# A single *Hox3* gene with composite *bicoid* and *zerknüllt* expression characteristics in non-Cyclorrhaphan flies

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**The members of the evolutionarily conserved Hox-gene complex, termed Hox genes, are required for specifying segmental identity during embryogenesis in various animal phyla. The *Hox3* genes of winged insects have lost this ancestral function and are required for the development of extraembryonic epithelia, which do not contribute to any larval structure. Higher flies (Cyclorrhapha) such as *Drosophila melanogaster* contain *Hox3* genes of two types, the *zerknüllt* type and the *bicoid* type. The *zerknüllt* gene is expressed zygotically on the dorsal side of the embryo and is required for establishing extraembryonic tissue. Its sister gene *bicoid* is expressed maternally and the transcripts are localized at the anterior pole of the mature egg. BICOID protein, which emerges from this localized source during early development, is required for embryonic patterning. All known direct *bicoid* homologues are confined to Cyclorrhaphan flies. Here, we describe *Hox3* genes of the non-Cyclorrhaphan flies *Empis livida* (Empididae), *Haematopota pluvialis* (Tabanidae), and *Clogmia albipunctata* (Psychodidae). The gene sequences are more similar to *zerknüllt* homologues than to *bicoid* homologues, but they share expression characteristics of both genes. We propose that an ancestral *Hox3* gene had been duplicated in the stem lineage of Cyclorrhaphan flies. During evolution, one of the gene copies lost maternal expression and evolved as *zerknüllt*, whereas the second copy lost zygotic expression and evolved as *bicoid*. Our finding correlates well with a partial reduction of *zerknüllt*-dependent extraembryonic tissue during Dipteran evolution.**

To trace the evolution of genetic interactions underlying early development, it is necessary to identify corresponding (orthologous) genes in different species (1, 2). The Hox genes provide an example in which orthologous genes can be identified unambiguously in different animal phyla (3), because the Hox genes are organized in a gene cluster termed Hox-gene complex (Hox-C), which is highly conserved in evolution (4). The conserved position of each Hox gene in the complex allows us to identify reliably its orthologue in other species, even if the conservation of the coding sequence is low. In insects (as in vertebrates), all the members of the Hox-C are involved in embryonic development. They encode homeodomain transcription factors, and most of them are required for differential segment identities (5–7). Three of the Hox-C genes of insects, namely *fushi tarazu*, *bicoid*, and *zerknüllt*, have evolved much more rapidly in both sequence and function than the remaining eight Hox genes (8–10). In *Drosophila*, all three have important roles during early development. *fushi tarazu* is required for establishing the boundaries between segments, *bicoid* for establishing the larval head and thorax, and *zerknüllt* for establishing extraembryonic tissue (11–13). Despite their very different functions, *zerknüllt* and *bicoid* are thought to derive from a common *Hox3* progenitor, which duplicated after the basal radiation of holometabolous insects but before the radiation of Cyclorrhaphan flies to which all known *bicoid* homologues are confined (8, 14–16). Cyclorrhaphan flies consistently contain two paralogous *Hox3* genes, one of the *bicoid* type and one of the *zerknüllt* type. In *Drosophila melanogaster*, an additional gene duplication affecting the *zerknüllt* gene took place and generated a third *Hox3* derivative, termed *zerknüllt 2* (17). However, in other Drosophilids no trace of a *zerknüllt* duplication has been found, suggesting that a second *zerknüllt* gene is specific to the *D. melanogaster* lineage (18, 19).

