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RESEARCH ARTICLE

Ancestral role of TNF-R pathway in cell differentiation in the basal metazoan *Hydra*

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ABSTRACT

Tumour necrosis factor receptors (TNF-Rs) and their ligands, tumour necrosis factors, are highly conserved proteins described in all metazoan phyla. They function as inducers of extrinsic apoptotic signalling and facilitate inflammation, differentiation and cell survival. TNF-Rs use distinct adaptor molecules to activate signalling cascades. Fas-associated protein with death domain (FADD) family adaptors often mediate apoptosis, and TNF-R-associated factor (TRAF) family adaptors mediate cell differentiation and inflammation. Most of these pathway components are conserved in cnidarians, and, here, we investigated the *Hydra* TNF-R. We report that it is related to the ectodysplasin receptor, which is involved in epithelial cell differentiation in mammals. In *Hydra*, it is localised in epithelial cells with incorporated nematocytes in tentacles and body column, indicating a similar function. Further experiments suggest that it interacts with the *Hydra* homologue of a TRAF adaptor, but not with FADD proteins. *Hydra* FADD proteins colocalised with *Hydra* caspases in death effector filaments and recruited caspases, suggesting that they are part of an apoptotic signalling pathway. Regulating epithelial cell differentiation via TRAF adaptors therefore seems to be an ancient function of TNF-Rs, whereas FADD–caspase interactions may be part of a separate apoptotic pathway.

KEY WORDS: Hydra, Apoptosis, TNF-R superfamily, FADD, Ectodysplasin

INTRODUCTION

The evolutionary origin of the tumour necrosis factor (TNF) receptor (TNF-R) signalling pathways is unclear. Recent work in the cnidarian *Acropora digitifera* indicated that it evolved in pre-Cambrian animals. Functional studies exposing human cells to *Acropora* TNF and *Acropora* cells to human TNF suggested that *Acropora* TNF signalling could be functionally linked to extrinsic apoptosis induction (Quistad et al., 2014; Quistad and Traylor-Knowles, 2016). In contrast, genetic approaches in early bilaterian model organisms, including *Caenorhabditis elegans* and *Drosophila melanogaster*, have so far not revealed any evidence for the existence of a functional extrinsic apoptosis induction pathway (Steller, 2008).

Mammalian TNF and TNF-R superfamilies have more than 40 members each, comprising TNF-R (also known as TNFRSF1), FasR (also known as CD95 or Fas), tumour necrosis factor related apoptosis inducing ligand receptor (TRAIL-R or TNFRSF10) and ectodermal dysplasia A (EDA)-receptor (EDAR) families of receptors, and TNF, FasL (also known as FASLG), nerve growth factor (NGF) and EDA families of ligands (Wajant, 2003). They function in apoptosis regulation, inflammation and morphogenesis.

In vertebrates, the EDAR pathway is involved in the development of ectodermal appendages like teeth, hair and glands in mammals, feathers in chicken, and scales in fish (reviewed in Lefebvre and Mikkola, 2014). The receptor is activated by the TNF family ligand EDA (reviewed in Mikkola, 2008). Ligand binding leads to nuclear factor κB (NF-κB) activation involving the adaptor molecules EDARADD, TRAF6 and IκB kinase (Sadier et al., 2014). Target genes of this pathway play a role in mediating morphological changes by modulating the actin cytoskeleton, and also in the switch from proliferation to growth arrest and differentiation (Kumar et al., 2001).

In humans, TNF-Rs can function as a death domain (DD) docking site for adaptor proteins, for example TRADD, which, upon binding, recruits the serine/threonine kinase RIP1 (also known as RIPK1) and TRAF2. This results in either NF-κB activation leading to cell proliferation or apoptosis by activating Jun N-terminal kinase (JNK) cascades or caspases. In the case of binding of a Fas-associated death domain protein (FADD) family member to the endosomal TNF-R, the complex recruits caspase-8 and induces the apoptotic signalling pathway (Mathew et al., 2009).

Apoptosis is regulated by proteases of the caspase family, consisting of initiator caspases and executioner caspases. Initiator caspases represent pro-caspases that are kept in an inactive conformation by characteristic pro-domains, for example, caspase recruitment domains (CARDs) or death effector domains (DEDs). Pro-caspase activation requires a scaffold (apoptosome), which brings several pro-caspase molecules into close proximity (Bao and Shi, 2007). Apoptosomes can be formed in the cytoplasm in response to intrinsic apoptosis inducers, or at the cell membrane in response to extrinsic apoptotic signals, which are propagated by 'death receptors' of the TNF-R superfamily (Salvesen and Riedl, 2008). Binding of TNF-like ligands induces receptor clustering and recruitment of adaptor molecules, for example, members of the FADD family, which crosslink intracellular receptor DDs with DEDs of pro-caspases, leading to auto-activation (Muzio et al., 1998).

In the cnidarian model organism *Hydra vulgaris*, apoptosis is important for cell number adjustment in response to nutrient supply, oogenesis and spermatogenesis (reviewed in Böttger and Alexandrova, 2007). Caspases and HyBcl-2-like proteins are involved in apoptotic processes in *Hydra* (Miller et al., 2000; Motamedi et al., 2019; Cikala et al., 1999). The *Hydra* genome also encodes homologues of the mammalian key players of the TNF signalling pathway and their downstream effectors for triggering the extrinsic apoptotic pathway, including TNF-R, FADD and DED caspase homologues (Lasi et al., 2010a; Wenger et al., 2014). In addition, a caspase homologue with a DD-like pro-domain (DD-

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Received 9 October 2020; Accepted 1 December 2020

caspase) has been described (Lasi et al., 2010a,b). Here, we investigate whether these TNF pathway components are involved in an extrinsic pro-apoptotic pathway by forming a death signalling module in *Hydra* or whether the *Hydra* TNF pathway is involved in different cellular mechanisms, for example, to facilitate differentiation.

