



Evolution of Developmental Control Mechanisms

Coral *emx-Am* can substitute for *Drosophila empty spiracles* function in head, but not brain developmentBeate Hartmann^a, Martin Müller^a, Nikki R. Hislop^b, Bettina Roth^a, Lucija Tomljenovic^b, David J. Miller^b, Heinrich Reichert^{a,*}^a Biozentrum, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland^b ARC Centre of Excellence for Coral Reef Studies and Comparative Genomics Centre, James Cook University, Townsville, Qld. 4811, Australia

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ABSTRACT

The *ems/Emx* genes encode homeodomain transcription factors that have conserved actions in anterior embryonic patterning in bilaterian animals ranging from insects to mammals. Recently, genes of the *ems/Emx* family have been identified in cnidarians raising the possibility that some of their developmental functions might be conserved throughout the Eumetazoa. To determine to what extent functions of a cnidarian *ems/Emx* protein have been retained across phyla, we carried out cross-phylum rescue expression experiments in which the coral *Acropora emx-Am* gene was misexpressed in *Drosophila ems* mutants. Our findings demonstrate that coral *emx-Am* can substitute for fly *ems* in embryonic head development and rescue the open head defect and the loss of segmental *engrailed* expression domains in *Drosophila ems* mutants. In contrast, the coral *emx-Am* gene can not substitute for fly *ems* in embryonic brain development. Even when a hexapeptide motif of the type present in the *Drosophila ems* gene is inserted into the coral *emx-Am* gene, rescue of the developmental brain defects in fly *ems* mutants fails. These findings have implications for understanding the evolutionary origins of head versus brain patterning mechanisms.

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Introduction

Comparative molecular analyses are providing increasing evidence for the evolutionary conservation of key developmental control genes involved in embryonic regionalization and patterning. This is exemplified by the genes of the *empty spiracles (ems/Emx)* family which play key roles in anterior embryonic patterning in animals ranging from insects to mammals. The *ems* gene was originally discovered in a *Drosophila* screen for zygotic patterning mutations and is a member of the cephalic gap gene group (Cohen and Jürgens, 1991; Cohen and Jürgens, 1990; Jürgens et al., 1984). It encodes a homeodomain containing transcription factor and was grouped into the “dispersed superclass” of homeobox genes (Gehring et al., 1994). It shares with the homeobox genes of the “complex superclass,” which includes the Hox genes, a hexapeptide motif of the core consensus sequence YPWL, located N-terminal of the homeodomain. The hexapeptide plays a role in the cooperative DNA binding of Hox proteins with the cofactor extradenticle (Exd) (Mann and Chan, 1996). However it is not known if the hexapeptide of the *Ems* protein serves a similar function.

During early embryogenesis, the *Drosophila ems* gene is first expressed at the early cellular blastoderm stage in a single circumferential stripe at the anterior end of the embryo. Later in embryogenesis, *ems* expression is detected in discrete ectodermal patches of the labral, antennal and intercalary segment of the anterior head as well as in lateral regions of ectodermal and neural cell patches in all trunk segments (Dalton et al., 1989; Walldorf and Gehring, 1992). The large antennal expression domain of the ectoderm gives rise to the *ems* expressing neuroblasts of the deutocerebral brain anlage and the smaller intercalary expression domain gives rise to the *ems* expressing neuroblast of the tritocerebral brain anlage (Urbach and Technau, 2003; Younossi-Hartenstein et al., 1996). Mutation of *ems* leads to a gap-like phenotype in the embryonic head, which includes deletions of cuticular and cephalic sensory structures in the ocular, antennal and intercalary segments, and results in an open-head phenotype (Cohen and Jürgens, 1990; Dalton et al., 1989; Jürgens et al., 1984; Walldorf and Gehring, 1992). Mutation of *ems* also results in deletion of deutocerebral and tritocerebral brain anlagen, which in turn leads to massive structural defects in the embryonic brain (Hirth et al., 1995). This brain phenotype is due to defective specification of the neuroectoderm in the antennal and intercalary domains of *ems* mutants and correlates with the absence of the proneural gene *lethal of scute*, which is thought to be required for neuroectodermal cells to adopt the competence to become neuroblasts (Younossi-Hartenstein et al., 1997).

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Comparative studies suggest that the expression pattern and the function of *ems/Emx* genes in anterior patterning are evolutionarily conserved throughout the Bilateria (reviewed by Lichtneckert and Reichert, 2005). Thus, the murine homologues of the *Drosophila ems* gene, *Emx1* and *Emx2*, are both expressed in the anterior brain and genetic loss-of-function analyses suggests that they are involved in anterior brain development (Pellegrini et al., 1996; Qiu et al., 1996; Simeone et al., 1992a,b; Yoshida et al., 1997). Further evidence for the functional equivalence of the *ems* and *Emx2* gene products comes from cross-phylum rescue experiments in which ubiquitous overexpression of a mouse *Emx2* transgene in an *ems* null mutant background rescues the brain phenotype of the mutant fly embryos (Hartmann et al., 2000). The notion of an evolutionarily conserved role of *ems/Emx* genes in anterior patterning of the brain and/or head during embryonic development is supported by expression data from a number of different vertebrate and invertebrate bilaterian species including mouse (Simeone et al., 1992a), chick (Bell et al., 2001), frog (Pannese et al., 1998), zebrafish (Morita et al., 1995), shark (Derobert et al., 2002), lamprey (Myojin et al., 2001), hemichordate (Lowe et al., 2003) and nematode (Aspöck et al., 2003). Indeed, *ems/Emx* genes appear to be such universal anterior markers that (together with the homeotic genes) they were included in the original description of the “zootype,” the universal genetic toolkit of animals (Slack et al., 1993).

ems/Emx genes have also been identified in the sister group of bilaterians, the Cnidaria (de Jong et al., 2006; Mokady et al., 1998). The Cnidaria are thought to represent one of the most ancient metazoan phyla and thus provide a useful outgroup for comparative studies of the molecular control of development in the more complex bilaterians. Among cnidarians, the Anthozoa, which includes the sea anemone *Nematostella* and the coral *Acropora*, is viewed as the most basal class, and these animals appear to reflect most faithfully ancestral characteristics (Bridges et al., 1992, 1995; Kortschak et al., 2003; Technau et al., 2005). Recently it has been demonstrated that in *Acropora millepora*, an *emx* gene is expressed in putative neurons in the aboral half of the planula larva (de Jong et al., 2006), which is the anterior region with respect to swimming direction. This apparent neuron-specific and axially restricted expression of the *Acropora emx* gene (*emx-Am*) in a developing cnidarian larva is reminiscent of the expression of *ems/Emx* genes in the anterior CNS and/or head systems of developing bilaterian embryos. This raises the question of whether the *ems/Emx* genes are part of an ancient axis- and/or CNS-specification system that predates the cnidarian/bilaterian split. If this were the case, then the protein encoded by the coral *emx-Am* gene might still retain conserved functions in development that could be uncovered in cross-phylum rescue experiments performed on a bilaterian such as *Drosophila*. Thus, a challenge now is to determine which functional aspects of the proteins encoded by the *ems/Emx* genes have been conserved and which aspects have diverged after the Cnidaria/Bilateria split.

