Original Article

SMED-TLX-1 (NR2E1) Is Critical for Tissue and Body Plan Maintenance in *Schmidtea mediterranea* in Fasting/Feeding Cycles

(nuclear receptor / regeneration / RNA interference / *Schmidtea mediterranea* / Tlx)

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Abstract. Nuclear receptors (NRs), or nuclear hormone receptors (NHRs), are transcription factors that regulate development and metabolism of most if not all animal species. Their regulatory networks include conserved mechanisms that are shared in-between species as well as mechanisms that are restricted to certain phyla or even species. In search for conserved members of the NHR family in *Schmidtea mediterranea*, we identified a molecular signature of a class of NRs, NR2E1, in the *S. mediterranea* genome and cloned its complete cDNA coding sequence. The derived amino acid sequence shows a high degree of conservation of both DNA-binding domain and ligand-binding domain and a remarkably high homology to vertebrate NR2E1 and *C. elegans* NHR-67. Quantitative PCR detected approximately ten-fold higher expression of *Smed-tlx-1* in the proximal part of the head compared to the tail region. The expression of *Smed-tlx-1* is higher during fed state than during fasting. *Smed-tlx-1* down-regulation by RNA interference affects the ability of the animals to maintain body plan and induces defects of brain, eyes and body shape during fasting and re-growing cycles. These results suggest that SMED-TLX-1 is critical for tissue and body plan maintenance in planaria.

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Abbreviations: DBD – DNA-binding domain, LBD – ligand-binding domain, NHRs – nuclear hormone receptors, NR(s) – nuclear receptor(s), PNR – photoreceptor nuclear receptor, RAR β2 – retinoid receptor β2.

Introduction

Nuclear receptors (NRs) are powerful regulators of animal development and metabolism. They are involved in the regulation of specific cell functions as well as integration of developmental and metabolic processes at the level of the organism. Their structure contains a highly conserved DNA-binding domain that is coordinated by two zinc ions in the form consisting of two "zinc fingers" and a carboxy-terminal ligand-binding domain composed of 12 helices. Several members of the NR family are hormonal receptors, such as steroid hormone and thyroid hormone receptors, retinoid receptors and vitamin D receptor. A growing number of NR family members are recognized as receptors capable to bind small molecules that, dependent on the receptor-ligand binding affinity, modulate the receptor transcriptional functions (Antebi, 2006; Kininis and Kraus, 2008; McEwan, 2009).

Ligands with very high affinity that fulfil the criteria of hormonally active compounds, usually derived from metabolites or molecules obtained with food, execute regulatory functions at the local or tissue-restricted level as well as at the level of the entire organism. Specific ligand-binding properties of many nuclear hormone receptors (NHRs) developed during evolution.

The superfamily of NRs includes members that are highly conserved in distant animal phyla as well as receptors that have apparently diversified (Escriva et al., 2000, 2004; Robinson-Rechavi et al., 2003). Some NRs, like RAR-related HR3 NRs, seem to have similar functions in insects and nematodes while other including the

multiplied HNF4-related NRs in *C. elegans* seem to have acquired new functions (Antebi, 2006).

Another class of NRs, RXRs, are conserved between fungi, *Cnidaria* and vertebrates, but their orthologue is missing in many nematode species (Antebi, 2006). In case of RXR, the ligand-binding specificity, its DNA--binding specificity and dimerization capabilities are conserved between *Cnidaria* and vertebrates (Kostrouch et al., 1998).

Here, we searched for conserved members of the NHR family in *S. mediterranea* and identified the molecular signature of a NR related to *C. elegans* NHR-67, insect tailless and vertebrate TLX. These NRs are classified as NR2E1 in the unified classification (Nuclear Receptors Nomenclature Committee, 1999).

S. mediterranea is becoming a very powerful model organism for its capabilities to regenerate tissues and especially to re-grow the entire organism from small fragments of the body. This requires complex rearrangement of tissues and restoration of new body plan from multipotent or totipotent cells, the neoblasts (Sanchez Alvarado et al., 2002; Cebria et al., 2007). This capability of regression and re-growing also takes place in adaptation of the animals to critical food restriction (Pellettieri et al., 2010).

The animals are able to sustain their existence under starvation (during which the number of cells and the animal size are decreasing) by utilization of their own tissues as energy supply, while protecting the pluripotent cells that are able to support the complete re-growing of animals when the food supply is restored. This process requires a complex regulatory network that sacrifices some cells while protecting other. Understanding a possible role of conserved NRs in this mechanism may reveal general relationships present in the NR regulatory network shared by Metazoan species.

Based on the molecular signature, we cloned the entire coding region of *S. mediterranea* NR2E1, the *Smed-tlx-1*, and performed its initial biological characterization. We show that NR2E1 is highly conserved between *Turbellaria* and vertebrates and is critical for the maintenance of tissue and animal integrity during fasting/feeding cycles.

