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The evolution and expression of panarthropod *frizzled* genes

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Abstract

Wnt signaling regulates many important processes during metazoan development. It has been shown that Wnt ligands represent an ancient and diverse family of proteins that likely function in complex signaling landscapes to induce target cells via receptors including those of the Frizzled (Fz) family. The four subfamilies of Fz receptors also evolved early in metazoan evolution. To date, Fz receptors have been characterised mainly in mammals, the nematode *Caenorhabditis elegans* and insects such as *Drosophila melanogaster*. To compare these findings with other metazoans, we explored the repertoire of *fz* genes in three panarthropod species: *Parasteatoda tepidariorum*, *Glomeris marginata* and *Euperipatoides kanangrensis,* representing the Chelicerata, Myriapoda and Onychophora respectively. We found that these three diverse panarthropods each have four *fz* genes, with representatives of all four metazoan *fz* subfamilies found in *Glomeris* and *Euperipatoides,* while *Parasteatoda* does not have a *fz3* gene, but has two *fz4* paralogues. Furthermore we characterized the expression patterns of all the *fz* genes among these animals. Our results exemplify the evolutionary diversity of Fz receptors and reveals conserved and divergent aspects of their protein sequences and expression patterns among panarthropods; thus providing new insights into the evolution of Wnt signaling more generally.

Introduction

In metazoans most key developmental processes from cell division to differentiation and death are regulated by Wnt signaling (Logan and Nusse, 2004). Wnt ligands bind to cell surface receptors including those encoded by *frizzled* (*fz*) genes (Bhanot et al., 1996;Dann et al., 2001). Fz proteins are seven-pass transmembrane domain receptors with an N-terminal cysteine rich domain (CRD), which regulates the specificity of interactions with Wnt ligands, and typically a C-terminal KTxxxW motif that is required for downstream interactions with the intracellular protein Dishevelled (Umbhauer et al., 2000;Wong et al., 2003;Huang and Klein, 2004;Tauriello et al., 2012). As well as their roles in the canonical Wnt pathway, Fz proteins are also involved in the planar cell polarity pathway, so called non-canonical Wnt pathways (including the Wnt/calcium pathway), and can be bound by other (i.e. non-Wnt) ligands (Veeman et al., 2003;Huang and Klein, 2004;van Amerongen and Nusse, 2009).

It has been shown that two *fz* genes found in sponges likely gave rize to four *fz* genes in the common ancestor of bilaterians: FZD I, II, II and IV (Schenkelaars et al., 2015). Lineage specify expansions followed, for example in mammals (FZD I = Fz1/2/3/6/7; FZD II = Fz5/8; FZD III = Fz9/10; FZD IV = Fz4). Moreover, metazoans had an ancestral repertoire of thirteen subfamilies of Wnt ligands, although there have been lineage specific expansions, for example in vertebrates, and the loss of particular ligands as seen in nematodes and insects (Prud'homme et al., 2002;van Amerongen and Nusse, 2009;Cho et al., 2010;Janssen et al., 2010;Schenkelaars et al., 2015). This considerable array of possible combinations of ligands and receptors likely underlies the diversity of roles played by Wnt signaling during metazoan development. Furthermore, it is thought that for many developmental processes landscapes of combinations of Wnt ligands and receptors are employed to achieve a robust outcome (van Amerongen and Nusse, 2009).

Fully understanding the evolution and function of Wnt signaling in metazoans requires characterizing the expression of key components such as the receptors and ligands in a wide diversity of lineages. In the arthropods and related phyla, the expression of Wnt ligand genes has been described in a range of species (Cho et al., 2010;Janssen et al., 2010;Murat et al., 2010;Hogvall et al., 2014;Janssen and Posnien, 2014), however the expression of the *fz* genes during embryogenesis has only been fully characterized in the insects *Drosophila melanogaster* and *Tribolium castaneum* (Bhanot et al., 1996;Kennerdell and Carthew, 1998;Beermann et al., 2011). Such studies suggest that the four ancient *fz* families were found in the common ancestor of arthropods but that there have also been lineage specific losses, like the loss of *fz3* in *Tribolium* (Beermann et al., 2011). In addition, analysis of *fz* expression and function in these insects clearly shows that many aspects of development employ multiple receptors, as well as more than one ligand (Bolognesi et al., 2008;Janssen et al., 2010;Beermann et al., 2011).

To provide a more comprehensive description of *fz* expression and evolution among arthropods, here we report our analysis of *fz* genes and their expression in representatives of the Myriapoda and Chelicerata, the pill millipede, *Glomeris marginata* (Dohle, 1964), and the common house spider, *Parasteatoda tepidariorum* (Hilbrant et al., 2012) respectively. Moreover, to provide a broader perspective to *fz* expression in the panarthropods (Onychophora + Arthropoda, Nielsen (1995), and see Edgecombe and Legg (2014) for a recent discussion), we also characterized the repertoire and function of these genes in the onychophoran *Euperipatoides kanangrensis* (Eriksson and Tait, 2012;Janssen and Budd, 2013).

Our study confirms that the common ancestor of the panarthropods contained copies of the four ancestral Fz subfamilies, but that some subfamilies have also been subject to loss or duplication in chelicerates. Analysis of the expression patterns of the four subfamilies of Fz genes during the embryonic development of *Euperipatoides, Glomeris* and *Parasteatoda* reveals both similarities and differences between these lineages. Our characterization of Fz gene expression patterns with respect to the known patterns of Wnt ligands in these animals also helps to better understand the Wnt landscapes that regulate their development.

Methods

Sequences and phylogenetic analysis

Fz sequences were recovered from published resources: *Euperipatoides kanangrensis* (Janssen and Budd, 2013); *Glomeris marginata* (Janssen and Posnien, 2014); *Parasteatoda tepidariorum* (Posnien et al., 2014); *Mesobuthus martensii* (Cao et al., 2013); *Ixodes scapularis* (Lawson et al., 2009): *Tribolium castaneum* (Richards et al., 2008); *Zootermopsis nevadensis* (Terrapon et al., 2014); *Strigamia maritima* (Chipman et al., 2014). *Pholcus phalangioides* sequences were recovered from a de novo assembled transcriptome, generated from a combination of different embryonic stages and three larval tissues including legs, the prosoma and the opisthosoma. Total RNA for larval tissue was extracted using the ZR Tissue & Insect RNA MicroPrep™ Kit (Zymo Research) and total RNA extraction of embryonic samples was performed using Trizol. The library for Illumina sequencing was generated using the standard protocol of the TruSeq RNA Sample Preparation Kit (v2, Illumina). All samples were pooled and sequenced on one lane of an Illumina Hiseq2000 sequencer, which resulted in 472,770,758 paired-end reads of 100 bp length each. The reads were quality trimmed and filtered as previously described (Posnien et al., 2014). All high quality reads were assembled into 444,103 transcripts using the *de novo* transcriptome assembler Trinity (version r2013_11_10, (Grabherr et al., 2011)). The GC content of this preliminary *de novo* assembly is 37.27%. The N50 is 1,517 bp with a median transcript length of 284 bp and an average transcript length of 670 bp.

To infer phylogenetic relationships, predicted Fz amino acid sequences were first aligned using MUSCLE (Edgar, 2004;Sievers et al., 2011) after which poorly aligned sites were masked using Gblocks v. 0.91b (Castresana, 2000) with relaxed selection criteria, both as implemented in Seaview v. 4.5.3 (Gouy et al., 2010).

Maximum-likelihood analysis was performed using RAxML v. 8 (Stamatakis et al., 2005), specifying an LT model of amino acid replacement and a gamma model of rate heterogeneity (+G) as selected by ProtTest v. 3.2 (Darriba et al., 2011). Nodal support was estimated with 500 bootstrap iterations.

Gene cloning

All gene fragments were isolated by means of PCR with gene specific primers. Fragments were cloned into pCR-II-TOPO or pCR4-TOPO vectors (Life Technologies). Sequences are available under accession numbers LN849082 (*Ek-fz1*), LN849083 (*Ek-fz2*), LN849084 (*Ek-fz3*), LN849085 (*Ek-fz4*), LN849078 (*Gm-fz1*), LN849079 (*Gm-fz2*), LN849080 (*Gm-fz3*), LN849081 (*Gm-fz4*). The *Parasteatoda* sequences are all available from the Assembled Searchable Giant Arthropod Read Database (ASGARD): (http://asgard.rc.fas.harvard.edu/) (Zeng and Extavour, 2012): *Pt-fz1* (Locus 3605), *Pt-fz2* (Locus 1), *Pt-fz4-1* (Locus 7239) and *Pt-fz4-2* (Locus 2608).

