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Co-option of an anteroposterior head axis patterning system for proximodistal patterning of appendages in early bilaterian evolution

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ABSTRACT

The enormous diversity of extant animal forms is a testament to the power of evolution, and much of this diversity has been achieved through the emergence of novel morphological traits. The origin of novel morphological traits is an extremely important issue in biology, and a frequent source of this novelty is co-option of pre-existing genetic systems for new purposes (Carroll et al., 2008). Appendages, such as limbs, fins and antennae, are structures common to many animal body plans which must have arisen at least once, and probably multiple times, in lineages which lacked appendages. We provide evidence that appendage proximodistal patterning genes are expressed in similar registers in the anterior embryonic neurectoderm of *Drosophila melanogaster* and *Saccoglossus kowalevskii* (a hemichordate). These results, in concert with existing expression data from a variety of other animals suggest that a pre-existing genetic system for anteroposterior head patterning was co-opted for patterning of the proximodistal axis of appendages of bilaterian animals.

Introduction

Since the advent of molecular biology, many morphological traits that are shared between disparate animal clades have been found to be controlled by conserved underlying genetic systems (McGinnis and Krumlauf, 1992; Holley et al., 1995; Bier, 1997; Silver and Rebay, 2005; Olson, 2006). Morphological novelty, on the other hand, involves the evolution of new traits that are often patterned by co-opted genes or genetic systems that originally performed other developmental functions. For example, eye spots on butterfly wings are patterned through the redeployment, in small foci corresponding to the eye spots, of genes that also control the growth and patterning of the entire insect wing (Keys et al., 1999; Carroll et al., 2008). Another example is seen in the redeployment of a few Hox genes to pattern the paired appendages of vertebrates; these genes having been co-opted from an ancestral role in patterning posterior structures on the main body axis of chordates (McGinnis and Krumlauf, 1992; Zakany and Duboule, 2007). In this study we wished to explore the origins of the proximodistal appendage patterning system.

The patterning of *Drosophila* appendages is a well studied system of proximodistal axis specification. Although numerous genes participate in patterning of *Drosophila* appendages, there is a core group of genes which is responsible for establishing the gross morphological divisions. The gene pair *buttonhead* (*btd*):*D-Sp1* [despite its name, *D-Sp1* gene is not an ortholog of vertebrate *Sp1*, being a member of the Sp8 family (Beermann et al., 2004), and it will hereafter be referred to as *D-Sp8*], and the genes *Distal-less* (*Dll*), *dachshund* (*dac*), and *homothorax* (*hth*), are expressed in and regulate the growth and boundaries of the distal, medial, and proximal appendage domains (Kojima, 2004). All of these genes encode DNA binding transcription factors, and we refer to them as the core proximodistal appendage patterning system.

In *Drosophila* embryos, *hth* and *btd* are both expressed at very early stages in the appendage primordia. *btd* and *D-Sp8* have overlapping functions in activating *Dll* transcription in embryonic thoracic appendage primordia (Estella et al., 2003). As the domains of *Dll* expressing cells expand, *hth* becomes excluded from a subset of these cells in response to repression by *Dll* (Bolinger and Boekhoff-Falk, 2005). Cells from these primordia go on to form the larval Keilin's organs and leg imaginal discs.

Early in leg imaginal disc development, as in the embryonic appendage primordia, cells are divided into two major domains by a central cluster of *Dll* expressing cells surrounded by *hth* expressing cells (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). As development progresses, *dac* expression comes on in a medial region of leg discs (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999), as well as in antennal discs (Dong et al., 2001). The expression domains of *Dll*, *dac*, and *hth* overlap at later stages of appendage disc development, although the genes also exhibit mutually repressive interactions in some cells of the leg discs (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999; Dong et al., 2001). In

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imaginal discs, the *btd* gene is expressed in a medial domain and is capable of activating *Dll*, *dac*, and *hth* when ectopically expressed (Estella et al., 2003). The developing *Drosophila* antenna has a slightly different imaginal disc expression profile than that of the leg, with the medial *dac* domain being smaller and *hth* expression overlapping that of both *dac* and *Dll* (Dong et al., 2001). However, expression domains of these genes still roughly correspond to the same proximodistal fates in both developing leg and antennae (Dong et al., 2001), and similarly ordered and overlapping expression domains of the core genes are conserved in many developing arthropod appendages (Angelini and Kaufman, 2005; Beermann et al., 2004; Schaeper et al., 2009).