Maternal *bicoid* transcripts are localized at the anterior pole of the embryo, but BICOID protein is distributed in an anterior-to-posterior concentration gradient across the early embryonic syncytium (20–22). The gene is required before gastrulation for the spatially restricted activation of segmentation genes such as *hunchback*, *Krüppel*, and *knirps* and for translational repression to prevent maternal *caudal* activity in the anterior portion of the embryo (23–28). *bicoid* is not expressed at other stages of the life cycle. The maternal expression of *bicoid*, as well as a role in anterior patterning, is conserved throughout Cyclorrhaphan flies (Fig. 1*A*, *B*) (8, 14, 29, 30). The zygotic *zerknüllt* transcripts and the ZERKNÜLLT protein are expressed on the dorsal side of the early embryo (11, 17). During precellular nuclear division cycles 11–13, *zerknüllt* is activated in a broad dorsal-on/ventral-off pattern by an unknown factor. Expression is subsequently maintained by DECAPENTAPLEGIC, but during cellularization in nuclear division cycle 14 expression is restricted to a narrow dorsal strip, where it is required for the specification of the extraembryonic anlage (31, 32). In addition, *zerknüllt* is expressed in a subset of pole cells (germ line), where its function remains obscure (11). *zerknüllt* is not expressed at other stages of the life cycle. In the lower Cyclorrhaphan fly *Megaselia abdita* (Phoridae), *zerknüllt* transcripts are restricted to the extraembryonic anlage/tissue, and neither the early broad expression nor the expression in the pole cells known from *Drosophila* is observed (Fig. 1 *C* and *D*) (15). Zygotic expression of *Hox3/zerknüllt* in the extraembryonic anlage/tissue is conserved throughout insects, although the topology of the anlage varies (10, 33). In addition, maternal expression of *Hox3/zerknüllt* has been reported in the locust *Schistocerca gregaria* (Orthoptera), a rather primitive winged insect (34). This finding raises the question of whether *bicoid* and *zerknüllt* evolved from a progenitor that had combined functions of the two genes in higher Diptera. However, in the beetle *Tribolium castaneum* (Coleoptera), *Hox3/zerknüllt* apparently is not expressed maternally (10, 34), raising a question as to the likely ancestral state of expression. Here, we describe *Hox3/zerknüllt* homologues from three basal Dipteran species, the dancefly *Empis livida* (Empididae), which belongs to the sister taxon of Cyclorrhaphan flies, the horsefly *Haematopota pluvialis* (Tabanidae), a very basal

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Abbreviation: Hox-C, Hox-gene complex.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AJ419659–AJ419661).

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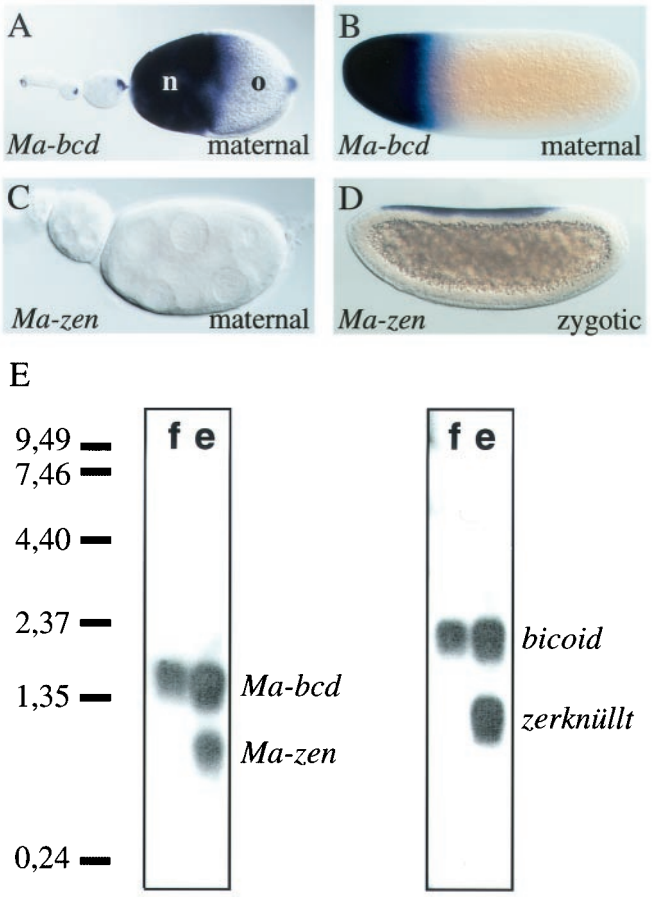


**Fig. 1.** Maternal expression of *bicoid* genes versus zygotic expression of *zerknüllt* genes in Cyclorrhaphan flies. (*A–D*) Transcript distribution of *Megaselia-bicoid* (*Ma-bcd*) and *Megaselia-zerknüllt* (*Ma-zen*) in egg chambers (*A* and *C*; n, nurse cells, o, oocyte) and embryos (*B* and *D*), respectively, by whole-mount *in situ* hybridization (15). Anterior is to the left and dorsal up. (*E*) Northern blot with poly(A)$^+$ RNA from *Megaselia* (*Left*) and *Drosophila* (*Right*): Samples of 1 $\mu$g per lane from females (f) or 1- to 4-h-old embryos (e) were loaded and hybridized against *Ma-zen* followed by *Ma-bcd* as control, or against *zerknüllt* followed by *bicoid* as control. Size markers on the left are given in kilobase pairs.

species in the suborder Brachycera, and the mothfly *Clogmia albipunctata* (Psychodidae). The results suggest that maternal *zerknüllt* expression was lost in the stem lineage of Cyclorrhaphan flies concomitantly with a reorganization of extraembryonic tissue.

