola. However, unlike *tdrd-7* genes, *osk* orthologs lack Tudor domains toward the C-terminus, and rather have a domain with structural similarity to SGNH/GDSL class hydrolases. Since such a domain is not found in *Tdrd-7* orthologs, it may be that *osk* arose by a fusion of a *tdrd7* paralog, and a gene possessing a hydrolase domain.

While proteins of the SGNH/GDSL hydrolase family are found in all insect species, Osk orthologs show no significant homology to these sequences in BLAST analyses (E-value cutoff = 10). Rather, the highest scoring non-Oskar BLAST hits for the C-terminal portion (i.e., excluding the first 100 amino acids) of Osk proteins are often SGNH/GDSL hydrolases of Bacteria (*e.g.*, Mp-Osk finds ZP_05979902.1 from *Subdoligranulum variabile* at an E-value of 0.17, and Cp-Osk findsYP_001491067.1 from *Aerobacter butzleri* at an E-value of 0.006). These observations raise the possibility that *osk* could have arisen by the combination of horizontal gene transfer from bacteria and gene fusion events. The fact that horizontal gene transfer from endosymbiotic bacteria occurs in insects is now well established [38,62], and a source for a potential horizontal transfer could be the endosymbions that are tightly associated with the early germ cells and gonads of many insect species (*e.g.*, [63,64]).

While the most parsimonious explanation for the observed distribution of *osk* orthologs among the Holometabola is that there was a single origin for this gene in a common ancestor of the holometabolous clade, we cannot formally exclude the possibility that the similarity in structure and function between the hymenopteran and dipteran Osk sequences was the result of two lineage specific events of convergent evolution responding to independent instances of selective pressure to establish cytoplasmic inheritance of germ line components. However, it seems highly unlikely that the molecular events required to invent a novel gene such as *osk* would occur in almost identical ways twice in evolution before a different solution is found, let alone the unlikelihood of such a gene being fixed in a population, and then subsequently integrated into a novel regulatory network.

However, the invention of a novel factor required for cytoplasmic inheritance of germ plasm components may not be an occurrence unique to the Holometabola. In zebrafish, the *bucky ball* gene has an *osk*-like function in generating maternal germ plasm, but is molecularly unrelated to *osk*, and is only found in vertebrate genomes [11,65]. This indicates that there is nothing intrinsic in the primary structure of Osk protein that is required for maternal assembly of germ plasm, and that there are many possible solutions to the problem of generating this substance. Further sampling of metazoan germ line establishment strategies will give insight into how common the generation of novel genes is in the process of evolving maternally generated germ plasm.

The origin of a protein regulatory network for restricting germ plasm production to the posterior pole

The process of maternal germ plasm assembly must be precisely controlled, and abnormalities in this process result in deep and sometimes spectacular consequences for the embryonic anterior-posterior axis [8]. Based on our results with *Nv*-bruno and *Nv-hyp-R*, a common mechanism to spatially regulate *osk* localization and translation was likely already present at the origin of the Holometabola. This, along with the fact that factors such as Vas and Tud have conserved roles downstream of Osk in *Nasonia*, indicates that a complex protein interaction network for localized production of germ plasm during oogenesis existed in a common ancestor of the Holometabola. This raises the question as to when during evolution has this network been assembled, and through which molecular mechanisms.

Proteins downstream of Osk, such as Tud, Vas, and Nos, have conserved roles in the specification and function of germ cells throughout the Metazoa, including those without maternal specification of the germ line [11], and therefore are able to function without Osk to generate germ cell characteristics. Similarly, the proteins upstream of Osk, such as Bruno, Hrp48, and Staufen, are also highly conserved throughout the metazoa, and have conserved functions in mRNA localization and translational control in a variety of cellular contexts outside of the germ line. Thus, Osk seems to have been intercalated between two ancient pre-existing regulatory networks. The position of Osk as the nexus between these two networks allows its specific and precisely controlled function in specifying the germ line fate.

The fact that both the up- and downstream networks were already well established before the evolution of *osk* indicates that relatively few evolutionary steps may have been required to integrate Osk between them. In addition, since Osk is at least partially derived from a *tdrd7/7*5-like gene, orthologs of which have well described functions in the germ line in vertebrates and invertebrates, the ancestral Osk may have been predisposed to interact with other germ plasm components.