Hydra polyps are composed of two epithelial monolayers, the ectoderm, shielding the organism from the outside, and the endoderm, covering the gastric cavity. These are separated by an extracellular matrix, the mesoglea. At the oral end there is a head with tentacles and a mouth opening and at the aboral end there is a peduncle (or foot) terminating in a basal disc. *Hydra* polyps have a high tissue turnover due to constant self-renewal of three stem cell lineages, including ectodermal and endodermal epithelial cells and pluripotent interstitial stem cells (David, 2012; Holstein et al., 1991). All *Hydra* epithelial cells contain muscle fibres making them epithelio-muscular cells. Interstitial stem cells reside in interstitial spaces between epithelial cells and give rise to nerve cells, gland cells and nematocytes. Epithelial cells at the tentacle bases and the basal disc stop dividing and differentiate into tentacle and basal disc cells, respectively. Thereby ectodermal tentacle cells enclose nematocytes and become so-called battery cells (Hufnagel et al., 1985). Battery cells are regularly lost from the tentacles and replaced with new cells arriving from the body column where the epithelium is growing by cell division. Some epithelial cells in the body column also acquire nematocytes.

We now show that the single *Hydra* TNF-R homologue (HyTNF-R) is very specifically expressed in epithelial cells enclosing nematocytes. These include all battery cells in tentacles and scattered epithelial cells of the body column. Phylogenetically, HyTNF-R is most similar to human EDAR, which is involved in epithelial cell differentiation. HyFADD adaptor proteins interact with *Hydra* DED and DD pro-caspases. Surprisingly, HyTNF-R does not recruit HyFADD proteins but most likely interacts with a TRAF-like adaptor and therefore is probably not involved in extrinsic cell death signalling.

RESULTS

Hydra TNF-R and TNF homologues are related to human EDAR and EDA

HyTNF-R

The previously described gene sequence encoding a *Hydra* TNF-R-like protein (GenBank accession GU121226, Lasi et al., 2010a) from *Hydra vulgaris* was amplified using *Hydra AEP* cDNA (GenBank accession MT905382) and analysed. The protein sequence has a signal peptide followed by two TNF-R domains with a characteristic cysteine pattern of six cysteine residues and a transmembrane domain. In the C-terminus a DD domain is present (Figs 1A and 2A). We compared the HyTNF-R protein sequences with those of all known human members of the TNF-R superfamily (Fig. 1A), which are characterized by two to four extracellular TNF-R repeats and an intracellular DD (Aggarwal, 2003). With two TNF-R repeats, HyTNF-R was most similar to human EDAR. Phylogenetic analyses of conserved DD sequences from HyTNF-R and all human TNF-Rs confirmed this (Fig. 1C). More extended phylogenetic analyses of HyTNF-R DD-sequences from further cnidarians (*Acropora digitifera* and *Nematostella vectensis*), *Danio rerio*, *Xenopus laevis*, *Xenopus tropicalis* and *Mus musculus* revealed two branches of cnidarian TNF-R DD sequences that were most closely related to vertebrate EDAR and NGF-R (p75) sequences (Fig. 3). One of these cnidarian branches only contains *Acropora* sequences. None of the cnidarian sequences branched with vertebrate TNF-R and death receptors. In order to find out whether HyTNF-R was the only superfamily member in *Hydra*, we performed BLAST searches with the whole sequence against NCBI and the *Hydra* Genome 2.0 server (https://arusha.nhgri.nih.gov/hydra/) but did not find any additional TNF-R receptor candidates. We repeated the search with the DD domain sequence and found three entries in the databases, with none of these having the characteristic domain structure of TNF-R. Finally, we searched with the DD sequence of HyFADD2. This revealed nine sequences, again with no similarities in domain structure to HyTNF-R (Fig. S1). Therefore, it appears that *Hydra* has only one bona fide TNF-R, in contrast to marine cnidarians (Fig. 3, Fig. S6).

HyTNF

Human EDAR is activated by EDA, a member of the TNF superfamily possessing a collagen domain in addition to its extracellular TNF domain (Mikkola and Thesleff, 2003). By BLAST searching the *Hydra* genome with the TNF domain sequence of human TNF, we found a putative *Hydra HyTNF* gene encoding a protein with C-terminal TNF- and collagen-like domains and an N-terminal transmembrane domain. This domain structure was the same as in human EDA, but different from the domain structures of human TNF, FasL or NGF, which all lack a collagen domain (Fig. 1B), (Swee et al., 2009). BLAST searches with the HyTNF domain sequence against the NCBI protein database and the *Hydra* Genome 2.0. did not reveal any additional TNF candidate sequences. Phylogenetic analysis of the conserved TNF domains of all human TNF-related sequences finds the *Hydra* TNF in one group with human EDA and TNFSF10 (Fig. 1D). An alignment of HyTNF with human EDA is shown in Fig. 2B.