To address this question, we carried out targeted gene expression experiments in which the coral *emx-Am* gene was expressed in appropriate embryonic domains of *Drosophila ems* mutants. Our findings demonstrate that *emx-Am* can indeed substitute for *ems* in epidermal head development and, thus, rescue the open head defect as well as restore the intercalary and antennal head segments of *Drosophila ems* mutants. The relative rescue efficiency of the *emx-Am* gene in these targeted gene expression experiments was comparable to that of the *Drosophila ems* gene. In contrast, the coral *emx-Am* gene was not able to rescue the brain defect of *Drosophila ems* mutants. Even when a hexapeptide motif of the type present in the *Drosophila ems* gene was inserted into the coral *emx-Am* gene, rescue of the developmental brain defects in fly *ems* mutants failed. The implications of these findings for understanding the evolutionary origins of head vs. brain patterning mechanisms in Eumetazoa are discussed.

Material and methods

Transgene constructs

Modified coral and fly *ems* constructs were generated from cDNA templates (in Bluescript) using the Stratagene QuikChange site-directed mutagenesis system under the manufacturers recommended conditions. In the case of the *Drosophila ems* cDNA, the YPWL amino acid motif that forms the core of the hexapeptide was mutated to AAAL using the primer 5'gatagctatcagctggccgcccgcctgctcagccccc3' and its reverse complement. The coral *Emx* protein lacks a definitive hexapeptide (de Jong et al., 2006); in this case, the amino acid sequence YPCA which occurs 28–31 residues N-terminal of the homeodomain was mutated to YPWL using the primer 5'cttttctattctatccgtggctgtaagctcatgatgtac3' and its reverse complement. In both cases the introduction of modifications was confirmed by DNA sequencing prior to cloning the cDNAs into pUAST. Both mutant and wild-type *Acropora emx* cDNAs were cloned in via the NotI and XhoI sites, and the corresponding *Drosophila ems* constructs were generated via the EcoRI site.

Fly husbandry

Flies were reared on standard corn meal medium at room temperature. Transgenic flies for the 3 P{w⁺m^c, UAS-cDNA} constructs were generated according to standard procedures (Rubin and Spradling, 1982). For UAS-*ems* and UAS-*emx-Am*, additional lines were generated by mobilization of an existing transgene with $\Delta 2-3$ transposase (Robertson et al., 1988). Linkage of each insert was determined by standard genetic crossing procedures.

For the rescue experiment, first, three double balanced stocks were established: (1) noc^{Sc^o}/CyO, LacZ; *ems*^{9H83} e/TM3, Sb e LacZ, (2) L/CyO, LacZ; *ems*^{9H83} e/TM3, Sb e LacZ and (3) L/CyO, LacZ; *ems*^{9Q64} e/TM3, Sb e LacZ. These stocks were used to establish all UAS-(cDNA)/CyO, LacZ; *ems*^{9H83} e/TM3, Sb e LacZ and UAS-(cDNA)/CyO, LacZ; *ems*^{9Q64} e/TM3, Sb e LacZ stocks used for this study. The *sca-Gal4* driver line (Klaes et al., 1994) of the genotype *sca-Gal4*; *ems*^{9H83} e/TM3, Sb e LacZ and the *nos-Gal4-3GCN4-Bcd3'UTR* driver line (Janody et al., 2000) of the genotype *nos-Gal4-3GCN4-Bcd3'UTR*; *ems*^{9H83} e/TM3, Sb e LacZ were obtained accordingly. The *sca-Gal4* driver is active during neuroectoderm specification and neuroblast formation (Sprecher et al., 2004). The *nos-Gal4-3GCN4-Bcd3'UTR* transgene allows expression of the Gal4-GCN4 protein as a maternal anterior gradient in the early *Drosophila* embryo (Janody et al., 2000). For rescue experiments, Gal4 driver virgins were collected and crossed to UAS-(cDNA)/CyO, LacZ; *ems*^{9H83} e/TM3, Sb e LacZ or UAS-(cDNA)/CyO, LacZ; *ems*^{9Q64} e/TM3, Sb e LacZ males. As negative control, Gal4 driver virgins were mated with *ems*^{9H83} e/TM3, Sb e LacZ or *ems*^{9Q64} e/TM3, Sb e LacZ males. Embryo collections of these crosses were done at 25 °C.

For targeted ectopic expression of *orthodenticle*, the responder line UAS-*otd* (Blanco et al., 2009) was used.

Immunocytochemistry and cuticular preparations

Embryos were dechorionated, fixed and labeled according to standard protocols (Patel, 1994; Therianos et al., 1995). Primary antibodies were rabbit anti-HRP 1:250 (Jackson Immunoresearch), mouse anti-en (undiluted, DSHB) and mouse anti- β gal 1:500 (Promega). Secondary antibodies were Alexa 488-conjugated goat anti-rabbit and Alexa 568-conjugated goat anti-mouse (Molecular Probes), both at 1:150. Immunolabeled embryos were mounted in one drop of Vectashield H-1000 (Vector) according to a procedure that allows for rotation of embryos, enabling us to score both brain hemispheres of a single embryo.

Cuticular preparations were made according to standard procedures (Nüsslein-Volhard et al., 1984).

Genetic rescue analysis

In genetic rescue experiments, *sca*-Gal4 or *nos*-Gal4 driven P{UAS-cDNA} activity in homozygous *ems* null mutants (*ems*^{9H83}/*ems*^{9H8} or *ems*^{9H83}/*ems*^{9Q64}) was confirmed by the absence of balancer-specific (CyO, *LacZ*; TM3, *LacZ*) β -gal immunoreactivity.