Material and Methods

Animals and animal cultures

Asexual strain of *Schmidtea mediterranea* was kindly provided by Dr. Francesc Cebria. Animals were kept in tap water (supplied by Veolia to the Prague 4 district from water supply facility Želivka). Tap water from this region proved to be more suitable for *S. mediterranea* than three bottled still water products including one declared as ideal for infants. Animals were fed in one-week intervals with beef liver tissue that was stored in frozen aliquots, de-frozen and briefly washed of blood before use. Approximately 1 g of liver tissue was used per 100 animals in 200 ml to 500 ml Beaker flasks. The water was filtered before use (45 μm filters Corning, Corning, NY) and kept for up to 14 days in open glass bottles.

Beaker flasks were exchanged every two weeks. Animals were kept in a partially shaded air-conditioned room at 20 °C and protected against direct light. To prepare larger quantities of animals, some animals were cut into several pieces and left to regenerate.

Total RNA preparation

Total RNA was prepared using two protocols. For cDNA preparation, the total RNA was prepared using proteinase K digestion, DNase treatment and RNA extraction as described (Brozova et al., 2006). Animals were anaesthetized and killed by fast freezing on dry ice. For reverse transcription – quantitative PCR, the total RNA was prepared both using the proteinase K – DNas RNA extraction protocol and extraction using Trizol (Invitrogen, Carlsbad, CA).

Reverse transcription

cDNA was prepared from 3 μg of total RNA using the SuperScriptII and/or SuperScriptIII kits from Invitrogen according to manufacturer's recommendations. To prepare cDNA intended for cloning the complete coding sequence of *Smed-tlx-1*, both random hexamers and poly-T primers were used. For quantitative RT-PCR only random hexamers were used.

Amplification of the complete coding sequence of *Smed-tlx-1* was done by the nested PCR strategy. Design of primers is indicated in Fig. 1. Primers for amplification of the predicted sequence available in SmedGD database (http://smedgd.neuro.utah.edu) (Sanchez Alvarado et al., 2002; Robb et al., 2008) were also used but did not yield an efficiently amplified fragment in nested PCR. We therefore employed the 3' RACE method from cDNA prepared using Transcriptor First Strand cDNA Synthesis Kit (Hoffmann-La Roche, Basel, Switzerland). For amplification of 5' ends of cDNAs, we used splice leader sequences SL1 and SL2 (Zayas et al., 2005). SL1: CGGTCTTATCGAAATCTATATAAATCTTATATG and SL2: CGGTCTTATCGAAATCTATATAAAAATT ATATG and primers designed according to a short segment that showed significant homology to most known TLX homologues.

The following primers were used for the cloning:

4-2010	ATGACAGTAACAAAGCAATCATTATTCAG
8-2010	GTACCTTGCAAGGTGTGTCAAGACCATTCA
9-2010	TTGCAAGGTGTGTCAAGACCATTCATCGG
13-2011	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
5-2011	TGGATCATGTCAGCAGACTTTGTCAG
19-2011	CATCTCTCTCATTTTAGGCAATCA
20-2011	CATGATTGAATGATTTTGAGTACAG
21-2011	TCATTTTAGGCAATCATATCCGATA
22-2011	TTGAATGATTTTGAGTACAGGCCGA

Cloning and sequencing

Amplified fragments were visualized on agarose gels stained with ethidium bromide, isolated using electro-

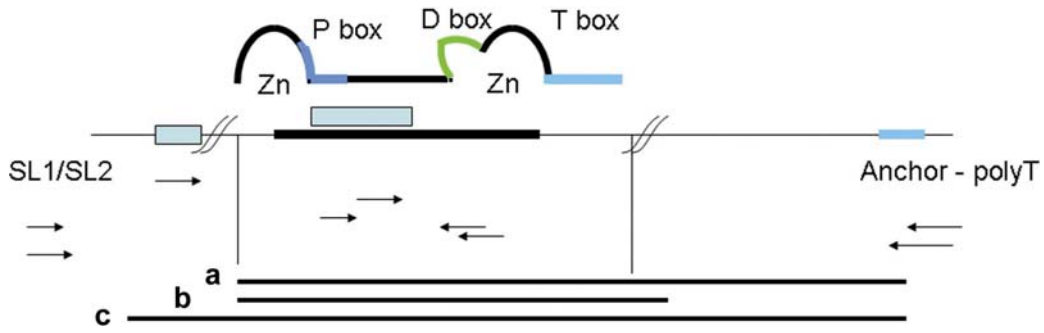

Fig. 1. Schematic representation of cloning strategy. The two structural motifs coordinated by zinc ions ("zinc fingers") of the DNA-binding domain are indicated by two arches; the P box, D box and T box are indicated. The region marked as thick line is the region identified as NR2E class of NHRs by computer searches with a high degree of probability. Primers designed for amplification of cDNA in 3' and 5' directions were designed and used in amplification PCRs as indicated. The 3' region was amplified using poly-T (Anchor) primer and 5' terminal region was amplified using primers derived from conserved sequences detected by computer alignments. Dark lines labelled as a, b and c represent amplified cDNA regions covering the region including the complete DBD and LBD (a), a short transcript that contains a stop codon and may code for a protein lacking most LBD (b) and the complete coding region of a cDNA containing the conserved 5' motif, complete DBD and complete LBD (c).

elution, ligated using pCR2.1 kit (Invitrogen) and transformed to Top10 competent bacteria (Invitrogen). Miniprep DNA was isolated and plasmid DNA assayed for the presence of inserts using DNA restriction and agarose gel electrophoresis. Candidate clones were sequenced in the DNA sequencing facility of the Institute of Inherited Metabolic Disorders by Sanger's method in an ABI Avant 3100 16 capillary sequencer (Applied Biosystems, Foster City, CA). All clones were sequenced minimally three times in both directions.