Investigation of genes underlying proximodistal development of vertebrate appendages has revealed that, despite structural dissimilarity to arthropod appendages, they develop under the control of a genetic patterning system that includes orthologs of *Drosophila btd*:*D-Sp8*, *Dll*, *dac*, and *hth* genes (Pueyo and Couso, 2005). Vertebrate *Sp8* genes are expressed in evolutionarily conserved patterns in distal ectoderm of limb buds, and knockdown of *Sp8* function in chick results in defects of limb outgrowth and patterning (Kawakami et al., 2004). *Dlx* family genes (*Dlx1*, *2*, *5* and *6*; *Dll* orthologs) are also expressed in distal ectoderm of mouse limb buds, and *Dlx5*:*Dlx6* double mutants have distal limb defects (Panganiban and Rubenstein, 2002; Kraus and Lufkin, 2006). *Dach1* (a *dac* ortholog) is expressed in a complex pattern in developing mouse limb buds, with a transient stage when expression is limited to anterior–medial limb bud cells (Hammond et al., 1998; Davis et al., 1999). *Meis1* (a vertebrate *hth* ortholog) is expressed in the proximal regions of vertebrate limb buds, and required for the normal development of the proximal domain of chick appendages (Mercader et al., 1999).

Available fossil data from the Pre-Cambrian does not allow us to be sure of the body plan of the last common ancestor of vertebrates and arthropods (Valentine, 2004). However, a synthesis of comparative morphology suggests that it either existed with rudimentary appendages or lacked them entirely (Shubin et al., 1997). The appendages of disparate extant bilaterian groups almost certainly evolved independently in multiple lineages subsequent to their divergence from a common ancestor which lacked appendages (Shubin et al., 1997). If animal appendages are not derived from a common ancestral appendage, the involvement of a common genetic system in proximodistal patterning could be due to random convergence of the same set of genes to pattern non-homologous appendages, or independent co-option of the same genetic system that functioned to pattern an ancestral structure shared by both vertebrates and arthropods (Panganiban et al., 1997; Davidson and Erwin, 2006; Tabin et al., 1999). Involvement of a genetic system in essential developmental roles (e.g. insect wing patterning) may make the regulatory interactions within the system resistant to change (Davidson and Erwin, 2006). This does not, however, preclude redeployment of such a patterning system using different genetic inputs and outputs, which could then contribute to novel morphological structures (Davidson and Erwin, 2006), such as butterfly wing eye spots (Keys et al., 1999).

It has been previously proposed that lateral appendages might have originated through the co-option of a pre-existing group of genes, including *Dll*, which controlled a rudimentary appendage-like outgrowth in the ancestor of vertebrates and arthropods (Tabin et al., 1999). It has also been proposed that the appendages of vertebrates and arthropods might be modified duplicates of the entire anteroposterior body axis (Minelli, 2000). This proposal is based in part on an ancestral role of Hox genes in patterning the main body axis, and