## Materials and Methods

**Species.** *Clogmia* (syn. *Telmatoscopus, Psychoda*) *albipunctata* Williston 1893 (Diptera; Psychodidae) and *Megaselia. abdita* Schmitz 1959 (Diptera: Phoridae) were reared in the laboratory. Our *Clogmia* and *Megaselia* strains were derived from samples collected by Klaus Sander (Albert-Ludwigs-Universität, Freiburg, Germany) in Turkey near Seljuk (Ephesus) and in Freiburg, respectively. *Clogmia* was kept on moist paper sprinkled with stinging-nettle powder, and synchronized *Clogmia* embryos were obtained after experimental egg activation in water (35). *Megaselia* was kept on wet paper towels sprinkled with aquarium fish food TetraRubin (Tetra, Melle, Germany). For a description of embryonic development of both species see refs. 36 and 37. *Haematopota pluvialis* L. 1758 (Diptera, Brachycera, Tabanidae) (38) and *Empis* (subgenus *Kritempis* Collin) *livida* L. 1758 (Diptera, Brachycera, Empididae) (39) were caught in the rural environment of Göttingen (Germany) and fed on honey water. To obtain developing ovarian egg chambers from *Haematopota*, females were fed on human blood and kept for 2–4 days at 21°C before the ovaries were dissected.

**Cloning of Homologues.** Homeobox fragments of *Hox3/zerknüllt* homologues were amplified from genomic DNA by PCR with use of the degenerate primer pairs AARMGIWSIMGIACNGCNTWYACNAGT/TTYTTRWAYTTCATICKICKRTTYTG for *Clogmia*, and CARCTBGTDGARCTIGARAAYGARTT/TTYTTRWAYTTCATICKICKRTTYTG for *Haematopota* and *Empis*. (PCR protocols are available on request.) cDNAs were obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE) by using for *Clogmia* the SMART RACE, and for *Haematopota* and *Empis* the Marathon cDNA Amplification Kits (CLONTECH). cDNA was prepared from ovarian poly(A)$^+$ RNA. Sequence alignments were performed by using CLUSTAL method alignment parameters in the MEGALIGN/DNASTAR software package.

**Whole-Mount *in Situ* Hybridization.** Egg chambers were dissected in PBS (140 mM NaCl/3 mM KCl/7 mM $Na_2HPO_4$/3 mM $NaH_2PO_4$, pH 7.8) and fixed in 178 $\mu$l of PBS/22 $\mu$l of formaldehyde solution (37%), 20 $\mu$l of dimethyl sulfoxide, and 500 $\mu$l of *n*-heptane for 25 min. *Clogmia* embryos were dechorionated with 50% commercial bleach for 2 min and fixed in 350 $\mu$l of PEMS (100 mM Pipes/2 mM $MgSO_4$/100 mM malic acid, pH 6.9), 45 $\mu$l of formaldehyde solution (37%), and 500 $\mu$l of *n*-heptane for 40 min. To remove the vitelline layer, the fixing solution was replaced by 500 $\mu$l of *n*-heptane, to which 500 $\mu$l of ice-cold methanol (−80°C) was added. After vigorous shaking, the embryos were washed with methanol (−80°C) and heated to +70°C. The temperature shock was repeated twice, and *in situ* hybridization was done with digoxygenin-labeled RNA antisense probes covering the ORFs of the respective genes following standard procedures as described (40).