The brain phenotype in *ems*^{-/-} embryos was scored as fully rescued, when there was no gap in the neuronal tissue between the protocerebral brain hemispheres and the subesophageal neuromeres. The open head phenotype in *ems*^{-/-} embryos was scored as fully rescued, when the brain lobe was not protruding out of the embryo and the dorsal ectodermal tissue appeared normal. Embryos of different genetic background were rated in a double blind experimental setup. A total of 50 hemispheres were judged for each experimental group as well as for the negative control group (*ems*^{9H83}/*ems*^{9H83} without rescue construct). Thus, we obtained a rescue score for each experimental group (RS) and a rescue score for the negative control group (RS_{*ems*-/-}), the latter reflecting the fact that there is no complete penetrance of the mutant phenotype. To correct for this incomplete phenotypic penetrance, the rescue scores were transformed into relative rescue efficiencies (RRE in %) according to the formula: $RRE = \frac{RS - RS_{ems-/-}}{50 - RS_{ems-/-}} * 100$. For statistical analysis we used the Fisher test. Values with $p \leq 0.01$ are referred to as significant.

A rescue of the head patterning defect in *ems*^{-/-} embryos was judged by scoring each embryonic hemisphere for the presence of the engrailed intercalary stripe, the antennal stripe or the engrailed head spot. A total of 50 hemispheres were judged for each experimental group as well as for the negative control group (*ems*^{9H83}/*ems*^{9Q64} without rescue construct). Thus, for each group the total number of observed head segments could vary between 0 and 150. For statistical analysis we used the independent *t* test. Values with $p \leq 0.01$ are referred to as significant.

Laser confocal microscopy

For laser confocal microscopy a Leica TCS SP microscope was used. Optical sections were taken approximately every 1 μ m, recorded in line average mode with picture size of 512 \times 512 pixels. Captured images from optical sections were arranged and processed using IMARIS (Bitplane). Figures were arranged and labeled using Adobe Photoshop.

Results

The coral emx gene does not rescue embryonic brain defects in Drosophila ems mutants

The *Drosophila ems* gene is a cephalic gap gene that is important for the development of the embryonic brain (Hartmann et al., 2000; Hirth et al., 1995; Urbach and Technau, 2003; Younossi-Hartenstein et al., 1996). The brain in *ems* null mutant embryos has a deletion in the deutocerebral and tritocerebral brain anlagen, which leads to a prominent gap devoid of neuronal cells between the remaining protocerebral brain hemispheres and the subesophageal neuromeres (Figs. 1A and B). We analysed the penetrance of this embryonic brain phenotype and found it present in 56% of the *ems* mutant embryos. For quantification of the following rescue experiments, a penetrance of 56% of the mutant brain phenotype is defined as a relative rescue efficiency of 0% (see Methods).

To determine if the brain phenotype can be rescued by targeted expression of the fly *ems* gene, we made use of the GAL4-UAS system (Brand and Perrimon, 1993). We generated two UAS responder lines

with independent inserts of an *ems* transgene in an otherwise homozygous *ems* mutant background (see **Material and Methods**). In order to target the *ems* transgenes to the early ectodermal tissue of the head, we used a *sca*-GAL4 driver (Klaes et al., 1994). In a wild-type background this results in expression of *ems* throughout the neuroectoderm starting at stage 9 and includes the anterior cephalic domain in which *ems* is normally expressed (data not shown). Targeted expression of the fly *ems* transgenes in an *ems* mutant background resulted in a significant rescue of the brain phenotype in both transgenic fly lines (see Fig. 2). The relative rescue efficiency was 57% for the UAS-*ems6* insertion line and 50% for UAS-*ems9*. In rescued embryos, contiguous neuronal tissue extended from the protocerebral hemispheres to the mandibular neuromere and prominent cellular gaps in this tissue were not observed (Fig. 1C, arrow).

To determine whether the brain phenotype in *Drosophila* embryos can be rescued by targeted expression of the coral *emx-Am* gene, we generated two UAS responder lines with independent insertions of an *emx-Am* transgene (see **Material and Methods**). The targeted expression of the *emx-Am* gene in *ems* mutant *Drosophila* embryos did not result in a significant rescue of the brain phenotype in either of the two insertion lines (see Fig. 2). The relative rescue efficiency was 4% for the UAS-*emx-Am5* insertion line and -11% for UAS-*emx-Am6*. In the majority of the transgenic embryos, large cellular gaps were observed between the remaining brain hemispheres and the subesophageal neuromeres (Fig. 1D, arrow).

The Drosophila ems gene without a normal hexapeptide domain rescues embryonic brain defects in ems mutants

The failure of the coral Emx protein to rescue the brain defects in *ems* mutants might be due to the fact that it lacks a definitive hexapeptide motif (de Jong et al., 2006); this motif is assumed to have a role in cooperative DNA binding of some Antp-superclass homeodomain proteins with the cofactor extradenticle (Mann and Chan, 1996). It is conceivable that this motif is essential for *ems* action in embryonic brain development. To test this, we generated UAS responder lines carrying an *emx-Am*^{+hp} transgene, which contained the coral *emx-Am* gene with an added hexapeptide motif (see **Material and Methods**).

However, even with the addition of a hexapeptide the coral *emx* transgene remained unable to restore the brain phenotype in *ems* mutant embryos (see Fig. 2). The relative rescue efficiency for UAS-*emx-Am*^{+hp} was 7%. Most mutant embryos still showed a prominent gap in the brain between the brain hemispheres and the subesophageal neuromeres (Fig. 1F, arrow).

Given that the presence or absence of the hexapeptide motif did not alter the rescue efficiency of the coral *emx-Am* gene, we wondered if the hexapeptide motif in the fly *ems* gene might be similarly dispensable for a rescue of the embryonic brain mutant phenotype. To investigate this, we generated UAS responder lines with independent inserts of an *ems*^{-hp} transgene in which the hexapeptide motif had been mutated (see **Material and Methods**).

Targeted expression of the fly *ems* gene without a normal hexapeptide motif in *ems* mutant embryos resulted in a significant rescue of the brain phenotype (see Figs. 2 and 1E). One insertion line (UAS-*ems*^{-hp3}) showed a relative rescue efficiency of 71% and a second insertion line (UAS-*ems*^{-hp2}) showed a relative rescue efficiency of 36% for the mutant phenotype. These findings indicate that the hexapeptide motif of the *ems* gene is not necessary for embryonic brain development.