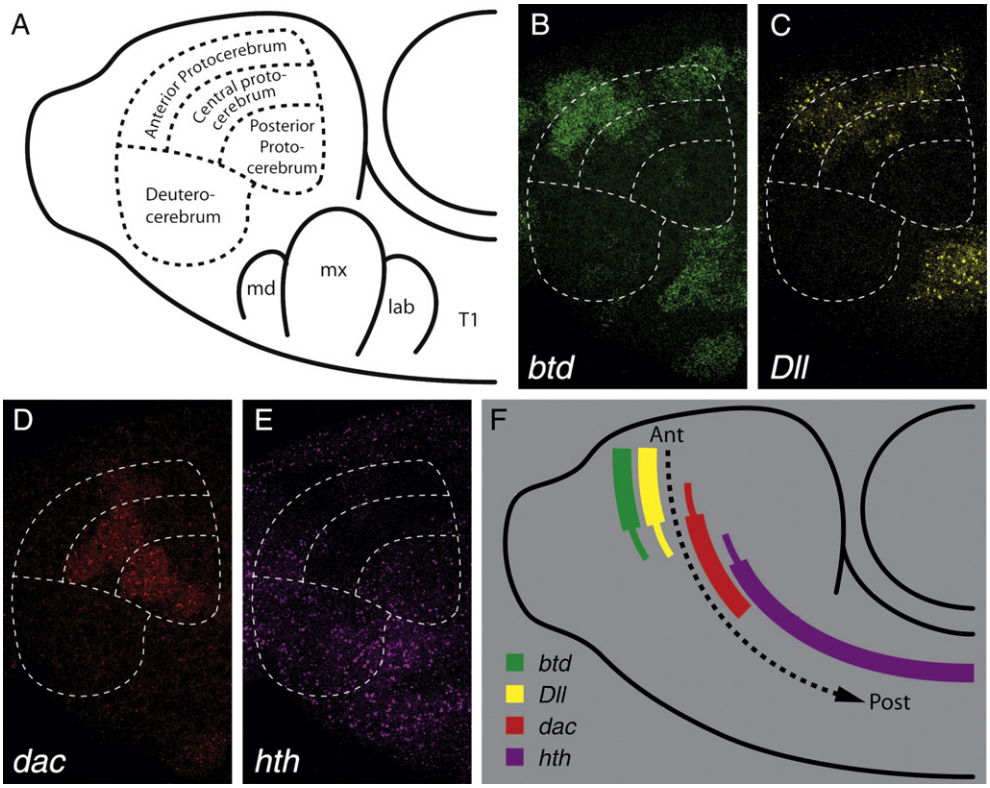

Fig. 1. Expression of core appendage patterning genes in the head neurectoderm of *Drosophila* embryos. Data are presented as maximum projections of confocal sections through embryonic procephalic neurectoderm of a stage 11 *Drosophila* embryo. (A) Schematic of the procephalic region of a stage 11 *Drosophila* embryo displaying subdivisions of the neurectoderm. Indicated are anterior, central, and posterior protocerebral and deuterocerebral regions of the head neurectoderm (adapted from (Younossi-Hartenstein et al., 1996)), as well as the mandibular (md), maxillary (mx), labial (lab), and first thoracic (T1) segments. (B) *btd* is transcribed mainly in the anterior protocerebral neurectoderm with a small region of expression in central regions. Also seen are expression domains outside the neurectoderm in antennal and maxillary segments. The *D-Sp8* transcription pattern is the same as *btd* in this region (data not shown). (C) *Dll* is transcribed in nearly the same neurectodermal pattern as *btd* at this stage. (D) *dac* is transcribed mainly in central and posterior protocerebral neurectodermal cells and overlaps with the posterior expression of *Dll* and *btd*. (E) *hth* is expressed in posterior protocerebral cells and deuterocerebral cells, overlapping with *dac* expression in its posterior expression domain. (F) Diagram indicating relative expression domains of *Dll*, *btd*, *dac*, and *hth* in the procephalic neurectoderm. Dashed arrow indicates general anterior to posterior orientation of the head neurectoderm.

the involvement of a subset of Hox genes in patterning the proximal–distal axis of vertebrate appendages (Zakany and Duboule, 2007). Hox genes do not have similar expression patterns in vertebrate and arthropod appendages, so for this and other reasons the model that the entire anteroposterior body axis patterning system is redeployed in most animal appendages (Minelli, 2000) is not well supported in our opinion. Our proposition relates an ancient conserved genetic system for patterning the anterior neurectoderm of animals to the proximodistal patterning of bilateral animal appendages.

A survey of previous research provides data from a few different animal groups on expression patterns of the core proximodistal appendage patterning genes in various tissues. We noticed that these genes, as well as other genes that are part of the proximodistal appendage patterning system in *Drosophila*, such as *aristaless* (*al*), *apterous* (*ap*), and *BarH1*, are expressed in discrete domains in the anterior embryonic neurectoderm of many chordates and arthropods (Supplementary material). We considered the hypothesis that a shared anteroposterior expression regimen of these genes in head neurectoderm might be common in bilateral animals. We wished to evaluate this hypothesis by testing the relative expression patterns of core appendage patterning genes in the anterior neurectoderm of *Drosophila* embryos, as well as in embryos of *Saccoglossus*, a basal deuterostome that lacks bilateral appendages.

