six3 acts upstream of foxQ2 in labrum and neural development in the spider Parasteatoda tepidariorum

Magdalena Ines Schacht 1,2 · Christoph Schomburg 1,3 · Gregor Bucher 1

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Abstract
Anterior patterning in animals is based on a gene regulatory network, which comprises highly conserved transcription factors like six3, pax6 and otx. More recently, foxQ2 was found to be an ancestral component of this network but its regulatory interactions showed evolutionary differences. In most animals, foxQ2 is a downstream target of six3 and knockdown leads to mild or no epidermal phenotypes. In contrast, in the red flour beetle Tribolium castaneum, foxQ2 gained a more prominent role in patterning leading to strong epidermal and brain phenotypes and being required for six3 expression. However, it has remained unclear which of these novel aspects were insect or arthropod specific. Here, we study expression and RNAi phenotype of the single foxQ2 ortholog of the spider Parasteatoda tepidariorum. We find early anterior expression similar to the one of insects. Further, we show an epidermal phenotype in the labrum similar to the insect phenotype. However, our data indicate that foxQ2 is positioned downstream of six3 like in other animals but unlike insects. Hence, the epidermal and neural patterning function of foxQ2 is ancestral for arthropods while the upstream role of foxQ2 may have evolved in the lineage leading to the insects.

Keywords Spiders · Labrum · RNAi · Gene interaction

Introduction
Anterior patterning in animals is based on an anterior gene regulatory network (aGRN), which is built by a conserved set of transcription factors and signalling pathways (Lowe et al. 2003; Marlow et al. 2014; Posnien et al. 2011; Sinigaglia et al. 2013; Steinmetz et al. 2010; Yaguchi et al. 2008; Tosches and Arendt 2013). However, functional studies revealed that the regulatory interactions within this ancestral patterning system have diverged between animals (Range and Wei 2016; Sinigaglia et al. 2013; Yaguchi et al. 2008; Kitzmann et al. 2017). One example is the function of the forkhead box transcription factor foxQ2. The forkhead box (Fox) family is a large family of transcription factors that holds many important regulatory roles in numerous developmental and homeostatic processes (Fritzenwanker et al. 2014; Uhlenhaut and Treier 2011; Benayoun et al. 2011; Carlsson and Mahlapuu 2002), and is widely represented in the animal kingdom (Kaestner et al. 2000). foxQ2 is a member of this transcription factor family, which is present in all major lineages of the metazoans (Chevalier et al. 2006; Sinigaglia et al. 2013; Marlow et al. 2014; Tu et al. 2006; Yu et al. 2003; Lee and Frasch 2004). In deuterostomes, such as the cephalochordate Branchiostoma floridae (B. floridae) and the echinoderm Strongylocentrotus purpuratus (S. purpuratus), foxQ2 is expressed at the anterior pole from the late blastula stage (B. floridae) or mesomeric cleavage stage (S. purpuratus) onwards. In both cases, transcripts are
still detected in larval stages in the anterior tip of the ectoderm (Yu et al. 2003; Tu et al. 2006; Yaguchi et al. 2008; Yankura et al. 2010; Range and Wei 2016). The three paralogs of foxQ2 in the hemichordate Saccoglossus kowalevskii (S. kowalevskii) also share a largely apical expression pattern, while exhibiting temporal differences (Fritzenwanker et al. 2014). A conserved apical expression of foxQ2 is also present in lophotrochozoans, such as the annelid Platynereis dumerilii (P. dumerilii) and the brachiopod Terebratalia transversa (T. transversa) (Santagata et al. 2012; Marlow et al. 2014), as well as in the early development of the insects Drosophila melanogaster (D. melanogaster) and Tribolium castaneum (T. castaneum) (Lee and Frasch 2004; Kitzmann et al. 2017). Additionally, in non-bilaterian species, such as the cnidarians Nematostella vectensis (N. vectensis) and Clytia hemispherica (C. hemispherica), foxQ2 expression is restricted to the aboral pole of the embryos (Chevalier et al. 2006; Sinigaglia et al. 2013), supporting the hypothesis of homology between the cnidarian aboral pole and the bilaterian anterior pole (Sinigaglia et al. 2013). Interestingly, while orthologs are found in fish, the foxQ2 gene was apparently lost from the genomes of amphibians and mammals.

