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Evolution of Developmental Control Mechanisms

Evolution of *nubbin* function in hemimetabolous and holometabolous insect appendages

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ABSTRACT

Insects display a whole spectrum of morphological diversity, which is especially noticeable in the organization of their appendages. A recent study in a hemipteran, *Oncopeltus fasciatus* (milkweed bug), showed that *nubbin (nub)* affects antenna morphogenesis, labial patterning, the length of the femoral segment in legs, and the formation of a limbless abdomen. To further determine the role of this gene in the evolution of insect morphology, we analyzed its functions in two additional hemimetabolous species, *Acheta domesticus* (house cricket) and *Periplaneta americana* (cockroach), and re-examined its role in *Drosophila melanogaster* (fruit fly). While both *Acheta* and *Periplaneta nub*-RNAi first nymphs develop crooked antennae, no visible changes are observed in the morphologies of their mouthparts and abdomen. Instead, the main effect is seen in legs. The joint between the tibia and first tarsomere (Ta-1) is lost in *Acheta*, which in turn, causes a fusion of these two segments and creates a chimeric *nub*-RNAi tibia–tarsus that retains a tibial identity in its proximal half and acquires a Ta-1 identity in its distal half. Similarly, our re-analysis of *nub* function in *Drosophila* reveals that legs lack all true joints and the fly tibia also exhibits a fused tibia and tarsus. Finally, we observe a similar phenotype in *Periplaneta* except that it encompasses different joints (coxa–trochanter and femur–tibia), and in this species we also show that *nub* expression in the legs is regulated by Notch signaling, as had previously been reported in flies and spiders. Overall, we propose that *nub* acts downstream of Notch on the distal part of insect leg segments to promote their development and growth, which in turn is required for joint formation. Our data represent the first functional evidence defining a role for *nub* in leg segmentation and highlight the varying degrees of its involvement in this process across insects.

Introduction

Insect appendages are immensely diverse and serve as a rich source for studying morphological evolution. In general terms, serially homologous appendages that originate from different segments exhibit the most obvious differences. For example, appendages such as fore and hind wings or mouthparts such as mandibles, maxillae, and labium are all characterized by distinct phenotypes. Based on a large body of evidence, from classic experiments in *Drosophila* to more recent studies in *Tribolium*, *Bombyx*, *Oncopeltus*, *Gryllus,* and *Acheta,* it is now well documented that these serial differences are mainly regulated by Hox genes (Abzhanov and Kaufman, 2000; Brown et al., 2000; Chesebro et al., 2009; Lewis, 1978; Lewis et al., 2000; Mahfooz et al., 2007; Masumoto et al., 2009; Rogers et al., 2002; Tomoyasu et al., 2005; Zhang et al., 2005). However, while much is understood regarding the genetic mechanisms that govern individual appendage identity, much less is known about the molecular basis of variation that exists within each appendage type. Thus, the genetic origins of many species-specific morphologies, such as the pigmentation, shape, and size of a fore wing or a fore leg remain largely unknown. A number of recent studies have provided support to the idea that developmental variation may be the leading cause of the large amount of phenotypic diversity observed between the appendages of even closely related species (Averof and Patel, 1997; Gompel et al., 2005; Mahfooz et al., 2007; Rogers et al., 2002; Tomoyasu et al., 2009; Wittkopp et al., 2002). In order to better understand this putative relationship between developmental and phenotypic variation, we chose to further investigate the functional role of the POU homeodomain gene *nubbin* (*nub*) in *Acheta* and *Periplaneta*, two basal hemimetabolous insect lineages.

Previous work has revealed that *nub* is an important developmental gene whose expression has been shown to be highly dynamic and variable throughout the appendages in several arthropod lineages (Abzhanov and Kaufman, 2000; Damen et al., 2002; Li and Popadić, 2004; Prpic and Damen, 2009). In *Drosophila melanogaster, nub* is expressed in the central nervous system (CNS), wing pouch and hinge (Billin et al., 1991; Cifuentes and Garcia-Bellido, 1997; Ng et al., 1995), and in regions along the developing leg near the future position of

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joints (Mirth and Akam, 2002; Rauskolb and Irvine, 1999). In another holometabolous species, *Bombyx mori* (silkworm), *nub* expression encompasses the entire wing disc (Kango-Singh et al., 2001). Recently, *nub* expression was evaluated in two basal insect lineages, *Thermobia* (firebrat) and *Periplanata* (cockroach), and in a moderately derived hemimetabolous species, *Oncopeltus* (milkweed bug) (Li and Popadić, 2004). In all three insects, *nub* displays a distinct, species-specific pattern in the head appendages and legs. In addition, a novel domain was observed in a posterior region of *Oncopeltus* embryos that corresponds to part of the future abdomen (Hrycaj et al., 2008). These results highlight a wide range of diversity in *nub* expression in insects and suggest that its level of functional divergence may be equally high.