Results and discussion

Genes of the core proximodistal appendage patterning system are expressed in a spatially and temporally complex manner during *Drosophila* development. However, in the anterior neurectoderm of *Drosophila* embryos, these genes are expressed in a clear anteroposterior order. We determined the relative expression patterns of *Dll*, *dac*, *hth*, and *btd*:*D-Sp8* using combinatorial in situ hybridizations, and analyzed their relative expression patterns in germband extended (stage 11) embryos (Figs. 1B–E). At this stage the procephalic neurectoderm can be divided into anterior, central, and posterior protocerebral areas, and a more posterior deuterocerebral area, with the most anterior cells being those flanking the dorsal midline of the procephalon and posterior cells located more ventrolaterally (Younossi-Hartenstein et al., 1996) (Fig. 1A). *btd*:*D-Sp8* and *Dll* are transcribed in overlapping patches covering most of the anterior protocerebral neurectoderm (Figs. 1B–C). The domain of *dac* transcription is mainly in central and posterior protocerebral neurectoderm, with small regions of overlap with *Dll* and *btd*:*D-Sp8* (Fig. 1D). *hth* transcripts are largely absent in anterior neurectoderm (Fig. 1E), and are completely excluded from domains which transcribe *Dll* and *btd*:*D-Sp8* (compare to Fig. 1C). The posterior protocerebral region contains cells which transcribe both *hth* and *dac*, but the majority of *hth* transcription is found in the deuterocerebrum and more posterior neurectoderm (compare Figs. 1D–E). Taken together, these data reveal an expression order of *btd*:*D-Sp8* and *Dll* in the most anterior neurectodermal cells, *dac* in medial cells, and *hth* in posterior cells, with small zones of overlap at the borders of the three major domains (Fig. 1F).

Using in situ hybridization, we also tested the expression patterns of the orthologous genes in *Saccoglossus* embryos ranging from gastrula to early gill slit stages. In post gastrulae, *Sp8* is transcribed at