foxQ2 is expressed in neurogenic regions of different species (Kitzmann et al. 2017; Hunnekuhl and Akam 2014; Marlow et al. 2014; Santagata et al. 2012). In P. dumerilii, foxQ2 and six3 are expressed in the apical plate including sensory-neurosecretory cells (Marlow et al. 2014). Similarly, in the myriapod Strigamia maritima (S. maritima), nested domains of foxQ2 and six3 expression were identified in neural precursors of the embryonic head (Hunnekuhl and Akam 2014). Both these transcription factors pattern anterior neurogenic domains during the embryonic development of the brachiopod T. transversa (Santagata et al. 2012). In the red flour beetle, Te-foxQ2-positive cells contribute to the central complex and the gene is required for central brain development (He et al. 2019). In T. castaneum, an extended GRN for anterior head development has been proposed in which Tc-six3 and Tc-foxQ2 hold upstream positions (Kitzmann et al. 2017). They mutually regulate each other forming an upstream core unit, which regulates several downstream targets. Those targets are required for the proper formation of anterior structures, like the head placode, neurogenic ectoderm, labrum and stomodeum, and are therefore important for the development of the head and brain (Kitzmann et al. 2017). Intriguingly, no or only mild epidermal effects were found in foxQ2 knockdown embryo in other animals, while in T. castaneum, a strong effect on epidermal patterning was observed (Kitzmann et al. 2017). This finding correlated with the downstream role of foxQ2 in most analysed species except for T. castaneum. However, it has remained unclear which of these aspects are ancestral for arthropods and which may be insect specific.

In order to gain insights from a basal euarthropod, we studied the foxQ2 expression, its RNAi phenotype and its interaction with six3 in the spider P. tepidariorum. We find that the epidermal labrum phenotype is conserved but that spider Pt-foxQ2 appears not to regulate either of the two Pt-six3 paralogs.

**Material and methods**

**Phylogenetic analysis**

The Pt-foxQ2 sequence (aug3.g4586.t1) was identified by performing a BLAST search using the translated nucleotide query of FoxQ2 from D. melanogaster (declared on FlyBase as fd102C, FlyBase ID: FBgn0039937) in the P. tepidariorum genomic gene predictions (Schwager et al. 2017). The 20 best-scoring sequences were combined with published protein sequences of FoxQ2, as well as the protein sequence of FoxP of D. melanogaster and Danio rerio, which were retrieved from Fritzenwanker et al. (2014). All protein sequences were subsequently used to perform a protein alignment with default settings using Clustal Omega (Sievers et al. 2011; Sievers and Higgins 2014). Phylogeny was inferred based on this alignment using MrBayes 3.2.0 (Ronquist et al. 2012), which subsequently used to perform a protein alignment with default settings using Clustal Omega (Sievers et al. 2011; Sievers and Higgins 2014). Phylogeny was inferred based on this alignment using MrBayes 3.2.0 (Ronquist et al. 2012), which was sampled from a total of 1836 runs using the Wag model of amino acid substitution.

**Animal culture and gene cloning**

Embryos and adults of P. tepidariorum were obtained from the stock in Göttingen. Spider were kept and fed as previously described (Turetzek et al. 2015; Pechmann et al. 2017). Embryos used for in situ hybridization (ISH) experiments were staged, collected and fixed as previously described (Hilbrant et al. 2012; Mittmann and Wolf 2012; Posnien et al. 2014). The P. tepidariorum genes six3.1 (NCBI GenBank: AB605265.1) and six3.2 (NCBI GenBank: AB605266.1) used in this work were already published in Schomburg et al. (2015). The identified Pt-foxQ2 sequence (NCBI GenBank: MN567069) was amplified from cDNA (fwd primer: 5’CGCTACTGACCAGAACCCTTT 3’; rev primer: 5’TAAAGGAAGCCGGATGACCA 3’) amplified a 979 bp long sequence) and cloned into the pJet1.2 vector (Thermo Fisher Scientific).

**Probe synthesis and in situ hybridization**

Pt-foxQ2 antisense mRNA probes for ISHs were synthesized from the entire cloned sequence (979 bp) with the DIG RNA labelling Mix using the T7 RNA polymerase (both Roche, DIG RNA labelling Mix with Cat.-No.: 11277073910 and T7 Polymerase with Cat.-No.: 10881767001). The Pt-six3.1
and Pt-six3.2 antisense mRNA probes were those used in Schomburg et al. (2015) and are of 1439 bp and 1328 bp length, respectively. ISHs and nuclear SYTOX® Green staining in P. tepidariorum embryos were carried out as described previously (Prpic et al. 2008; Pechmann and Prpic 2009).