Quantitative PCR was done using the Universal Probe Library technique (Hoffmann-La Roche, Basel, Switzerland) and in a LightCycler 1.2 instrument equipped with the LightCycler 4.1 software (Hoffmann-La Roche).

The conditions for amplifications were used as follows: pre-denaturation cycle at 95 °C for 10 min, 45 cycles consisting of steps of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 1 s. Then the reactions were cooled at 37 °C for 15 s. The fluorescence was detected at the end of each 60 °C step. All UPL probes were detected using 480–530 nm channel.

For *Smed-tlx-1*, primers #7523 (tctgctcgatctctgtttaacact) and #7524 (agcaccgacacgatctcttt) and probe #47 were used. For normalization of results the *Ura4* mRNA was used, which was amplified with primers #7561(gcc tgctcaaacgcagttat) and #7562 (atggtaaatgcggccttaaa) and probes #53 and #11. Standard curves were prepared as described (Vohanka et al., 2010).

RNA interference was induced using the standard *C. elegans* protocol (Timmons et al., 2001; Liby et al., 2006). The sequence covering the complete DNA binding domain and 2/3 of the ligand-binding domain (LBD) (*Sac*I – *Xho*I) was cloned into the L4440 vector and transformed into HT115 bacteria, both kindly provided by Dr. Andrew Fire. Production of dsRNA was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO) for 4 h, bacteria were pelleted at 1,500 *g* at 4 °C, kept refrigerated at 4 °C and used for the maximum of two weeks. For induction of RNA interference, bacteria were fed to animals in a mixture containing one part of homogenized beef liver tissue. Liver homogenate was prepared using a 10 ml glass homogenizer from 1 g of liver tissue supplemented with 500 µl deionized autoclaved water, the homogenate was centrifuged in 1.5 ml aliquots in Eppendorf tubes at 10,000 *g* for 7 min, clear supernatant was discarded and the upper portion of the pellet containing small cellular fractions used for bacteria/DNA mixtures. Some cultures were fed using pelleted bacteria without the liver extract.

Control samples were prepared in a similar way using the bacteria producing non-specific dsRNA from the empty L4440 vector.

In vivo imaging

Animals were viewed using an Olympus SZX10 (Olympus Czech Group, Prague, Czech Republic) stereo microscope equipped with an Andor Clara CCD camera (Andor Technology, Belfast, Northern Ireland).

Informatics and modelling was performed using the following sites: http://www.mat.univie.ac.at/~neum/protein.html; http://www.biochem.ucl.ac.uk/~shepherd/old-2001/bioinf-prot-pred.html; www.ebi.ac.uk/Tools/msa/clustalw2/; www.phylogeny.fr.

Protein sequences were downloaded from UniProt, NCBI, Oma and PDB databases (www.uniprot.org, www.ncbi.nlm.nih.gov, www.omabrowser.org and www.rcsb.org).

Sequences were aligned with T-coffee (http://www.tcoffee.org (Notredame et al., 2000)).

Results

I. Cloning S. mediterranea Smed-tlx-1

In search for the members of nuclear receptors in *S. mediterranea*, we identified a partial DNA-binding do-