The localization of osk likely also had an evolutionary antecedent, as the presence of posteriorly localized patterning factors has been detected in some hemimetabolous species, *e.g.*, [64,66]. Since germ cells arise at the posterior pole just after gastrulation in some hemimetabolous species [16,67,68], it is
possible that factors that predispose posterior nuclei to take germline fate are also localized at the posterior pole in these species. The molecular nature of any such factor, and whether its role is direct or indirect, remains to be determined. Testing the function and regulation of orthologs of genes both up- and downstream of osk in hemimetabolous, and other holometabolous, insect species should give insights into the functioning of the ancestral germline regulatory network, and could provide further clues as to how osk could have been integrated into it.

Materials and Methods

A BLAST based strategy was used to identify potential osk orthologs in sequenced insect genomes (see Table S1). The following databases were searched: for *Bombyx mori*, Silkworm Genome Assembly at silkdb.org [69]; for *Triolium castaneum*, BeetleBase3_NCBI_DB at beetlebase.org [70]; for *Nasonia vitripennis*, Nasonia Scaffolds Assembly Nci1_1.0 at hymenopteragenome.org/nasonia/ [71]; For *Apis mellifera*, Scaffolds Assembly 2 at hymenopteragenome.org/beecase/; for *Acrlyosiphum pisum*, genome (reference only) at http://www.ncbi.nlm.nih.gov/projects/genome/seq/FlashGen/FlashGen.cgi?taxid = 7029; for *Rhodinus prolixus*, Harpoptathus saltator and Canponus fluvius, species-specific Whole-genome shotgun reads (wgs) databases were selected at blast.ncbi.nlm.nih.gov; for *Culex pipiens* selected at blast.ncbi.nlm.nih.gov; for Specific Whole-genome shotgun reads (wgs) databases were searched genome/seq/BlastGen/BlastGen.cgi?taxid = 7029; for *Drosophila melanogaster*, *S. bruno* and *D. persimilis* Assembly Cpiq1-Johannesburg Strain, Supercontigs at http://cequinque-fasciatus.vectorbase.org/Tools/BLAST/, and for *Pediculus humanus*, Assembly PhumU1, Supercontigs - USDA Strain at http://phumanus.vectorbase.org/Tools/BLAST/. These databases were queried using tblastn with default parameters (except the E-value cut off was raised to 10 where necessary) with the following Oskar sequences in the genomes of insects (see Table S1). The parameters employed can be found in the Materials and Methods section. Red boxes indicate a hit against a putative osk ortholog, blue boxes indicate hits against non-Oskar tejas/lotus domain containing genes. N/A indicates that no hits were obtained using and E-value cutoff of 10. Only hits with E-values less than one are shown, except where the best hit in the searched genome for a particular Osk ortholog is greater than one. The values in the first row of each genome searched are the E-values of the best hit, and any other hit with an E-value less than 1, returned by the corresponding Osk ortholog. In the second row of each searched genome field, the Genbank or genome database accession number of either a predicted gene corresponding to the genomic hit, or, if no gene is predicted, the genomic coordinates are shown. The best, and significant hits were then used as queries against the *Drosophila* genome, and the resulting CG identifiers are shown in the third row under each genome searched, and the E-values of the matches are shown on the fourth row. Since the *Nasonia* and *Messor* Osk sequences can detect the rapidly diverging osk sequence of *D. melanogaster*, we would expect that these sequences should be shown in the genomes of insects. As, *Bombyx*, and *Triolium*, were present, unless the evolution at the osk loci species were independently accelerated in each of their lineages beyond the rate seen in the fly. Due to the nature of whole genome shotgun sequencing, we cannot exclude that genomic regions including osk orthologs were coincidently missed in the genomes where no osk is found. In these cases the *Culex* sequences did not give significant results, and the results shown are from using the Osk ortholog from the closely related mosquito species *Aedes aegypti*. Using the *Aedes* sequence in other genomes did not give significantly different results. The genomic region surrounding the region showing homology to Osk was used as input into FgenesH using the *Apis* model at http://linux1.softberry.com to predict an Osk sequence, that was then used as a query against the fly genome. The genomic region surrounding the region showing homology to Osk was used as input into FgenesH+ using the *Apis* model and Nv-Osk protein sequence at http://linux1.softberry.com to predict an Osk sequence, which was then used as a query against the fly genome.