**dsRNA synthesis and RNA interference**

The complete cloned sequence for Pt-foxQ2, Pt-six3.1 and Pt-six3.2 (see details above) was amplified using vector specific primers combined with the T7 promoter sequence (5′GAAT TGTAATACGACTCActATAGG3′) in standard PCR and served as a template for the in vitro transcription. Double stranded RNA (dsRNA) of each gene was synthesized using the Ambion® T7 MEGAscript® Kit (Life Technologies, Carlsbad, CA, USA). Precipitation of the transcribed dsRNAs and the injection procedure was performed as described previously (Akiyama-Oda and Oda 2006; Turetzek et al. 2015). For Pt-foxQ2, Pt-six3.1 and Pt-six3.2, 2.5 μl of dsRNA (4 μg/μl) was injected into sexually mature adult female spiders using borosilicate injection needles. Females were treated with 5 injections in total, whereby females were injected every second day and mated after the third injection. The same injection regime was applied for the negative control spiders that were injected with injection buffer only. Previous studies used GFP as a control injections which result in very few unspecific effects in RNAi embryos (Schwager et al. 2009; Paese et al. 2018). Therefore, we decided to use injection buffer only. Each female was kept and fed in the same way. The first four cocoons of each spider were collected, opened and kept in test tubes provided with a damp tissue to maintain humidity, essential for proper development. Half of each cocoon was fixed, whereas the other half could develop. During development, the embryos were checked regularly under the stereomicroscope and individual embryos of each cocoon were analysed in Voltalef® oil. All embryos were screened for any visible phenotypes. For the analysis of the embryos, batch 2–4 were merged because they had similar strong effects while batch 1 showed less penetration.

**Microscopy and imaging**

Images of P. tepidariorum embryos were taken with a Leica M205 FA binocular (Leica Microsystems, Wetzlar, Germany) and recorded with the ImagePro v.7.0 software. Images were taken with fluorescent and white light simultaneously. After white correction, the green SYTOX® appears light blue, while the DIG staining appears dark blue, thus allowing staining and morphology to be shown in the same picture. Images were corrected for colour values and brightness with the Adobe Photoshop® CS6 software (version 9.0).

**Results**

**P. tepidariorum possesses one homolog of foxQ2**

Large-scale duplication of genes in spiders has been reported in P. tepidariorum before (Pechmann et al. 2015; Turetzek et al. 2015; Schomburg et al. 2015; Schwager et al. 2017; Leite et al. 2018). Therefore, we wanted to identify the total number of foxQ2 orthologs in P. tepidariorum. We performed a multiple sequence alignment with FoxQ2 protein sequences of different metazoan species and generated a phylogenetic tree based on Bayesian analysis. The phylogenetic tree (Fig. 1) shows that the spider’s translated transcript aug3.g4586.t1 clusters with all FoxQ2 sequences from the different species and is therefore indeed the spider foxQ2 ortholog. None of the other identified P. tepidariorum sequences cluster with Pt-FoxQ2 or any other analysed metazoan FoxQ2 sequence, indicating that there is only one homolog of foxQ2 in the spider (shown in red, Fig. 1).