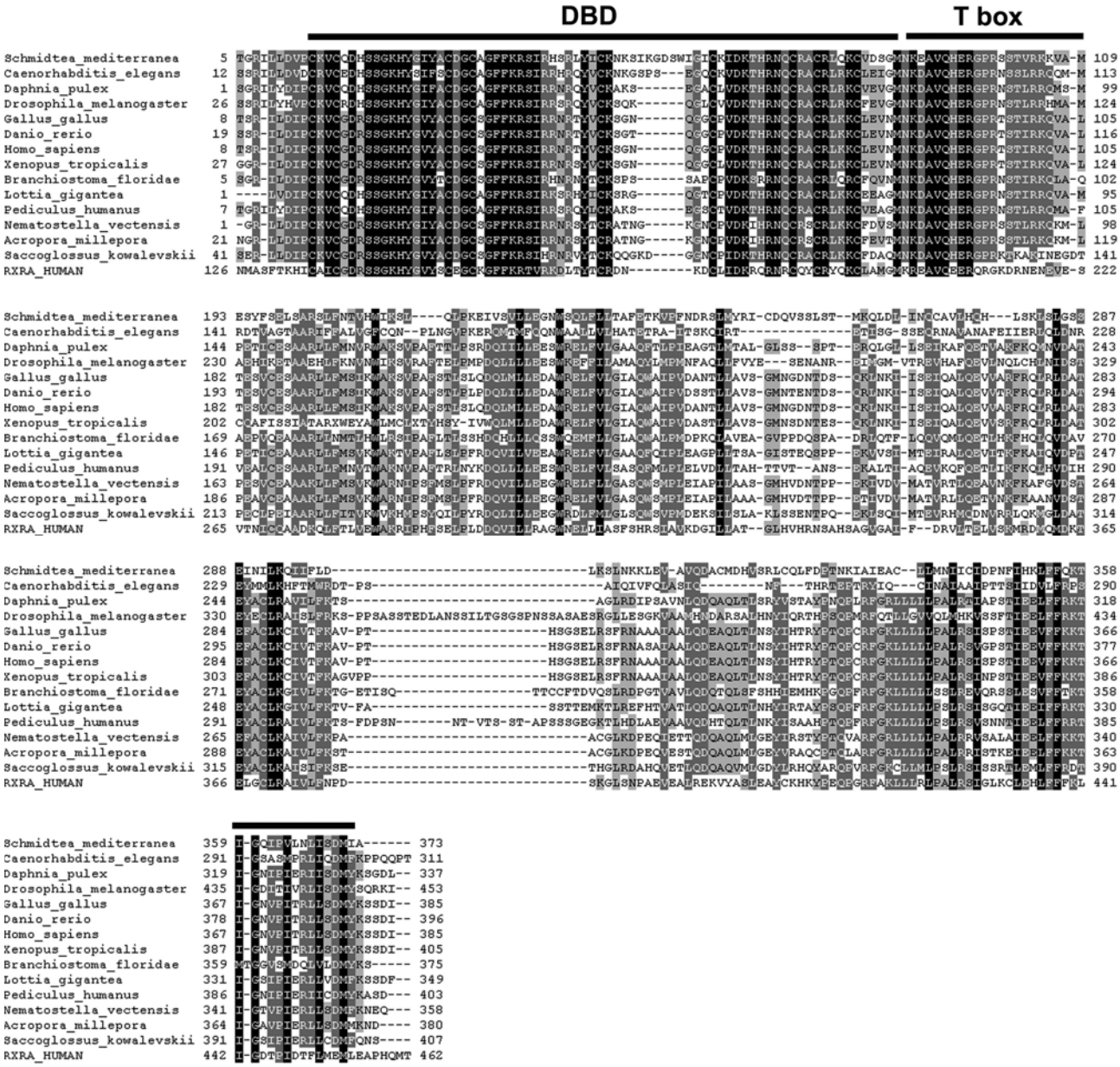

Fig.2. Sequence alignment of SMED-TLX-1 with orthologues from selected species and human retinoic acid receptor RXR-α of known 3D structure. Only conserved regions from DBD and LBD domains are shown, the sequences of non-conserved linker are omitted.

Protein sequences from UniProt, NCBI, Oma and PDB databases: *Schmidtea_mediterranea* SMED-TLX-1, *Caenorhabditis_elegans* NHR67_CAEEL, *Daphnia_pulex* E9FSF9_DAPPU, *Drosophila_melanogaster* TLL_DROME, *Gallus_gallus* NR2E1_CHICK, *Danio_rerio* Q6DEH0_DANRE, *Homo_sapiens* NR2E1_HUMAN, *Xenopus_tropicalis* XP_002937209.1, *Branchiostoma_floridae* C3XUQ3_BRAFL, *Lottia_gigantea* LOTGI05609, *Pediculus_humanus* E0VN83_PEDHC, *Nematostella_vectensis* A7SD90_NEMVE, *Acropora_millepora* Q95WG4_ACRMI, *Saccoglossus_kowalevskii* XP_002736110.1, RXRA_HUMAN PDB 3dzy.

main (DBD) sequence of the NR2 class of NRs in chromosomal region v31.016829..1526.2067. The sequence was included in a predicted transcript classified as TLX homologue in the genome browser SmedGD. In accordance with the proposed *S. mediterranea* nomenclature, we named the gene *Smed-tlx-1*. However, the predicted sequence differed on both 5' and 3' ends substantially from TLX homologues known from many animal species sequenced so far. In order to amplify larger segments of cDNA, we designed primers from the predicted form of *Smed-tlx-1* cDNA and attempted to amplify larger segments by reverse-transcription PCR. Only primers designed according to the apparently conserved sequence of the DNA-binding domain and first part of the hinge region led to amplification of the predicted sequence. For full cDNA amplification, we used the 3' RACE method (with the poly-A-derived anchor primer). We hypothesized that the predicted sequence containing codons of the first two cysteines of the DBD may be divided into two separate exons and we used the se-

