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The bHLH transcription factor hand is required for proper wing heart formation in *Drosophila*

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ABSTRACT

The Hand basic helix–loop–helix transcription factors play an important role in the specification and patterning of various tissues in vertebrates and invertebrates. Here, we have investigated the function of Hand in the development of the *Drosophila* wing hearts which consist of somatic muscle cells as well as a mesodermally derived epithelium. We found that Hand is essential in both tissues for proper organ formation. Loss of Hand leads to a reduced number of cells in the mature organ and loss of wing heart functionality. In wing heart muscles Hand is required for the correct positioning of attachment sites, the parallel alignment of muscle cells, and the proper orientation of myofibrils. At the protein level, α-Spectrin and Dystroglycan are misdistributed suggesting a defect in the costameric network. Hand is also required for proper differentiation of the wing heart epithelium. Additionally, the *handC-GFP* reporter line is not active in the mutant suggesting an autoregulatory role of Hand in wing hearts. Finally, in a candidate-based RNAi mediated knock-down approach we identified Daughterless and Nautilus as potential dimerization partners of Hand in wing hearts.

Introduction

Hand proteins are members of the Twist-family of class II basic helix–loop–helix (bHLH) transcriptional regulators implicated in the development of various tissues in invertebrates as well as in vertebrates reviewed in (Barnes and Firulli, 2009). Higher vertebrates possess two paralogous *hand* genes, *hand1* and *hand2*, which are expressed in many different cell types and are involved in the development of the neural crest, placenta, limbs, neurons, and heart (Cross et al., 1995; Cserjesi et al., 1995; D'Autreaux et al., 2007; Doxakis et al., 2008; Howard et al., 2000; Riley et al., 1998; Srivastava et al., 1995; Srivastava et al., 1997). Mostly due to defects in cardiac development, *hand* null mutations or loss of function cause embryonic lethality in zebrafish, chick and mouse (Firulli et al., 1998; Riley et al., 1998; Srivastava et al., 1995; Srivastava et al., 1997; Thomas et al., 1998; Yelon et al., 2000). In contrast, *Drosophila* harbors only a single *hand* gene and null mutants survive at significant percentage into adulthood. Mutant animals have either no or reduced lymph glands and display late morphogenesis defects in cardiac tissue and the gut (Han et al., 2006; Lo et al., 2007). Adult individuals lacking *hand* reveal a significantly shortened lifespan, most likely due to a failure in food uptake caused by midgut blockage by excessive development of ectopic peritrophic membrane and due to malformation of the dorsal vessel (Lo et al., 2007). In vertebrates as well as in *Drosophila*, *hand* genes are known to be expressed in myogenic cells, e.g., in cardiomyocytes of the early heart field in mice embryos and in all cardiomyocytes from embryogenesis until adulthood in *Drosophila*. Hand is also found in vertebrate smooth muscles and in *Drosophila* circular visceral muscles. Until now, the only example that demonstrates a role for Hand in somatic muscle development comes from the nematode *C. elegans* (Fukushige et al., 2006). Homozygous *hnd-1* (*hand*) or *hlh-1* (*myoD*) mutant worms are viable and fertile. In contrast, a significant percentage of *hnd-1*/*hlh-1* double mutant worms die during embryogenesis with severe muscle differentiation defects. Genetic interaction assays let the authors conclude that striated muscle differentiation in *C. elegans* depends on partially redundancy of *hlh-1*, *hnd-1*, which act together with *unc-120* (SRF-related). This result is intriguing because it indicates a potentially conserved role of Hand also in somatic muscles cells. In *Drosophila*, so far, the only somatic muscles that express *hand* are the muscles found in wing hearts. These organs are located at the entry point of the posterior wing veins into the scutellum and act as suction pumps that draw hemolymph from the wings into the body cavity. Tightly

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associated with these muscle cells is an epithelial cell layer that acts as a back-flow valve and separates the muscle cells from the body cavity (Lehmacher et al., 2009; Tögel et al., 2008; Tögel et al., 2013). Our work shows that the transcription factor Hand is required for the establishment of the proper cell number in the mature organs. Furthermore, it is essential for the internal and external organization of the wing heart muscle cells, including the orientation of myofibrils, the position of attachment sites in the epidermis, and the overall alignment of muscle cells. However, initial steps of organ formation are unaffected in *hand* null mutants. Loss of *hand* has no impact on the specification of embryonic wing heart precursors or on their proliferation during larval and pupal stages or on the formation of syncytial muscle cells. The observed defects in sum cause insufficient wing heart activity, which ultimately leads to a wing maturation defect and flightlessness of the animals. Our results presented herein provide the first example of *hand* function in *Drosophila* muscles with somatic characteristics, thereby supporting the hypothesis that Hand proteins might play a conserved role in the somatic mesoderm (Fukushige et al., 2006).

Material and methods

Drosophila strains

w^{1118} was used as wild-type. All *handC-GFP* reporter lines used herein were previously described (Sellin et al., 2006; Tögel et al., 2008). The $hand^{173}$ null mutant allele was kindly provided by Frasch (Lo et al., 2007). Gal4 drivers were: *prc-Gal4* (Chartier et al., 2002) from M. Semeriva, *Mef2-Gal4* from H. Nuygen, and *handC-Gal4* (our laboratory). *UAS-P35, UAS-mCD8::GFP*, *Df(3R)Exel6195* (*nau*, BL7674), *Df(3R)BSC490* (*nau*, BL24994), *Df(2R)ED2098* (*cag*, BL9277), *Df(2R) BSC314* (*cag*, BL24340), *Df(2L)BSC342* (*da*, BL24366), *Df(2L)BSC209* (*da*, BL9637) and nej^{3} (BL3729) were obtained from the Bloomington stock center. RNAi lines for knock-down of *cag*, *CG7911*, *da*, *hand*, *Hey*, *Lmpt*, *nau*, *nej*, *Nlp*, *Pka-C1*, *Pkc53E*, *pnr*, *PP2A-B′* were obtained from the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007).