**Pt-foxQ2 shows conserved expression in the anterior region of the embryo**

Previous studies in different metazoan species showed that foxQ2 is expressed in the anterior regions during embryonic development (Yu et al. 2003; Lee and Frasch 2004; Tu et al. 2006; Marlow et al. 2014; Fritzenwanker et al. 2014; Kitzmann et al. 2017) but expression analysis of foxQ2 in chelicerates had been missing. Here, we analysed the expression of Pt-foxQ2 during spider embryogenesis (see Mittmann and Wolff, 2012 for detailed description of morphology during embryogenesis and Schomburg et al. 2015 for description of anterior structures for embryonic stages 10 to 13). We found that expression of Pt-foxQ2 started at stage 7, when the germ band became evident, spanning the anterior rim of the germ band as a broad stripe (arrowhead in Fig. 2a; see Fig. S1 for stages 3–6, devoid of Pt-foxQ2 staining). At stage 8, Pt-foxQ2 was bilaterally expressed in the anterior part of the pre-cheliceral region in one larger domain (white arrowheads in Fig. 2b) and an emerging smaller domain posterior-laterally to it (black arrowheads in Fig. 2b). Expression of Pt-foxQ2 showed a tripartite domain on each side of the pre-cheliceral region at stage 9 with an additional domain on both sides of the anterior non-neurogenic ectoderm (asterisks in Fig. 2c; the non-neurogenic ectoderm in the pre-cheliceral lobes is all ectoderm along the rim of the head lobes outside of the anterior and lateral furrows. This area is free of neural precursor invagination sites—see Fig. S2 for a scheme of a stage 11 embryo and Schomburg et al. 2015 for extensive description from stage 10 to 14). At stage 10, these three domains were localized in a different pattern. Transcripts of Pt-foxQ2 in the non-neurogenic ectoderm became localized more anteriorly (asterisks in Fig. 2d–f), while the median
domains split into three distinct domains (white arrowheads in Fig. 2d). The lateral expression in the neurogenic ectoderm splits into two domains between the edge of the head lobes and the prospective labrum (black arrowheads in Fig. 2d). At stage 11, the most lateral expression domains remained at the end of the anterior furrow (black arrowheads in Fig. 2e), while the median expression domain shifted posteriorly along with the stomodeum and labrum. The former domains fused into the median expression domain shifted posteriorly along with the stomodeum at earlier stages (white arrowheads in Fig. 2e, f). During stage 13, the non-neurogenic ectoderm started to grow over the brain region (Fig. 2g), and the prospective labrum (black arrowhead in Fig. 2f), while the other expression was found in a complex spotted pattern in the brain region and at the base of the labrum (black arrowhead in Fig. 2h). Although it may appear as if expression of Pt-foxQ2 could be found in the chelicerae (dotted outlines in Fig. 2h), the expression was in fact located underneath the chelicerae and corresponded to the domains located next to the stomodeum at earlier stages (white arrowheads in Fig. 2e, f).

The same was true for the complex expression in the neurogenic ectoderm, which is likely only expressed in the neurogenic ectoderm, but not in the epidermal tissue above. The complex expression pattern showed that Pt-foxQ2 was expressed in the anterior tip of the early germ band and later in the neurogenic and non-neurogenic ectoderm and the labrum.

**Downregulation of Pt-foxQ2 results in labrum defects**

In most species, foxQ2 is downstream of six3 in the aGRN. In contrast, mutual activation was found in *T. castaneum* indicating a gain of an upstream role in the aGRN (Kitzmann et al. 2017). Therefore, we sought to identify the level of conservation of this genetic interaction between these two transcription factors in the spider. There are two paralogs of six3 in *P. tepidariorum* (*Pt-six3.1* and *Pt-six3.2*) and their embryonic expression patterns had been described previously...
At late embryonic stages (stage 10–12), Pt-six3.1 is expressed in the neurogenic ectoderm and the labrum. Transcripts of Pt-six3.2 are detected in the labrum, stomodeum and in the anterior median region of the head. At stage 13, Pt-six3.2 is expressed in the primordia of the lateral eyes and in cells underneath the non-neurogenic ectoderm (Schomburg et al. 2015).

We performed parental RNAi (pRNAi) to downregulate the expression of Pt-foxQ2 and analyse the function of this transcription factor during embryogenesis. Likewise, we analysed the function of Pt-six3.1 and Pt-six3.2 by RNAi to obtain information about a possible interaction between Pt-foxQ2 and Pt-six3.1/six3.2. The analysis included data from females (cocones 1–4 from 3 different females) that were either injected with dsRNAs (Pt-foxQ2, Pt-six3.1 or Pt-six3.2), or with injection buffer (negative control), and from one female spider that was not injected at all (wild type) (supplemental material Tab. S1). Each cocoon was checked for viable and dead embryos (referred to as Pt-geneX RNAi embryos) and they were scored for morphological defects. We found that Pt-foxQ2 RNAi, Pt-six3.1 RNAi and Pt-six3.2 RNAi embryos showed no cuticle defects. They were able to develop until the embryo started to eclose. However, most of the embryos were unable to hatch from the egg membrane indicating an essential function of those genes. Instead, the membrane remained intact and the embryos stayed at this developmental stage for several days before drying out. Fifty-nine percent of the Pt-foxQ2 RNAi embryos did not hatch, and the equivalent phenotype was observed for 43% in Pt-six3.1 RNAi, and for 24% in Pt-six3.2 RNAi embryos in the first cocoon. This phenotype increased in subsequent cocoons (99–100% for Pt-foxQ2 RNAi embryos, 70–84% for Pt-six3.1 RNAi embryos, 66–73% for Pt-six3.2 RNAi embryos) (Fig. 3a). Three to 36% of the negative control embryos did not hatch or were dead (Fig. 3a) which was similar to the rate observed in previous studies (Turetzek et al. 2015). While we did not observe cuticle phenotypes in any of our treatments, we did find morphological differences in the Pt-foxQ2 RNAi embryos at stages 10 (77% in cocoon 1 and 88% in cocoon 2–4) and 11 (40% in cocoon 1 and 50% in cocoon 2–4). In 38% (cocoon 1) and in 45% (cocoon 2–4) of the injected embryos, the labrum was strongly reduced or completely missing (Fig. 3b; Fig. 4b–h).