Embryo collection, fixation and staging

Mature females of *Glomeris* were collected from February to May in the Reichswald forest (Nordrhein-Westfalen, Germany) near Kranenburg. They were kept in plastic boxes filled with decomposing beech leaves and moist soil at approximately 20°C. Under these conditions females lay eggs for several weeks. Pregnant females of *Euperipatoides* were sampled between August and October in the Kanangra Boyd National Park, NSW, Australia, and were then kept at approximately 8°C in plastic containers filled with moist moss. Under these conditions they survive for at least several month when fed with small crickets once a week. The *Parasteatoda* culture at

Oxford Brookes University was founded by spiders from a strain collected and maintained in Göttingen. Adults were kept separately in plastic vials at 25°C and fed with crickets. *Pholcus* spiders were obtained from the laboratory stock in Göttingen (Pechmann et al., 2011), and maintained like *Parasteatoda*, except adults were and fed with large flies like *Musca.* Embryos were collected, fixed and stored as described in Janssen et al. (2004) for *Glomeris,* in Janssen et al. (2015b) for *Euperipatoides* and in Akiyama-Oda and Oda (2003) for *Parasteatoda* with slight modifications. *Glomeris, Euperipatoides* and *Parasteatoda* embryos were staged according to Janssen et al. (2004), Janssen and Budd (2013), and Mittmann and Wolff (2012) respectively.

Whole mount in situ hybridization and nuclear staining

In situ hybridization of *Euperipatoides* and *Glomeris* embryos was performed as described in Janssen et al. (2015b) and Prpic and Tautz (2003), respectively. Embryos were hybridized with Digoxigenin-labeled RNA probes at 62°C for at least 16 hours. *Parasteatoda* in situ hybridization was carried out after the whole-mount protocol for spiders (Prpic et al., 2008) with minor modifications. Primers used to generate probes are available on request.

Nuclear staining was performed by incubation in 1 μg/ml 4-6-diamidin-2-phenylindol (DAPI) in phosphate-buffered saline with 0.1% Tween-20 for 30 minutes.

Data documentation

Embryos were analyzed under a Leica dissection microscope equipped with a Leica DC100 (*Glomeris* and *Euperipatoides*) or with a Jenoptik ProgRes C3 (*P.*

tepidariorum) digital camera. Linear corrections of brightness, contrast, and colour values were performed with Adobe Photoshop.

Results and Discussion

Phylogeny of the panarthropod frizzled receptors

Coding sequences of all putative Fz receptors recovered from genomic or transcriptomic resources for ten panarthropod species were mostly complete. Exceptions include the Mm-fz1 sequence, which appears to be truncated after transmembrane domain 5. Gm-fz2, Zn-fz3 and Mm-fz4-1 sequences are also possibly incomplete at the N-terminus (Fig. S1).

Phylogenetic reconstruction using maximum likelihood recovered four distinct orthology groups: FzI, FzII, FzIII and FzIV (Fig. S2). This is in concordance with previous findings in other metazoans (Schenkelaars et al., 2015). To avoid insect overrepresentation and potential disrupting effects of the derived Dm-fz3 and Dm-fz4 sequences, *Drosophila* sequences were omitted from our final analysis (Fig. 1). This had little influence on the topology of FzI, II and IV orthology groups, but led to clustering of the *Glomeris* Gm-fz3 sequence with the other non-onychophoran sequences within the FzIII orthology group.

In all investigated species, a single FzI orthologue was identified. FzII orthologues are present in the genomic resources of all species, except for the genome of the scorpion *Mesobuthus*. FzIV orthologues were also found in all investigated species. Moreover, it appears that a duplication of FzIV can either be traced to the base of the Arachnopulmonata (Scorpiones and Tetrapulmonata - including spiders) (Sharma et al., 2014), or happened independently in spiders and scorpions (Figs 1 and 2). The only orthology group that showed gene loss in representatives of both the Chelicerata and Mandibulata was FzIII (Figs 1 and 2).

Fz sequence variation

Fz receptors contain a conserved amino-terminal extracellular region that mediates binding to Wnt ligands: the cysteine-rich domain (CRD) (Figs 3 and S1). The highest sequence similarity among panarthropod Fz CRD domains was found in the FZI orthology group (only nine sites with less than 60% consensus), whereas the FZIII CRD sequences are most divergent (30 sites with less than 60% consensus) (Fig. 3). Moreover, the *Drosophila* Dm-fz1/3/4 sequences stand out due to their relatively large number of deviations from the consensus of their respective orthology groups (Fig. 3). This is consistent with long branches observed for these *Drosophila* sequences in our phylogenetic reconstruction (Fig. S2), and is an indication that insights gained from functional studies of Fz receptors in *Drosophila* might not be representative of their function in other panarthropods.

A second functional domain is the KTxxxW motif, which is typically found directly after the seventh transmembrane domain (Huang and Klein, 2004). In *Drosophila* and vertebrates, this intracellular motif has been associated with binding to proteins of the Dishevelled family. Alignment of the KTxxxW motif from panarthropods (Fig. 3, positions 548-566) shows high sequence similarity in the FzI/II orthology groups, and less similarity for the FzIII/IV sequences. Interestingly, as with *Drosophila* Dm-fz3 and Dm-fz4, the tryptophan of this motif has been lost in both of the spider *Parasteatoda* Pt-fz4 paralogues, which could indicate a change or loss of the binding affinity of this motif in these two proteins. The tryptophan residue has, however, been retained in most other panarthropods FzIII/IV sequences, including the spider *Pholcus* (Figs 3 and S1).

Embryonic expression of fz1 *genes*

We identified orthologues of the FzI subfamily in *Euperipatoides* as well as the two arthropods, *Glomeris* and *Parasteatoda* (Figs 1 and 2).

In early embryonic stages of *Euperipatoides, Ek-fz1* is expressed ubiquitously except for the posterior segment addition zone (SAZ), but is then upregulated in a segmental pattern in transverse stripes (Fig. 4A). In the head lobes, expression is observed in a patch-like domain at the posterior margin of each of the two hemispheres (Fig. 4A,B). Later, expression disappears from the tip of the outgrowing appendages (Fig. 4C,D) and cells in the nervous system express *Ek-fz1* at higher levels (Fig. 4C). This expression profile persists through to later developmental stages (Fig. 4E).

Gm-fz1 is expressed ubiquitously and uniformly at all investigated stages of embryogenesis in *Glomeris* (Fig. 5A-D). In *Parasteatoda*, *Pt-fz1* is also probably ubiquitously expressed at low levels, but the most prominent expression of *Pt-fz1* expression starts at stage 9.1 in the ventral neuroectoderm (Fig. 6), which then persists through subsequent stages. However, no specific spatial expression of *Pt-fz1* was detected before or after this stage of embryogenesis in *Parasteatoda*.

In the early embryos of both *Drosophila* and *Tribolium,* the *fz1* genes are initially uniformly expressed (Park et al., 1994;Müller et al., 1999;Beermann et al., 2011). While this ubiquitous expression persists in *Tribolium* embryos (Beermann et al., 2011), the expression of *Drosophila* Fz resolves into broad stripes between the Engrailed (En) expressing cells (Müller et al., 1999). In both of these insects, Fz appears to function together with the respective Fz2 receptor to regulate segment formation via the Wg ligand (Chen and Struhl, 1999;Müller et al., 1999;Bolognesi et al., 2008;Beermann et al., 2011). The expression of *fz1* genes in *Euperipatoides, Glomeris* and *Parasteatoda* is consistent with a role for *fz1* genes in segmentation across the panarthropods.

Drosophila fz is also expressed in other tissues including the imaginal discs and central nervous system (CNS) (Park et al., 1994). However, *fz* mutants can produce viable adult flies that only exhibit defects in planar cell polarity (reviewed in Strutt, 2003). This suggests that, like during segmentation (see above), this receptor functions in combination with other Fz proteins in a range of roles in *Drosophila*, and given its widely distributed expression in *Euperipatoides, Glomeris* and *Parasteatoda*, this is probably the case in other panarthropods.

Embryonic expression of fz2 *genes*

We identified orthologues of the FzII subfamily in all three of the panarthropods that we investigated (Figs 1 and 2).

In *Euperipatoides, Ek-fz2* is strongly expressed during embryogenesis in the ventral region of the head lobes, including the mouth that forms from the anterior of the mouth-anus furrow (Chen and Struhl, 1999, and see below). Interestingly, tissue anterior to the head lobes proper also expresses *Ek-fz2* (Fig. 4F,F’). This remains the only detectable expression (Fig. 4G,H) until stage 20 when dot-like domains appear in an anterior to posterior order at the base of the walking limbs, and in a small domain ventrally at the base of the third and fourth walking limb (Fig. 4I). The latter expression may be correlated with the development of the nephridia, which are more pronounced in these two segments (Janssen et al., 2015a).

In *Glomeris*, *Gm-fz2* is expressed in an anterior domain covering approximately 30% of the early post-blastoderm stage embryo (Fig. 5E). At later stages this domain includes the anterior and median region of the developing head lobes including the stomodeal region (Fig. S3A-C). At approximately stage 0.5, a faint transverse stripe of expression appears (Fig. S3A). This stripe likely corresponds to expression in the mandibular segment as is obvious in later stages (cf. panels A and B in Fig. S3). Within the mandibular segment, expression is restricted to the anterior of the developing mandibles (Figs. 5F and S3B,C). The dorsal segmental tissue that forms at stage 3 (Mayer, 2006) expresses *Gm-fz2* in a complex pattern (Figs 5F-H and S3C), and *Gm-fz2* is also expressed in the midgut from stage 5 onwards (Fig. 5G).