To control for the specificity of the rescue effects and rule out the possibility that other classes of homeobox genes might rescue the developmental defect, we analysed the ability of *orthodenticle* (*otd*) to rescue the brain phenotype of *ems* mutant embryos. *otd* is another cephalic gap gene containing a homeobox. It is expressed at the early cellular blastoderm stage in a single circumferential stripe which partially overlaps with the *ems* expression domain. Later, *otd*

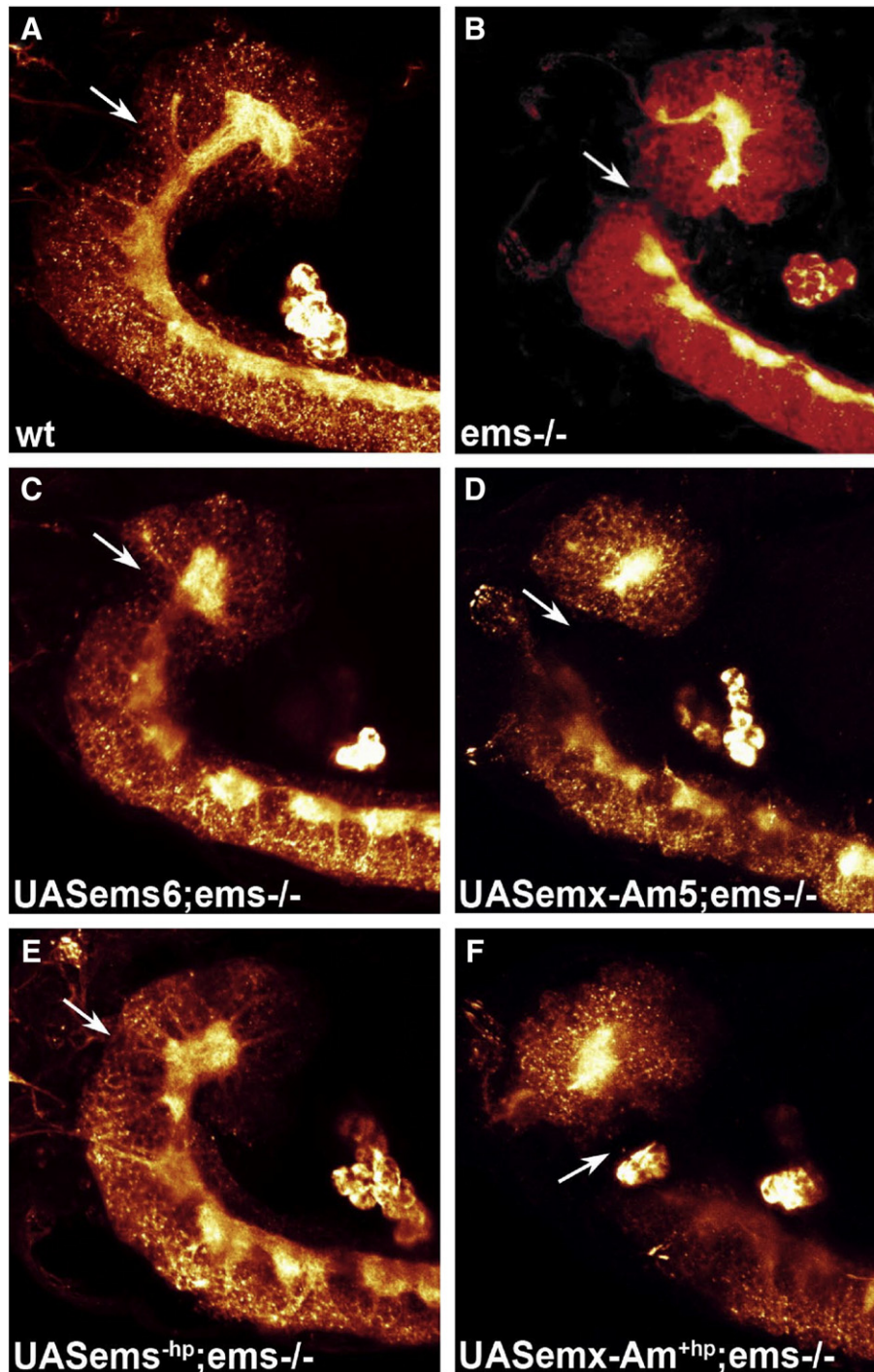


Fig. 1. The coral *emx* gene does not rescue the fly *ems* mutant brain defect. (A–F) Reconstructions of lateral confocal microscopy sections of stage 15 embryos; anti-HRP immunolabeling; anterior of body axis to the left. (A) Wild type; (B) *ems* null mutant; a gap devoid of neuronal cells appears between the remaining protocerebral brain anlage and the subesophageal neuromeres (arrow, compare to A); (C) *ems* null mutant carrying a *sca*-GAL4/UAS-*ems6* rescue construct; (D) *ems* null mutant carrying a *sca*-GAL4/UAS-*emx-Am5* rescue construct; (E) *ems* null mutant carrying a *sca*-GAL4/UAS-*ems*^{hp3} rescue construct; and (F) *ems* null mutant carrying a *sca*-GAL4/UAS-*emx-Am*^{hp} rescue construct. (C and E) Both, targeted expression of the fly *ems* gene or the fly *ems* gene with a mutated hexapeptide are able to rescue the *ems* mutant brain phenotype. No gap is observed in the brain (arrow) and the neuronal tissue extends from the protocerebral neuromere to the mandibular neuromere. (D and F) Neither targeted expression of the coral *emx-Am* gene nor the coral *emx-Am* gene with an added hexapeptide motif result in a rescue of the *ems* mutant brain defect. Most mutant embryos still show a prominent gap in the brain between the protocerebral neuromere and the subesophageal neuromeres (arrow).

expression in the head is found in the antennal and preantennal neuroectoderm as well as in many neuroblasts delaminating from these regions (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Urbach and Technau, 2003). Targeted misexpression of *otd* under the control of the *sca*-GAL4 driver in *ems* mutants did not result in a rescue of the brain phenotype (see Fig. 2). The relative rescue efficiency for this line was 11%.

The coral emx gene rescues the embryonic open-head defect in Drosophila ems mutants

In addition to its role in embryonic brain development, *ems* is also involved in proper embryonic development of the head ectoderm, and in *ems* mutants embryonic head development is severely perturbed (Cohen and Jürgens, 1990; Dalton et al., 1989;