HyTNF-R protein is localised in epithelial cells that incorporate nematocytes

In order to investigate the function of HyTNF-R, we used an antibody that specifically recognised HyTNF-R on western blots and by immunofluorescence. Immunofluorescence on *Hydra* whole mounts revealed staining of scattered ectodermal epithelial cells in the body column and of all battery cells in the tentacles (Fig. 4A–D). A closer look at the epithelial cells in the body column showed that each of them had incorporated one or two nematocytes (Fig. 4D,E). This is illustrated by co-staining with an antibody against the minicollagen component of the nematocyte wall (Tursch et al., 2016) (Fig. 4F–I). Nematocytes are clearly recognisable with this antibody staining and showed the typical half-moon shaped nuclei of nematocytes harbouring these capsules (see magnifications of tentacle cell in Fig. 4J–M and of body column cells in Fig. S2A–H). All HyTNF-R-stained epithelial cells had normal nuclei without any signs of apoptosis (Fig. S2J–M).

When we expressed HyTNF-R–GFP in single *Hydra* cells via biolistic transformation, GFP signals were found in vesicular structures in the cytoplasm and at the plasma membrane. We did not find any signs of apoptotic cells by examining DAPI staining of 50 HyTNF-R–GFP-positive epithelial cells (Fig. 5A, left hand panel, compare with HyFADD -expressing cell with apoptotic nucleus in white box, Fig. 5B, right hand panel).

Comparing the pattern of HyTNF-R-positive cells on the body column and in tentacles with the pattern of apoptotic cells in untreated *Hydra* polyps stained with Acridine Orange does not reveal any similarity, as indicated in Figs S7A and S2N,O (see also Böttger and Alexandrova, 2007). Acridine Orange-labelled cells are found sparsely and slightly clustered in the body column and at the tentacle tips [apoptotic cells fluoresce green after being ingested by endodermal epithelial cells (Cikala et al., 1999)], whereas HyTNF-

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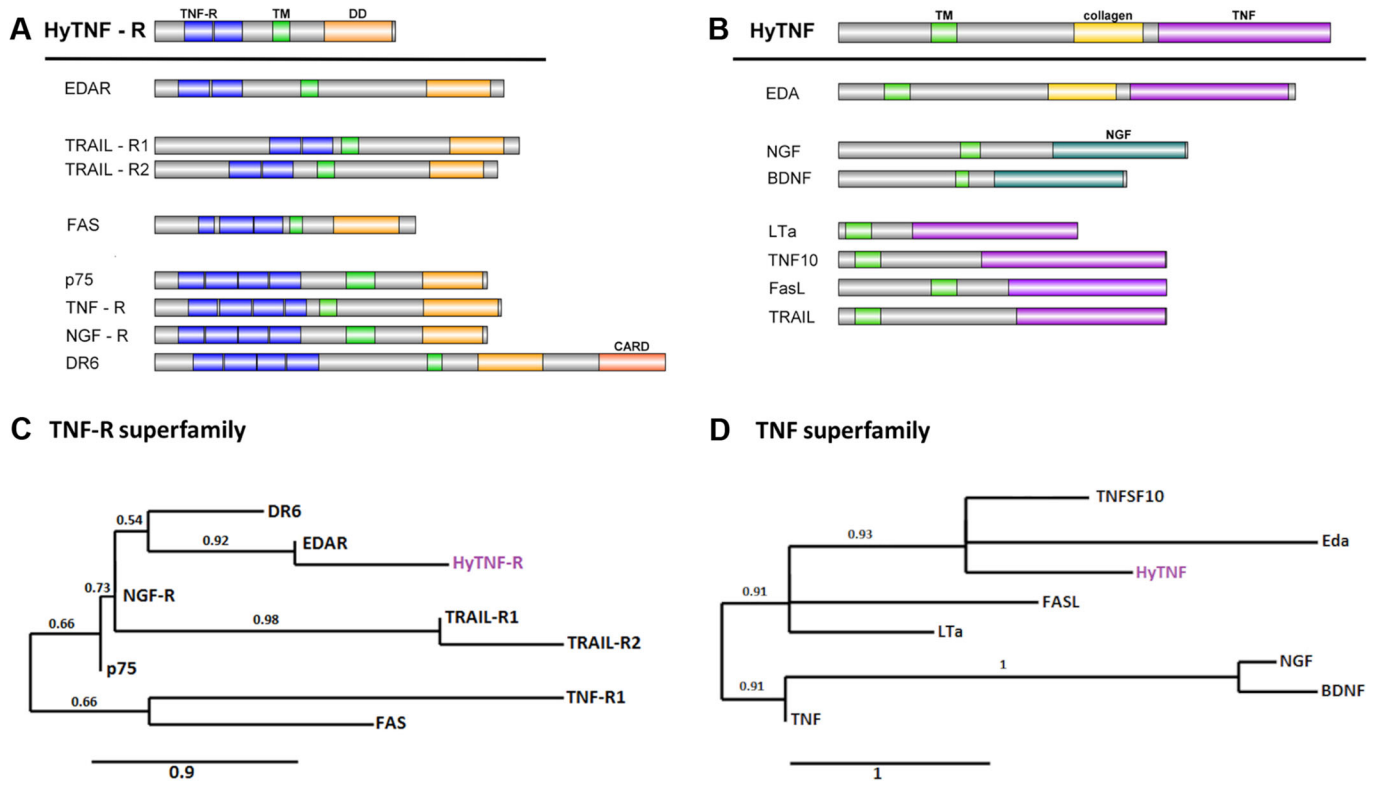

Fig. 1. Comparison of Hydra TNF-R and TNF with human homologues. (A) Comparison of domain structures of human DD-domain-containing TNF-R superfamily members with HyTNF-R. (B) Comparison of domain structures of human TNF superfamily members with HyTNF. TNF-R domains, blue; transmembrane domains (TM), green; DD domains, orange; CARD domain, red; TNF domain, pink; collagen domain, yellow; NGF domain, dark green. (C) Phylogenetic tree based on DD domain sequences from human TNF-R superfamily members, as in A, and HyTNF-R. (D) Phylogenetic tree based on TNF/NGF domain sequences from human TNF superfamily members and HyTNF. Bootstrap support is shown at nods; branch length represents evolutionary distance proportional to the number of substitutions per site (scale bars).