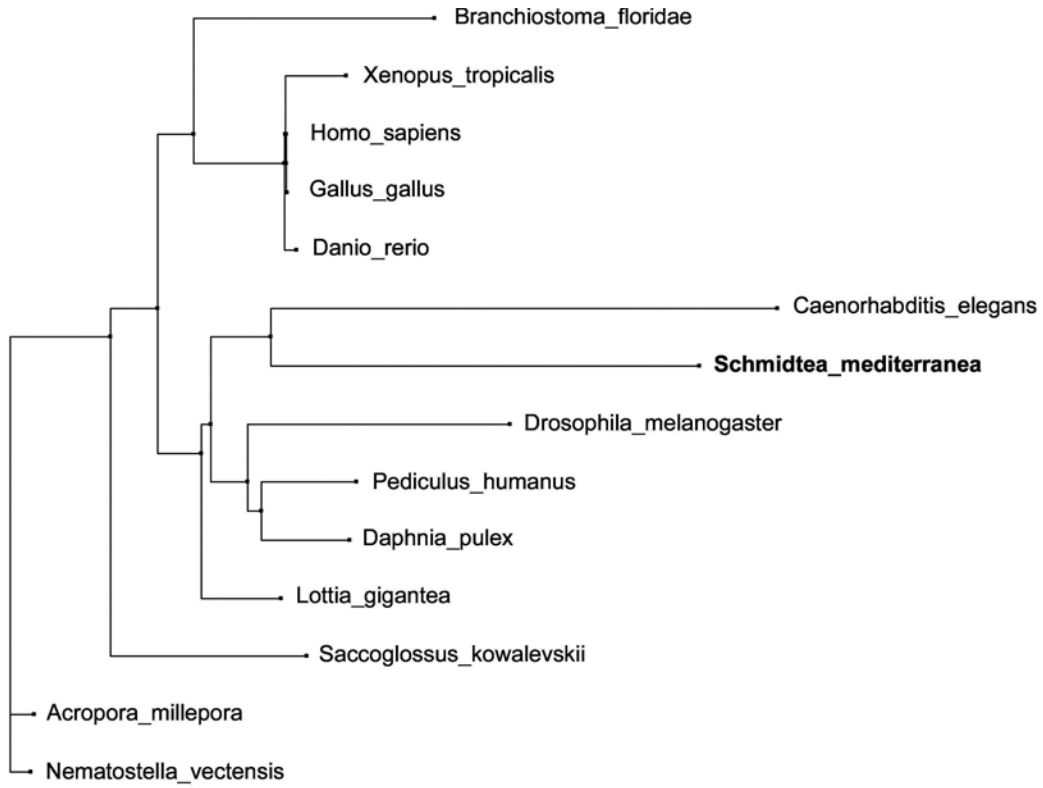

Fig. 3. Phylogenetic tree derived from the alignment in Fig. 2. Close relation of SMED-TLX-1 to representative NR2E1 family members from various phyla are shown. *S. mediterranea* SME-TLX-1 is positioned next to C. *elegans* homologue NHR-67.

quence positioned more toward the 3' direction for amplification of the sequence in both directions. The strategy for amplification is shown in Fig. 1. For amplification of the 5' end of cDNA, we attempted to use splice leader sequences known to exist in *S. mediterranea* and primers derived from various regions recognized as similar to known TLX homologues. This strategy led to cloning of a sequence that shows signs of mature spliced message as well as high similarity to known tlx homologues. The sequence was deposited to GenBank and is available under the Accession Number JQ085483.

The sequence alignment shows a remarkable conservation of the derived amino acid sequence between the SMED-TLX-1 and other members of the NR2E family (Fig. 2). Both the DNA-binding domain and ligand-binding domain show a high degree of conservation. The DBD clearly indicates that the SMED-TLX-1 differs, together with other NR2E class members, from other NRs. The second zinc finger is broader, the D box contains amino acid insertions longer than in most other NRs, and the sequence corresponding to T box also clusters NR2E proteins together. The molecular signature of SMED-TLX-1 thus has the following form: C-X2-C-X13-C-X2-C-X18-C-X13C-X9-C-X2-C-X4-C-X3-GM.

Comparison of the SMED-TLX-1 sequence with other members of NR2 family members indicated that TLX in *S. mediterranea* is related to vertebrate orthologues as well as to the *C. elegans* orthologue, NHR-67 (Fig. 3). The sequences in Fig. 2 are all first mutual blast hits. Comparison of SMED-TLX-1 with representatives of TLX homologues, photoreceptor nuclear receptor (PNR) and *C. elegans* genes NHR-67, FAX-1 and NHR-111 shows that SMED-TLX-1 clusters clearly with TLX, not with the PNR group of genes (Fig. 3).

Contrary to most members of the NR superfamily of proteins, SMED-TLX-1 contains 13 amino acids in the D box region. This region contains seven amino acids in vertebrate Tlx and in *Drosophila* Tll. Comparison with the known 3D structure of the retinoic acid receptor RXR-α (Fig. 4) shows, however, that this inclusion should not interfere with DNA binding of the receptor.

With 12 helices conserved, the overall structure of LBD keeps the molecular signature of NRs, and remarkably conserved is also the carboxy terminus of the sequence, suggesting the conserved properties in binding of interacting proteins (Fig. 2).