**Northern Hybridization.** Total RNA was isolated from dissected ovaries by using TRI REAGENT solution (WAK-Chemie Medical, Bad Soden, Germany) following the producer's instructions. Poly(A)$^+$ RNA was prepared from total RNA according to the instructions of the Oligotex mRNA Kit (Qiagen, Hilden, Germany). One microgram of *Megaselia* mRNA, 1 $\mu$g of *Drosophila* mRNA, 350 ng of *Clogmia* mRNA, 500 ng of *Haematopota* mRNA, and 750 ng of *Empis* mRNA were separated in a denaturing 1% agarose gel together with the 0.24- to 9.5-kb RNA Ladder (Life Technologies, Rockville, MD). Separated RNA was transferred to nylon membrane (Hybond-N+, Amersham Pharmacia) (41) and hybridized in Rapid-hyb buffer (Amersham Pharmacia) at 65°C for several hours to radio-labeled DNA probes. Nonspecifically bound probe molecules were removed by washing in 0.2× SSC (3 mM sodium citrate/30 mM NaCl, pH 7) and 0.1% SDS at 65°C for 30 min.

## Results and Discussion

**Isolation of *Hox3* Genes from Lower Diptera.** A survey of the literature on *bicoid* and *zerknüllt* gene expression suggested that in Cyclorrhaphan flies *bicoid* homologues are expressed only maternally, whereas *zerknüllt* homologues are expressed only zygotically (see Introduction). We confirmed lack of maternal *zerknüllt* expression in *Megaselia* (Fig. 1 *C–E*) and *Drosophila* (Fig. 1*E*; data not shown) by whole-mount *in situ* staining of ovarian egg chambers and Northern blotting experiments. Next we asked how *Hox3* genes are expressed in lower (non-Cyclorrhaphan) flies. To identify *Hox3* genes in lower Diptera, we took a PCR-based approach by using a variety of degenerate primers to amplify both *bicoid* and *zerknüllt* homologous DNA fragments from the mothfly *Clogmia* (Psychodidae), the horsefly

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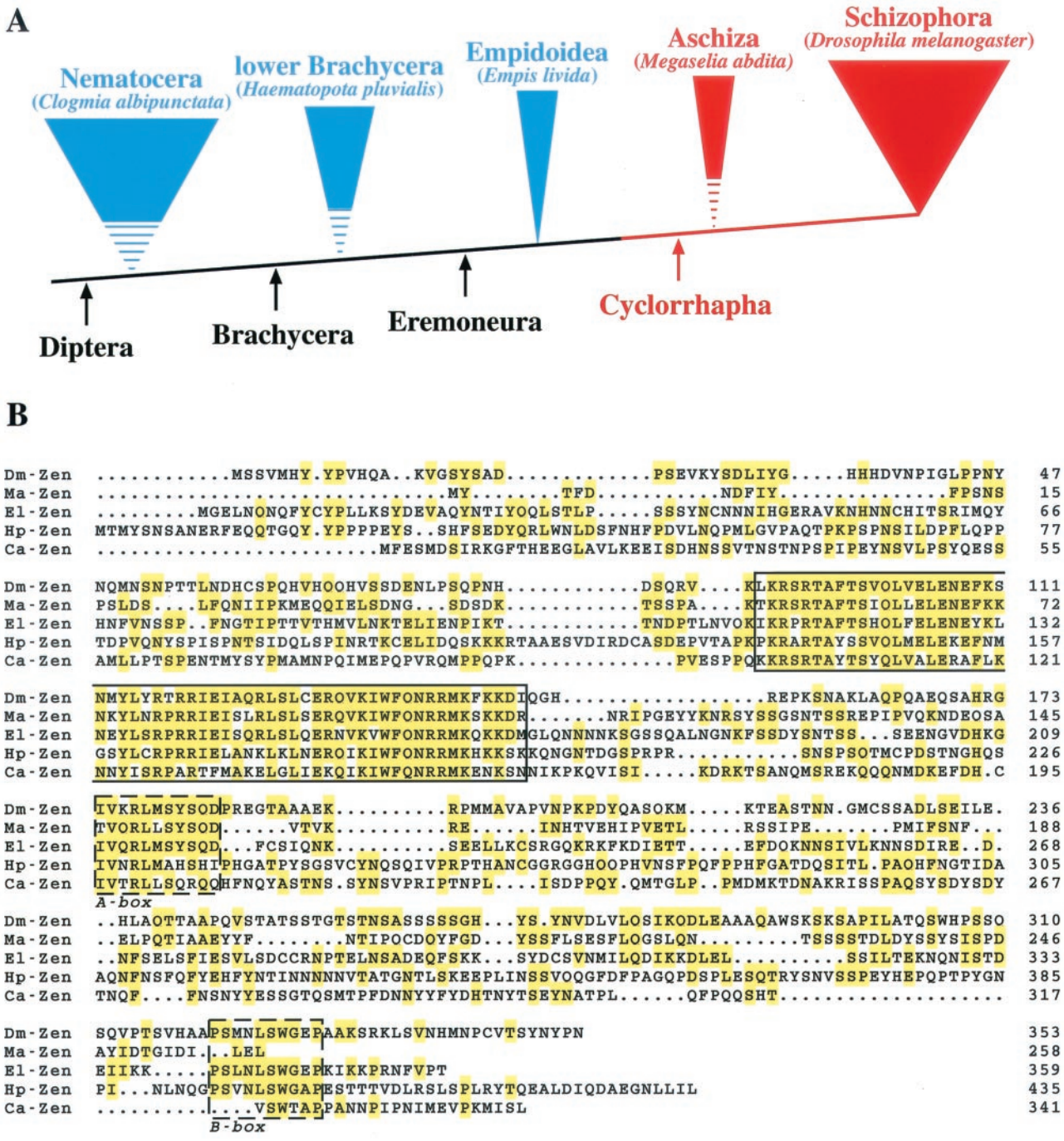