In *Parasteatoda*, *Pt-fz2* expression commences at stage 5 in a broad ring around the germ disc (Fig. 6B). After radial symmetry is broken (Akiyama-Oda and Oda, 2003), *Pt-fz2* continues to be expressed in an anterior stripe (Fig. 6C), which widens at a later stage, but the expression intensity remains comparable. At stage 8.1 *Pt-fz2* appears to be expressed at the anterior border of the germ band and in the forming L1 to L4 segments, although the expression in the L2-L4 segments is much broader than in L1 (Fig. 6D). At stage 8.2, when prosomal segmentation has become morphologically visible and the first opisthosomal segment (O1) has formed, *Pt-fz2* is expressed in the anterior compartment of each segment and in the segmental grooves (see Fig. 6E). There is also a narrow *Pt-fz2* expression domain at the anterior margin of the germ band (see Fig. 6E). However, no expression is observed in the SAZ. Subsequently, *Pt-fz2* expression in each segment contracts to the segmental grooves, and the anterior-most band expands to a wedge shaped domain in the anterior precheliceral lobes, partially encompassing the stomodaeum (Fig. 6F). Strong expression is also observed in the ventral neuroectoderm and the dorsal periphery of each segment (see Fig. 6F).

In *Drosophila* embryos, Dfz2 is also initially expressed in a broad domain but is absent from the anterior and posterior poles (Bhanot et al., 1996). Dfz2 expression then develops into broad segmental stripes posterior to En expressing cells, but unlike Fz, this expression does not quite extend as far as the *wg* expressing cells (Bhanot et al., 1996;Müller et al., 1999).

In *Tribolium*, like *Drosophila, Glomeris,* and *Parasteatoda, Tc-fz2* is also initially expressed in a broad presegmental region that resolves into segmental stripes anterior to *wg* expression (with the exception of the more complex pattern of *Gm-fz2*), and like in *Parasteatoda* and *Glomeris*, expression of *Tc-fz2* in *Tribolium* is absent from the SAZ (Beermann et al., 2011).

Taken together, comparison of the expression of *fz2* genes across arthropods suggests that this member of the Fz family was employed in segmentation in the common ancestor of these animals. In developing insect segments it is most likely that Fz2 is bound by Wg, however, in *Parasteatoda* the most probable candidates are *Wnt5, Wnt8* and *Wnt16* because *wg* is not expressed in an obvious segmental pattern in this spider (Janssen et al., 2010). Moreover, given the lack of a segmental pattern of expression of *fz2* in *Euperipatoides* (Fig. 4*),* it remains unclear if this gene played a similar role in the ancestor of panarthropods. It is also evident from our results that the anterior expression of *fz2* in the median head region is conserved in *Glomeris*, *Euperipatoides*, *Tribolium* and *Parasteatoda* and therefore probably represents a conserved ancestral expression domain of this gene.

Embryonic expression of fz3 *genes*

We identified orthologues of the FzIII subfamily in both *Euperipatoides* and *Glomeris*, but like *Tribolium*, *Parasteatoda* lacks a *fz3* gene (Figs 1 and 2).

Ek-fz3 is expressed segmentally in the form of transverse stripes (Fig. 7A). These stripes are level with the position of the limb primordia (Fig. 7A). The tips of outgrowing limbs, however, are free from expression (Fig. 7D,E). Transcription in the head lobes is restricted to lateral tissue (Fig. 7A,B,E) and complements that of *Ek-fz2*. Expression in the trunk segments complements that of *Ek-fz4* (see below). The SAZ does not express *Ek-fz3*, but a faint signal is visible in the anus (Fig. 7D,E). At later developmental stages, expression is seen in the mouth (Fig. 7E).

In *Glomeris* we did not detect any specific staining for *fz3* in embryos younger than stage 3. From this stage onwards, *Gm-fz3* is expressed in the form of two dots in each eye field (Fig. 5I,J). At later developmental stages, expression appears in the lateral region of the anal valves (Fig. 5K,L). Detection of *Gm-fz3* transcripts requires prolonged staining time, therefore, it is unclear whether this gene is expressed ubiquitously at a low level, or if this weak signal represents background staining. It is possible that *Gm-fz3* is also expressed in the developing gut (Fig. 5).

The expression of *fz3* has been investigated previously in *Drosophila*, but it is not completely straightforward to relate these patterns to those we have observed in *Glomeris* and *Euperipatoides*. *Drosophila fz3* is expressed in segmentally reiterated stripes during later embryogenesis like that observed in *Euperipatoides*, as well as, for example, in the brain, eye and leg discs, and anal tissue like *Glomeris* and/or *Euperipatoides* (Sato et al., 1999;Sivasankaran et al., 2000). This may indicate that *fz3* played an ancestral role in tissues like the nervous system, eye and appendage development, however, a survey of a wider range of panarthropod species that still possess a member of the FzIII subfamily is required to address this more fully.

Embryonic expression of fz4 *genes*

We identified single copies of FzIV subfamily genes in *Euperipatoides* and *Glomeris,* and two *fz4* paralogues in *Parasteatoda* (Figs 1 and 2).

The early expression of *Ek-fz4* is comparable to that of *Ek-fz1. Ek-fz4* is expressed in a segmental fashion. Within the head lobes, the primordia of the frontal appendages, however, are free from expression as are the eyes and a wedge-shaped domain ventral to the eyes (Fig. 7F). At later stages it becomes clear that segmental expression is initially located between the outgrowing limbs (Fig. 7G). Expression in the limb buds is restricted to anterior and posterior proximal tissue (Fig. 7G-I). Tissue ventral to the base of the limbs no longer expresses *Ek-fz4* at these later stages, or only weakly (cf. Fig. 7H and I). As the ventral nervous system grows out in the process of ventral closure, tissue ventral to the bases of the limbs remains free of transcripts (Fig. 7J).

At early stages, *Glomeris Gm-fz4* is expressed in all tissues except for a distinct region in the anterior head, possibly the eye-field (Fig. 5M). Expression is then upregulated in segmental stripes in the SAZ and newly formed segments, but the anal valves remain free of expression throughout development (Fig. 5M-P).

Interestingly, *Gm-fz4* is also expressed in the dorsal extraembryonic tissue in form of an anterior cap (Fig. S3 H-J). At stage 3, the anterior of the head lobes are free of expression. With the exception of the labrum, there is no detectable expression in the distal part of all appendages (Fig. S3D-G). At all later stages, *Gm-fz4* is strongly expressed in the midline (Fig. 5N-P), but only at around stage 3 do longitudinal stripes of expression appear on either side of the midline (Fig. 5N). In dorsal tissue, two domains per segmental unit exhibit recognizably higher expression (Fig. 5N-P).

In *Parasteatoda*, *Pt-fz4-1* expression commences at stage 8.2 in a stripe at the posterior of the O2 segment and in a stripe at the anterior portion of the SAZ (see Fig. 8A). Faint *Pt-fz4-1* expression can also be detected in the mesoderm of the forming limbs and in a distinct domain on each of the precheliceral lobes, at stage 9.1 (Fig.8B). Subsequently, *Pt-fz4-1* expression becomes stronger and more broadly expressed in the limb mesoderm and expands also in the ventral neuroectoderm (see Fig. 8C). The expression in the precheliceral lobes continues during stage 9.2 (see Fig.8C). Then during stage 12, *Pt-fz4-1* is strongly expressed in the limb mesoderm, in the labrum and in the mesoderm of the opisthosomal segments (see Fig. 8D). As the brain differentiates, expression refines to the anterior border of the precheliceral lobes: the anlage of the prosomal shield that overgrows the brain in the following stages (Wolff and Hilbrant, 2011;Mittmann and Wolff, 2012) (Fig. 8D).

Pt-fz4-2 expression arises in an anterior stripe at stage 6 (Fig. 9A). Compared to the anterior *Pt-fz2* stripe at a similar stage (see Fig. 6C), this *Pt-fz4-2* expression domain is narrower (Fig 9A,B). Later, at stage 8.2, *Pt-fz4-2* is expressed in the segmental grooves in the prosomal and opisthosomal segments and in a ring around the future labrum (Fig. 9C). The expression of *Pt-fz4-2* retracts to the dorsal periphery of each segment at stage 9.2 and the domain around the labrum becomes more defined (Fig. 9D). *Pt-fz4-2* is also strongly expressed in the limb and opisthosomal mesoderm at stage 12 (Fig. 9E). Anteriorly, *Pt-fz4-2* expression continues in the labrum, and also appears in a domain directly anterior to it (Fig. 9E).

Interestingly, the two *fz4* paralogues in *Parasteatoda* exhibit both similarities and differences in their expression. For example, both are expressed in the labrum and in similar patterns in the extended walking legs, but *fz4-1* is expressed earlier in the nervous system and head lobes, while *fz4-2* is expressed segmentally including in anterior segments (Figs 8 and 9). Moreover, *fz4-2* expression appears in restricted domains at the segmental borders and the forming limbs compared to *fz4-1*, which is more broadly expressed within the segments, limbs and the developing nervous system. These results may suggest that these two paralogues have been subject to subfunctionalisation (Force et al., 1999;Lynch and Force, 2000).

It is clear from our comparative analysis and from previous work in *Drosophila* and *Tribolium* (Janson et al., 2001;Beermann et al., 2011) that genes of the FzIV family likely play a diverse range of roles during the development of panarthropods. However, comparable patterns emerge among these arthropods, which suggest that these genes were involved in nervous system development, and possibly segment formation, as well as perhaps limb development more widely among panarthropods.