Supporting Information

Figure S1 Expression of components of the maternal germ plasm regulatory network in *Nasonia* ovarioles. A: *Nasonia* vasa expression. B: *Nasonia* tudor expression. C: *Nasonia* bruno expression. D: *Nasonia* hrp48 expression. Arrows in A indicate the higher levels of expression in the most anterior nurse cells. Scale bar represents 0.1 mm. All ovarioles are oriented with anterior up.

Table S1 Identification of Oskar orthologs in insect genomes. Potential osk orthologs were searched for in the genomes of insects using BLAST. Details of the sequences and databases used and the parameters employed can be found in the Materials and Methods section. Red boxes indicate a hit against a putative osk ortholog, blue boxes indicate hits against non-Oskar tejas/lotus domain containing genes. N/A indicates that no hits were obtained using and E-value cutoff of 10. Only hits with E-values less than one are shown, except where the best hit in the searched genome for a particular Osk ortholog is greater than one. The values in the first row of each genome searched are the E-values of the best hit, and any other hit with an E-value less than 1, returned by the corresponding Osk ortholog. In the second row of each searched genome field, the Genbank or genome database accession number of either a predicted gene corresponding to the genomic hit, or, if no gene is predicted, the genomic coordinates are shown. The best, and significant hits were then used as queries against the *Drosophila* genome, and the resulting CG identifiers are shown in the third row under each genome searched, and the E-values of the matches are shown on the fourth row. Since the *Nasonia* and *Messor* Osk sequences can detect the rapidly diverging osk sequence of *D. melanogaster*, we would expect that these sequences should be shown in the genomes of insects. As, *Bombyx*, and *Triolium*, were present, unless the evolution at the osk loci species were independently accelerated in each of their lineages beyond the rate seen in the fly. Due to the nature of whole genome shotgun sequencing, we cannot exclude that genomic regions including osk orthologs were coincidently missed in the genomes where no osk is found. In these cases the *Culex* sequences did not give significant results, and the results shown are from using the Osk ortholog from the closely related mosquito species *Aedes aegypti*. Using the *Aedes* sequence in other genomes did not give significantly different results. The genomic region surrounding the region showing homology to Osk was used as input into FgenesH using the *Apis* model at http://linux1.softberry.com to predict an Osk sequence, that was then used as a query against the fly genome. The genomic region surrounding the region showing homology to Osk was used as input into FgenesH+ using the *Apis* model and Nv-Osk protein sequence at http://linux1.softberry.com to predict an Osk sequence, which was then used as a query against the fly genome.
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Author Contributions

Conceived and designed the experiments: JAL OO AK. Performed the experiments: JAL OO AK. Analyzed the data: JAL OO AK EA CD SR. Contributed reagents/materials/analysis tools: JAL OO AK EA CD SR. Wrote the paper: JAL OO AK EA CD SR.

