Insertional mutagenesis screening identifies the *zinc finger homeodomain 2* (*zfh2*) gene as a novel factor required for embryonic leg development in *Tribolium castaneum*

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Abstract The genetic control of leg development is well characterized in the fly *Drosophila melanogaster*. These control mechanisms, however, must differ to some degree between different insect species to account for the morphological diversity of thoracic legs in the insects. The legs of the flour beetle *Tribolium castaneum* differ from the *Drosophila* legs in their developmental mode as well as in their specific morphology especially at the larval stage. In order to identify genes involved in the morphogenesis of the *Tribolium* larval legs, we have analyzed EGFP enhancer trap lines of *Tribolium*. We have identified the *zfh2* gene as a novel factor required for normal leg development in *Tribolium*. RNA interference with *zfh2* function leads to two alternative classes of leg phenotype. The loss of a leg segment boundary and the generation of ectopic outgrowths in one class of phenotype suggest a role in leg segmentation and segment growth. The malformation of the pretarsal claw in the second class of phenotype suggests a role in distal development and the morphogenesis of the claw-shaped morphology of the pretarsus. This suggests that *zfh2* is involved in the regulation of an unidentified target gene in a concentration-dependent manner. Our results demonstrate that enhancer trap screens in *T. castaneum* have the potential to identify novel gene functions regulating specific developmental processes.

Keywords *Tribolium castaneum* · Leg morphogenesis · Appendage diversity · Genetic control · Insect morphology

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Introduction

The genetic control of leg development has been studied in some detail in the fly *Drosophila melanogaster*. The primordia of the legs are specified in the *Drosophila* embryo by Wingless (Wg) signaling (e.g., Cohen et al. 1993). The leg-promoting function of Wg is counteracted on the dorsal side by Decapentaplegic (Dpp) signaling and on the ventral side by Egfr signaling, leading to the specification of the leg primordia on the ventral–lateral side of the body (Kubota et al. 2000, 2003; Goto and Hayashi 1997). The development of the proximal-distal axis of the legs is orchestrated by Wg signaling together with Dpp signaling. These two signaling pathways activate several target genes [e.g., *Distal-less* (*Dll*), *dachshund* (*dac*)] in a concentration-dependent manner (e.g., Lecuit and Cohen 1997). These target genes pattern the leg along its proximal–distal axis and coordinate the formation of the leg segment boundaries and the growth of the leg segments (podomeres) by instructing the Notch signaling pathway to be active at the future leg joints (Rauskolb and Irvine, 1999).

Many aspects of the genetic control of leg development in *Drosophila* are conserved in other arthropod species.

For example, the expression and function of the *Dll* gene (e.g. Abzhanov and Kaufman 2000; Prpic et al. 2003; Schoppmeier and Damen 2001; Beermann et al. 2001), and the control of leg segmentation and podomere growth by Notch signaling (Prpic and Damen 2009) are highly conserved. On the other hand, the morphology of arthropod legs differs quite substantially between species. This indicates that other aspects of the *Drosophila* leg developmental mode are not conserved and are responsible for the morphological differences between the species. The identification of novel leg genes in species other than *D. melanogaster* can serve as a first step towards a better understanding of the developmental basis of leg diversity in the arthropods. We are studying leg development in the red flour beetle *Tribolium castaneum*. *Tribolium* differs from *Drosophila* in the mode of leg development and in the specific morphology of the legs. Especially in the larva, the legs deviate from the insect typical composition as they lack a separate tibia, lack separate tarsal segments, and have an undivided, claw-shaped pretarsus.

In order to identify genes that play a role in *Tribolium* leg development we took advantage of enhancer trap lines produced during the GEKU insertional mutagenesis screen conducted in a collaborative effort by four laboratories in Germany and the USA. Details about this screen will be published elsewhere (Trauner et al., manuscript in revision). We have analyzed the enhancer trap lines and have searched specifically for lines expressing the reporter gene [enhanced green fluorescent protein, (EGFP)] in the thoracic legs of *Tribolium*. This paper reports on the gene *zinc finger homeodomain 2* (*zfh2*) that was identified in this process and that was shown to have two striking alternative phenotypes in the legs, linking it to distal development as well as leg segmentation.

Materials and methods

Characterization of the transposon insertion site

Genomic DNA from imaginal (adult) beetles was isolated by maceration and DNA precipitation. An aliquot of the genomic DNA was digested with Bsp143I, and the fragments were circularized by ligation. Inverse PCR was performed on this template using piggyBac specific primers. The obtained fragments were sequenced directly and the sequence was then compared with the fully sequenced genome of *T. castaneum* by BLAST similarity analysis (Altschul et al. 1997). The sequence obtained from the region flanking the transposon insertion site has been deposited with the EMBL nucleotide database (accession number FN395289).