II. Smed-tlx-1 expression is regulated in response to fasting/feeding state

In order to assess the expression of *Smed-tlx-1*, we designed primers according to the Roche Universal Library strategy. For analysis we used the region localized in LBD. In animals kept under once a week feeding cycle and expression analysis just before the new feeding, the *Smed-tlx-1* was found to be relatively abundantly expressed in both the proximal part of the body and the tail region, suggesting an expression pattern not restricted to the brain region. Nevertheless, the expression

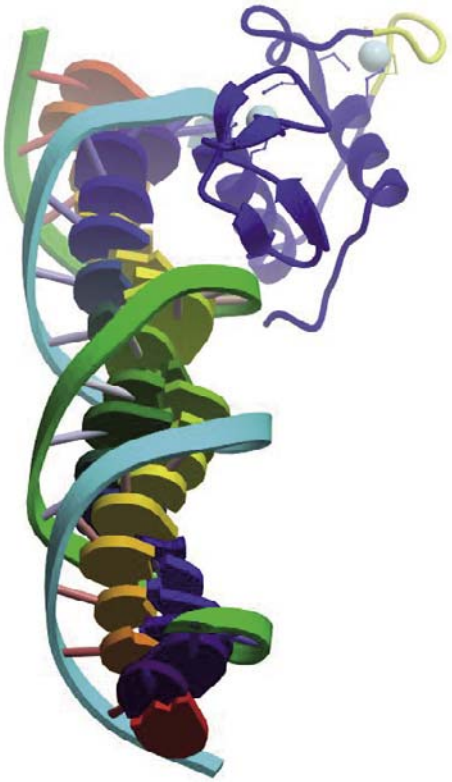

Fig. 4. 3D structure of the DNA-binding domain of the human retinoic acid receptor RXR-α (Chandra et al., 2008). The yellow loop indicates the D box where the greatest variability among the SMED-TLX-1 orthologues exists (cf. Fig. 2 first row, sequence 50-64, CKNKSIKGDSWG-IC).

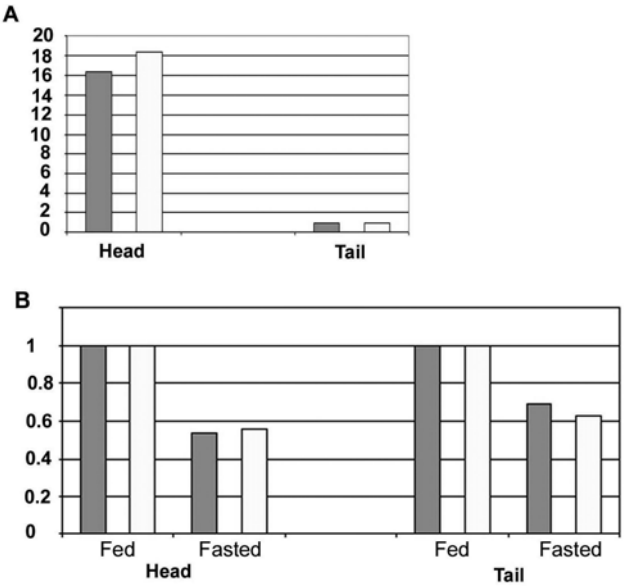

Fig. 5. Expression analysis of *Smed-tlx-1* in RT-qPCR. A – comparison of *Smed-tlx-1* expression in the most proximal part of the head and most distal part of the tail. Two different regions, primer pairs and UPL probes were used (dark and pale columns). The analysis shows high *Smed-tlx-1* expression in the region containing brain and eyes and well-detectable expression of *Smed-tlx-1* in the tail region. B – analysis of *Smed-tlx-1* expression in fed (set as 1) and fasting animals. The two-probe strategy shows expression of full-length cDNA in both states and elevated expression during the animal growing (fed) state in both head and tail regions.

in the head was approximately 10 to 20 times higher compared to the tail region (Fig. 5 A).

Next, we analysed the expression of *Smed-tlx-1* during the fasting and fed states. *Smed-tlx-1* appeared to be expressed in both states, with accented expression following feeding (Fig. 5 B).

III. SMED-TLX-1 is critical for tissue maintenance and integrity of the organism in feeding/fasting cycles

For initial analyses of SMED-TLX-1 biological functions, we employed the RNAi method shown previously to be effective in induction of gene loss of function in *S. mediterranea*. We used the same setting as for *C. elegans* except that the bacteria producing dsRNA were in some experiments mixed with liver homogenate. Several feeding schemes were used.