References

1. Extavour CG, Akam M (2003) Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development 130: 3809–3818.
2. Extavour CGM (2007) Evolution of the bilaterian germ line: lineage origin and modulation of specification mechanisms. Integrative and Comparative Biology 47: 770–785.
3. Schwall F (1985) Insect Morphogenesis. Sauer HW, ed. Basel. Karger.
4. Hegner RW (1914) Studies on germ cells. I. The history of the germ cells in insects with special reference to the Keimbaehn-determinants. II. The origin and significance of the Keimbaehn-determinants in animals. Journal of Morphology 25: 375–369.
5. Mahowald AP (2001) Assembly of the Drosophila germ plasm. International Review of Cytology - a Survey of Cell Biology Vol 203: 187–213.
6. Hegner RW (1911) Experiments with Chrysoamelid Beetles. III. The Effects of Killing Parts of the Eggs of Lepitostarea decemlinata. Biological Bulletin 20: 232–251.
7. Illmensee K, Mahowald AP (1974) Transplantation of Posterior Polar Plasm in Drosophila. Induction of Germ Cells at the Anterior Pole of the Egg. Proceedings of the National Academy of Sciences of the United States of America 71: 1016–1020.
8. Ephrussi A, Lehmann R (1992) Induction of Germ-Cell Formation by Oskar. Nature 358: 387–392.
9. Rongo C, Lehmann R (1996) Regulated synthesis, transport and assembly of the Drosophila germ plasm. Trends in Genetics 12: 102–109.
10. Thomson T, Lasko P (2005) Tudor and its domains: germ cell formation from a Tudor perspective. Cell Research 15: 281–291.
11. Even-Campen B, Schwager EE, Extavour CGM (2010) The Molecular Machinery of Germ Line Specification. Molecular Reproduction and Development 77: 3–18.
12. Raz E (2000) The function and regulation of vasa-like genes in germ-cell development. Genome Biol 1: REVIEWS1017.
13. Klag J (1977) Differentiation of primordial germ cells in the embryonic development of Thermobia domestica, Pack. (Thysanura): an ultrastructural study. Journal of Embryology and Experimental Morphology 38.
14. Mito T, Nakamura T, Sarashina I, Chang CC, Ogawa S, et al. (2008) Dynamic expression patterns of vasa during embryogenesis in the cricket Gryllus bimaculatus. Developmental Genes and Evolution 218: 381–387.
15. Chang CC, Dearden P, Akam M (2002) Germ line development in the grasshopper Schistocerca gregaria: vasa as a marker. Developmental Biology 252: 100–110.
16. Mellany H (1935) Memoirs: The Early Embryonic Development of Rhodinus prolixus (Hemiptera, Heteroptera). Quarterly Journal of Microscopical Science 2: 71–90.
17. Baur T, Braendle C, Shingleton A, Sink G, Kambhampati S, et al. (2003) A comparison of parthenogenetic and sexual embryogenesis of the pea aphid Acyrthosiphon pisum (Hemiptera : Aphididae). Journal of Experimental Zoology Part B-Molecular and Developmental Evolution 295B: 59–81.
18. The International Aphid Genomics Consortium (2010) Genome Sequence of the squash bug, Anasa tristis. Nature 467: 100–118.
19. Miura T, Braendle C, Shingleton A, Sisk G, Kambhampati S, et al. (2003) A comprehensive survey of developmental genes in the pea aphid, Acyrthosiphon pisum: frequent lineage-specific duplications and losses of developmental genes. Insect Molecular Biology 12: 47–62.
20. Richards S, Gibbas RA, Weinstock GM, Brown SJ, Denell R, et al. (2008) The genome of the model beetle and pest Tribolium castaneum. Nature 452: 949–953.
21. Weinstock GM, Robinson GE, Gibbas RA, Wetter KC, Evans JD, et al. (2006) Insights into social insects from the genome of the honeybee Apis mellifera. Nature 443: 931–945.
22. Nagy L, Riddiford L, Kiguchi K (1994) Morphogenesis in the Early Embryo of the Lepidopteran Bombyx-Mori. Developmental Biology 165: 137–151.
23. Schroder R (2006) vasa mRNA accumulates at the posterior pole during blastoderm formation in the flour beetle Tribolium castaneum. Development Genes and Evolution 216: 277–283.
24. Nelson JA (1915) The Embryology of the Honey Bee. Princeton: Princeton University Press. pp 282.
25. Dearden PK, Wilson MJ, Sablan L, Osborne PW, Havler M, et al. (2006) Patterns of conservation and change in hox-like developmental genes. Genome Research 16: 1356–1364.
26. Dearden PK, Wilson MJ, Sablan L, Osborne PW, Havler M, et al. (2006) Comprehensive survey of developmental genes in the pea aphid, Acyrthosiphon pisum: frequent lineage-specific duplications and losses of developmental genes. Insect Molecular Biology 19: 47–62.
27. Shigenobu S, Bickel RD, Brisson JA, Butts T, Chang CC, et al. (2010) Expression pattern of vasa-like vumLVG protein and its implications in germ cell development. Developmental Genes and Evolution 216: 94–99.
28. Bull AL (1982) Stages of Living Embryos in the Jewel Wasp Mormoniella-Nasonia/Vitripennis-Walker) (Hymenoptera, Pteromalidae). International Journal of Insect Morphology & Embryology 8: 27–47.
29. Nakao H, Hatayama M, Lee JM, Shimoda M, Kanda T (2006) Expression pattern of vasa-like vumLVG protein and its implications in germ cell development. Developmental Genes and Evolution 216: 94–99.
30. Miura T, Braendle C, Shingleton A, Sisk G, Kambhampati S, et al. (2003) A comprehensive survey of developmental genes in the pea aphid, Acyrthosiphon pisum: frequent lineage-specific duplications and losses of developmental genes. Insect Molecular Biology 12: 47–62.
55. Hay B, Jan LY, Jan YN (1990) Localization of Vasa, a Component of Drosophila Polar Granules, in Maternal-Effect Mutants That Alter Embryonic Anteroposterior Polarity. Development 109: 425–433.