Molecular cloning, parental RNA interference (RNAi) and whole-mount in situ hybridization

Based on the published genome sequence of *T. castaneum* (*Tribolium* Genome Sequencing Consortium 2008), a fragment of *zfh2* was amplified by PCR from cDNA (prepared from embryos aged 0 to 48 hours) using the primers G03891_fw (TTA GCA TCG GGA AGA CTG GGA) and G03891_rev (GGC TTG GTT GTA GGC CAT GTC). The obtained fragments were cloned and sequenced according to standard methods. Probes for whole-mount in situ hybridization and double-stranded RNA were synthesized based on the fragment isolated with the primers given previously. Whole-mount in situ hybridization was performed according to the published protocol (Prpic et al. 2001). Parental RNAi was performed according to the previously published protocol (Bucher et al. 2002) except that imaginal not pupal beetles were used for injection.

Results

Analysis of the enhancer trap line Goe-08115

The screen for enhancer trap lines that express *EGFP* in the legs identified, among other lines, the line Goe-08115. In this line, EGFP protein is detected in embryos shortly before hatching (Fig. 1a) and in larvae (Fig. 1b). *EGFP* is expressed in the claw and distal end of the tibiotarsus, and in a dorsal spot spanning the trochanter and proximal femur of all thoracic legs. There is also expression in the mandible. In situ hybridization with an *EGFP* probe revealed that EGFP mRNA is already expressed in elongating embryos (Fig. 1c). The embryonic mRNA pattern is similar to the later protein pattern and consists of a distal domain and a proximal dorsal spot in all thoracic legs and an expression domain in the mandible.

Inverse PCR on genomic DNA prepared from this line and subsequent sequencing identified 324 bp of flanking sequence (see Materials and methods) of the inserted transposon. This sequence matched to the genome sequence of *T. castaneum* (*Tribolium* Genome Sequencing Consortium 2008) on chromosome 3 from base pairs 24899583 to 24899906 (refering to genome release 2005 Oct 11) thus identifying the insertion site of the transposon in the genome. Genboree analysis shows a number of predicted genes (GLEAN gene prediction algorithm) in the vicinity of the transposon insertion site. In the area of 100 kb left and 100 kb right of the transposon insertion site, 15 genes are predicted by GLEAN (Fig. 1d). We have searched for a *Drosophila* homolog of each of these predicted genes and have tried to predict the possible function of these genes in order to identify the most promising candidate gene for the
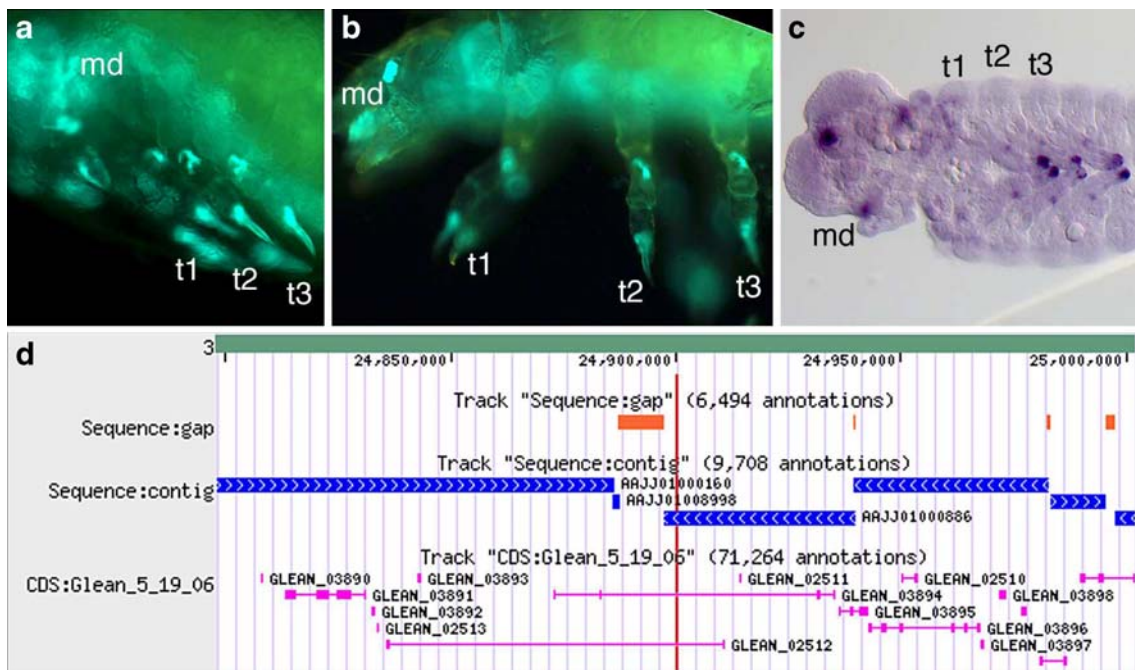
Fig. 1 Localization of the transposon insertion site in the *Tribolium* line Goe-08115. **a**, **b** Detection of EGFP fluorescence (*bright green*) in late embryonic stages (**a**) and larvae (**b**). **b** Detection of EGFP mRNA (*dark purple*) in an embryo at the mid-germ band retraction stage. **d** Screen shot of the genome browser window (at http://www.hgsc.bcm.tmc.edu) identifying annotated details around the transposon insertion site. The frame of the screen shot is centered on the insertion site [i.e. the insertion site is in the middle of the image (*red vertical line*)]. The green bar at the top gives the base pair count on chromosome three of *T. castaneum*. The *blue bar* gives the continuous sequence data information revealing several small gaps (*orange color*) that have been bridged by genome-mapping data during assembly. The *pink bars* at the bottom give the locations of the GLEAN predicted genes around the transposon insertion site. The analysis of these GLEANs is shown in Table 1. Abbreviations: *md* mandible, *t1–t3* thoracic leg 1 to 3. The anterior end of the animal is to the *left* in panels a–c