To date, the function of *nub* in insects has only been described in *Drosophila* and *Oncopeltus*. In flies, *nub* is recognized for its role in growth and proximal–distal patterning of wings (Cifuentes and Garcia-Bellido, 1997; Neumann and Cohen, 1998; Ng et al., 1995), and maturation of neuroblast cells (Bhat and Schedl, 1994). Aside from the brief reference to causing "shortened and gnarled" legs in *nub* hypomorphic mutants, *nub* function in fly leg development is undocumented (Cifuentes and Garcia-Bellido, 1997). In *Oncopeltus*, however, *nub* is necessary for proper development of the antennae and labium in the head and for the growth of the femoral segment in all three pairs of legs (Hrycaj et al., 2008). It also has a novel function in the abdomen where it represses limb formation by controlling the Hox gene *abd-A*. To determine whether, and to what degree, these roles are conserved in other insects, we performed a detailed analysis of *nub* expression and function in *Acheta*. In addition, we extended our previous expression studies in *Periplaneta* (Li and Popadić, 2004) by performing functional analysis and reassessed the role of *nub* in *Drosophila* leg development. Our study shows that *Acheta nub* mRNA accumulates in the head appendages, legs, and abdomen in a pattern that is different from *Oncopeltus* and highlights the presumptive leg joints. Subsequent maternal RNA interference (RNAi) experiments show that while *nub*-depleted first nymphs exhibit crooked antennae, their gnathal appendages (mandibles, maxillae, and labium) are unaffected. Interestingly, *nub* does not have any discernable role in the abdomen, despite its expression in this region. The most prominent feature of *nub* RNAi phenotype is observed in the thorax, where all three pairs of legs are severely undersized due to reduced trochanter (Tr) and femur (Fe), and to fusion between the tibia (Ti) and first tarsomere (Ta-1). *Periplaneta nub* expression also accumulates in legs near the presumptive joints and *nub*-depleted cockroaches exhibit an *Acheta*-like phenotype of fused segments (Cx/Tr and Fe/Ti). Our analysis of *Drosophila nub* null mutants reveals fusions between leg segments, as well as the loss of claws and all joints, except some between tarsal sub-segments. To our knowledge, these results constitute the first functional evidence of the involvement of *nub* in insect leg segment development and demonstrate the varying degrees of plasticity in its contribution to joint formation.

Materials and methods

Insect cultures

A. domesticus were raised at room temperature on a diet of fresh lettuce leaves supplemented by dry cat food and water. The laid eggs were collected on a daily basis and incubated at 30 °C in a moist environment for all experiments.

P. americana were originally purchased from Carolina Biological Supply Company (Burlington, USA) and were maintained in the laboratory under conditions previously described in Hrycaj et al. (2010). The laid oothecae (egg cases) were handled in the same way as *Acheta* eggs.

Drosophila nub null individuals used in this study were generated and described by Hrycaj et al. (2008). Briefly, nub^{E37}/*CyO-ftzlacZ* flies were crossed to either *Df(2L)GR4/CyO* or *Df(2L)prd1.7/CyO* (Bloomington Stock Centre). The *nub* mutant class was identified as pharate adults by virtue of its number and wing phenotype. The *odd-lacZ* line was donated by T. Kline (Dusseldorf, Germany) and both live pharates and freshly emerged imagos were dissected and stained to reveal ß-gal activity as described in Couso et al. (1994). The *pdm2* mutants used were $pdm2^{\{XP\}d09994}$ (Bloomington Stock Centre).

Generation of Acheta nub cDNA

The total RNA isolation and synthesis of cDNA, RT-PCR, and cloning were carried out according to the previously described protocols (Li and Popadić, 2004). Briefly, degenerate primers targeting the highly conserved amino acid motifs EQFAKT (5′-GGAATTCGARCARTT YGCIAARAC-3′) and KEKRINP (5′-GCTCTAGAGGRTTIATICKYTTY-CYTT-3′) were used to generate a 387 bp long PCR fragment of *Acheta nub* that was then cloned into a pCR4-TOPO vector and verified by sequencing (GenBank sequence accession number HQ543076). The nucleotide sequences from ten clones were compared with each other and to other previously described *nub* orthologs. No evidence of paralogous copies was found.

In situ hybridization and immunostaining

The synthesis of digoxigenin-labeled antisense *nubbin* RNA probes and in situ hybridization procedure were performed as described in Li and Popadić (2004). *Ubx* expression was detected using the mouse monoclonal antibody FP 6.87 (1:8; donated by R. White) according to the protocol by Mahfooz et al. (2004). Zeiss Axiophot and Leica TCS SP2 laser confocal scanning microscopes were used to take images of in situ hybridization- and antibody-stained embryos, respectively.

For *Drosophila* antibody staining, pupae were collected and staged from the *odd-lacZ* stock, dissected, and stained as described in Galindo et al. (2005). The antibodies used were: anti-Nub (1:10; donated by S. Cohen) and anti-ßgal (1:1000; Sigma, St. Louis, MO, USA, catalog #94644).

RNA interference (RNAi)

To analyze *nub* function, we injected adult *Acheta* females with *nub* double-stranded RNA (dsRNA) of two different lengths according to the maternal RNAi methodology described in Mahfooz et al. (2007). Both *nub* dsRNA transcripts generated essentially the same RNAi phenotypes. Specifically, 6 µl of *nub* dsRNA at a concentration of 2.5 µg/µl was injected into the abdomens of female crickets using a Hamilton syringe with a 32-gauge needle. Following injections, they were placed in separate containers and reared with wild type males. The eggs were collected daily and incubated at 30 °C. Some of them were left undisturbed, while those intended for in situ and antibody staining were dissected at various stages of development. Both the embryos that died before completing embryogenesis (approximately 95% development) and those that emerged into first nymphs were scored for *nub* RNAi phenotypes. We examined a total of 1061 embryos and first nymphs and placed them into two different classes depending on the phenotypic severity (class I — strong and class II — moderate). For double RNAi experiments, *Acheta* females were injected with equimolar amounts of *nub* and *Ubx* dsRNA that was previously generated by Mahfooz et al. (2007). To control for nonspecific side effects of RNAi, we analyzed the progeny of crickets injected with a 375 bp fragment of GFP dsRNA. All GFP-treated embryos and first nymphs were indistinguishable from wild type controls.

To generate RNAi phenotypes in *Periplaneta*, nine fertilized adult females were injected with *nub* dsRNA according to the methodology