R-positive cells are much more abundant, evenly distributed over the lower two thirds of the body column and present in all tentacle cells. We therefore consider it unlikely that the presence of HyTNF-R in nematocyte bearing epithelial cells is connected with apoptosis.

We then tested whether HyTNF-R could be associated with tentacle differentiation. Hence, we treated animals with the GSK-β inhibitor Alsterpaullone (ALP, as described by Broun et al., 2005). This leads to the formation of ectopic tentacles on the body column (Fig. S7B). Even by early stages, these new tentacles incorporate nematocytes and are strongly stained with anti-TNF-R antibody (Fig. S7C–F).

HyTNF-R interacting proteins

In order to find proteins that interact with HyTNF-R we used the anti-TNF-R antibody for immunoprecipitation of *Hydra* cell lysates and subjected the precipitate to qualitative mass spectrometric analysis. Fig. 6B shows HyTNF-R protein in the precipitate on the western blot. Fig. 6A lists 20 selected proteins that were identified with a probability score of 100% in the samples of proteins co-precipitated with anti-HyTNF-R antibody, but not with the control antibody. Functional classification indicated co-precipitation of a *Hydra* TRAF6-like protein, and a homologue of an IκB kinase subunit. Furthermore, these co-precipitated proteins could potentially interact with HyTNF-R during its biosynthesis, trafficking and endocytotic recycling. In addition, three cytoskeletal proteins associated with actin or tubulin dynamics and two extracellular matrix (ECM) proteins were found. HyFADD was not detected (Fig. 6A, for a full list, see Table S1).

We next investigated *Hydra* TRAF proteins as possible HyTNF-R adaptors. Therefore, we cloned the sequences of HyTRAF6 (T2M357_HYDVU) and HyTRAF4 (which we identified by additional database search; https://www.uniprot.org/uniprot/T2M357; GenPept XP_004207529.1) from *Hydra* cDNA. Sequence analyses and protein domain searches revealed that they have an N-terminal ring finger, two TRAF-type zinc fingers, and a meprin and TRAF homology (MATH) domain, which is responsible for the interaction of TRAFs with receptors (Fig. 6C). Additionally, TRAF6 has a coiled coil region. Comparison of HyTRAF4 and HyTRAF6 domain structures revealed similarity with human TRAF2, 3, 5 and 6. Furthermore, phylogenetic analyses comparing the protein sequences of the conserved MATH domains from human and *Hydra* showed that human TRAF4 and TRAF6 are most closely related to HyTRAF4 and HyTRAF6, respectively, whereas all other human TRAF-sequences (TRAF1, 2, 3 and 5) form a separate group, indicating that they might have evolved later (Fig. 6D). This is supported by a previous study, which had suggested TRAF4 and TRAF6 as the founding members of the family (Chung et al., 2002). The large differences in the domain structures of TRAF proteins probably reflect variation in signalling cascades to which they can be linked.

In summary, these data suggest that *Hydra* TNF and TNF-R are similar to mammalian EDA and EDAR and that HyTNF-R could potentially use HyTRAF4 and HyTRAF6 proteins as adaptors. We propose that HyTNF-R has a function for epithelial cell differentiation into battery cells, but not in apoptosis. In order to search for alternative possibilities for an extrinsic apoptotic pathway in *Hydra*, we investigated *Hydra* FADD proteins.