**Fig. 2.** (*A*) The phylogenetic relationship of all higher level taxa of Diptera including an estimated 125,000 species (43, 44). Relative species abundance in each taxon is indicated by the width of each triangle. Arrows indicate strongly supported monophyletic groups. Monophyly of Aschiza, lower Brachycera, and Nematocera is not or not strongly supported and these taxa might be paraphyletic. Cyclorrhapha are marked in red. (*B*) Alignment of the deduced amino acid sequences of *Hox3*/*zerknüllt* ORFs from *D. melanogaster* (Dm) (20), *M. abdita* (Ma) (15), *E. livida* (El), *H. pluvialis* (Hp), and *C. albipunctata* (Ca). Identical amino acids are underlaid in yellow; dots indicate gaps. The homeodomain is boxed; two conserved regions in the C-terminal portion, A-box and B-box, are framed with a dotted line. The numbers at the right margin refer to the last amino acid in each row.

*Haematopota* (Tabanidae), and the dancefly *Empis* (Empididae) covering a wide range of non-Cyclorrhaphan Diptera (Fig. 2*A*). In each species, we identified a single *Hox3* homeobox fragment corresponding to a *zerknüllt*-type gene. cDNAs covering all the ORFs of the genes were obtained by 5′ and 3′ rapid amplification of cDNA ends on templates prepared from ovaries. The cDNAs were 1,243 bp (*Clogmia*), 2,458 bp (*Haematopota*), and 1,681 bp (*Empis*) in length and are available under GenBank accession numbers AJ419659, AJ419660, and AJ419661, respectively. The predicted amino acid sequences of the full-length proteins were used in protein database searches with BLAST software (42) and produced highest scores with vertebrate *HoxA3* genes (*Haematopota*) or insect *zerknüllt* genes (*Empis*, *Clogmia*). Alignment of the predicted amino acid sequences revealed two conserved sequence stretches C-terminal to the homeodomain (Fig. 2*B*). These results establish that the newly identified sequences are