Surprisingly, a strong effect of *Smed-tlx-1* inhibition appeared during the fasting period following the single feeding with the dsRNA-producing bacteria. The most apparent effect was shrinking the head area of inhibited worms (Fig. 6 A, B, C, E). Animals also showed slow movement and changes of the body shape in the form of strictures or even defects that were seen in both head and tail areas. Further culture of RNAi-affected animals led to substantial recovery of the affected worms, but huge morphological defects were observed especially in the head area (Fig. 6 G, H). They included smaller and malformed eyes and irregular shape of the head. Some animals lacked one or both eyes and animals with three underdeveloped eyes were also seen (Fig. 6 J, K). Repeated feeding with dsRNA-producing bacteria led to gross defects and death of most animals. Some animals also developed abnormalities in the tail area. They included irregularities of the body shape and protrusions in the form of thin tail-like projections on the dorsal side of the body. The critical function of SMED-TLX-1 in the regressive phase of the fasting-feeding cycles was more obvious when longer periods of starvation were applied following the RNAi. There was no effect of unspecific dsRNA produced by bacteria in control cultures (Fig. 6 D, F, I), except occasionally observed thin tail-like projections.

Discussion

SMED-TLX-1 is a conserved member of the NR family of proteins

In this work we identified the sequence coding for a nuclear receptor from the class NR2E1 in *Schmidtea*

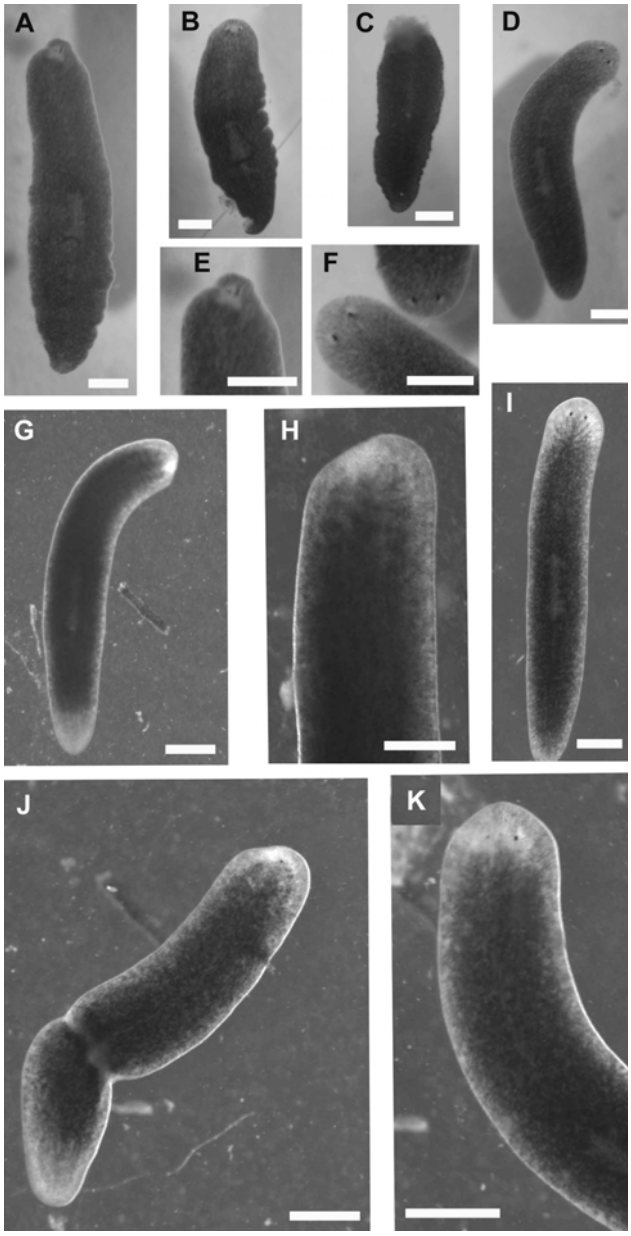

Fig. 6. *In vivo* imaging of *S. mediterraneae* inhibited for *Smed-tlx-1* function by RNAi (panels A, B, C, E, G, H, J and K) and control animals (panels D, F and I). During the starvation period following the RNAi induced by feeding, animals developed morphological defects in head areas (panel A and detail shown in panel E), defects in head as well as in tail areas (B), including complete disintegration of the head regions (C). After re-feeding animals developed irregularly, some with missing eyes, irregular shape of proximal body part (G) and irregular texture of internal structures in the proximal body part (H). Frequently, irregular development of eyes was observed (J, K). Bars represent 500 μm.

mediterranea. The NR2E1 class of NRs include insect tailless and nematode NHR-67 and homologues of these NRs in many clads, including chordates, arthropods, gastropods, as well as *Cnidaria*. The coding region of *Smed-tlx-1* amplified from total RNA using specific primers in one direction and non-specific primers for the poly-adenylation tail of mRNA seems to be expressed at high quantities, since the fragment of cDNA covering the open reading frame of this mRNA is efficiently amplified from total RNA preparations in single PCR reactions employing specific primers. In contrast, the predicted isoform of mRNA was not amplified even in two successive PCRs with specific primers for both directions. The length of the derived protein is rather small, including only 373 aa, which contrasts with *Drosophila* and nematode homologues that have longer carboxy-regions and the length of 452 and 416 aa, respectively. Analysis of sequence homology, however, indicates that both *Drosophila* and nematode proteins have a longer sequence at the carboxy terminus extending beyond a conserved motif found in most if not all TLX orthologues. It is worth noting that about 100 aa C-terminal extension present in *Caenorhabditis* (*Caenorhabditis elegans, C. remanei, C. briggsae, C. brenneri*) is highly homologous within this group but exists in no other orthologues.