In *Tribolium*, *Tc-fz4* appears to function together with *Tc-fz1* to regulate leg development most likely by binding Wg, although other Wnt ligands could be involved (Bolognesi et al., 2008;Grossmann et al., 2009;Beermann et al., 2011). Similarly, Wg is a likely candidate for binding the Fz4 receptors in the developing appendages of *Parasteatoda,* although *Wnt5, Wnt8, Wnt11-2* and *Wnt16* are also expressed during limb bud formation and/or appendage elongation in this spider (Janssen et al., 2010).

The function of Fz receptors

Despite the striking expression patterns of *fz* genes in animals in many cases the specific functions of most Fz receptors remain enigmatic because knockout or knockdown appears to have no phenotypic consequences. This may be in part because these receptors have overlapping or possibly even redundant functions in certain contexts. For example, as described above, in *Drosophila, fz* and *fz2* appear to have overlapping functions (Bhat, 1998;Kennerdell and Carthew, 1998;Chen and Struhl, 1999;Müller et al., 1999). Similarly in *Tribolium* knockdown of both *fz1* and *fz2* is required to induce germ band phenotypes and while RNAi against *Tribolium fz1* alone affects leg development, knockdown of either *Tribolium fz2* and fz4 alone has no detectable effect (Beermann et al., 2011). Furthermore, *Drosophila fz3* mutants are viable and fertile and unlike *Dfz2*, this *Drosophila fz* does not appear to function together with *fz1* (Sato et al., 1999).

It is also clear that Fz receptors can play more subtle roles perhaps to modulate Wnt signaling rather than transduce an all-or-nothing switch. For example *Dfz3* is not a very effective transducer of Wg and may attenuate Wg activity, as well as receiving other Wnt ligands (Sato et al., 1999).

These findings are consistent with Fz receptors and Wnt ligands acting in complex combinatorial landscapes rather than simple linear pathways to modulate cell-to-cell communication to regulate gene expression, cell fate and ultimately development (van Amerongen and Nusse, 2009). It was previously shown in *Parasteatoda* and *Tribolium* that *Wnt8* is required for the development of the SAZ and production of posterior segments (Bolognesi et al., 2008;McGregor et al., 2008). However, many other Wnts are expressed posteriorly in arthropods (Bolognesi et al., 2008;Janssen et al., 2010;Hayden and Arthur, 2014;Hayden et al., 2015), and our *fz* expression analysis suggests that these ligands work through multiple Fz receptors including Fz1 in and proximal to the SAZ in panarthropods.

Interestingly there is increasing evidence that Fz receptors may interact with other ligands and signaling pathways. For example, it was shown in vertebrates that Fz4 is bound by the Norrin ligand, which also antagonizes BMP signaling (Xu et al., 2004;Deng et al., 2013).

Therefore Fz receptors are probably part of much larger integrated signaling landscapes in some developmental contexts. In order to fully understand the role as well as functional evolution the of *fz* genes in other panarthropods, systematic knockdown of these genes individually and in all combinations in a range of different development stages and tissues in models such as *Parasteatoda,* where functional tools are available (Hilbrant et al., 2012), is required.

Conclusions.

We have shown that the panarthropod *fz* genes show complex patterns of gene expression and divergence in the protein sequences that they encode. However, our analysis has allowed the identification of the possibly ancestral roles for some of these receptors in specific aspects of development such as Fz2 genes in segmentation in arthropods. Further disentangling the roles of these important receptors in animal development requires further comparative analysis of their expression and detailed dissections of their function in different animals and developmental contexts.

Conflict of Interest Statement

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

This study was conceived by AM, MH and RJ. All authors contributed to the acquisition of the data. AM, MH, RJ and AS wrote the manuscript with contributions to drafts by MW, NT, GG, NP and MH. The final version of the manuscript was approved by all authors. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figure legends

Figure 1. Maximum-likelihood tree inferred from the analysis of conserved regions in *frizzled* genes of nine selected panarthropod species (Fig. S1 shows our results from the same species while including *Drosophila*). Node values indicate bootstrap support. Ek, *Euperipatoides kanangrensis*; Gm, *Glomeris marginata*; Pt, *Parasteatoda tepidariorum*; Mm, *Mesobuthus martensii*; Is, *Ixodes scapularis*: Tc, *Tribolium castaneum*; Zn, *Zootermopsis nevadensis*; Sm, *Strigamia maritima*; Pp, *Pholcus phalangioides.*

Figure 2. Panarthropod *frizzled* genes. The four *frizzled* orthology groups are represented by coloured boxes. The absence of a box indicates the loss of a particular *frizzled* orthologue. Duplicated *frizzled* genes are represented by two overlapping boxes. Phylogenetic relationships of the species included are indicated by the tree on the left.

Figure 3. Sequence alignments of the cystein rich domain (CDR) and KTxxxW motif of arthropod Frizzled proteins. 60% consensus sequences, based on the alignment of the Frizzleds from all ten species depicted in figure 2, are shown in bold type separately for orthology groups FzI-IV. Individual sequences are shown for *Euperipatoides kanangrensis* (Ek), *Glomeris marginata* (Gk), *Parasteatoda tepidariorum* (Pt) and *Drosophila melanogaster* (Dm). Chelicerate fz3, for which no *Parasteatoda* sequence was found, is represented by the *Pholcus phalangioides* sequence (Pp-fz3). Cysteine residues are shaded grey in CRD consensus sequences. Black font indicates deviation from the consensus sequence. Red, green and blue

fonts are used to highlight the Lysine, Threonine and Tryptophan residues in the KTxxxW motif. Amino acid positions are indicated according to Dm-fz.

Figure 4. Expression of *Euperipatoides fz1* and *fz2*

In all panels anterior is to the left. Panels **A-E** show expression of *Ek-fz1.* Panels **F-I** show expression of *Ek-fz2*. Panels **A** and **C**, lateral views. Panels **B, E-I,** ventral views. Panels **D,** dorsal view. Panel **F′** shows the DAPI counterstaining of the embryo shown in F (same position). Developmental stages are indicated. Asterisks in panels **A, B** and **E** mark expression at the posterior rim of the head lobes. Arrows in panel **C** point to clusters of cells in the ventral nervous system with upregulated expression. The asterisk in panel **F** marks expression in extraembryonic tissue. Asterisks in **I** mark expression in the ventral nervous system at the base of the walking limbs. The arrow in panel **I** points to dot-like expression in the walking limb L4. Note that this expression is restricted to L4 and L5. Abbreviations: fap, frontal appendage; hl, head lobe; j, jaw; L, walking limb; saz, segment addition zone; sp, slime papilla; st, stage.

Figure 5. Expression of *Glomeris frizzled* genes

In all panels anterior is to the left. Ventral views, except for panels **D** and **H** (lateral views). Developmental stages are indicated. **A-D** Ubiquitous expression of *Gm-fz1.* **E-H** Expression of *Gm-fz2*, **I-L** Expression of *Gm-fz3*. **M-P** Expression of *Gm-fz4*. The arrow in panel **N** points to the midline; the arrowhead points to expression parallel to the midline. Asterisks and filled circles (•) in panel **O** mark two domains of expression in every dorsal segmental unit of the trunk. The arrowhead in panels **F-H** marks dorsal expression. The asterisk in **G** marks expression in the midgut.

Abbreviations: an, antennal segment; av, anal valve; h, head; md, mandibular segment; oc, ocular region; saz, segment addition zone, st, stage; T, trunk segment.

Figure 6. Expression of *Parasteatoda fz1 and fz2*

In all panels anterior is to the left. **A** *Pt-fz1* expression, from left to right, in a ventral view of the posterior prosomal germ band, a ventral view of the opisthosomal germ band and a lateral view. **B-E** *Pt-fz2* expression. **B** flatmounted germ disc. **C, D** Early germ band (anterior prosoma). **E** Germ band (anterior prosoma, posterior prosoma and lateral view). **F** Germ band (anterior prosoma, posterior prosoma and opisthosoma/SAZ view) .The arrow in **D** indicates the expression domain along the anterior border of the germ band. The arrow in **F** marks expression at the stomodaeum. Developmental stages are indicated. Abbreviations: L, walking limb; O, opisthosomal segment; Ch, cheliceres; Pp, pedipalps; PcL, precheliceral lobe; Sto, stomodaeum; st, stage.

Figure 7. Expression of *Euperipatoides fz3* and *fz4*

In all panels anterior is to the left. Panels **A-E** show expression of *Ek-fz3*. Panels **F-J** show expression of *Ek-fz4*. Panels **B, E, I, J**, ventral views. Panels **A, C, D, F, G,** lateral views. Panel **H**, dorsal view. Developmental stages are indicated. The arrow in panel **A** and the asterisks in panels **F** and **G** mark the primordium of the frontal appendages. Abbreviations as in Fig. 3 and a, anus; e, eye; m, mouth.