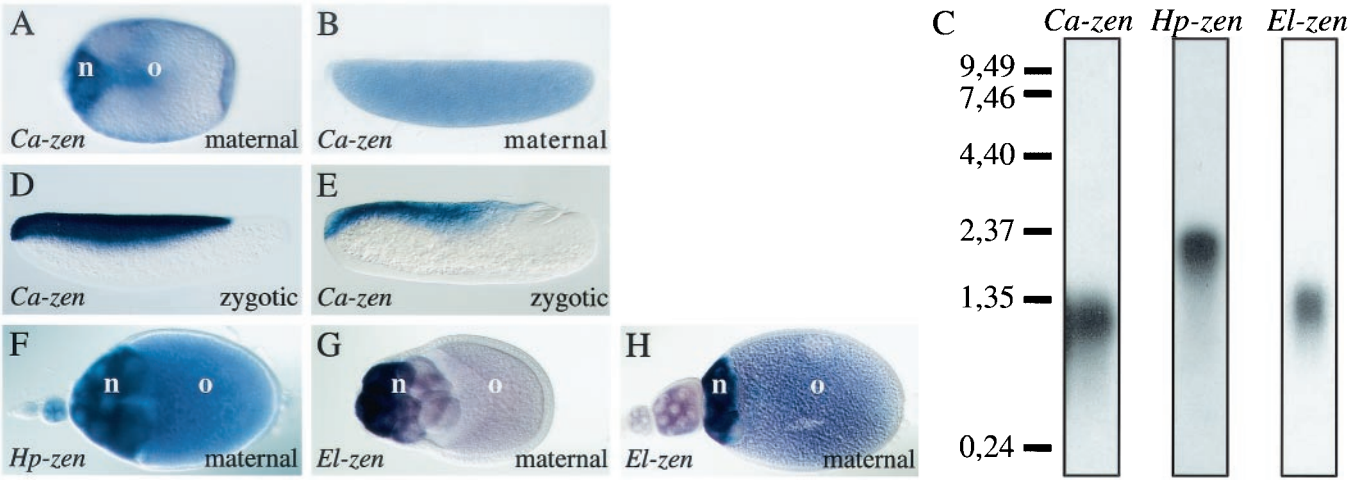


**Fig. 3.** Expression of *Hox3/zerknüllt* genes in lower Diptera. (*A* and *B*) *Ca-zen* in nurse cells (n) and oocyte (o) of an ovarian egg chamber (*A*) and an early embryo (*B*). (*C*) Northern blot of ovaries from *Clogmia* (*Left*), *Haematopota* (*Middle*), and *Empis* (*Right*) hybridized against *Ca-zen*, *Hp-zen*, and *El-zen*, respectively. (*D*, *E*) Zygotic expression of *Ca-zen* in *Clogmia* embryos before (*D*) and during gastrulation (*E*). (*F–H*) Egg chambers of *Haematopota* (*F*) and *Empis* (*G* and *H*) stained with *Hp-zen* and *El-zen*, respectively. In *G*, the somatic epithelium of the ovarian egg chamber has been damaged allowing for a space between the nurse cells and the oocyte. Embryos are oriented with anterior to the left and dorsal up.

direct *zerknüllt* homologues referred to as *Ca-zen* for *Clogmia*, *Hp-zen* for *Haematopota*, and *El-zen* for *Empis*. PCR fragments of the *bicoid* type were not found, although 11 degenerate primers against conserved sequence stretches were tried in various combinations on genomic and cDNA. The findings suggest there are no direct *bicoid* homologous genes in any of these three species. Thus, the newly identified *Hox3* sequences could be orthologous to both *zerknüllt* and *bicoid* if both genes originated from a gene duplication in the stem lineage of Cyclorrhaphan flies, or they could be orthologous to *zerknüllt* only if the duplication occurred before the radiation of Diptera. Gene trees with the homeodomains of *zerknüllt* and *bicoid* homologues from Cyclorrhapha and other Hox genes were already known to support a *bicoid/zerknüllt* kinship (ref. 15; data not shown). When comparing *Hox3/zerknüllt* from a wider selection of insect species, the relationship of *labial* (*Hox1*) and *proboscipedia* (*Hox2*), *bicoid* homologues, and *Hox3/zerknüllt* homologues is not resolved because of the diverged *Hox3/zerknüllt* sequences. Therefore, we assumed that *Hox3* was duplicated after the basal radiation of Diptera and asked whether expression of *Ca-zen* in *Clogmia* differs from *zerknüllt* expression in Cyclorrhaphan flies.

**Maternal and Zygotic Expression of *Ca-zen*.** *Ca-zen* is strongly expressed in the germ-line cells of ovarian egg chambers. The transcripts are detected in the nurse cells ("n", Fig. 3*A*) and the oocyte ("o", Fig. 3*A*), and seem to be evenly distributed in the early embryo (Fig. 3*B*). To corroborate maternal expression of *Ca-zen*, we performed a Northern blot analysis with mRNA prepared from ovaries. A single band of expected size is obtained after hybridization with a *Ca-zen* probe (Fig. 3*C*). Zygotic expression of *Ca-zen* starts during cellularization of the blastoderm in an anterior and dorsal domain, which corresponds to the anlage of extraembryonic tissue (Fig. 3 *D* and *E*). Extraembryonic *Ca-zen* expression is maintained during gastrulation, but no zygotic *Ca-zen* expression outside the extraembryonic anlage/tissue is observed. The expression pattern of *Ca-zen* differs in two important ways from conserved *zerknüllt* expression in Cyclorrhaphan flies (Fig. 1*D*). First, *Ca-zen* is expressed maternally, whereas in Cyclorrhapha *zerknüllt* genes are expressed strictly zygotically. Second, zygotic *Ca-zen* expression extends to the anterior tip of the cellular blastoderm, whereas *zerknüllt* expression in Cyclorrhapha is restricted to a narrow dorsal strip at the same developmental stage. Thus, *Ca-zen* in *Clogmia* combines expression characteristics of *bicoid* and *zerknüllt* in Cyclorrhapha (Figs. 1 *A–D* and 3 *A–D*).