Several insertions can be found in the *Schmidtea*, *Drosophila* and *Caenorhabditis* TLX homologues (Fig. 2), including the insertion in the D box region of the DBD. Comparison with the known structures of members of the NR family of proteins indicates that the insertions do not break the basic three-dimensional structure of these receptors.

SMED-TLX-1 *is important for keeping the body plan and tissue integrity during the regression and growth phases of planarian life*

In this work, we cloned and partially characterized a member of the NR2E class of NHRs in *Schmidtea mediterranea*. Analysis of its primary sequence as well as its three-dimensional model indicate a very high degree of homology between its counterparts in various Metazoan species including coral, arthropods, nematodes and chordates. The DBD of SMED-TLX-1 is conserved to the extent that suggests conserved binding to the response elements similarly as shown in the case of nematode orthologue NHR-67 (DeMeo et al., 2008). Expression of *Smed-tlx-1* is augmented in the proximal part of the body but is not restricted to this region. In keeping with this, the developmental consequences of *Smed-tlx-1* inhibition by RNAi leads to multiple phenotypic changes including shrinkage of the brain area, body shape defects, and in the following regeneration phase defects of head morphology, defective development of eyes, slow movement and defects of the body shape in the tail area.

S. mediterranea is able to cope with food restriction by resorbing its own tissues, while the stem cells and tissues important for normal planarian life sustain functionality and allow the animal to re-grow when the food supply is restored (Pellettieri et al., 2010). This process includes apoptotic cell death and removal of dead cells by phagocytosis. The survival of cells that support the organism functionality and next regeneration includes

anti-apoptotic protein BCL2. This basic mechanism is reminiscent of rearrangement of tissues during amphibian metamorphosis (Das et al., 2002; Nakajima et al., 2005; Rowe et al., 2005) as well as development of organs during chordate ontogenesis (Nagasawa et al., 1997).

The close relationship between SMED-TLX-1 and its nematode, insect and chordate homologues (NHR-67, tailless and Tlx) indicate that other mechanistic relationships may also be conserved

The DBD is nearly constant (Fig. 2), indicating that it might bind to a very similar DNA sequence in all organisms. The insect *tailless* and vertebrate TLX are predominantly transcriptional repressors and both regulate development by affecting additional nuclear receptors; *tailless* represses *knirps* (Moran and Jimenez, 2006) and TLX retinoid receptor β2 (RAR β2) (Kobayashi et al., 2000).

The search for homologues in *C. elegans* indicates that the functions supported by NR2E class members may have diversified between several NHRs; NHR-67 is the most obvious orthologue of TLX/tailless in *C. elegans*. Other homologues found in the *C. elegans* genome are FAX-1, NHR-111 and NHR-239 and possibly other NHRs.

NHR-67 regulates proper development of vulva cells in a regulatory circuit based in inhibitory function of NHR-67 and COG-1 (Fernandes and Sternberg, 2007). The circuit includes another NR, the NHR-113. This NHR is highly conserved between nematode species but is only distantly related to ERR and RARs (Ririe et al., 2008).

NHR-67 deficiency affects L3 and L4 stage development probably by affecting the cell migration programe, left-right specification and timing (Sarin et al., 2009).

NHR-67 controls specification of gustatory neurons and affects the left/right (L/R) asymmetric subtype diversification. NHR-67 positively regulates the expression of a sensory neuron-type-specific selector gene, *che-1*, which encodes a zinc-finger transcription factor. The neuronal function of NHR-67 is broader and covers many neuronal types (Kato and Sternberg, 2009).

Although *tailless*/TLX seem to be primarily repressors, the existence of a direct transcription activation function is well documented. TLX activates NAD^+-dependent histone deacetylase directly by acting on sirt1 promoter (Iwahara et al., 2009).

In addition to this, TLX is recruited to the Oct-3/4 promoter in hypoxia and augments its expression. This induces proliferation of progenitor cells and preserves their pluripotency (Chavali et al., 2010).

The DBD of SMED-TLX-1 is almost identical to human TLX with the exception of the D box, which contains 13 amino acids compared to vertebrate TLX that has only 7 aa in the corresponding region. The *C. elegans* homologue, NHR-67, possesses 10 aa in this region. The model based on the known structure of related NHRs (reference to the structure see Fig. 4) suggests that this variable insertion is unlikely to affect the DNA binding or dimerization. Its location is distant from the DNA-binding region (Fig. 5) and the dimerization occurs through LBD (not shown in Fig. 5). It may, however, affect protein-protein interactions with other yet unknown proteins. The LBD of SMED-TLX-1 is also highly conserved. The LBD possesses all 12 helices, and the helix 12 involved in cofactor binding is conserved (Fig. 2).

The remarkable conservation of SMED-TLX-1 suggests that additional mechanisms functioning in *S. mediterranea* tissue maintenance and renewal may also be conserved between *Turbellaria* and vertebrates.

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