Figure 8. Expression of *Parasteatoda fz4-1*

In all panels anterior is to the left. **A** Germ band (ventral opisthosomal/SAZ view). **B** Germ band (anterior (left) and posterior (right) prosoma). **C** Germ band (from left to right: posterior opisthosoma/SAZ and anterior prosoma, posterior prosoma and anterior opisthosoma). **D**. Retracting germ band (anterior prosoma, posterior prosoma, opisthosoma). The white arrows in **A** indicate the O1 and O2 segments; the black arrowheads mark *Pt-fz4-1* expression in O2 and the SAZ. Developmental stages are indicated. Abbreviations as in Fig. 6. Lb, labrum; T, telson.

Figure 9. Expression of *Parasteatoda fz4-2*

In all panels anterior is to the left. **A, B** Early germ band (anterior prosoma). **C** Germ band (from left to right: anterior prosoma, posterior prosoma and opisthosoma/SAZ). **D**. Germ band (posterior opisthosoma/SAZ and anterior prosoma, posterior prosoma and anterior opisthosoma) **E**. Retracting germ band (anterior prosoma, posterior prosoma and opisthosoma). The arrows in panels **C, D, E** indicate expression in the labrum. Developmental stages are indicated. SAZ, segment addition zone, further abbreviations as in Fig. 6.

Supplementary data

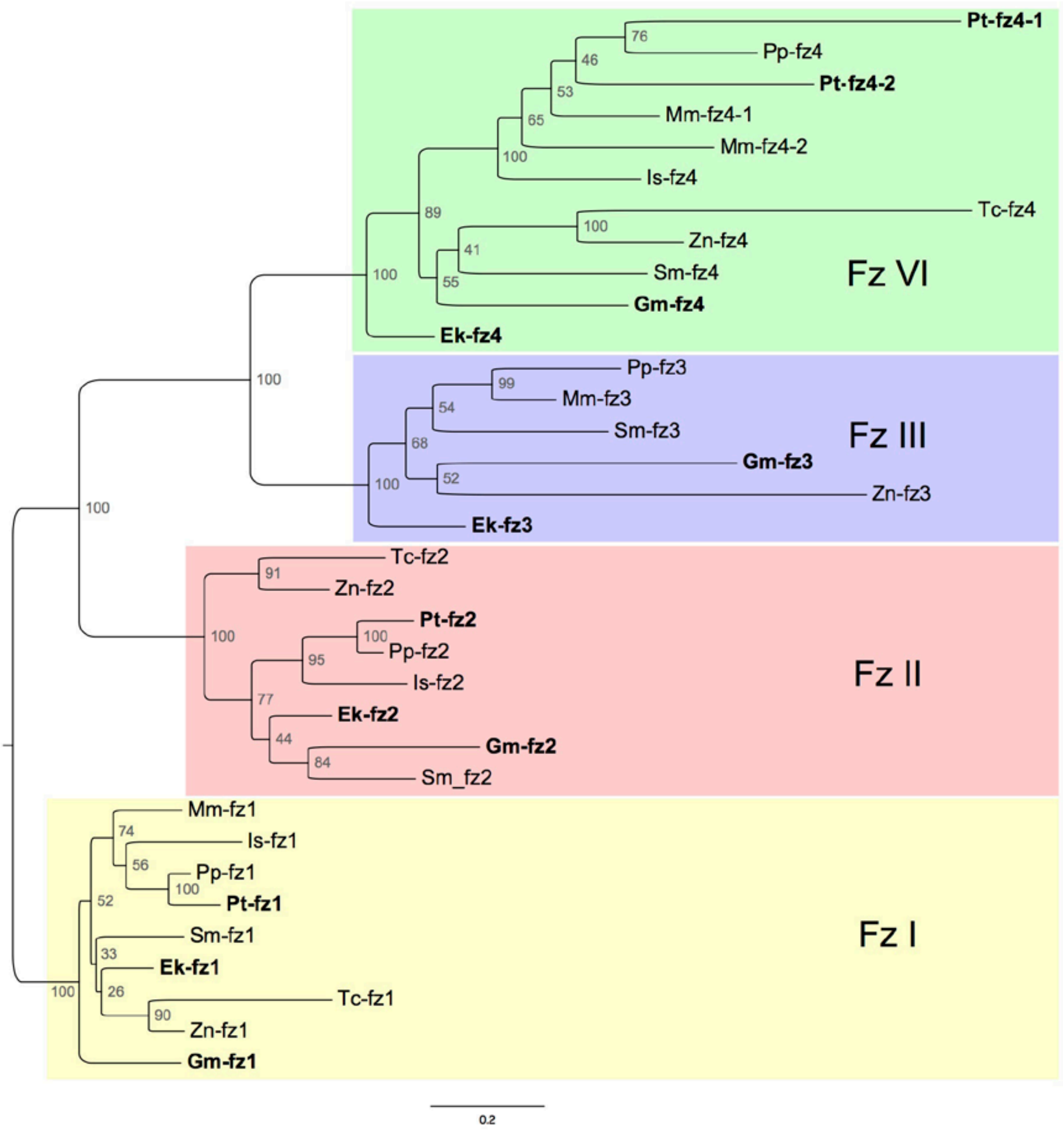
Figure S1. Multiple sequence alignment of all Frizzled sequences found in genomic resources for ten selected panarthropod species including *D. melanogaster.*

Figure S2. Maximum-likelihood tree inferred from the analysis of conserved regions in Frizzled sequences of 10 selected panarthropod species, including *D. melanogaster*.

Figure S3. Additional aspects of *Glomeris fz2* and *fz4* expression

Panels A-C (ventral views; anterior to the left). Panels D-G (dissected appendages, anterior views). H-J (lateral views; anterior to the left). Arrows in panels H-J point to a sharp border of expression within the extraembryonic tissue. The outline of the embryos proper is indicated (dashed lines). The asterisk in H marks the border of expression in embryonic tissue. Abbreviations: an, antenna; md, mandible; mx, maxilla; L walking limb; lr, labrum; oc, ocular region; S, stomodaeum; saz, segment addition zone.

Figures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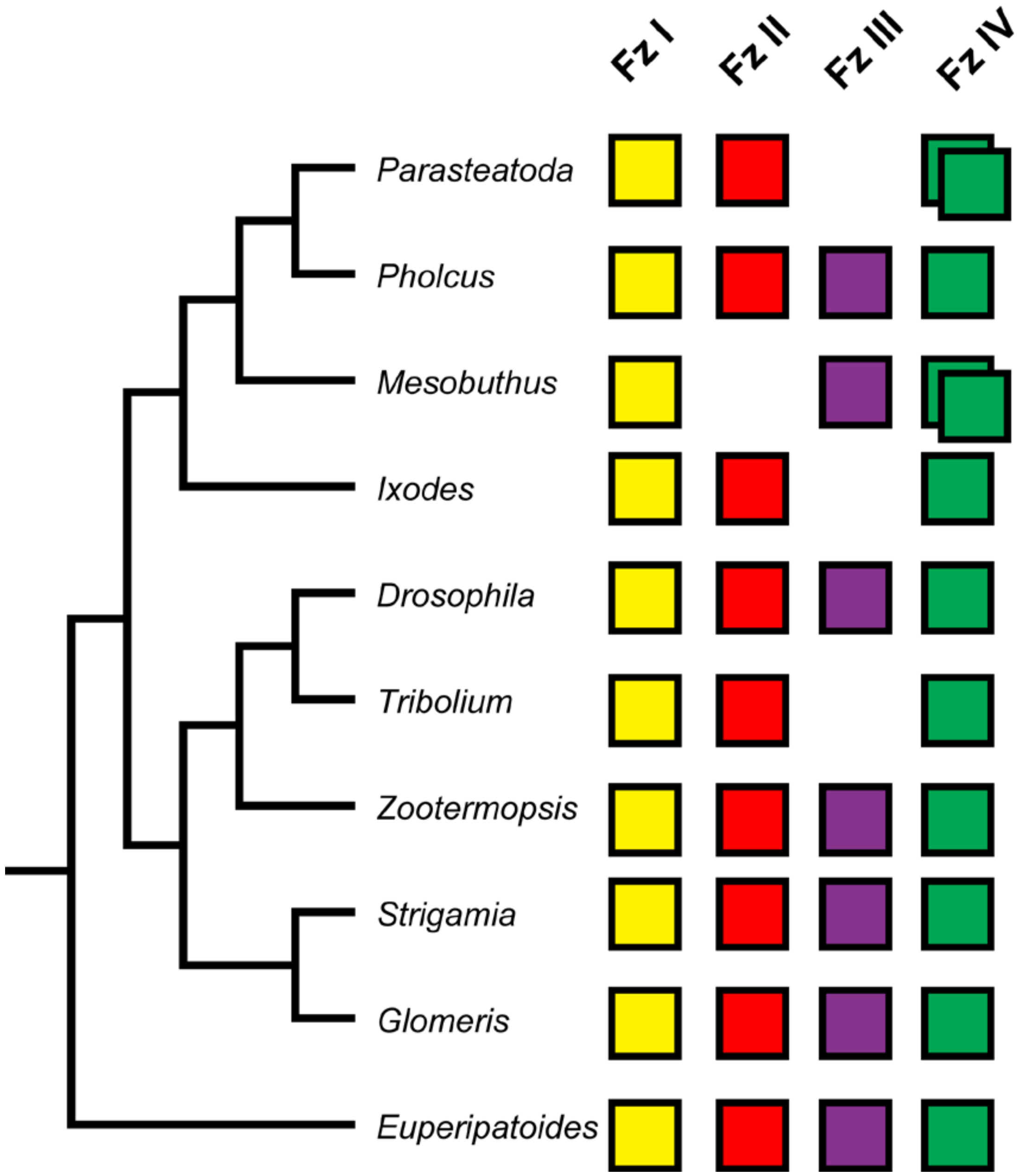
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Fz III
Fz IV
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Mesobuthus
Ixodes
Drosophila
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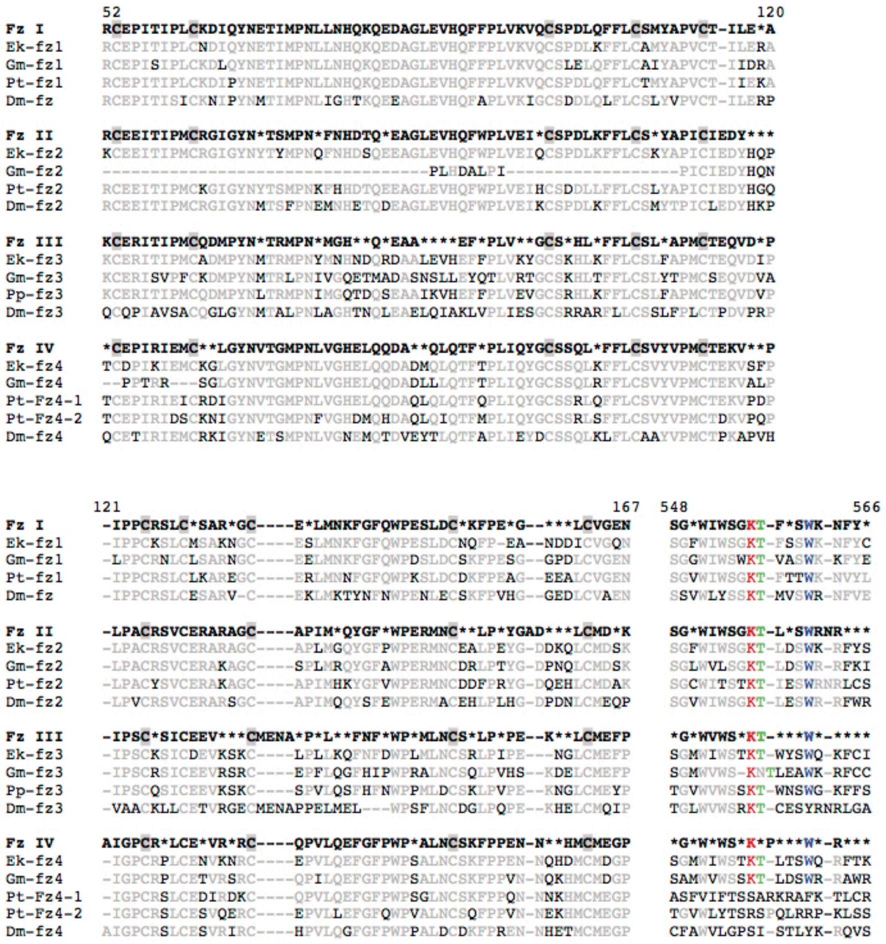
Strigamia
Glomeris
Euperipatoides