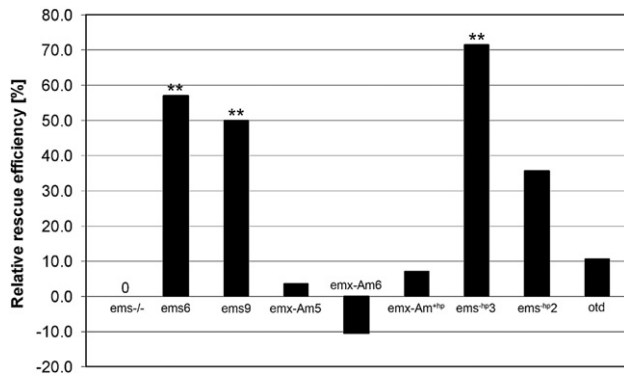


Fig. 2. Relative rescue efficiency of embryonic brain defect in *ems* null mutants for different rescue constructs. The relative efficiency of rescue is shown for the fly *ems* gene (two independent insertion lines; *ems6* and *ems9*), the coral *emx-Am* gene (two independent insertion lines; *emx-Am5* and *emx-Am6*), the coral *emx-Am* gene with an added hexapeptide motif (*emx-Am*^{hp}), the fly *ems* gene with a mutated hexapeptide motif (two independent insertion lines; *ems*^{-hp3} and *ems*^{-hp2}) and, as a negative control, for the *otd* gene. All genes were expressed in the *ems* mutant under the control of the same *sca*-GAL4 driver line. See Methods for explanation of relative rescue efficiency. A significant rescue resulted from targeted expression of the fly *ems* gene. A rescue of the brain defect is also achieved by targeted expression of the fly *ems* gene with a mutated hexapeptide motif. Neither targeted expression of the coral *emx-Am* gene nor the coral *emx-Am* gene with an added hexapeptide motif, nor targeted expression of *otd* result in a rescue of the *ems* mutant brain defect. Bars with two stars represent significant (with $p < 0.01$) differences in relative rescue efficiency values compared to *ems*^{-/-}.

Jürgens et al., 1984; Walldorf and Gehring, 1992). Accordingly, analysis of cuticular preparations from *ems* mutants reveals a gap-like, open-head phenotype in which the anterodorsal head ectoderm is defective (Figs. 3A and B). Associated with the defective ectodermal tissue in the dorsal head are perturbed head involution and protrusion of the brain hemispheres out of the embryo proper (Figs. 3C and D). We analysed the penetrance of this open-head phenotype and found it present in 66% of the homozygous *ems* mutant embryos. For quantification of the following genetic rescue experiments, we define a penetrance of 66% of the mutant head phenotype as a relative rescue efficiency of 0% (see Methods).

To ensure that the open-head phenotype could be rescued by targeted expression of the fly *ems* gene we again used the *sca*-GAL4 driver line together with the two UAS responder lines that had independent inserts of an *ems* transgene. Targeted expression of the fly *ems* transgene in an *ems* mutant background resulted in a significant rescue of the open-head phenotype in both transgenic fly lines (see Fig. 4); the relative rescue efficiencies were 97% (UAS-*ems9*) and 85% (UAS-*ems6*). In rescued embryos the anterodorsal head ectoderm was completely intact and the brain hemispheres remained contained within the embryo proper (Fig. 3E).

To examine whether the open-head phenotype in *Drosophila* embryos can be rescued by targeted expression of the coral *emx-Am* gene, we used the *sca*-GAL4 line to drive expression of the two UAS responder lines that had independent inserts of an *emx-Am* transgene. Remarkably, targeted expression of the *emx-Am* gene under the control of the *sca*-GAL4 driver in *ems* mutant *Drosophila* embryos resulted in rescue of the open-head phenotype with near equivalent efficiency to that of the *Drosophila* gene (see Fig. 4). Thus relative rescue efficiencies of 82% (UAS-*emx-Am5*) and 70% (UAS-*emx-Am6*) were determined for the two insertion lines. In general, *ems* mutant embryos rescued by the coral *emx* genes were indistinguishable from those rescued by the fly *ems* gene (compare Figs. 3E and F) with respect to the head ectodermal phenotype. Thus, in contrast to its inability to replace the *Drosophila* *ems* gene in embryonic brain development, the coral *emx-Am* gene might be able to replace the *ems* gene in embryonic head development.

The *Drosophila* *ems* gene without a normal hexapeptide domain rescues the embryonic open-head defect in *ems* mutants

Since the coral *emx-Am* gene lacks the hexapeptide motif the rescue result reported above suggests that this motif might also be dispensable for proper *ems* protein function during head development. To test this, we made use of the two UAS responder lines with independent inserts of an *ems*^{-hp} transgene with a mutated hexapeptide motif. Targeted expression of the mutated fly *ems* transgene under the control of a *sca*-GAL4 driver in *ems* mutant *Drosophila* embryos rescued the open-head phenotype with the same efficiency as the wild-type gene (see Fig. 4). One insertion line (UAS-*ems*^{-hp3}) showed complete rescue (100% relative rescue efficiency), and the second insertion line (UAS-*ems*^{-hp2}) showed a relative rescue efficiency of 97%. These findings indicate that the hexapeptide motif is not necessary for the action of the *ems* gene in embryonic development of the head ectoderm.

To control for the specificity of this rescue effect we again analysed the ability of *orthodenticle* (*otd*) to rescue the open-head defect of *ems* mutant embryos. Targeted misexpression of an *otd* transgene under the control of the *sca*-GAL4 driver in *ems* mutants resulted in a relative rescue efficiency of 33% which was not statistically significant (at the $p < 0.01$ level) (see Fig. 4).

The coral *emx* gene rescues embryonic head patterning defects in *Drosophila* *ems* mutants

The ability of the coral *emx-Am* gene to rescue the open head defect suggests that the coral protein can replace the fly protein in embryonic head development. *Ems* is necessary for the establishment and correct development of the ocular, antennal and intercalary segments of the developing head (Cohen and Jürgens, 1990; Dalton et al., 1989; Jürgens et al., 1984; Walldorf and Gehring, 1992). We next wanted to test whether the coral *emx-Am* gene is able to rescue the development of these three head segments. For this, we focused on the pattern of *engrailed* (*en*) expression in the head of early embryos, which in the wild-type characteristically delimit the different cephalic segments (Cohen and Jürgens, 1990; Schmidt-Ott and Technau, 1992; Schock et al., 2000). As expected, in the wild-type *en* expression domains corresponding to each cephalic segment can be clearly identified, while in *ems* mutant embryos expression of *en* is lacking in the intercalary and antennal segments and in a portion of the preantennal (ocular) region (Figs. 5A and B). In order to target the *ems* or *emx-Am* transgenes at an early embryonic time point, we used a *nos*-Gal4 driver (Janody et al., 2000, see Methods). In a wild-type background this resulted in expression of *ems* exclusively and throughout the cephalic region starting from early blastoderm until stage 11 of embryogenesis (data not shown). Targeted expression of the fly *ems* transgenes with and without a hexapeptide motif in an *ems* mutant background resulted in a significant rescue of the head segmentation defect as indicated by wild-type-like *en* expression patterns in the transgenic lines UAS-*ems9*, UAS-*ems6*, UAS-*ems*^{-hp3} and UAS-*ems*^{-hp2} (see Fig. 6). In rescued embryos, *en* expression was restored in the intercalary and antennal segments as well as in the preantennal domain which is marked by the *en* head spot (Fig. 5C). Remarkably, targeted expression of the *emx-Am* gene under the control of the *nos*-GAL4 driver in *ems* mutant *Drosophila* embryos also resulted in a significant rescue of the head segment defect as monitored by *en* expression domains (Figs. 5D and 6). In embryos of both transgenic lines (UAS-*emx-Am5* and UAS-*emx-Am6*) the *en* intercalary spot and the *en* antennal stripe were restored. The domain of the *en* antennal stripe appeared broader than in the fly rescue and was arranged in a more vertical orientation compared to the horizontal orientation of the antennal stripe in the wild type. This might reflect a slight disarrangement of the ectodermal tissue, but could also indicate a rescue of the preantennal head spot which is