**Interaction between Pt-foxQ2, Pt-six3.1 and Pt-six3.2**

In order to test whether Pt-foxQ2, Pt-six3.1 and Pt-six3.2, respectively, interact with one another, we performed Pt-foxQ2 in situ hybridisations in Pt-six3.1 RNAi and Pt-six3.2 RNAi embryos and vice versa. We found RNAi against Pt-foxQ2 had no effect on the expression of either of the Pt-six3 paralogs, with the exception of the labrum, which normally expresses both Pt-six3.1 and Pt-six3.2, but was missing after Pt-foxQ2 RNAi (Fig. 4; note that the embryo in b has an

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**Fig. 2** Embryonic Pt-foxQ2 expression. After initial expression as a stripe at the anterior end of the embryo (white arrowhead in a), Pt-foxQ2 is found in several dynamic domains in the pre-cheliceral lobes (b–f). White and black arrowheads and asterisks mark the corresponding domains in all panels. From stage 11 onward, the head epidermis grows over the neurogenic ectoderm (anterior furrow (AF) in e). At later stages, Pt-foxQ2 transcripts are found in the labrum and the brain (g, h). Expression is not in the chelicerae but below of them (black arrowhead in h). All embryos shown in frontal view. Developmental stages are indicated in the lower right-hand corner. AF: anterior furrow; Pp: pedipalps; Ch: chelicerae; L1: first walking leg)
overall weaker staining). The morphological defect served as internal control that the RNAi experiment had worked efficiently in these embryos. However, we cannot exclude that the level of downregulation may have been insufficient for an effect on Pt-six3.1 and Pt-six3.2 expression.

In the converse experiment, RNAi against neither Pt-six3.1 nor Pt-six3.2 showed any apparent morphological differences between wild-type and injected embryos (Fig. 5). The expression of Pt-foxQ2, however, was altered. At stage 10, the most anterior of the tripartite median domain (white arrowheads in Fig. 2d) was clearly missing after injection with either Pt-six3.1 or Pt-six3.2 dsRNA (asterisks in Fig. 5a, c). At the later stages (Fig. 5b, d), it was difficult to make exact statements about the nature of the changes of expression in the RNAi phenotype, due to the complex dynamic of the expression patterns and the morphological rearrangements during those stages. However, it is clear that the Pt-foxQ2 expression domain around the stomodeum was reduced in the RNAi experiments, compared to the wild-type staining (white arrowheads in Fig. 2e). The domain was probably also lacking at stage 11, but this was less clear because at that stage, the two domains had fused in wild type (Fig. 2e).

![Fig. 4](image-url) Pt-six3 expression in Pt-foxQ2RNAi embryos. Expression of Pt-six3.1 in Pt-foxQ2RNAi embryos (a–d). Expression of Pt-six3.2 in Pt-foxQ2RNAi embryos (e–h). There were no qualitative changes in expression of Pt-six3 paralogs in Pt-foxQ2RNAi embryos (note that the overall staining in embryo b is weaker due to experimental conditions). After Pt-foxQ2 RNAi, the labrum (dotted lines in all panels) was reduced or missing leading to a secondary reduction of the Pt-foxQ2-positive tissue. All embryos shown in frontal view. Developmental stages are indicated in the lower right-hand corner, the stained gene in the lower left-hand corner and treatment in the upper right-hand corner.
Pt-foxQ2 as a conserved anterior marker and important factor in labrum and neural development