enhancer trap (Table 1). Seven GLEAN predicted genes did not produce a significant match in the GenBank database. This could indicate that these genes are *Tribolium*-specific or that these predictions are erroneous. The remaining GLEAN-predicted genes correspond to homologous genes in *Drosophila*, three of which are of unknown function while the remaining genes code for proteins predicted to be cytoplasmic, membrane-bound, or spliceosomal (Table 1). Only a single GLEAN gene, *GLEAN_03891*, is predicted to encode a nuclear protein containing zinc fingers and homeodomains. BLAST comparison of the sequence of *GLEAN_03891* with the *Drosophila* genome identifies the putative *Drosophila* ortholog *zinc finger homeodomain 2* (*zfh2*; CG1449). This orthology assessment has also been corroborated by reciprocal BLAST analysis comparing the sequence of CG1449 to the *T. castaneum* genome.

Expression of *zfh2* from *T. castaneum*

We have then isolated the *zfh2* gene from *Tribolium* and analyzed its embryonic expression pattern. During early germband development *zfh2* is expressed in the head lobes and in a separate domain in the growth zone at the posterior end (Fig. 2a, b). In the elongating germ band a strong expression in the entire ventral nervous system appears and the expression in the brain becomes more restricted to several cell groups (Fig. 2c, d; Fig. 3a, b). The developing appendages all express *zfh2*. In the antenna and the thoracic legs, *zfh2* is expressed strongly and homogeneously in the distal half of the appendages (Fig. 3a–c). In the labial and maxillary appendages *zfh2* expression is restricted to the distal parts of the palps. The labral buds show a distal spot of *zfh2* expression, and the mandibles express the gene weakly along their ventral edge (Fig. 2c). In the retracting germ band the expression of *zfh2* in some tracheal openings appears (Fig. 2e), and in the fully retracted embryo *zfh2* is expressed in all tracheal openings, and a stronger expression in the mandibles appears (Fig. 2f). The expression level in the central nervous system increases during germ band elongation. In fully retracted embryos, *zfh2* marks parts of the central nervous system including the two hemispheres of the protocerebrum, segmental ganglia, connectives and commissures. The thoracic legs in fully retracted embryos show several expression domains (Fig. 3d): a proximal ventral stripe (which is weak in the first thoracic segment and stronger in thoracic segment 2 and 3) and a proximal dorsal spot, two ring-shaped but punctate medial domains, and a stronger domain in the leg tips.

Table 1 All genes predicted by the GLEAN algorithm to be located in the vicinity of the transposon insertion site in the line Goe-08115

GLEAN number	*D. melanogaster* ID	Cellular component (www.flybase.org)
02508	CG3757	Cytoplasm, extracellular
02509	CG3757	Cytoplasm, extracellular
02510	CG32597	Unknown
02511	No significant similarity	–
02512	No significant similarity	–
02513	No significant similarity	–
03890	No significant similarity	–
03891[a]	CG1449	Nucleus
03892	No significant similarity	–
03893	No significant similarity	–
03894	CG5339	Unknown
03895	CG9657	Membrane
03896	CG32604	Spliceosome
03897	No significant similarity	–
03898	CG1629	Unknown