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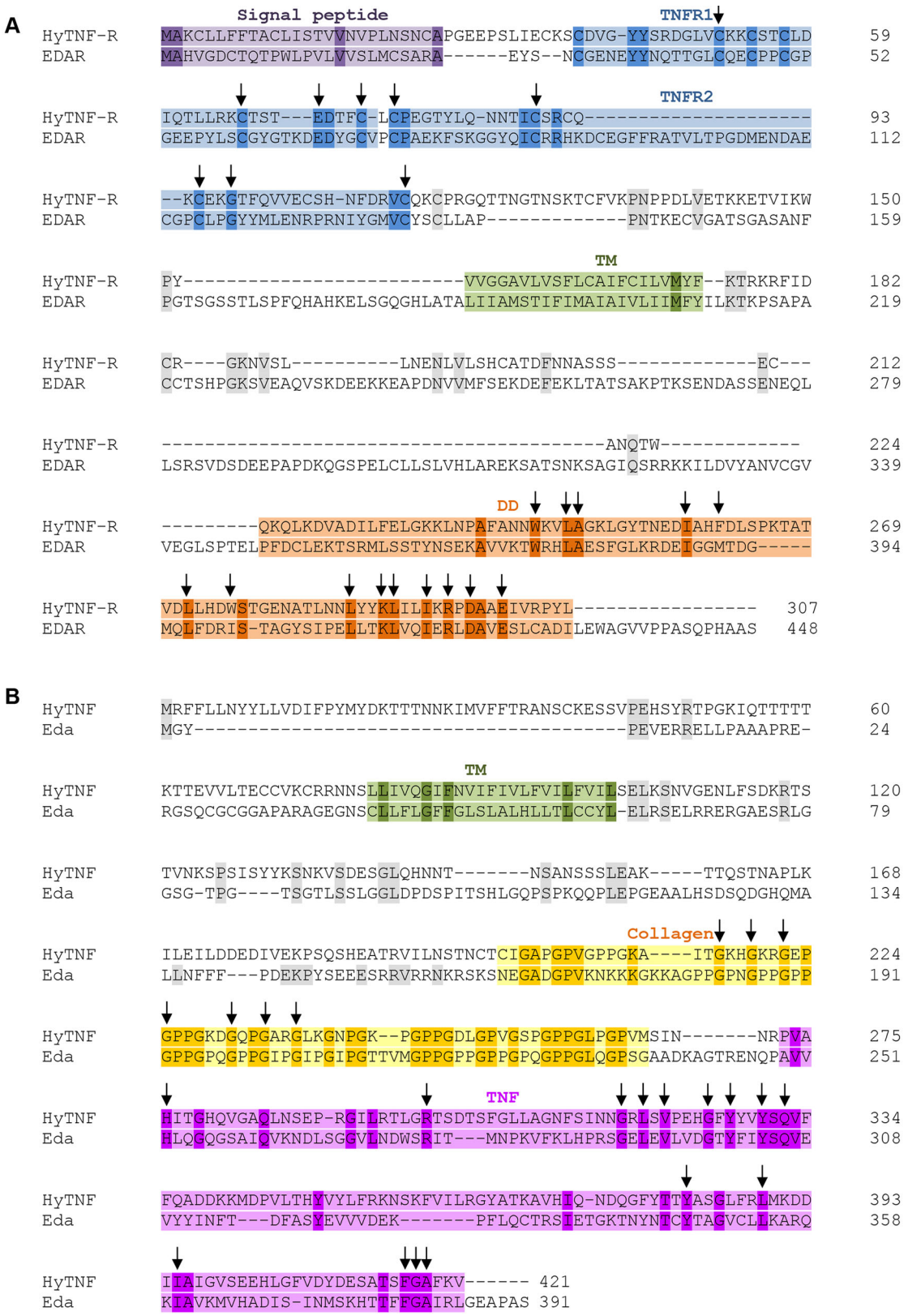

Fig. 2. Sequence alignments of *Hydra* and human TNF-R and TNF sequences. (A) Alignment of HyTNF-R and human EDAR sequences. Signal peptides, purple; TNF-R repeats, blue; transmembrane (TM) domains, green; DD domains, orange; identical amino acids outside conserved domains (grey). Evolutionary conserved amino acids of TNF-R repeats and DD domains according to the NCBI-CD description are highlighted (black arrows). (B) Alignment of HyTNF and human EDA-sequences: TM, green; collagen domain, yellow; TNF domain, pink. Identical amino acids are highlighted, evolutionary conserved amino acids are indicated with black arrows.

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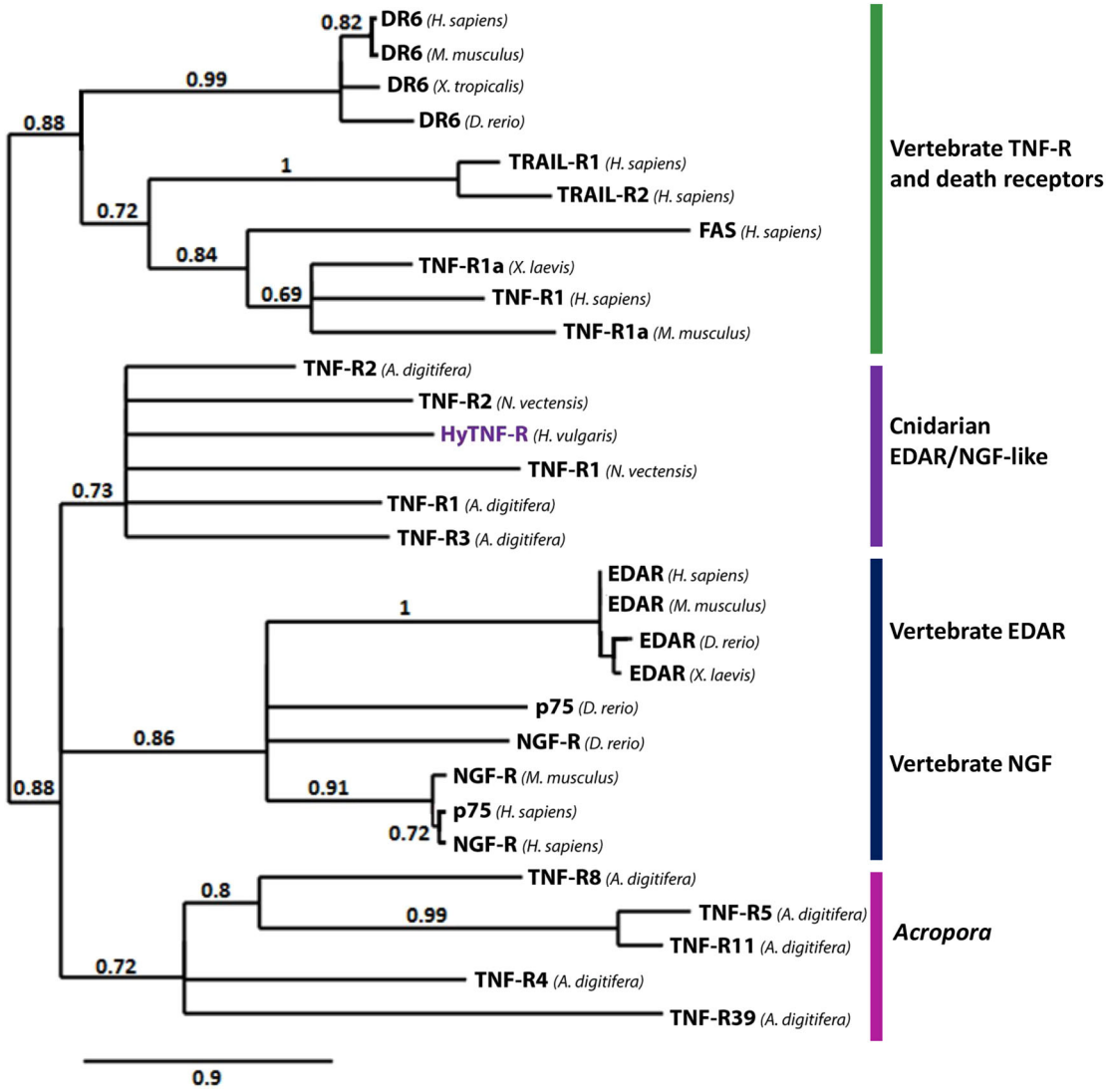