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Pt-fz1        RCEPITIPLCKDIPYNETIMPNLLNHQKQEDAGLEVHQFFPLVKVQCSPDLQFFLCTMYAPVCT-IIEKA
Dm-fz         RCEPITISICKNIPYNMTIMPNLIGHTKQEEAGLEVHQFAPLVKIGCSDDLQLFLCSLYVPVCT-ILERP

Fz II         RCEEITIPMCRGIGYN*TSMPN*FNHDTQ*EAGLEVHQFWPLVEI*CSPDLKFFLCS*YAPICIEDY***
Ek-fz2        KCEEITIPMCRGIGYNYTYMPNQFNHDSQEEAGLEVHQFWPLVEIQCSPDLKFFLCSKYAPICIEDYHQP
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Dm-fz2        RCEEITIPMCRGIGYNMTSFPNEMNHETQDEAGLEVHQFWPLVEIKCSPDLKFFLCSMYTPICLEDYHKP

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Pt-Fz4-2      TCEPIRIDSCKNIGYNVTGMPNFVGHDMQHDAQLQIQTFMPLIQYGCSSRLSFFLCSVYVPMCTDKVPQP
Dm-fz4        QCETIRIEMCRKIGYNETSMPNLVGNEMQTDVEYTLQTFAPLIEYDCSSQLKLFLCAAYVPMCTPKAPVH


             121                                            167   548              566
Fz I          -IPPCRSLC*SAR*GC----E*LMNKFGFQWPESLDC*KFPE*G--***LCVGEN   SG*WIWSGKT-F*SWK-NFY*
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Pt-fz1        -IPPCRSLCLKAREGC----ERLMNNFGFQWPKSLDCDKFPEAG--EEALCVGEN   SGVWIWSGKT-FTTWK-NVYL
Dm-fz         -IPPCRSLCESARV-C----EKLMKTYNFNWPENLECSKFPVHG--GEDLCVAEN   SSVWLYSSKT-MVSWR-NFVE

Fz II         -LPACRSVCERARAGC----APIM*QYGF*WPERMNC**LP*YGAD***LCMD*K   SG*WIWSGKT-L*SWRNR***
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Ek-fz3        -IPSCKSICDEVKSKC----LPLLKQFNFDWPLMLNCSRLPIPE---NGLCMEFP   SGMWIWSTKT-WYSWQ-KFCI
Gm-fz3        -IPSCRSICEEVRSRC----EPFLQGFHIPWPRALNCSQLPVHS--KDELCMEFP   SGMWVWS-KNTLEAWK-RFCC
Pp-fz3        -IPSCQSICEEVKSKC----SPVLQSFHFNWPPMLDCSKLPVPE--KNGLCMEYP   TGVWVWSSKT-WNSWG-KFFS
Dm-fz3        -VAACKLLCETVRGECMENAPPELMEL---WPSFLNCDGLPQPE--KHELCMQIP   TGLWVWSRKT-CESYRNRLGA

Fz IV         AIGPCR*LCE*VR*RC----QPVLQEFGFPWP*ALNCSKFPPEN-N**HMCMEGP   *G*W*WS*K*P***W*-R***
Ek-fz4        -IGPCRPLCENVKNRC----EPVLQEFGFPWPSALNCSKFPPEN-NQHDMCMDGP   SGMWIWSTKT-LTSWQ-RFTK
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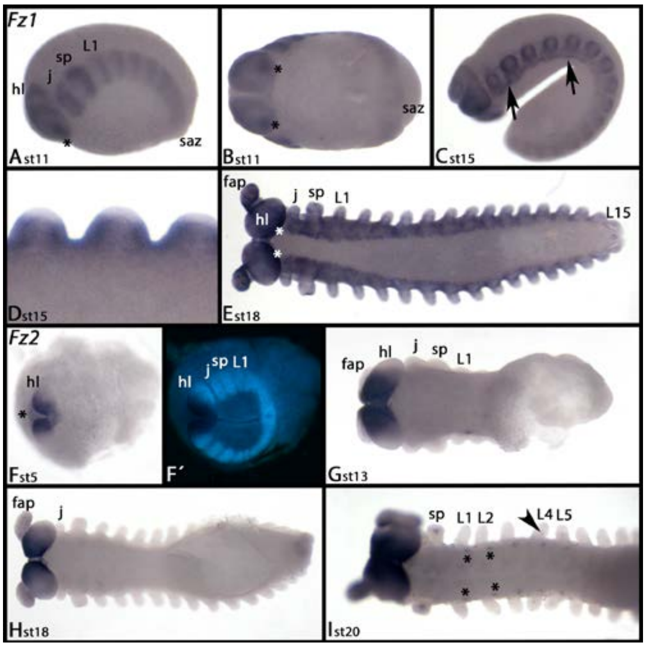