The numbers of the GLEAN-predicted genes are given in the first column. The second column gives the orthologous genes, if any, from *D. melanogaster* [identified by their annotation ID ("CG number")] as inferred from BLAST similarity analysis in the *D. melanogaster* genome. "*No significant similarity*" indicates that the GLEAN-predicted gene does not bear similarity to any gene in GenBank. The third column gives the predicted intracellular localization of the gene product as stated in the "cellular component" field on the Flybase Web Service (Drysdale and FlyBase Consortium 2008)

[a] The GLEAN-predicted gene corresponds to *zfh2* and was selected for further study

The dotted appearance of the two medial rings indicates that the expression in these dots might be correlated with peripheral nervous system development probably of sensory organs in the vicinity of the leg joints. The expression pattern of *zfh2* thus consists of the enhancer trap pattern plus additional expression domains not detected in the enhancer trap pattern (see Discussion).

RNAi with zfh2 in Tribolium leads to leg development defects

We have then tested the function of the *zfh2* gene during *Tribolium* leg development using RNAi. Surprisingly, two different classes of phenotype were present after *zfh2* RNAi. One class comprises legs with fused leg segments (femur and tibiotarsus are fused) and sometimes, ectopic outgrowths (see arrows in Fig. 4a–j). This was the more frequent phenotype—61% of all scored larvae [$n=144$ (100%)] belonged to this class. We term this phenotype the "femorotarsus" phenotype. The second class of phenotype comprises legs with a reduced and rounded claw but otherwise normal morphology (Fig. 4k–n). This phenotype occurred in 13% of all scored larvae. We term this phenotype the "smallclaw" phenotype.

The weakest forms of the "femorotarsus" phenotype have all podomeres, but the femur is visibly shorter than wildtype, and the joint between femur and tibiotarsus is not fully formed (Fig. 4a, arrowhead). In other cases, the shortened femur and the tibiotarsus are fully fused (Fig. 4b). In stronger cases, femur and tibiotarsus are not only fused but this large fusion podomere is bent and enlarged ventrally (Fig. 4c, d), and sometimes this enlargement is forming a small outgrowth (Fig. 4e). The formation of ectopic outgrowths can also affect the dorsal side (Fig. 4f–h) or dorsal and ventral side at the same time (Fig. 4j). In rare cases, two separate tips on the same side are present (Fig. 4i) suggesting the presence of two outgrowths but which could also be caused by tumorous growth of a single outgrowth.

The "smallclaw" phenotype is relatively uniform. All leg segments (except for the claw) are present and have their normal length and morphology (Fig. 4k–n). The claw is shortened and is rounded at the end. The length of this malformed claw shows only little variation (Fig. 4k–n).

Discussion

Comparison of the zfh2 genes of Tribolium and Drosophila

The *zfh2* gene has been previously identified in *Drosophila*. The protein encoded by this gene is unusual in having 16 zinc fingers and three homeodomains in a single protein (Fortini et al. 1991). Conceptual translation of the predicted