Fig. 3. Phylogenetic tree of the TNF-R superfamily in cnidarian and vertebrate species. A MUSCLE alignment of DD domains of TNF-R superfamily members from *Homo sapiens* (*H. sapiens*), *Mus musculus* (*M. musculus*), *Danio rerio* (*D. rerio*), *Xenopus laevis* (*X. laevis*), *Xenopus tropicalis* (*X. tropicalis*), *Acropora digitifera* (*A. digitifera*), *Nematostella vectensis* (*N. vectensis*) and *Hydra vulgaris* (*H. vulgaris*) was used for tree construction. Bootstrap support is shown at nods; branch length represents evolutionary distance proportional to the number of substitutions per site (scale bars). Colour bar sections indicate grouping of vertebrate and cnidarian receptors.

A potential HyFADD-DED/DD-caspase-mediated cell death pathway

HyFADD1 and HyFADD2 form death effector filaments

We cloned two potential FADD-encoding genes from *Hydra* cDNA and named the encoded proteins HyFADD1 (GenPept XP_002166467.1) and HyFADD2 (GenPept XP_002166848.2). Both HyFADD protein sequences contained a DD followed by a DED (Lasi et al., 2010a). This domain structure is identical with that of human FADD, but different from the domain structures of human TRADD and EDARADD (data not shown). When we biolistically transfected *Hydra* cells with plasmids encoding GFP-tagged HyFADD1 and HyFADD2, we did not see any epithelial cells expressing these proteins. In the case of HyFADD1, we pictured two cells with HyFADD–GFP signals, which were clearly apoptotic (Fig. 5B; Fig. S4). These experiments suggest that HyFADDs might induce apoptosis in *Hydra* cells. We then expressed GFP-tagged HyFADD1 and HyFADD2 in human HEK cells. These transfected cells accumulated both HyFADD proteins in long cytoplasmic fibres. For HyFADD1, these resembled a cage around one half of the nucleus. HyFADD2 filaments were very elongated throughout the cytoplasm (Fig. 5C). Similar fibres had previously been observed with human FADD proteins in HeLa cells. These had been designated death effector filaments (DEFs; Siegel et al., 1998).

HyDDCasp and HyDEDCasp are recruited to HyFADD1 and HyFADD2 death effector filaments

We then investigated the distribution of two *Hydra* caspases, HyDEDCasp and HyDDCasp (Lasi et al., 2010b) in human HEK cells. HyDEDCasp has one DED in its N-terminal region and therefore it is similar to mammalian caspase 8. HyDDCasp has a DD (Lasi et al., 2010a). DDs are not typical for animal caspase pro-domains but they occur in some meta-caspases of plants and fungi. Expression of HA-tagged versions of these *Hydra* caspases in human HEK cells revealed a uniform punctate pattern in the cytoplasm for both (Fig. 7A,D). This pattern changed dramatically when either caspase was co-expressed with HyFADD1 or