Immunohistochemistry and histology

Primary antibodies used were mouse anti-Arm 1:3 (N2 7A1, supernatant, DSHB), anti-Dlg 1:3 (4F3, supernatant, DSHB), anti-βPS-Integrin 1:3 (CF.6G11, supernatant, DSHB), anti-Prc 1:2 (EC11, supernatant, DSHB), anti-α-Spectrin 1:30 (3A9, supernatant, DSHB), and rabbit anti-Dg 1:200 (Deng et al., 2003). Rabbit anti-GFP 1:1000 was obtained from Abcam (ab6556). Secondary antibodies (Dianova) were used 1:100 for Cy2-conjugated, and 1:200 for Cy3-conjugated antibodies. ATTO-655 (ATTO-Tec) and TRITC (Fluka) conjugated phalloidin was used 1:20 and 1:200-1:300, respectively, to stain actin filaments. Histological semi-thin sections and transmission electron microscopy was performed as previously described (Lehmacher et al., 2009).

Time lapse studies

For imaging of pupae, a small glass bottom dish for upright microscopy was used upside down. The lid of the dish was glued as bottom to a microscope slide, while the bottom with the cover slip was used as top. After removal of the puparium with fine forceps, the pupa was adhered with a drop of Voltalef S10 oil at its dorsal side in the region of the thorax to the cover slip from the inside. A commercial rubber band was used to tightly fit the bottom into the lid. This allowed for the use of oil immersion objectives and prevented dehydration of the pupa. Additionally, moistened stripes of filter paper were put inside the dish to ensure sufficient ambient humidity (Tögel et al., 2013). Time-lapse images were recorded at about eight-minute intervals for at least 30 h using a 25 × (NA 0.8) planapochromatic objective on a Zeiss 5 Pa laser scanning confocal microscope. Movies were created from maximum projections of each obtained stack.

Temperature shift experiment

Fly stocks were reared at room temperature. After selection of the appropriate virgin females and males, the flies were put in vials and immediately transferred to either 18 °C or 29 °C to ensure that even mating takes place at the desired temperature. Offspring were then kept at one temperature till they reached a specific developmental stage and were then transferred to the other temperature until eclosion took place. For staging of pupae at different temperatures, the table from Atreya and Fernandes was used (Atreya and Fernandes, 2008). In general, development takes twice as long at 18 °C than at 29 °C.

Preparations and image analysis

Pupae were fixed with their puparium on double sided scotch tape in lateral position and cut in halves with a razor blade. The dorsal half was then immediately transferred to PBS for preparation. The internal tissues as well as large parts of the thoracic musculature were removed with fine forceps. Thereafter pupae were transferred to a reaction tube for fixation and subsequent antibody staining as described in (Monier et al., 2005). For RT-PCR, five pupae carrying the *hand*C-GFP construct were dissected as mentioned above. In addition, all green fluorescing tissues were carefully removed except for the wing heart progenitors in the thorax. The dorsal carcasses were then used for total RNA preparation (PeqGold total RNA kit, peqlab). Confocal images were collected with a Zeiss 510 Meta or a Zeiss 5 Pa laser-scanning microscope. For epifluorescence and bright field microscopy, a Leica MZ16FA stereomicroscope or a Zeiss Axioscope 2 microscope was used, both equipped with UV-illumination and a digital camera. Image capturing was performed with the software package AnalySIS (SIS software) or Axiovision (Zeiss).

RT-PCR and northern blot

Total-RNA (PeqGold total RNA kit, peqlab) from dissected tissues was treated with DNase I (Invitrogen) according to the manufacturer's instructions and used as a template for cDNA synthesis (AMV First Strand cDNA Synthesis Kit for RT-PCR, Roche). Primer pairs for amplifying the basic amplicons were: atgtttaagaattccgttgcc (*hand* forward, fw), gatgcccaaacatcttgtggc (*hand* reverse, rv), tgctggtggctcaacgtcaac (*daughterless* fw), gcccattccgagtccgtccgc (*daughterless* rv), atgcgagtgcgggaaatggtg (*nautilus* fw), gctcaaactgggcgccaggtt (*nautilus* rv), ccggcggcggcaacggatccg (*nejire* fw), aacggctcctccgccgccagc (*nejire* rv), atggaggtaatccagtcccag (*cag* fw), gcctcctcggtggaatagata (*cag* rv). Nested primers were: ttgacgtgcgaatactcaacc (*hand* fw), caacccgtgcggcccttggtc (*hand* rv), actcgctgcaacaaaaggaat (*daughterless* fw), ctggtgaacctgctgctgctg (*daughterless* rv), gagcggcttccatctcagcca (*nautilus* fw), gtcgccatcgcgtgtggtact (*nautilus* rv), gaacgggtggcgacgacggca (*nejire* fw), tccaacgccaccagctcctgc (*nejire* rv), gagcgaaacaagctatcggtt (*cag* fw), ggctctatcagagtcacgggg (*cag* rv). Amplification products of the expected size (about 450 bp) were cloned into the pGEM-T easy vector (Promega) and sequenced. Northern blots were conducted with total-RNA (15 μg/lane) according to standard protocols at a hybridization temperature of 67 °C.

Yeast two-hybrid assay

Vectors encoding either the Gal4 binding domain (Gal4-BD, based on pGBK-T7), the Gal4 activation domain (Gal4-AD, based on pGAD-T7), or the distinct fusion constructs were transformed into the