Fig. 2 Expression of *zfh2* in *Tribolium* embryos. **a**, **b** In early germ band elongation stages, *zfh2* is expressed in the head lobes and in the posterior portion of the embryos. **c** Expression in the appendages is restricted to the distal ends in fully elongated embryos. **d**, **e** germ-band retraction, the expression in the central nervous system becomes stronger and more refined. Near the end of germ band retraction (*e*) the expression in the spiracles (tracheal openings) appears in some segments. (f) In fully retracted embryos, the spiracles of the meso- and metathoracic segment and the abdominal segments express *zfh2*, as well as the central nervous system and the appendages. Abbreviations: *hl* head lobes, *gz* growth zone, *lr* labrum, *md* mandible, *mx* maxilla. *lb* labium, *t1–t3* thoracic legs 1 to 3, *cns* central nervous system, *sp* spiracle. Anterior is to the left in all panels
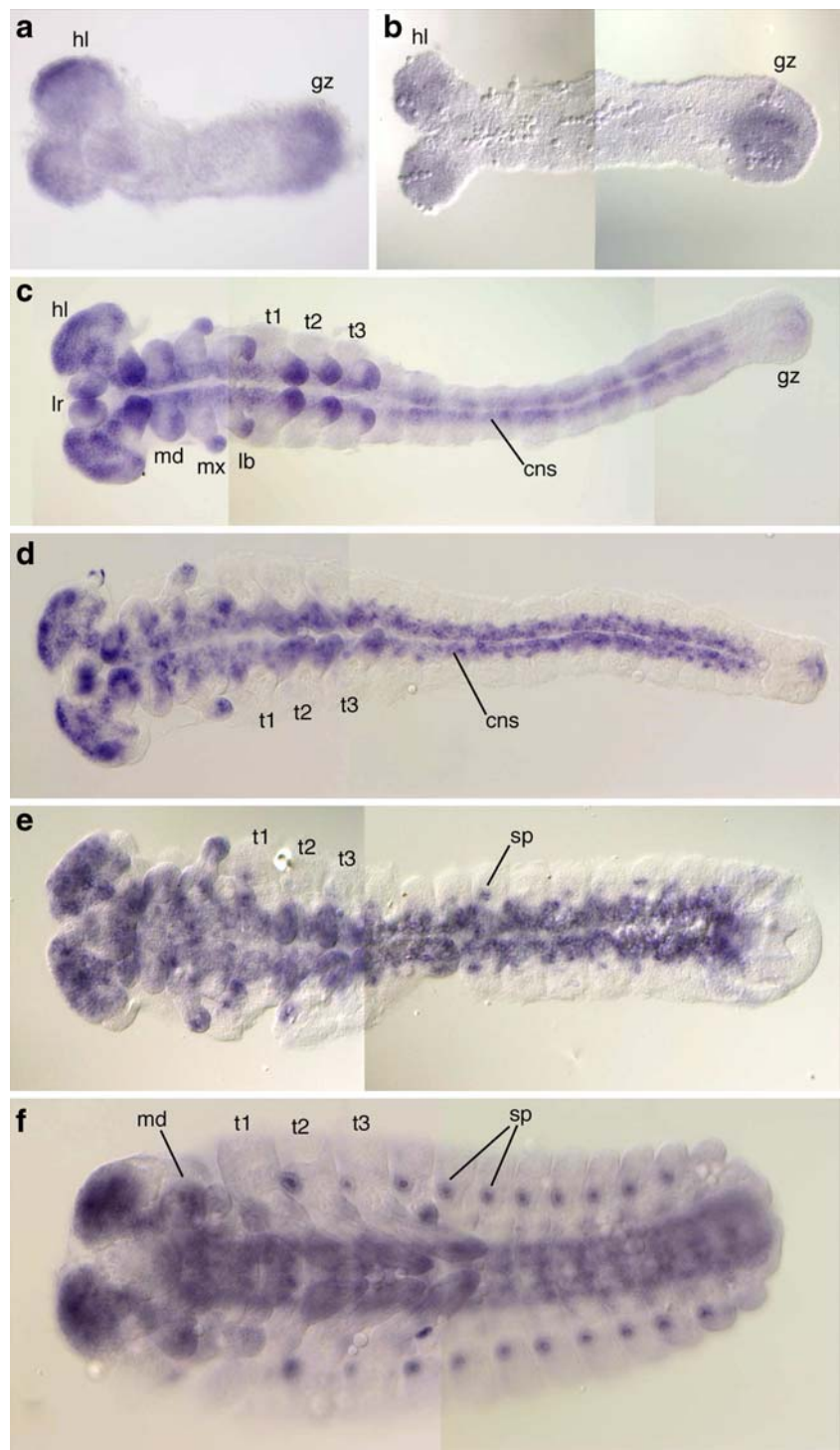

Tribolium zfh2 gene homolog (*GLEAN_03891*) reveals a similar structure of multiple putative DNA-binding domains. However, *Tribolium* Zfh2 has 23 zinc fingers and four homeodomains (not shown), and thus is more similar to the mammalian homolog of Zfh2, the AT motif-binding factor 1 (ATBF-1; Morinaga et al. 1991; Hashimoto et al. 1992) that has the identical number of zinc fingers and

homeodomains in its longest isoform (Miura et al. 1995; Kawaguchi et al. 2001).

The embryonic expression of *zfh2* is very similar in *Drosophila* (Lai et al. 1991) and *Tribolium*. Both genes are mainly expressed in the brain and the ventral nerve cord. In addition, *Drosophila zfh2* is expressed in the hindgut primordium which might correspond to the posterior