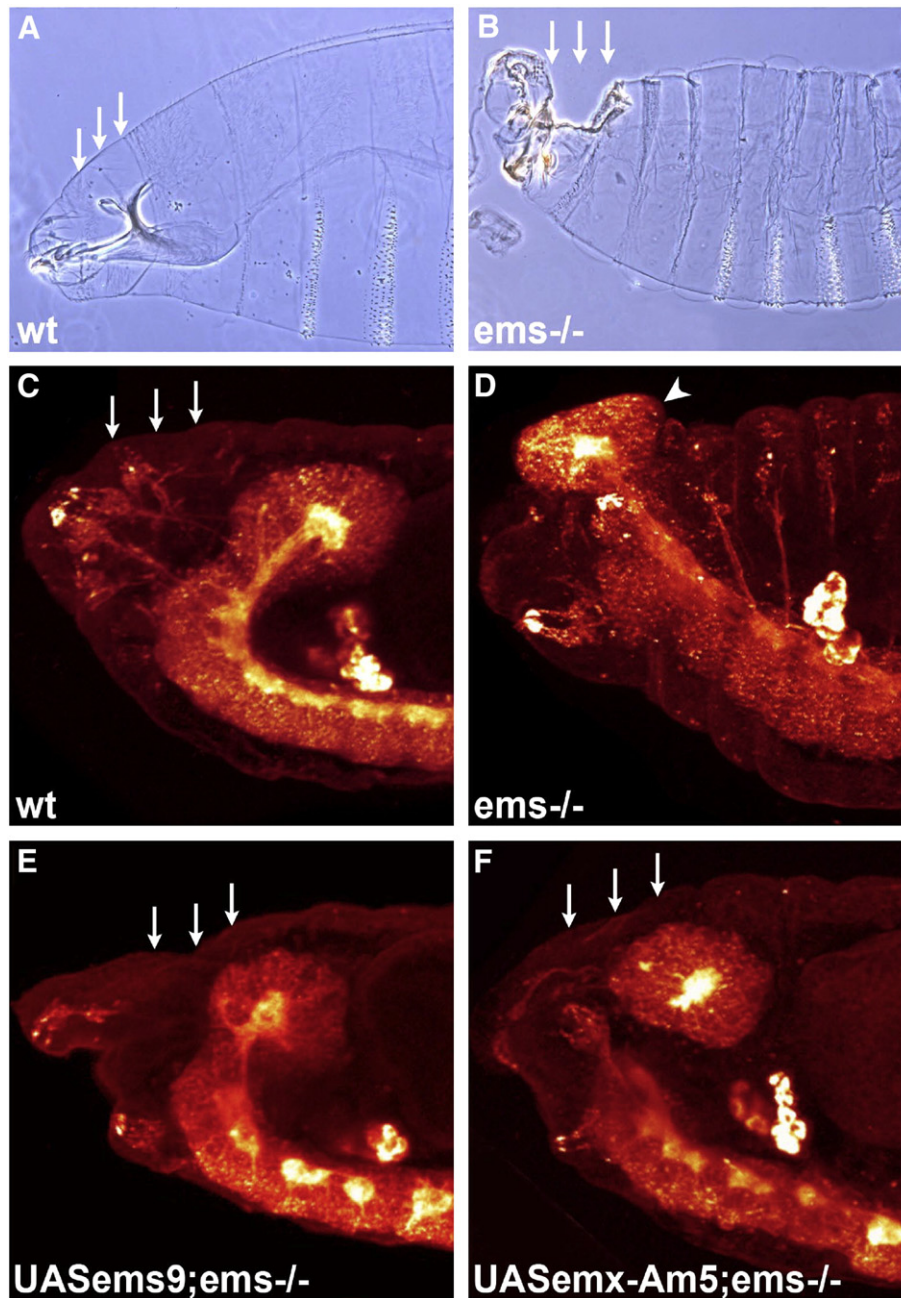


Fig. 3. The coral *emx* gene rescues the fly *ems* mutant head defect. (A and B) Cuticular preparations of late embryos; lateral views of anterior part of animal. (A) Wild type; (B) *ems* null mutant; *ems* mutants reveal a gap-like, open-head phenotype in which the anterodorsal head ectoderm is defective (arrows in B, compare to A). (C–F) Reconstructions of lateral confocal microscopy sections of stage 15 embryos; anti-HRP immunolabeling; anterior of body axis to the left. (C) Wild type; anterodorsal head ectoderm is intact (arrows) and brain hemispheres are contained within embryo proper; (D) *ems* null mutant; at the position of the defective head ectoderm the brain hemispheres protrude out of the embryo proper (arrowhead, compare position to arrows in B); (E) *ems* null mutant carrying a *sca*-GAL4/UAS-*ems9* rescue construct; and (F) *ems* null mutant carrying a *sca*-GAL4/UAS-*emx-Am5* rescue construct. Both, targeted expression of the fly *ems* gene or the coral *emx-Am* gene result in a rescue of the *ems* mutant head defect; the head ectoderm is intact (arrows in E and F) and brain hemispheres remain contained within embryo proper.

fused to the antennal stripe. Targeted expression of the *otd* gene in *ems* mutant *Drosophila* embryos did not rescue the head segment defect (Fig. 6). We conclude that in contrast to its inability to replace the *Drosophila ems* gene in embryonic brain development, the coral *emx* gene is able to replace the *ems* gene in embryonic head patterning.

