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Neuronal function of *Tbx20* conserved from nematodes to vertebrates

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Abstract

The Tbx20 orthologue, *mab-9*, is required for development of the *Caenorhabditis elegans* hindgut, whereas several vertebrate Tbx20 genes promote heart development. Here we show that Tbx20 orthologues also have a role in motor neuron development that is conserved between invertebrates and vertebrates. *mab-9* mutants exhibit guidance defects in dorsally projecting axons from motor neurons located in the ventral nerve cord. *Danio rerio* (Zebrafish) tbx20 morphants show defects in the migration patterns of motor neuron soma of the facial and trigeminal motor neuron groups. Human *TBX20* is expressed in motor neurons in the developing hindbrain of human embryos and we show that human *TBX20* can substitute for zebrafish tbx20 in promoting cranial motor neuron migration. *mab-9* is also partially able to rescue the zebrafish migration defect, whereas other vertebrate T-box genes cannot. Conversely we show that the human *TBX20* T-box domain can rescue motor neuron defects in *C. elegans*. These data suggest the functional equivalence of Tbx20 orthologues in regulating the development of specific motor neuron groups. We also demonstrate the functional equivalence of human and *C. elegans Tbx20* T-box domains for regulating male tail development in the nematode even though these genes play highly diverged roles in organogenesis.

Keywords: T-box; Axon guidance; Neuron; C. elegans; Zebrafish

Introduction

T-box transcription factors, unified by a common DNA binding domain, or T-box, are an important group of developmental control genes (Bollag et al., 1994; Papaioannou, 2001). These factors are found in all metazoan phyla and have considerable medical significance, as T-box gene mutations are associated with a number of human developmental syndromes. At least 18 T-box genes have been identified in vertebrates, which can be broadly organised into 5 subfamilies: T (includes *T* and *Tbx19*), Tbx1 (includes *Tbx1*, *Tbx10*, *Tbx15*, *Tbx18*, *Tbx22* and *Tbx20*), Tbx2 (includes *Tbx2*, *Tbx3*, *Tbx4* and *Tbx5*), Tbx6 (includes *Tbx6* and *Tbx16*) and Tbr1 (includes *Tbr1*, *Eomesodermin* and *Tbx21*) (Naiche et al., 2005). Invertebrates differ widely in their T-box gene complements, with *Drosophila melanogaster* containing 8 recognizable T-box genes and *Caenorhabditis elegans* containing 21. While the Drosophila T-box genes tend to have obvious vertebrate counterparts, it is not so easy to assign the *C. elegans* genes to particular subfamilies. Indeed, there are only 4 cases where orthology is clear: there is a representative of the Tbx20 branch of the Tbx1 subfamily (*mab-9*; Woollard and Hodgkin, 2000), a member of the Tbx2 subfamily (*tbx-2*, required for pharyngeal and foregut development; Roy Chowdhuri et al., 2006; Smith and Mango, 2007), a second member of the Tbx1 subfamily (*mls-1*, isolated in a screen for mutants with defects in muscle cell fate determination; Kostas and Fire, 2002), and an orthologue of the divergent ascidian

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as-T2 gene (*tbx-7*, function unknown). There are no members of the T, Tbx6 or Tbr1 subfamilies in *C. elegans*. Given that representatives of these families are present in Drosophila (and that Drosophila and Caenorhabditis are both ecdyzosoans) the most likely explanation is that these genes were present in a common ancestor but have been lost in the worm. It is also possible that a higher evolutionary rate in the nematode genome has obscured clear orthologous relationships.

One of the most highly conserved of the *C. elegans* T-box genes is the Tbx20 orthologue *mab-9*, making the Tbx20 subfamily one of the very few pan-metazoan orthologous groups of T-box genes. In vertebrates, as well as in Drosophila, Tbx20 genes are implicated in heart development (Miskolczi-McCallum et al., 2005; Stennard et al., 2005). Homozygous null *Tbx20* mice die at mid-gestation due to defective hearts, which fail to loop and which display many morphological and molecular abnormalities, including a severely compromised cardiac transcription programme, reduced expansion of cardiac progenitors and a block to heart chamber differentiation (Stennard et al., 2005). As well as having a major role in early heart specification, experiments with heterozygous mutant mice have also revealed a requirement for *Tbx20* in adult heart integrity and contractile function (Stennard et al., 2005). Heart defects have also been reported in zebrafish embryos treated with tbx20-specific morpholinos (Szeto et al., 2002). In Drosophila, there are two Tbx20 orthologues, *midline* and *H15*, and both of these genes have been shown to be required for heart specification and morphogenesis (Miskolczi-McCallum et al., 2005). Although the Drosophila "heart" is a simple tube that pumps hemolymph, and the vertebrate heart is a multi-chambered complex organ, it is clear that the genes driving heart cell fate determination in vertebrates and Drosophila are conserved. This strongly supports the hypothesis that vertebrate and arthropod hearts are homologous structures.