Fig. 3 Details of *zfh2* expression in *Tribolium* embryos. **a**, **b** Gelatine–albumine cross-sections through the thorax (**a**, nearly transversal section) and the head (**b**, sagittal section) of a mid-elongation stage embryo. Strong expression is seen in the ventral nervous system and in patches in the head lobe and also at the tip of the appendages. **c**, **d** Dissected legs showing a single distal domain during germ-band elongation (**c**) and several additional leg domains in fully retracted stages (**d**). The two ring domains are denoted by "*1*" and "*2*", the persisting distal domain is labeled "*3*". The *arrows* point to a dorsal and a ventral proximal domain. Please note that the strong expression in the pleuropodium is an artifact seen with any probe at this developmental stage. Abbreviations, see Fig. 2. Additional abbreviations: *vns* ventral nervous system, *hl* head lobe, *ic* tissue of the intercalary segment, *an* antenna *pl*, pleuropodium
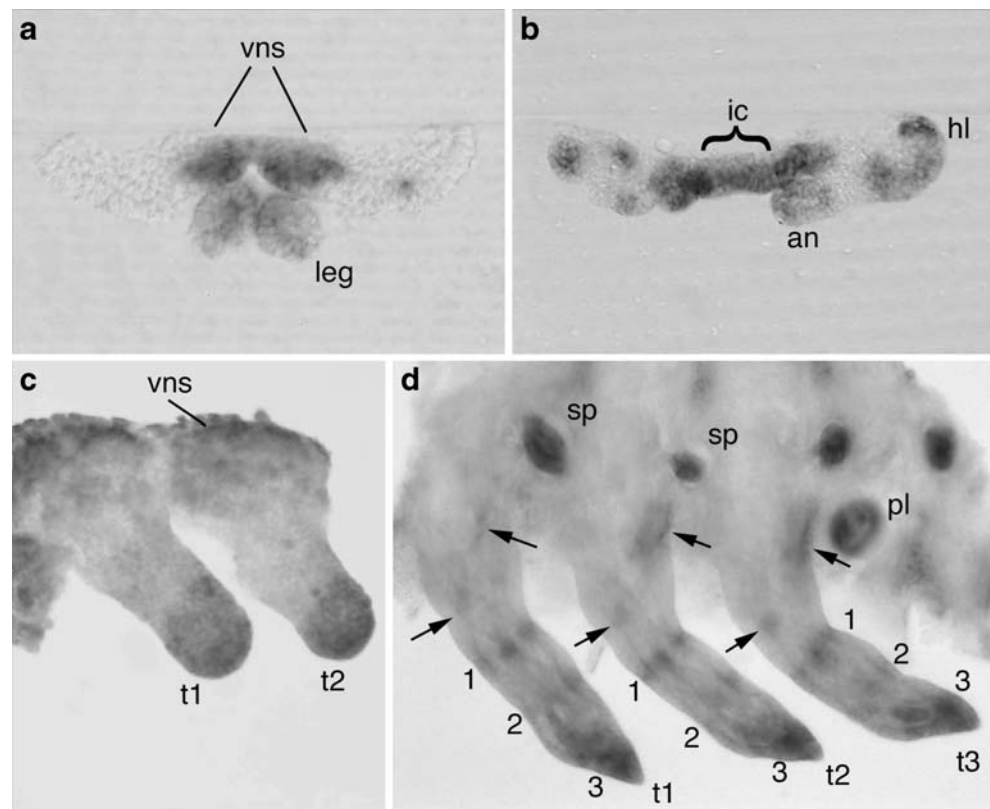
expression in the growth zone in *Tribolium*. The *Drosophila* gene is known to be required for nervous system development (Lundell and Hirsh 1992), but a function of *zfh2* in *Drosophila* leg development has not been reported. However, *zfh2* has been demonstrated to play a role in proximal–distal axis formation in the wing disk (Whitworth and Russell 2003). Interestingly, during wing development, *zfh2* is regulated downstream of Wingless and Nubbin, which are also known to have roles in distal leg development and leg segmentation, respectively (e.g., Lecuit and Cohen 1997; Rauskolb and Irvine 1999), thus possibly linking *zfh2* to exactly those processes that are disturbed in leg development of *Tribolium* RNAi phenocopies.

Regulation of *zfh2* in *Tribolium*

The enhancer element that has been trapped in the line Goe-08115 obviously is not responsible for producing the entire expression pattern of *zfh2* in *Tribolium* because the EGFP expression pattern in Goe-08115 is only a subset of the expression pattern of *zfh2* as detected with a *zfh2*-specific RNA probe. We conclude from this that the enhancer of *zfh2* consists of (at least 2, likely more) separate elements for the expression in the nervous system, tracheal openings and appendages. Even the expression domains in the appendages appear to be regulated through different enhancer elements: The enhancer element trapped

in line Goe-08115 activates expression in the leg tips as well as in the proximal dorsal spot. Additional domains activated by this enhancer element are in the labrum and in the mandibles. The two ring-shaped domains in the legs, however, must be activated through a different enhancer element. These two separate enhancer elements responsible for activation of *zfh2* in the distal and in the ring-shaped domains, respectively, are not only responsible for activating *zfh2* at different locations, but also at different time points. The distal domain element appears to be activated early in development, whereas the ring-shaped domains element is active when the legs are already grown out considerably.