**Maternal Expression of *Hox3/zerknüllt* Was Lost in Cyclorrhaphan Flies.** To determine the relevance of maternal *Hox3/zerknüllt* expression for reconstructing the evolution of *bicoid,* it is important to know whether maternal expression of *Hox3/zerknüllt* homologues occurs throughout lower Diptera. Therefore, we analyzed the expression of *Hp-zen* and *El-zen* in ovarian egg chambers of each species by whole-mount *in situ* hybridization and Northern blot analysis. *Hp-zen* transcripts are strongly detected in the nurse cells and in the oocyte of *Haematopota* ovarian egg chambers (Fig. 3*F*), and a single band of expected size is detected in Northern blots by using ovarian mRNA and a labeled *Hp-zen* probe (Fig. 3*C*). In ovarian egg chambers of *Empis*, *El-zen* transcripts are first detected in distal nurse cells (Fig. 3*G*). Only toward the end of oogenesis are transcripts also found in the proximal nurse cells and in the oocyte (Fig. 3*H*). A projection of these results on the phylogenetic tree of Diptera suggests that maternal expression of *Hox3* genes of the *zerknüllt* type is lost in the stem lineage of Cyclorrhaphan flies. This conclusion obviously depends on the resolution of the phylogenetic tree of Diptera. Therefore, it is important to note that the monophyly of the higher ranked taxa Schizophora, Cyclorrhapha, Empidoidea, and Brachycera is strongly supported (43, 44). In addition, this tree is supported by the fossil record of flies, because the appearance of these taxa in the fossil record matches their appearance as inferred from the phylogenetic tree (45).

**Correlation between the Reorganization of Extraembryonic Tissue and Loss of Maternal *Hox3/zerknüllt* Expression during Dipteran Evolution.** The maternal expression of all three newly identified *Hox3/zerknüllt* homologues implies selection for this trait in lower Diptera and release from a corresponding specific constraint in Cyclorrhapha. To understand better the changing developmental constraints during the evolution of Diptera, we compared embryonic development throughout this taxon. Embryonic development in Diptera seems rather uniform and resembles that of *Drosophila*. However, an important difference within Diptera occurs with respect to extraembryonic tissue

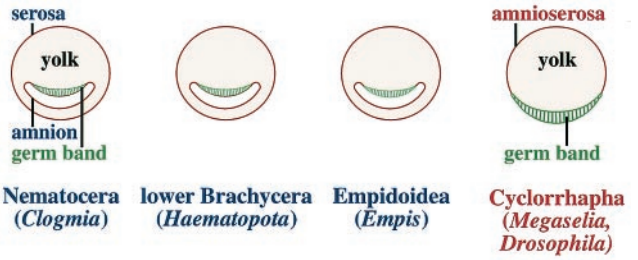


**Fig. 4.** Schematic cross-sections of Dipteran embryos showing extraembryonic epithelia. Cyclorrhaphan (red) and non-Cyclorrhaphan Diptera (blue) differ with respect to the formation of an amniotic cavity and the serosa. The ventral side of the germ band is covered by these tissues only in non-Cyclorrhaphan Diptera.

organization (Fig. 4). The establishment of extraembryonic tissue requires the activity of a *Hox3/zerknüllt* gene not only in *Drosophila* (17, 46, 47), but most likely in all winged insects (10, 34). In species of several insect orders and, in particular, in all non-Cyclorrhaphan flies analyzed so far, including the three species of this study, extraembryonic tissue consists of an amnion and a serosa. These two epithelia do not contribute to the embryo proper but transiently wrap the embryo (Fig. 4) (36, 48–50). In contrast, Cyclorrhapha including *Megaselia* and *Drosophila* develop without such wrapping, and the extraembryonic tissue is reduced to a transient dorsal epithelium, termed amnioserosa (Fig. 4) (15, 49, 51) and, as recently discovered in *Drosophila*, some additional cells surrounding the yolk (52). Thus, the transition in extraembryonic tissue organization in the stem lineage of Cyclorrhaphan flies occurred in a period when maternal expression was lost in the *zerknüllt*-type *Hox3* genes.