In *C. elegans*, *mab-9* mutants were originally isolated by virtue of their male tail defects (Hodgkin, 1983). *mab-9* is required for the correct specification of cell fate in the hindgut (rectum) such that in the absence of MAB-9 the male-specific blast cell B takes on the fate of its anterior neighbour Y and likewise, F takes on the fate of U (Chisholm and Hodgkin, 1989). As B and F blast cell fates are absolutely required to build the internal structures of the male tail (in particular the sclerotised spicule used for sperm transfer during mating), *mab-9* mutant males are incapable of mating and have gross morphological abnormalities. Hermaphrodites also have an abnormal hindgut because B and F are rectal epithelial cells that have a structural role in hermaphrodites, and therefore they tend to be constipated (Woollard and Hodgkin, 2000). There is no obvious analogy between heart and rectum, despite the fact that both organs are biological oscillators (Iwasaki and Thomas, 1997), therefore it would seem that *Tbx20* has acquired novel functions over the course of evolution associated with the generation of morphological diversity. It has been argued that the *C. elegans* pharynx may be the analogue of the vertebrate heart, as both function as pumps that do not require nervous system input and which express distinct sets of muscle proteins (Avery and Horvitz, 1989; Haun and Okkema, 1998). Indeed, the vertebrate heart specification gene *nkx2.5* can substitute for the *C. elegans* pharynx development gene *ceh-22* (Haun and Okkema, 1998). However, we could find no obvious pharyngeal role for *mab-9*. It is intriguing, however, that another T-box family member, *tbx-2*, is known to be required for both *C. elegans* pharynx formation and vertebrate heart development (Harrelson et al., 2004; Cai et al., 2005; Roy Chowdhuri et al., 2006; Smith and Mango, 2007).

Intriguingly, *mab-9* mutants are also uncoordinated (Unc), especially for backwards movement, suggesting that there may be other sites of *mab-9* function in *C. elegans*. The backwards Unc phenotype was initially thought to be a secondary consequence of the hindgut abnormalities, but the observation that *mab-9* is expressed in the nervous system (Woollard and Hodgkin, 2000), together with the fact that alleles of *mab-9* were isolated in a screen for mutants with defects in axon guidance (Huang et al., 2002), points to a more specific role. This is interesting in light of the fact that Tbx20 in vertebrates is expressed in the nervous system in addition to the heart (Ahn et al., 2000). For example, tbx20 in zebrafish is expressed in cranial motor neurons and in mouse *Tbx20* is expressed in the hindbrain during motor neuron cell migration and additionally in dorsally directed axons (Ahn et al., 2000; Kraus et al., 2001; Meins et al., 2000). Furthermore, there is new evidence to suggest defects in motor neuron differentiation and migration in *Tbx20* knockdown mice (Song et al., 2006; Takeuchi et al., 2005).

In this report we have examined the function of *Tbx20* in *C. elegans* and vertebrates in order to explore whether *Tbx20* orthologues share an ancient conserved function in nervous system development. In addition, we tested *C. elegans* and vertebrate *Tbx20* genes for functional conservation in cross-species rescue experiments to test whether the orthologous genes are functionally interchangeable despite their different roles in organ development. *In situ* hybridization experiments in human embryos and in equivalent developmental stage mouse embryos as well as in zebrafish reveal *Tbx20* expression within motor neurons associated with cranial nerve V and VII. To gain insight into the possible function of *Tbx20* in vertebrate motor neurons, tbx20 specific morpholinos were utilized to disrupt tbx20 expression in zebrafish. Injection of tbx20 morpholino affected the migration and medio-lateral position of the cranial nerve nuclei nV and nVII in a dose-dependent fashion, indicating that tbx20 is essential for correct motor neuron migration in zebrafish. In *C. elegans* we find that *mab-9* mutants display specific axon guidance defects in all the subsets of motor neurons in which *mab-9* expression is observed. Therefore, *Tbx20* is required in both vertebrates and invertebrates for aspects of motor neuron migration. Finally, we show that the human *TBX20* T-box gene is capable of rescuing *Tbx20* associated phenotypes in both zebrafish and *C. elegans*, thus suggesting that *Tbx20* orthologues are functionally interchangeable across a wide range of metazoan phyla.

Materials and methods

C. elegans strains, genetics and microscopy

All strains used were derived from the wild-type (WT) Bristol strain N2. Routine maintenance of worms and genetic manipulations such as crosses were