Interpretation of the leg phenotypes

The two different phenotypes obtained after RNAi require separate interpretation because they appear to be independent from each other and no intermediates between the two have been observed. The "smallclaw" phenotype suggests that the loss of *zfh2* leads to difficulties with distal patterning. The *zfh2* gene is expressed strongly in the leg tips and the "smallclaw" phenotype suggests that it is necessary there for the normal development of the distal-most podomere, the pretarsus (claw). However, the claw is not lost, only its morphology is disturbed, suggesting that *zfh2* is not at the top level in the genetic hierarchy that determines pretarsal fate. Alternatively, the full effect of
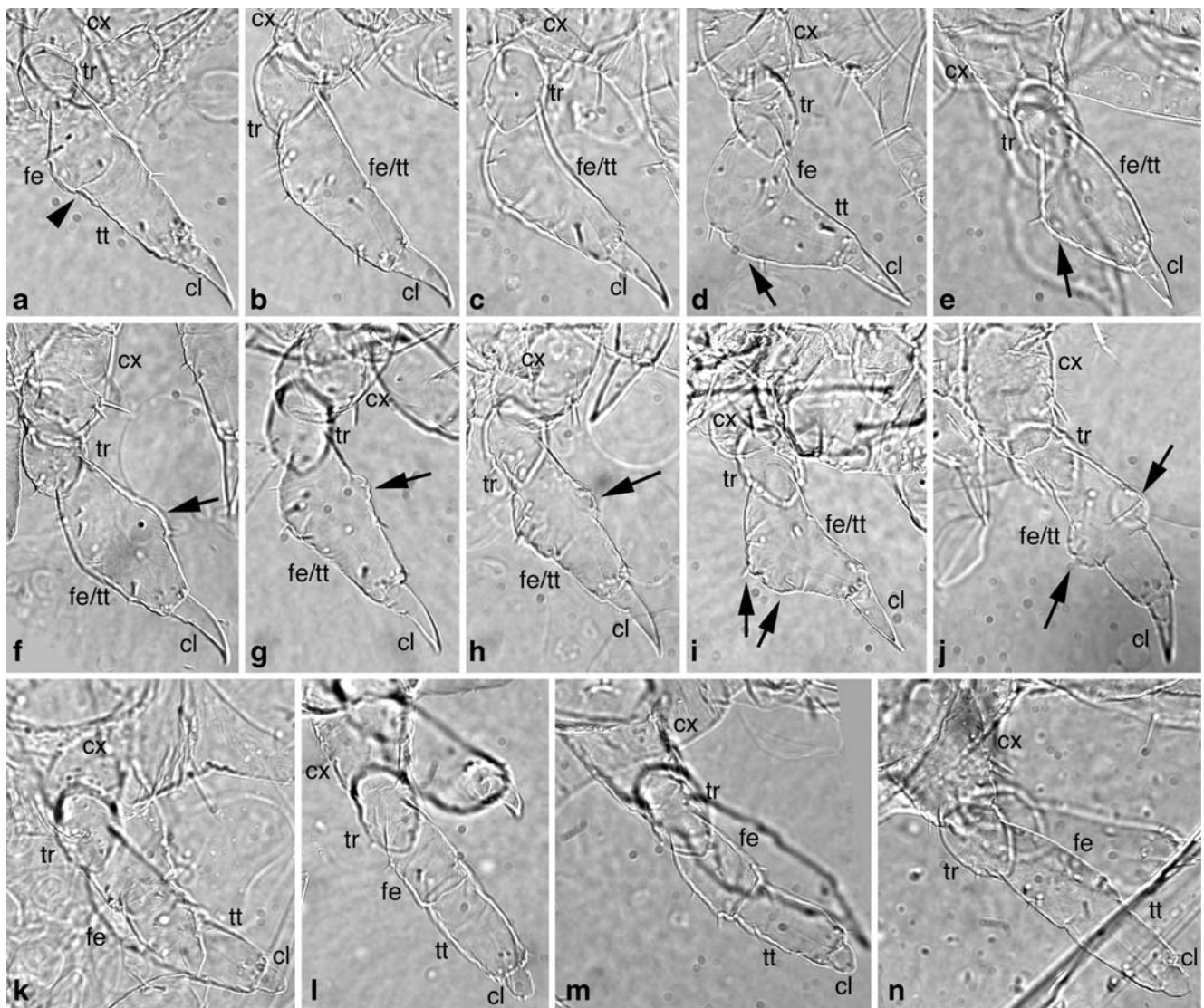
Fig. 4 Spectrum of leg phenotypes obtained after RNAi with *zfh2*. **a–j** The fusion of femur and tibiotarsus and the formation of ectopic outgrowths characterizes the "femorotarsus" phenotype. The weakest form of this phenotype has a shortened femur, but retains a malformed joint between femur and tibiotarsus (**a**, *arrowhead*). Stronger phenotypes have fully fused femur and tibiotarsus, a ventral bend of different severity **b**, **c** and can have ectopic outgrowths (*arrows* in **d–j**). **k–n** The segmental composition is normal in the "smallclaw" phenotype. The claw abnormally rounded and short (**k**, **l**) to very short (**m**, **n**). Abbreviations: *cx* coxa, *tr* trochanter, *fe* femur; *tt* tibiotarsus, *cl* claw (pretarsus)

loss of *zfh2* might be masked by redundancies (e.g., paralogous genes).