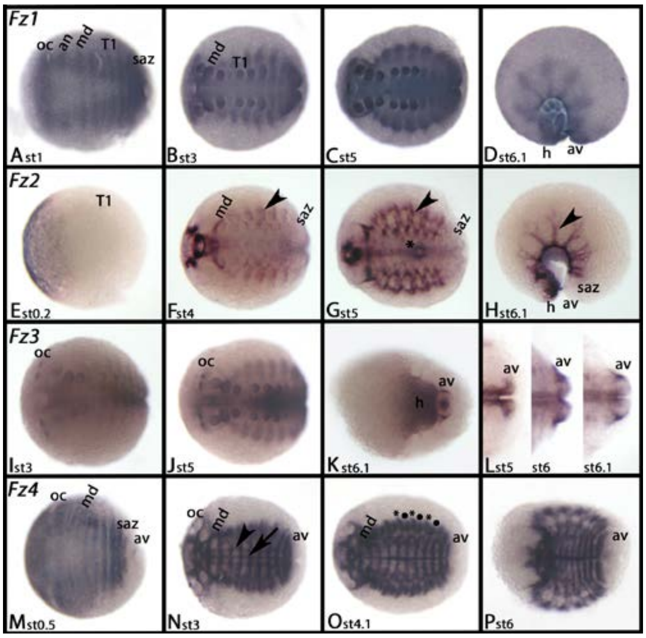
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saz
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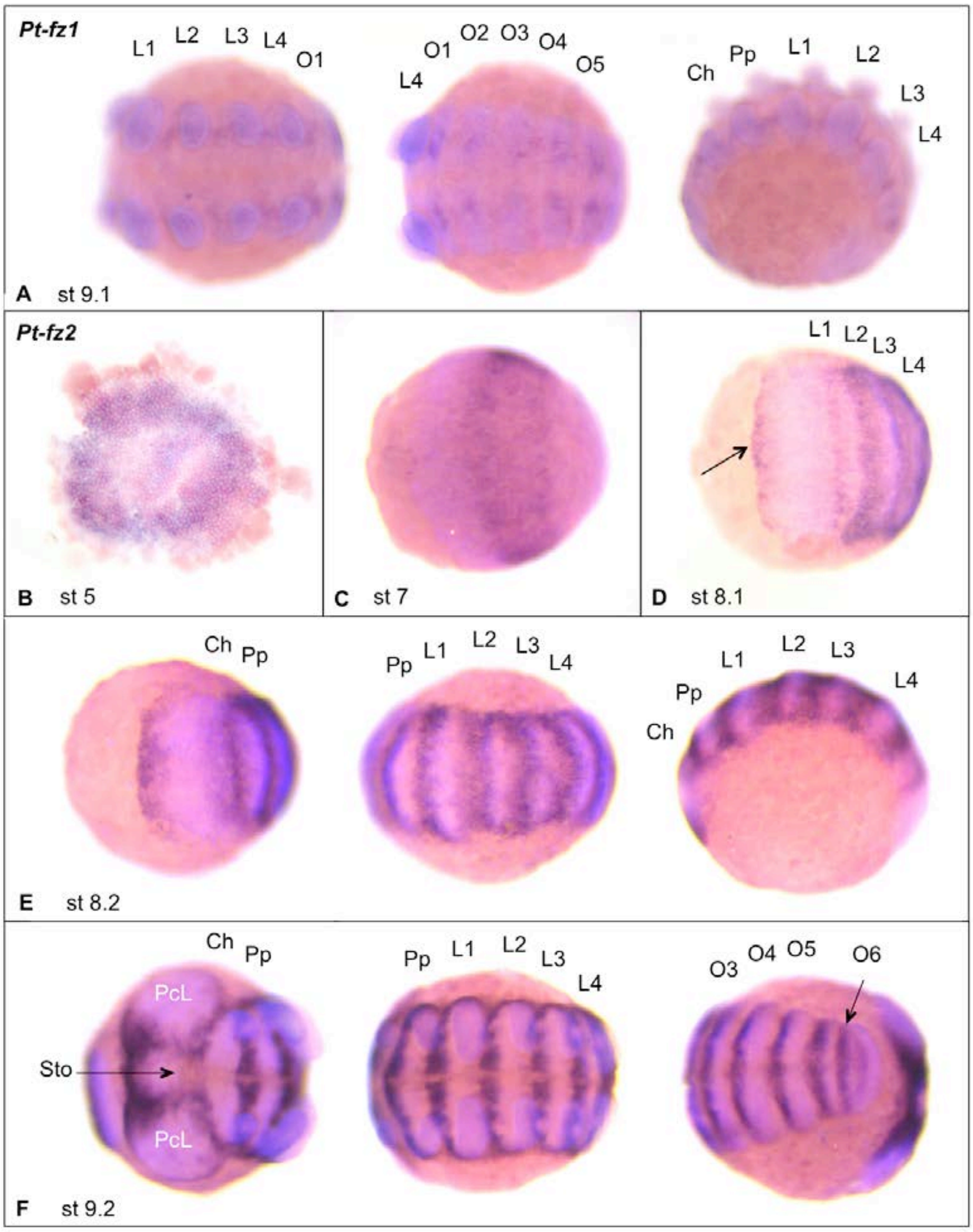
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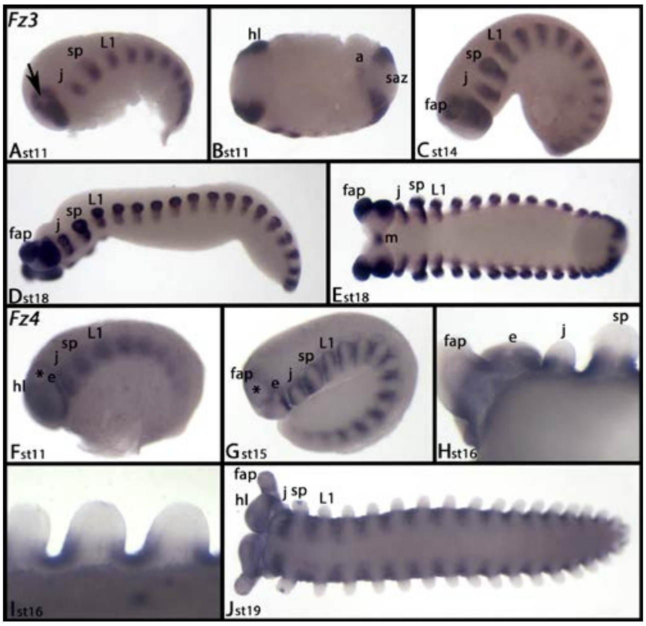
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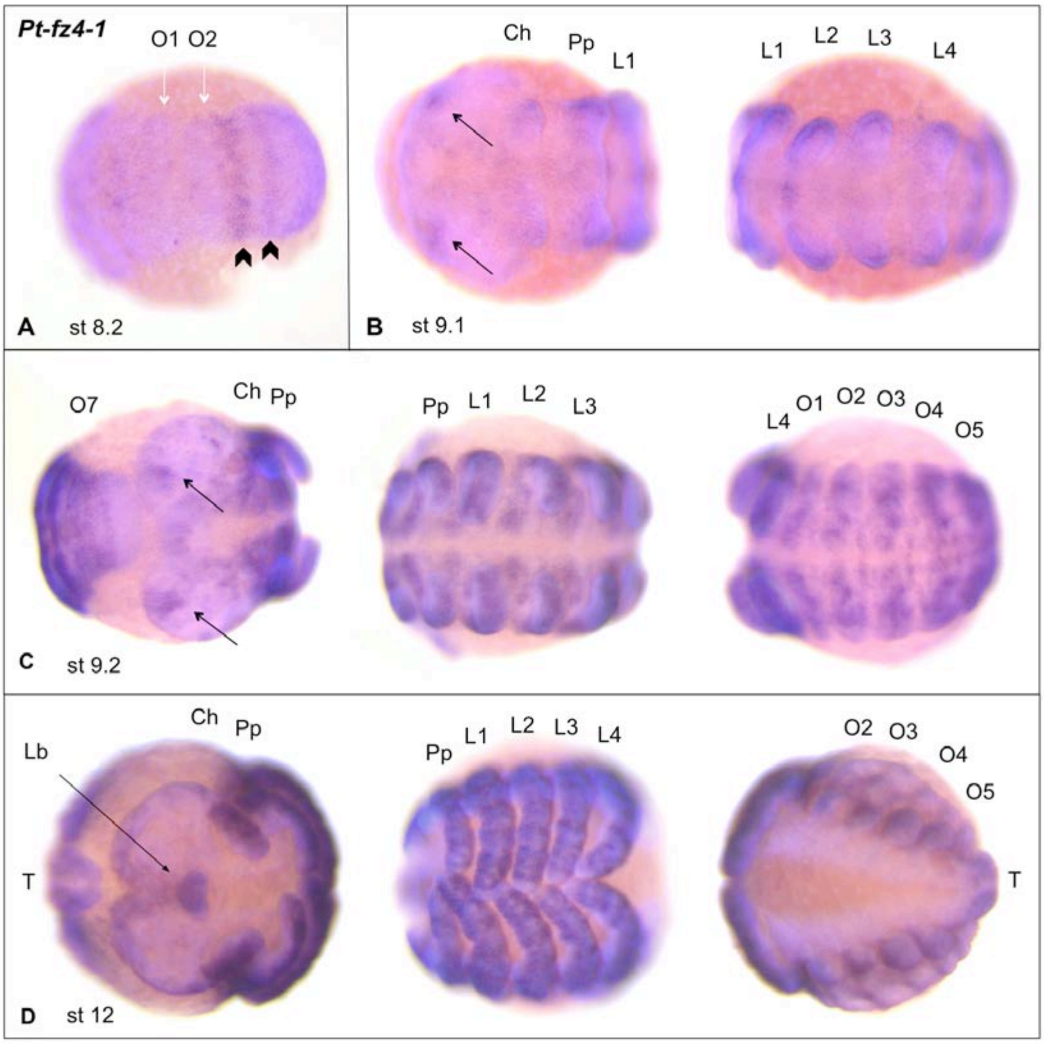