Discussion

The *ems/Emx* genes are key elements of the conserved molecular genetic mechanisms that control the patterning and specification of the anteroposterior body axis and neuraxis in bilaterians. Although

the roles of these patterning genes are well understood in *Drosophila* and mouse, their evolutionary origins are equivocal (Holland, 2000; Martindale, 2005). Given that the Cnidaria are the sister group to the Bilateria (Medina et al., 2001), a comparative analysis of cnidarian vs. bilaterian *ems/Emx* gene homolog function is likely to be informative as to the origin of bilaterian patterning mechanisms. In this report we have used targeted gene expression experiments in *Drosophila* to demonstrate the cross-phylum functional conservation of the cnidarian *emx-Am* gene. Our findings indicate that *emx-Am* can substitute for *ems* in embryonic head development and rescue the open head defect as well as cephalic segmental patterning as indicated by *en* expression domains in *Drosophila ems* mutants. In contrast, the

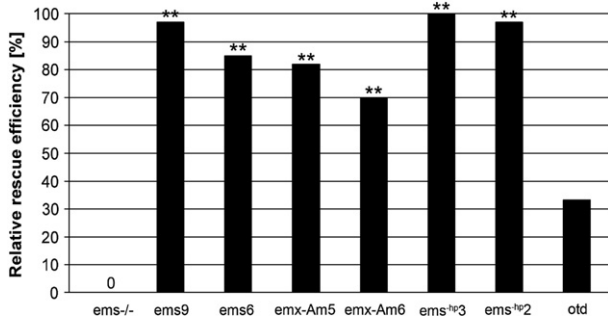


Fig. 4. Relative rescue efficiency of embryonic head defect in *ems* null mutants for different rescue constructs. The relative efficiency of rescue is shown for the fly *ems* gene (two independent insertion lines; *ems9* and *ems6*), the coral *emx-Am* gene (two independent insertion lines; *emx-Am5* and *emx-Am6*), the fly *ems* gene with a mutated hexapeptide motif (two independent insertion lines; *ems*^{hp3} and *ems*^{hp2}) and, as a negative control, for the *otd* gene. All genes were expressed in the *ems* mutant under the control of the same *sca*-GAL4 driver line. See Methods for explanation of relative rescue efficiency. A significant rescue (with $p < 0.01$; bars with two stars) resulted from targeted expression of the fly *ems* gene, the coral *emx-Am* gene and the fly *ems* gene with a mutated hexapeptide motif. Targeted expression of *otd* did not result in a significant rescue of the *ems* mutant phenotype ($p = 0.02$).

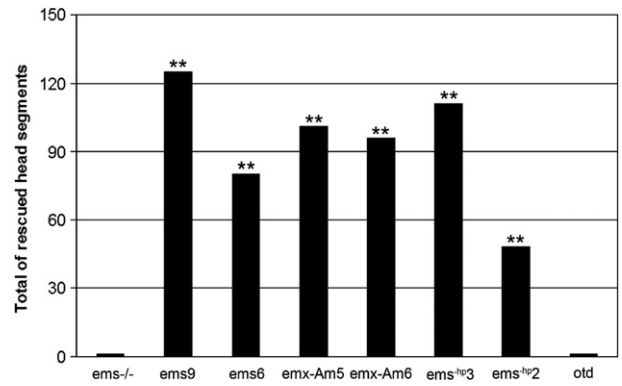


Fig. 6. Total number of rescued head segments as judged by *en* expression pattern in *ems* null mutants for different rescue constructs. The number of rescued head segments is shown for the fly *ems* gene (two independent insertion lines; *ems9* and *ems6*), the coral *emx-Am* gene (two independent insertion lines; *emx-Am5* and *emx-Am6*), the fly *ems* gene with a mutated hexapeptide motif (two independent insertion lines; *ems*^{hp3} and *ems*^{hp2}) and, as a control, for the *otd* gene. All genes were expressed in the *ems* mutant under the control of the same *nos*-GAL4 driver line. A significant rescue (with $p \leq 0.01$; bars with two stars) resulted from targeted expression of the fly *ems* gene, the coral *emx-Am* gene and the fly *ems* gene with a mutated hexapeptide motif. Targeted expression of *otd* did not rescue the *ems* mutant phenotype.