The "femorotarsus" phenotype is more difficult to interpret. The *zfh2* gene is expressed in two rings in the legs and one of these rings (ring 2 in Fig. 3d) is at the presumptive junction of the femur and the tibiotarsus consistent with a function of *zfh2* in the morphogenesis of this joint in the larval leg. Loss of *zfh2* is therefore likely to interfere with joint formation and thus might cause the fusion of the two leg segments observed in the "femorotarsus" phenotypes. In *Drosophila* and the spider *Cupiennius salei*, proper formation of the leg joints is also necessary for proper growth of the leg segments themselves (de Celis et al. 1998;

Rauskolb and Irvine 1999; Bishop et al. 1999; Prpic and Damen 2009). It is unclear whether podomere growth and joint formation are also coupled in *Tribolium*, but if so, it could explain the shortening of the femur in the "femorotarsus" phenotypes. These morphological changes indicate a role of *zfh2* in leg segmentation. However, the enlargement and the ectopic outgrowths in the "femorotarsus" phenotypes are unexpected and do not seem to be leg segmentation defects. Rather, they appear to be defects with proximal–distal axis formation by inducing supernumerary proximo–distal axes at the fused junction between femur and tibiotarsus. Regeneration experiments in other insect species have suggested that, in the podomeres, a

gradient of positional information exists that is used to restore the lost appendage parts during regeneration (Bohn 1970a, 1976; Bulliere 1971; Nakamura et al. 2007). However, experimentally joining two leg portions that have different positional values at the joining point results in reversed regeneration or supernumerary proximal–distal axes (Bohn 1970a, 1970b; Mito et al. 2002). This is probably because the joining of incompatible positional values disturbs normal gradient formation that is necessary for the formation of a single proximal–distal axis. The factors involved in these gradients are not all identified, and it is unclear whether these processes that guide regeneration in larval stages play a role in embryonic development as well. However, the reduction of femur size and the fusion of femur and tibiotarsus in the *Tribolium* "femorotarsus" phenotype might join incompatible positional values in these podomeres and thus lead to disturbances in gradient formation for proximal-distal axis development, which in turn could lead to supernumerary outgrowths in the area of the joining. Undoubtedly, more work is necessary to investigate pattern formation by morphogen gradients in the appendages of *Tribolium*.

Possible origins of the alternative phenotypes after *zfh2* RNAi

An unexpected finding is the existence of the two alternative leg phenotypes "femorotarsus" and "smallclaw" without any intermediate or combined phenotypes. These phenotypes could be caused by small differences in the effectivity of the RNAi in the respective appendage. Two alternative results depending on the absolute concentrations or the ratio of two factors are known from the morphogen regulation of target genes (*dachshund, Distal-less*) during *Drosophila* leg development (Estella and Mann, 2008). Zfh2 may regulate the expression of one or several target genes that act as morphogens. Alternatively, the target gene(s) may have a composite regulatory region in which several elements compete for Zfh2 binding to either repress or activate the target gene, with different outcomes of the competition in claw cells and in joint cells, respectively. Interfering with *zfh2* expression might disrupt this sensitive competition system in different ways in the different cell types and at different RNAi efficiencies.

Another possibility is that *zfh2* RNAi interferes directly with allometric leg growth. This primary defect could lead to a secondary disturbance of the leg patterning network, for example because there is not enough space for the regular number of joints at the required distances. The attempts of the regulatory network to compensate for the defect could lead to two different outcomes because they are the only two stable alternatives to the wildtype situation.

Finally, we note that recent work has shown that the translation of *Drosophila zfh2* is regulated by the micro-RNA *miR-276a,b* (Stark et al. 2005) and thus by an RNAi-related mechanism. We think that it is possible, that RNAi interferes with miRNA regulation of *zfh2* to lead to the two observed alternative outcomes.

The occurrence of two alternative phenotypes after RNAi is, to our knowledge, unprecedented. Clearly, the possible explanations given in the discussion above are just attempts to provide first ideas about how this phenomenon could be brought about. More work is thus required to investigate further the possible miRNA regulation of *Tribolium zfh2* and the interactions and regulatory targets of this multidomain protein.

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