56. Kim-Ha J, Kerr K, Macdonald PM (1995) Translational Regulation of Oskar Messenger-Rna by Bruno, an Ovarian Rna-Binding Protein, Is Essential. Cell 81: 403–412.

57. Huynht JR, Munro TP, Smith-Lièvre K, Lepesant JA, Johnston DS (2004) The Drosophila hnrNPA/B homolog, Hrp48, is specifically required for a distinct step in osk mRNA localization. Developmental Cell 6: 625–635.

58. Yano T, Lopez de Quinto S, Matsui Y, Shevchenko A, Shevchenko A, et al. (2004) Hrp48, a Drosophila hnrNPA/B homolog, binds and regulates translation of oskar mRNA. Developmental Cell 6: 637–648.

59. Dowton M, Austin AD (1994) Molecular Phylogeny of the Insect Order Hymenoptera - Apocritan Relationships. Proceedings of the National Academy of Sciences of the United States of America 91: 9911–9913.

60. Bünning J (1994) The insect ovary. London: Chapman & Hall.

61. Achtelig M, Krause G (1971) Experiments on Uncleared Egg of Pimpla-Turionellae L. (Hymenoptera) for Functional Analysis of Oosome Region. Wilhelm Roux Archiv Fur Entwicklungsmechanik Der Organismen 167: 164–&.

62. Hotopp JCD, Clark ME, Oliveira DCSG, Foster JM, Fischer P, et al. (2007) Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. Science 317: 1753–1756.

63. Koch A (1931) Die Symbiose von Oryzaephilus surinamensis L. (Cucujidae, Coleoptera). Zoomorphology 23: 389–424.

64. Sander K (1969) Specification of the basic body pattern in insect embryogenesis. Advances in Insect Physiology, JE Treherne, MJ Berridge, VBWiglesworth, eds. Academic Press 12: 125–235.

65. Bontems F, Stein A, Marlow F, Lyautet J, Gupta T, et al. (2009) Bucky Ball Organizes Germ Plasm Assembly in Zebrafish. Current Biology 19: 414–422.

66. Lall S, Ludwig MZ, Patel NH (2003) Nanos plays a conserved role in axial patterning outside of the Diptera. Curr Biol 13: 224–229.

67. Heming BS (1979) Origin and Fate of Germ-Cells in Male and Female Embryos of Haplotriplris-Verbaaci (Osborn) (Insecta, Thysanoptera, Philorotheidae). Journal of Morphology 160: 323–&.

68. Johannsen OA, Butt FH (1941) Embryology of Insects and Myriapods. New York: McGrav-Hill.

69. Duan J, Li RQ, Cheng DJ, Fan W, Zha XF, et al. (2010) SilkDB v2.0: a platform for silkworm (Bombyx mori) genome biology. Nucleic Acids Research 38: D453–D456.

70. Kim HS, Murphy T, Xia J, Caragea D, Park Y, et al. (2010) BettleBase in 2010: revisions to provide comprehensive genomic information for Tribolium castaneum. Nucleic Acids Research 38: D437–D442.

71. Munoz-Torres M, Reese J, CP C, AK B, JP S, et al. (2010) Hymenoptera Genome Database: integrated community resources for insect species of the order Hymenoptera. Nucleic Acids Research.

72. Lynch JA, Peel AD, Drechsler A, Averof M, Roth S (2010) EGF Signaling and the Origin of Axial Polarity among the Insects. Current Biology 20: 1042–1047.

73. Hanys-Nakamura K, Kobayashi S, Nakamura A (2004) Germ cell-autonomous Wunen2 is required for germline development in Drosophila embryos. Development 131: 4545–4553.

74. Khila A, Abouheif E (2009) In situ hybridization on ant ovaries and embryos. Cold Spring Harb Protoc. 2009: pdb prot5250.

75. Wheeler WC, Whiting M, Wheeler QD, Carpenter JM (2001) The phylogeny of the extant hexapod orders. Cladistics 17: 113–169.