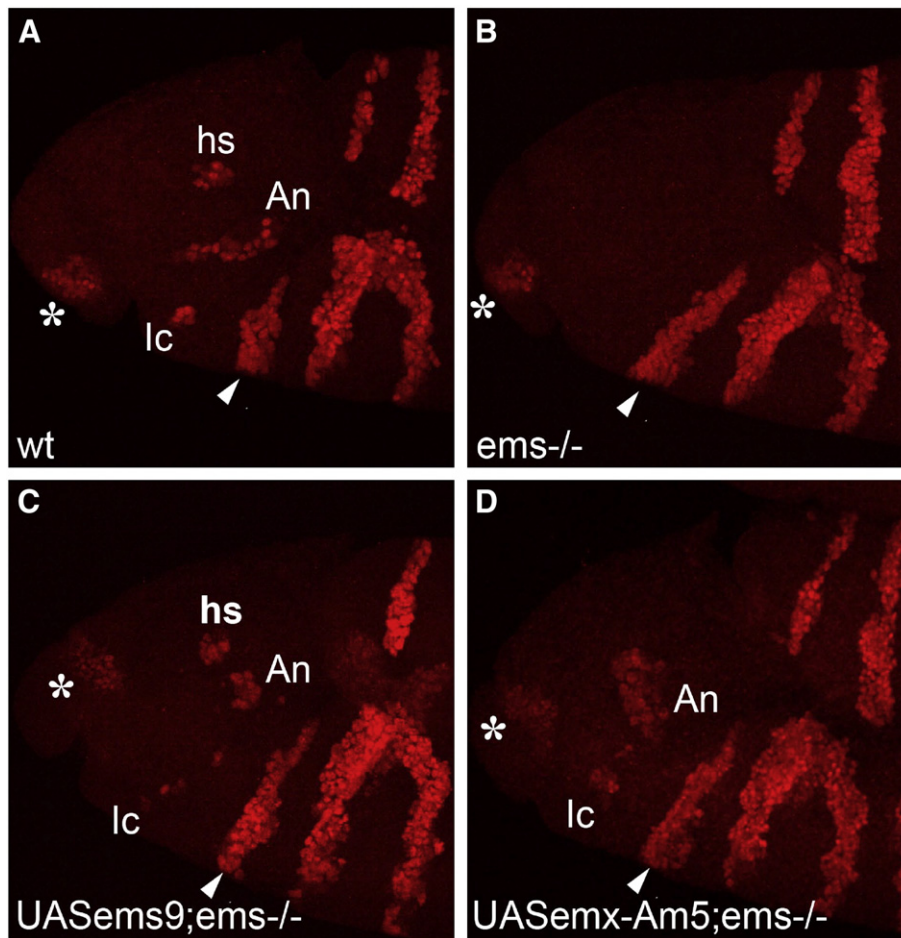


Fig. 5. The coral *emx* gene rescues the loss of specific head segments in *ems* mutant flies. (A–D) Reconstructions of lateral confocal microscopy sections of stage 11 embryos; anti-*en* immunolabeling; anterior of body axis to the left, arrowhead indicates mandibular segment, star indicates labral segment. (A) Wild type; in the head *en* is expressed in the labral, maxillary, mandibular, intercalary (lc) and antennal segment (An), a preantennal region as indicated by the *en* head spot (hs) and the labral segment. (B) *ems* null mutant; loss of *en* expression in three head segments; the preantennal head spot, the intercalary and antennal segment. *en* expression in the labral and the three gnathal segments is unaffected. (C) *ems* null mutant carrying a *nos*-GAL4/*UAS-ems9* rescue construct; *en* expression is restored in the *en* head spot, the antennal and intercalary segment. (D) *ems* null mutant carrying a *nos*-GAL4/*UAS-emx-Am5* rescue construct. Targeted expression of the coral *emx-Am* gene results in a rescue of the *ems* mutant head defect in the intercalary and antennal segment. *en* expression in the antennal segment is broader than in wild type which might be due to a fusion of the preantennal head spot with the antennal stripe.

cnidarian *emx-Am* gene cannot rescue the embryonic brain defect of *Drosophila ems* mutants, even when a hexapeptide motif of the type present in the *Drosophila ems* gene was inserted into the cnidarian gene.

Although there appears to be no simple relationship between the major longitudinal axis of the cnidarians (oral-aboral) and the anteroposterior axis in bilaterians, the ectodermal expression patterns of the coral *emx-Am* gene and the fly *ems* gene are comparable in that both are axially regionalized. The *ems* gene is expressed in a regionally restricted manner in the anterior cephalic ectoderm of the fly embryo (Dalton et al., 1989; Walldorf and Gehring, 1992). The *emx-Am* gene is regionally expressed in the ectoderm in the aboral half to two thirds of the planula larva (de Jong et al., 2006). The cross-phylum rescue experiments reported here imply that in addition to their comparable expression patterns, these genes are functionally related. Implied conservation of function of Ems/Emx proteins has previously been shown between insects and vertebrates and between insects and nematodes (Aspöck et al., 2003; Hartmann et al., 2000). These findings suggest that functional conservation of the Ems/Emx proteins might predate the origins of the Bilateria. The *Acropora* to *Drosophila* cross-phylum rescue experiments presented here now suggest that the functional aspects of the proteins utilized in *Drosophila* to mediate anterior cephalic development has been conserved by Ems/Emx proteins from the very beginning of eumetazoan evolution to the present. This is remarkable not only because of the large evolutionary distance between cnidarians and insects but also because the former do not display cephalization. Furthermore, the fact that these proteins act as transcription factors implies that a subset of their downstream targets, be these genes or protein cofactors, may also have been evolutionarily conserved. Thus, the simplest explanation of the cross-phylum rescue data presented here is that at least some components of the anteroposterior specification systems known from Bilateria coexisted in the common ancestor of modern cnidarians and bilaterians but reached their present sophistication in bilaterian cephalization only after evolutionary separation and divergence (Hobmayer et al., 2000).

The nervous system-specific expression of both cnidarian *emx-Am* (de Jong et al., 2006) and *Drosophila ems* genes (Hartmann et al., 2000; Hirth et al., 1995; Urbach and Technau, 2003; Younossi-Hartenstein et al., 1996) could indicate that the two genes might also have comparable and evolutionarily conserved roles in nervous system development. However, our cross-phylum rescue experiments provide no support for this. Thus, while a general ectodermal function may be conserved between the *Acropora* and *Drosophila ems/Emx* genes, the lack of rescue in these experiments suggests that the roles of these genes in central nervous system development may not be conserved. It is highly likely that the emergence of the first nervous system predated the evolutionary divergence of Bilateria and Radiata, which is likely to have been in late pre-Cambrian time, given the fact that neurons and nervous systems are present in both. However, in terms of complexity there are marked differences between the two animal groups. Cnidarian nervous systems are relatively simple, indeed, the nerve nets of sessile anthozoan such as corals are the simplest known nervous systems of extant animals (Bullok and Horridge, 1965; Mackie, 2004). In contrast, the central nervous system of an insect like *Drosophila* is a highly complex structure composed of hundreds of thousands of neurons that are interconnected in exquisitely organized ganglionic neuropil structures (Burrows, 1996; Strausfeld, 1976). Failure of the *Acropora* protein to rescue the fly brain phenotype may therefore reflect the greater number of regulatory interactions that must be mediated by the *Drosophila* protein in constructing a highly complex centralized nervous system. Indeed it seems reasonable to assume that many of these interactions, protein–DNA or protein–protein, evolved in the lines leading to animals with complex brains after their separation from the lines leading to animals with simple nerve nets.

Surprisingly, in our experiments the hexapeptide motif was not required for rescue of either the cephalic ectoderm or the embryonic nervous system phenotypes, suggesting that it might be redundant for normal Ems function. There are precedents for this. For example, mutation of the AbdA hexapeptide does not appear to alter its binding site specificity and this motif is dispensable with respect to AbdA epidermal functions (Merabet et al., 2003). Whilst the classical function of the hexapeptide is thought to be in modification of the binding site specificity of Hox proteins by recruiting the Exd/Pbx proteins (Chan et al., 1994, 1995; Johnson et al., 1995), other findings demonstrate this hexapeptide-mediated interaction with cofactors has additional complexity. For example, in the Hox protein Lab, the hexapeptide appears to inhibit Lab function by inhibiting DNA binding. This is because Lab proteins, in which this motif was deleted or mutated, bind DNA with higher affinity than do proteins with an intact hexapeptide. Moreover, this hexapeptide mutant of Lab had an increased ability to activate transcription in vivo (Chan et al., 1996). In this case, Exd appears to induce a conformational change in Lab, thereby overcoming the negative function of the hexapeptide. If the hexapeptide of the Ems protein functions similarly, then mutating the hexapeptide should increase the ability of Ems to activate its transcriptional targets. This would explain why rescue experiments with *ems* transgenes with or without a hexapeptide lead to similar results. Alternatively, the fact that the Ems hexapeptide is dispensable in our rescue experiments might be an artefact of driving high-level pan-neural expression via the *sca-GAL4* line. In the case of Ubx, the hexapeptide appears to provide an interface for the assembly of transcription repression complexes; Ubx lacking the hexapeptide can form repression complexes, but much higher concentrations are required (Tour et al., 2005).

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