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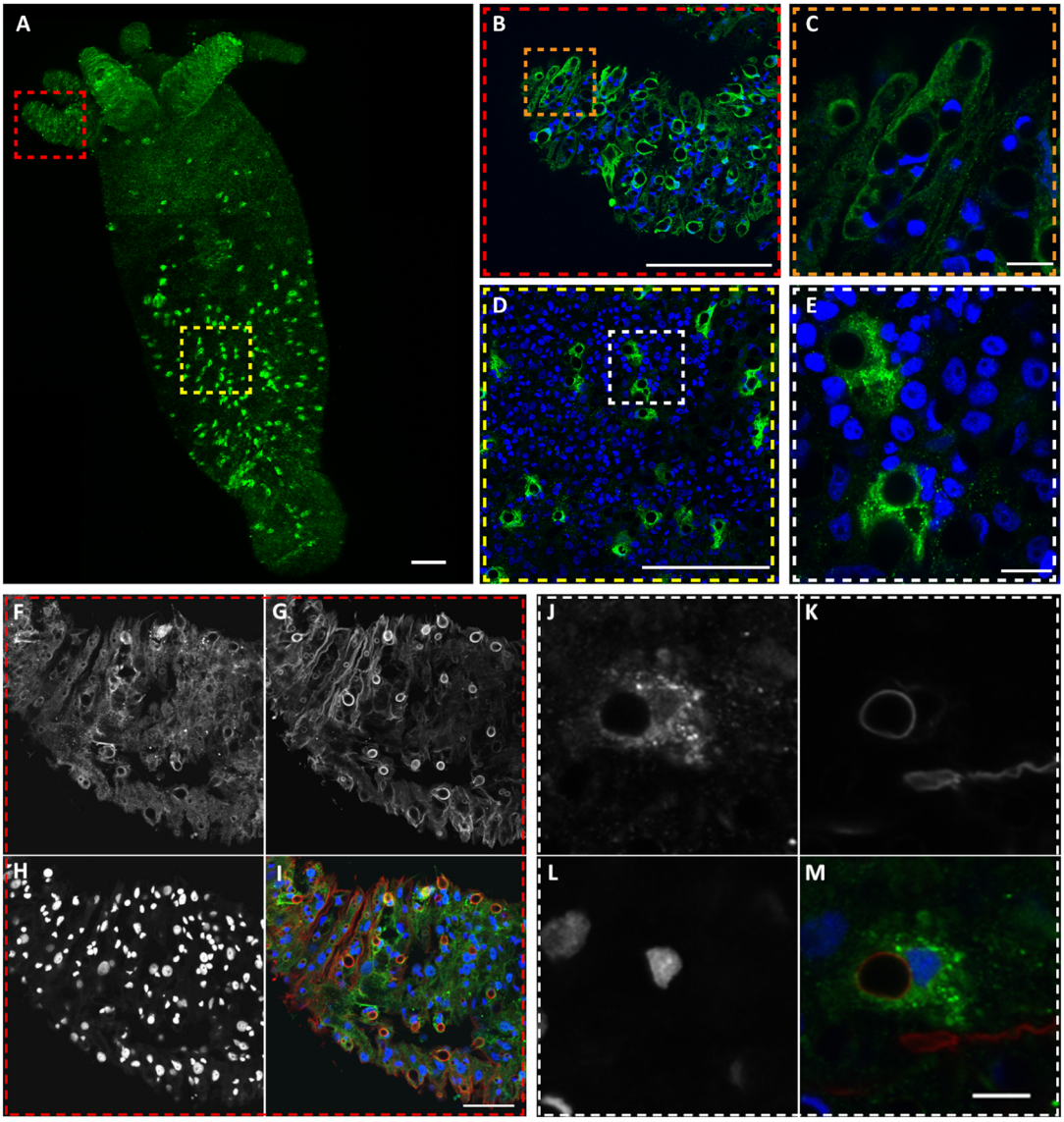

Fig. 4. Localisation of HyTNF-R. Laser confocal microscopy images of *Hydra* whole mounts stained with anti-HyTNF-R antibody (green), DNA-stain DAPI (blue) and anti-minicollagen antibody (N-CRD, Tursch et al., 2016, red). (A) Whole polyp, anti-TNF-R. (B–E) anti-TNF-R and DAPI [(B) tentacle, (C) battery cell, (D) part of the body column, (E) two ectodermal epithelial cells with incorporated nematocytes]. (F–M) Co-staining with anti-TNF-R, anti-N-CRD and DAPI [(F–I) tentacle, (J–M) epithelial cell on the body column]. (F,J) Anti-TNF-R, (G,K) anti-N-CRD, (H,L) DAPI, (I,M) merged. Images are stacks of an average of 15 confocal sections, coloured boxes indicate magnified regions. Scale bars: 100 µm (A,B,D,F–I), 10 µm (C,E,J–M).

HyFADD2. In those conditions, the caspases appeared associated with HyFADD death effector filaments, demonstrating that both HyFADD proteins were able to recruit them (Fig. 7B,C,E,F). This suggested that both HyFADD1 and HyFADD2 can interact with HyDEDCasp and HyDDCasp. Occasionally, we observed apoptosis in HEK cells, when HyDEDCasp was expressed, either alone or together with HyFADD1 or HyFADD2 (Fig. S5A) suggesting that HyDEDCasp, like its human homologue, might induce apoptosis. However, this effect was not as pronounced as we had previously seen when human caspase 8 was expressed in HEK cells (Lasi et al., 2010b) and could not be quantified. Auto-processing of the HyDEDCasp with its only DED might not be as efficient in human cells as it is with human caspase 8.

*Hy*TNF-R does not recruit HyFADD1 and HyFADD2

Next, we co-expressed HA-tagged HyTNF-R with GFP-tagged HyFADD1 and HyFADD2 in HEK cells. HyTNF-R was localized in apparent clusters at the plasma membrane and near the nucleus (Fig. 7G). Co-expression of both HyFADD1 and HyFADD2 did not change this distribution of HyTNF-R (Fig. 7H,I). Both HyFADD-proteins were localised in DEFs, as observed before (Fig. 7H,I) showing no indication of an interaction between HyFADD proteins and HyTNF-R. This supports the hypothesis that HyTNF-R does not take part in a caspase-mediated cell death pathway. Co-expression of HyTNF-R with HyTRAF6 resulted in similar expression patterns (Fig. 7J). In summary, HyTNF-R co-expression studies suggest that interaction of HyTNF-R with HyTRAF6 is more likely than with HyFADDs.