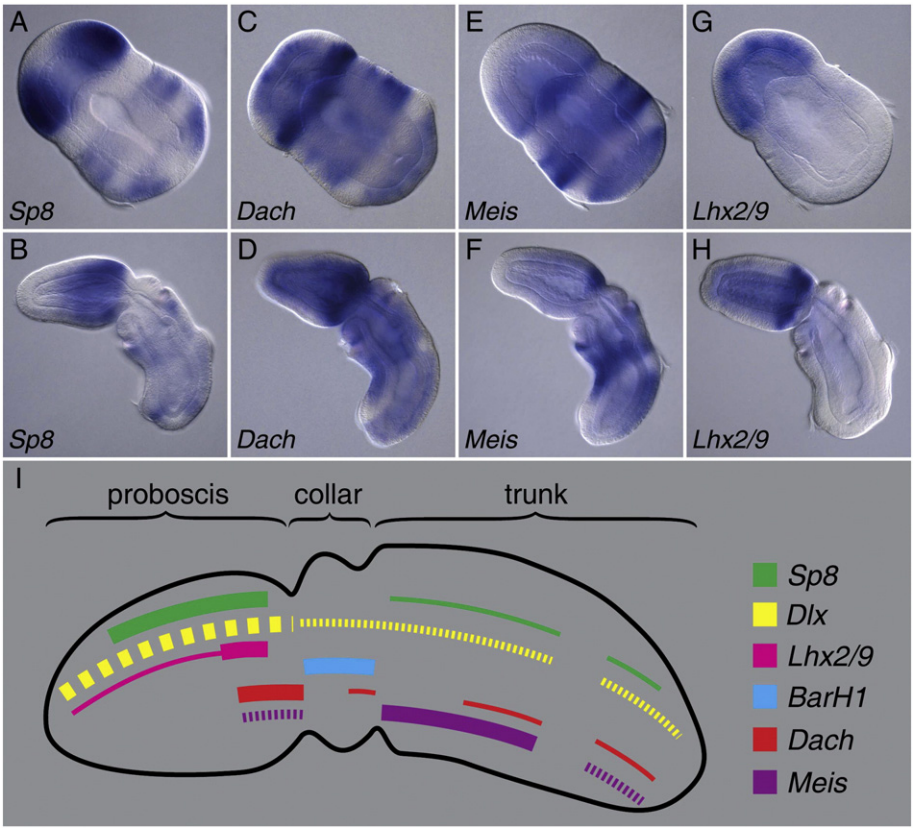

Fig. 2. Expression of core appendage patterning genes in *Saccoglossus* embryos. Data are presented as saggital optical sections of in situs with anterior to the upper right of each panel of post gastrula (A, C, E, and G) and one gill slit stage embryos (B, D, F, and H). (A, B) High levels of *Sp8* transcripts could be detected in two broad lateral patches in the proboscis. Expression is absent from both the dorsal and ventral midlines and the most anterior region of the proboscis. *Sp8* transcripts are additionally detected at low levels in two broad lateral stripes in the trunk ectoderm with both dorsal and ventral midlines and ciliated band free of expression. (C, D) *Dach* is expressed at high levels in an ectodermal stripe just anterior to the collar. Additional low level ectodermal expression is detected throughout much of the embryo. (E, F) *Meis* is expressed strongly in the trunk ectoderm excluding the ciliated band and at the base of the proboscis at early developmental stages, which then subsequently refines to a strong dorsal domain. (G, H) At post gastrula stage *Lhx2*/*9* is expressed throughout the proboscis ectoderm. By one gill slit stage expression becomes restricted mainly to a strong stripe at the base of the proboscis. (I) A schematic combining these data with previously published expression data (Lowe et al., 2003) for *Dlx* and *BarH1* indicating the relative levels and anteroposterior extents of neurectodermal expression of the appendage patterning genes. Dashed lines indicate expression in a subset of cells for the indicated anteroposterior domain.

high levels in the anterior third of the embryonic neurectoderm, and to a lesser degree in multiple medial to posterior stripes (Fig. 2A). In embryos at the one gill slit stage, expression is similar with the major expression confined to the proboscis (Fig. 2B). The *Saccoglossus dac* ortholog (*Dach*) is transcribed at high levels in a neurectodermal stripe just anterior to the collar, and at low levels throughout most of the rest of the embryo, both in post gastrula (Fig. 2C) and one gill slit stage embryos (Fig. 2D). *Meis*, the ortholog of *hth*, is transcribed at high levels in a broad band in the trunk neurectoderm, as well as in two dorsal patches—one just anterior to the collar; the other in the posterior trunk (Figs. 2E–F). The *Saccoglossus Lhx2/9* gene, orthologous to *apterous*, is transcribed throughout the proboscis neurectoderm in late gastrulae (Fig. 2G) and then becomes restricted mainly to a strong stripe just anterior to the collar (Fig. 2H). Along with previously documented expression patterns for the *Saccoglossus BarH1* and *Dlx* orthologs (Lowe et al., 2003) we provide an expression model (Fig. 2I) summarizing the transcription domains of all of these genes.

Based on the above data and previously published work (Supplementary material), we estimated the ancestral expression domains of the anteroposterior head patterning system (Fig. 3A), and compared them to the approximate domains of the proximodistal appendage patterning system in the developing *Drosophila* leg (Fig. 3B). We propose that a "head-appendage" genetic patterning system, consisting of the *btd/Sp8*, *Dll/Dlx*, *dac/Dach* and *hth/Meis* genes (and likely other genes, some of which are shown in Fig. 3), was present in a bilaterian ancestor that lacked appendages, where the system functioned to pattern the anteroposterior head axis. The evolution of this system may even have contributed to the process of cephalization in early animals. Subsequently, this system was co-opted to pattern the proximodistal axis of bilateral appendages through modification of input and output connections.