Here, we present the first expression and functional study of a foxQ2 homolog in chelicerates. Like in all metazoan animals analysed so far (Yu et al. 2003; Tu et al. 2006; Yaguchi et al. 2008; Sinigaglia et al. 2013; Chevalier et al. 2006), it is expressed as a patterning gene in the aGRN in the early embryo. Later in development, Pt-foxQ2 is expressed in various regions of the neurogenic ectoderm, which suggests its involvement in neural development might be a conserved aspect among arthropods (Kitzmann et al. 2017; Hunnekuhl and Akam 2014). Furthermore, Pt-foxQ2 has a similar effect on the development of the labrum as seen in T. castaneum (Kitzmann et al. 2017). This hints at a conserved role for foxQ2 in the development of epidermal head structures in arthropods in contrast to other metazoan lineages where gene knockdown did not reveal strong epidermal defects (Yaguchi et al. 2008; Sinigaglia et al. 2013).

The two Pt-six3 paralogs appear to compensate for each other functions in P. tepidariorum

Loss of Pt-six3.1/3.2 function in P. tepidariorum showed no deleterious effect in the anterior median region in contrast to T. castaneum, where this whole region including the labrum is deleted (Posnien et al. 2011). This was unexpected because in all animals studied so far, six3 acted upstream to or on the same level as foxQ2. However, P. tepidariorum has two six3 paralogs with overlapping expression in the anterior median region (compare Fig. 4a and e), which could lead to a compensatory effect. One hint for compensatory function is the elevated portion of hatched animals in both Pt-six3.1/3.2 treatments (about 30% in the second clutch) compared to the Pt-foxQ2 treatment (1% in the second clutch). Unfortunately, double RNAi has so far not been successful in P. tepidariorum.

An evolutionary playground of anterior interactions

foxQ2 and six3 are both prominent factors in anterior development and are commonly co-expressed during embryogenesis and show quite different interactions in different animal species (Fritzenwanker et al. 2014; Hunnekuhl and Akam 2014; Marlow et al. 2014; Martín-Durán and Hejnol 2015; Santagata et al. 2012; Sinigaglia et al. 2013; Tü et al. 2006; Wei et al. 2009; Range and Wei 2016; Kitzmann et al. 2017). In all studied animals but in insects, six3 is clearly upstream of foxQ2 in the aGRN. In T. castaneum, in contrast, Tc-foxQ2 gained a more upstream role activating Tc-six3. Hence, both genes activate each other and knockdown of either gene leads to similar epidermal phenotypes (i.e. loss of the labrum) (Kitzmann et al. 2017). It had remained unclear whether this interaction was conserved in arthropods or whether it evolved later in the lineages leading to the insects.

As expected, regulation of Pt-foxQ2 by Pt-six3.1/3.2 was observed in P. tepidariorum. However, the RNAi experiments did not provide any evidence for Pt-foxQ2 being required for Pt-six3.1/3.2 expression as the expression of both Pt-six3 paralogs remained unchanged in Pt-foxQ2RNAi embryos, except for a secondary reduction due to the...
missing tissue of the labrum. We cannot exclude that we do not see an effect on Pt-six3.1/six3.2 expression because the RNAi embryos might still have residual transcripts sufficient for this activation. However, the strong RNAi effect on the labrum argues against this scenario. Hence, assuming that a Pt-foxQ2 does not activate nor repress Pt-six3.1/six3.2 in P. tepidariorum, we suggest that the spider situation reflects the ancestral condition (i.e. six3 upstream of foxQ2) while in the lineage leading to the insects foxQ2 gained a more upstream role by activating six3 expression.

**Conclusion**

Our expression analysis adds Pt-foxQ2 to the list of genes active in the early anterior neurogenic ectoderm in spiders. Our knockdown experiments identify both Pt-foxQ2 and Pt-six3.1/six3.2 as genes required for labrum development in *P. tepidariorum* (Fig. 6). This indicates that the epidermal patterning function of foxQ2 is an ancestral feature of arthropods. Further, we propose that Pt-six3.1/six3.2 is upstream with respect to Pt-foxQ2 (Fig. 6), although we cannot rule out that Pt-foxQ2 RNAi knockdown was not strong enough to see an effect on the other genes. We propose that the interactions of the spider aGRN are conserved with those of other animals and suggest that foxQ2 orthologs gained a more upstream role rather recently in the lineage leading to the insects.

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