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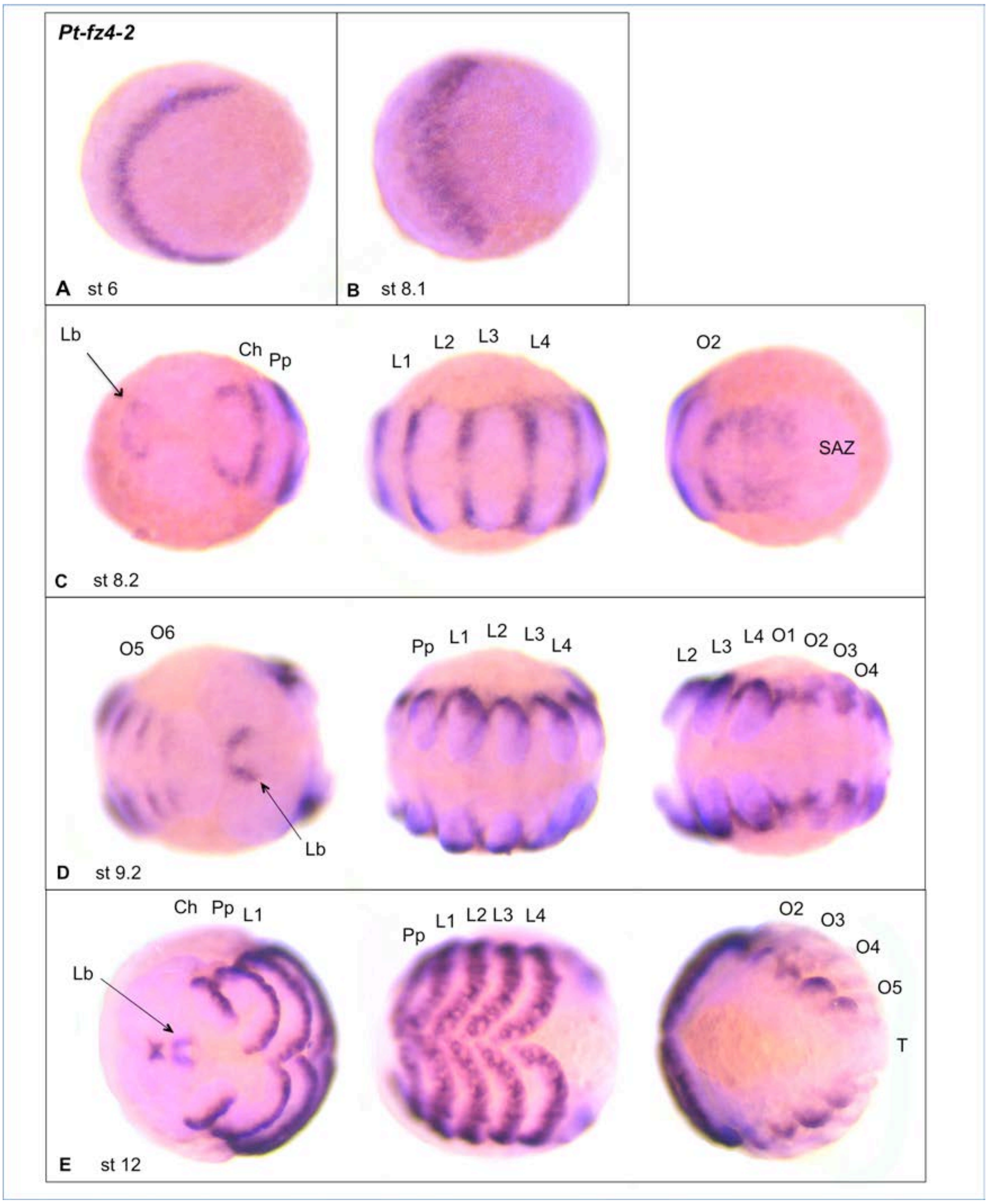
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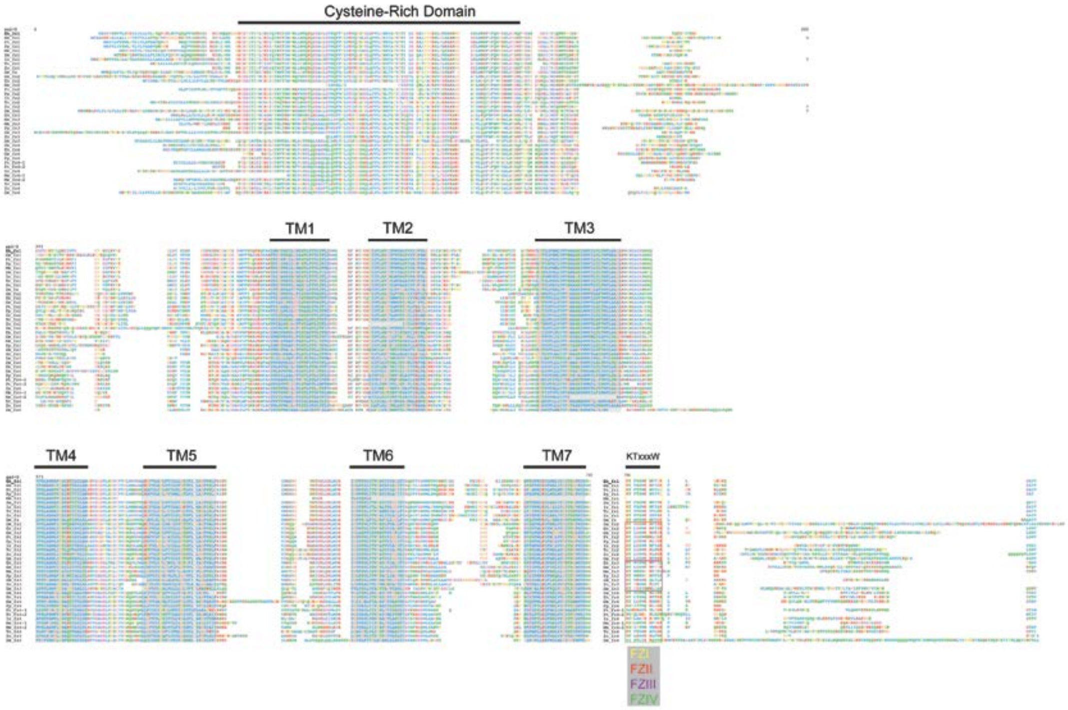
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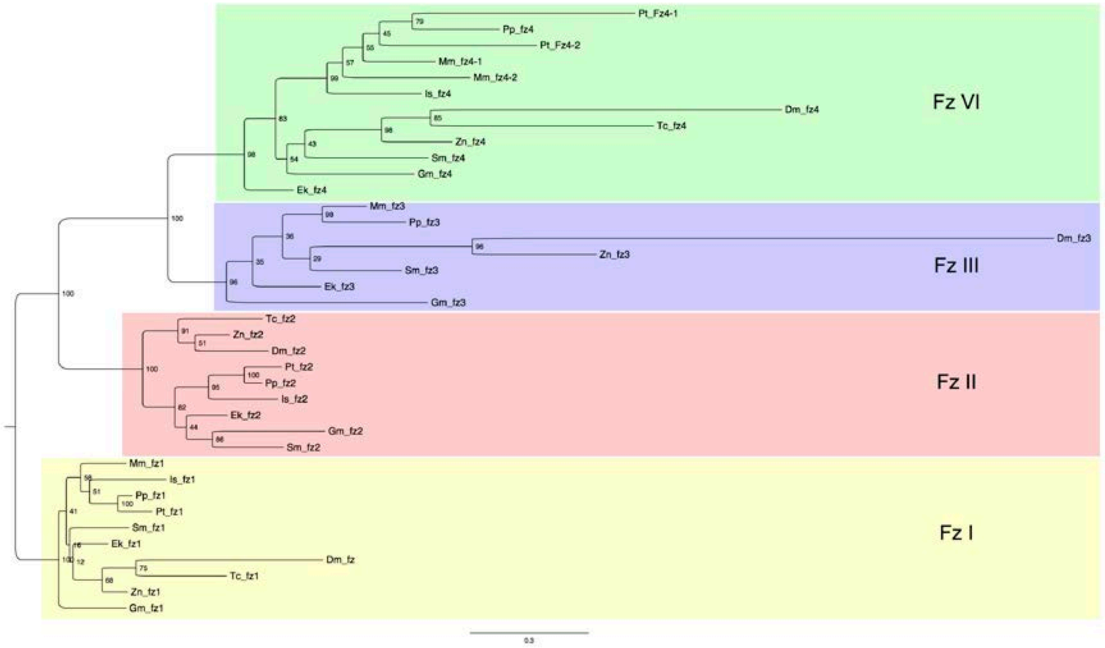
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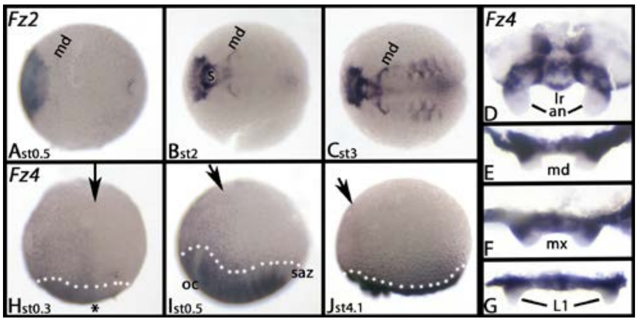
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