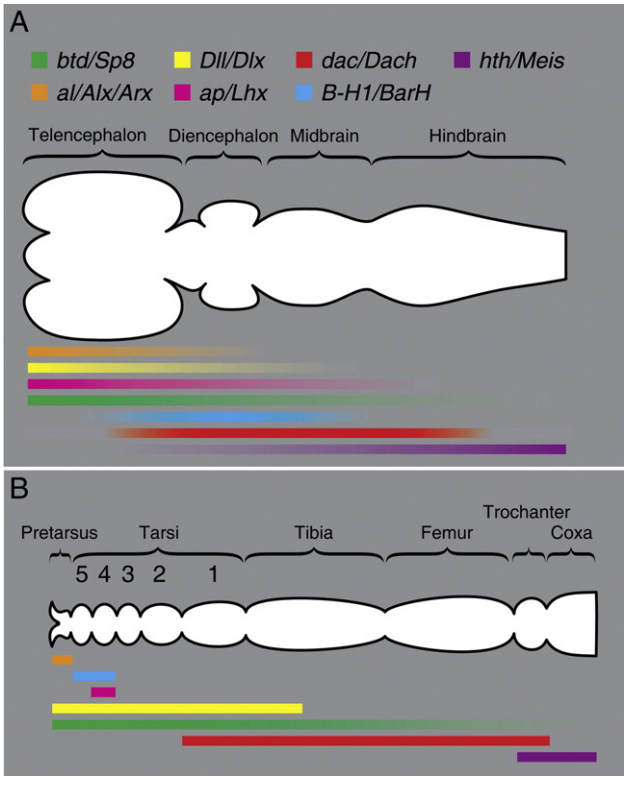

Fig. 3. A schematic diagram comparing expression of core appendage patterning genes in limbs and anterior neurectoderm. (A) Estimated ancestral expression patterns in embryonic anterior neurectoderm (based on conserved domains of expression) is displayed on a generalized diagram of a chordate brain. (B) Expression of proximodistal appendage patterning genes is displayed on a diagram of an adult *Drosophila* leg (adapted from (Kojima, 2004)).

After a system for anteroposterior head patterning had been co-opted for proximodistal appendage patterning, it could be used to specify and diversify the pattern of many body wall outgrowths (e.g. sensory structures, locomotory appendages, external genitalia, feeding appendages, etc.) through changes in the system. These could include variations in the regulatory relationships and expression patterns of the core appendage patterning genes, as well as further modifications of input and output connections (Dong et al., 2001). Consistent with this theory, inputs into this system during *Drosophila* appendage formation, such as *Dpp* and *wg*, are not conserved in this role among insects (Angelini and Kaufman, 2005). At least part of the same system has apparently been co-opted for the development of beetle horns (Moczek et al., 2006; Moczek and Rose, 2009), an appendage-like body wall outgrowth, long after the evolutionary advent of bilateral appendages. This patterning system may be poised for co-option in part by changing the expression pattern of *btd/Sp8* class genes, as driving ectopic expression of *btd* in small patches in *Drosophila* larval imaginal discs results in a crude recapitulation of the entirety of the expression pattern of the core genes, as well as creating ectopic appendage structures (Estella et al., 2003). It will be interesting to study the expression patterns of the head-appendage patterning genes in other branches of the evolutionary tree, especially in cnidarians, acoel flatworms, and lophotrochozoans.

Materials and methods

Drosophila in situ hybridizations were performed as in Kosman et al. (2004). *btd* antisense probes were made from a 2.6 kb genomic fragment starting 49 bp 5′ of the coding region. *Dll* antisense probes were made from a 1.4 kb EcoRI cDNA fragment (Cohen et al., 1989). *dac* antisense probes were made from a genomic PCR fragment cloned into pCRII (Invitrogen), the primers for the *dac* fragment were: 5′ AAGCAAAGTATAGAACGGATTAGCA 3′; 5′ TCCAACGAATCTTTCACTTCG 3′. *Saccoglossus* in situ hybridizations were performed as in Lowe et al. (2004). Antisense probes for *Sp8*, *Lhx2/9*, *Dach*, and *Meis* were made from *Saccoglossus kowalevskii* cDNAs (Freeman et al., 2008), accession numbers NM_001168189, NM_001164971, NM_001164944, and GU384871 respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.04.022.

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