**Model for the Evolution of *bicoid* and *zerknüllt*.** On the basis of our findings, we propose that *bicoid* and *zerknüllt* evolved in the stem lineage of Cyclorrhaphan flies from a *Hox3* gene with maternal and zygotic expression, which is still found in non-Cyclorrhaphan Diptera (Fig. 5). In the common progenitor, zygotic activity was required for extraembryonic development, a feature conserved by the Cyclorrhaphan *zerknüllt* genes. Maternal activity of *Hox3/zerknüllt* homologues is not understood currently and will require adopting methods for gene inactivation in non-Cyclorrhaphan Diptera (29, 53). However, because maternal expression of *Hox3/zerknüllt* homologues is conserved in all non-Cyclorrhaphan Diptera analyzed so far, maternal activities of these genes are important for development. We obviously still lack an understanding of how maternal *Hox3/zerknüllt* activity turned into maternal *bicoid* activity, one of the problems being the different DNA- and RNA-binding properties of BICOID compared with all other Hox genes (28, 54–59). In *Drosophila*, ectopic expression of *zerknüllt* induces extraembryonic tissue (60) and ectopic expression of *bicoid* induces anterior embryonic structures (61, 62). Thus, both genes have counteracting effects and cannot convert their respective activities in the same spatial domain of the embryo. Therefore, a separation of the functional expression domains of *bicoid* and *zerknüllt* in time and space, as well as selective loss of maternal

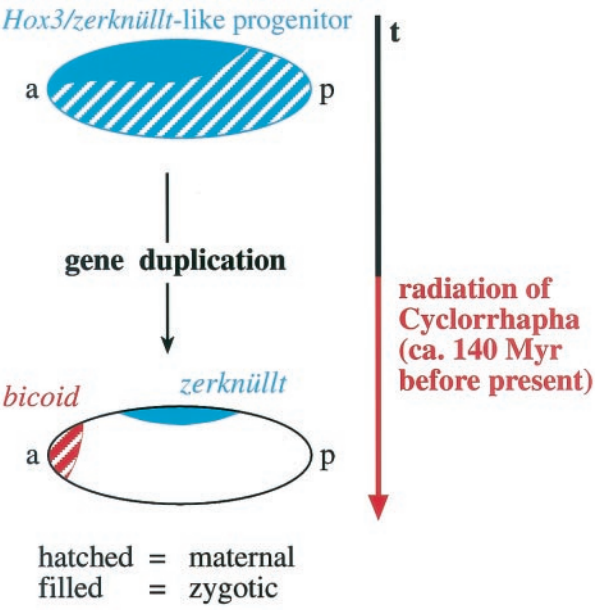


**Fig. 5.** Hypothetical scenario of the evolution of *bicoid* and *zerknüllt*. The common ancestral gene is thought to have been expressed maternally (hatched) providing transcripts throughout the early embryo, and zygotically (filled) in the extraembryonic tissue anlage. After duplication, presumably in the stem lineage of Cyclorrhaphan flies, zygotic expression is lost in the *bicoid* daughter and maternal expression is lost in the *zerknüllt* daughter. Opposing functions of *bicoid* and *zerknüllt* (see text) evolved after the spatial separation of expression domains, caused by the anterior localization of *bicoid* and the reduction of the extraembryonic anlage/tissue. a, anterior; p, posterior.

versus zygotic enhancer elements, seems to be an important prerequisite for subsequent divergent evolution of both genes in Cyclorrhapha. We suggest that anterior localization of *bicoid*, which is based on specific sequence elements in the 3′ untranslated region of the gene (63), and respecification of anterior blastoderm toward an embryonic fate were important steps toward this goal. In summary, the key features of our model are as follows: a single *Hox3* gene with maternal and zygotic activity is present in the stem lineage of Diptera; it was duplicated in the stem lineage of Cyclorrhapha, giving birth to maternal *bicoid* and zygotic *zerknüllt*; and the functional evolution of BICOID-specific DNA- and RNA-binding properties became possible after the reduction of the extraembryonic anlage/tissue. It will be challenging to test this model at the levels of genomics, developmental genetics, and morphology.

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