Cell type-specific mRNA expression

In order to confirm that the indicated protein interactions and the resulting hypotheses for TNF- and FADD-related pathways in *Hydra* are realistic, we analysed the cell types in which DEDCasp, FADD1, FADD2, TRAF4, TRAF6, TNF and TNF-R mRNAs are

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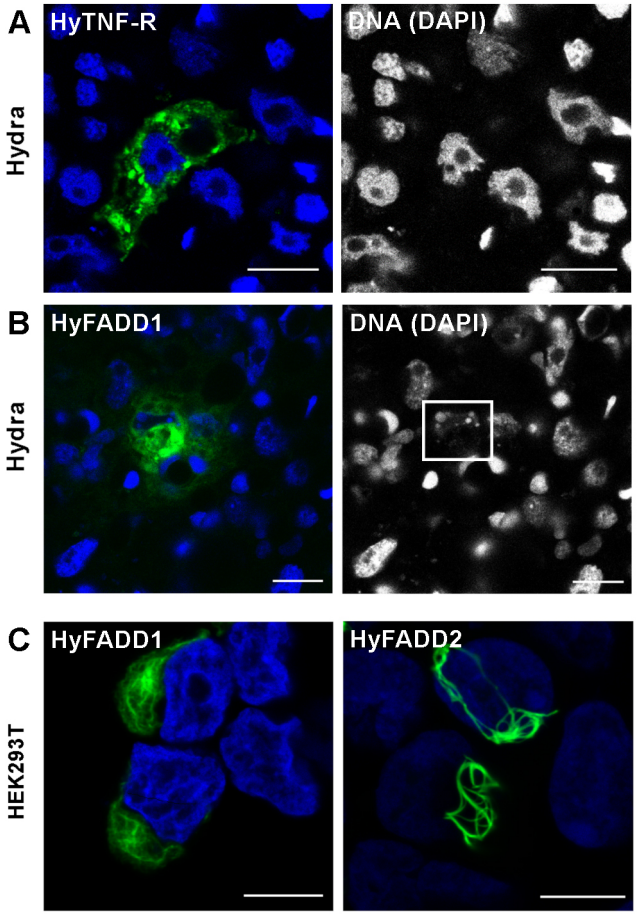

Fig. 5. Overexpression of proteins in *Hydra* and in human HEK293T cells. Laser confocal microscopy images of (A) GFP-tagged HyTNF-R and respective DAPI stained nuclei, (B) GFP-tagged HyFADD1 in *Hydra* cells after biolistic transformation and associated DAPI-stained nuclei (a HyFADD1-positive cell with apoptotic nucleus is shown in the white rectangle), and (C) GFP-tagged HyFADD1 and HyFADD2 in HEK293 T cells. Merged images of GFP and DAPI signals and DAPI signals are shown. GFP, green; DAPI, blue. Scale bars: 10 µm.

expressed. We made use of the recently published *Hydra* gene expression atlas and extracted data for these genes (Siebert et al., 2019). This is shown in Fig. 8A. In accordance with antibody staining, HyTNF-R is most intensely expressed in a fraction of ectodermal epithelial cells in the body column (ecEp stem cells 2) and in all ectodermal battery cells. Less expression was documented in ecEp/nem integration doublets, ectodermal cells in the head and in the endoderm (enEp stem cell 1) and almost no expression in interstitial cells, including male and female germ cells. HyTNF is expressed at high levels in few cells of all cell types. HyTRAF4 is strongly expressed only in ectodermal battery cells. Weaker expression is found in ectodermal epithelia cells/nematocyte integration doublets and in mature nematocytes. HyTRAF6 is very strongly expressed in female nurse cells and weakly in all epithelial cell types. HyFADD1 and HyFADD2 are mainly found in ectodermal and endodermal cell types and only smaller amounts in interstitial cells. Both are also expressed in female nurse cells. HyDEDCasp is almost exclusively expressed in nurse cells. Expression data for HyDDCasp were not available.

From these data we conclude that (1) expression data correlate with antibody staining for HyTNF-R, (2) adaptor proteins including HyTRAFs and HyFADDs are also present in HyTNF-R expressing cells, and (3) TNF-R expression is not specifically associated with male and female germ cells that frequently undergo apoptosis in *Hydra* as part of their differentiation program (Böttger and Alexandrova, 2007; Kuznetsov et al., 2001; Technau et al., 2003). Interestingly, HyFADDs, HyTRAF6 and HyDEDCasp are strongly expressed in nurse cells, which represent a special differentiation state in the female germ line. They undergo morphological changes during oogenesis, and induce an apoptotic programme, which is arrested after they have been ingested by the maturing oocyte (Alexandrova et al., 2005).

DISCUSSION

The induction of intrinsic apoptotic pathways follows a similar molecular logic in vertebrates and invertebrates including the pre-bilaterian phylum of cnidaria (Lasi et al., 2010a). Components of extrinsic apoptotic pathways including TNF-Rs, TNFs, FADDs and caspase 8 are also conserved from cnidarians to vertebrates; however, it is not clear yet whether they function in apoptosis induction in invertebrates. In this work, we analysed these molecules in *Hydra*, and we propose that HyTNF-R is not involved in apoptosis but has a morphogenetic function for the differentiation of specific nematocyte-incorporating epithelial cells. Furthermore, we suggest a HyFADD-dependent caspase activation pathway, which may be apoptotic, but is not triggered by HyTNF-R (Fig. 8B).

TNF-R signalling in mammals is very complex with large ligand and receptor superfamilies and multiple adaptor molecules feeding into different pathways to induce or prevent apoptosis or to direct specific differentiation events (Mathew et al., 2009). In higher invertebrates, including ascidians and sea urchins, several TNF and TNF-R superfamily members have been identified, which are involved in inflammation and immune response (Parrinello et al., 2008; Romero et al., 2016). For lower invertebrates, the situation is complex. In the nematode *C. elegans* neither TNF- nor TNF-R-encoding genes have been found. In the insect *Drosophila*, a TNF-R homologue (Wengen) and a TNF homologue (Eiger) have been described (Kauppila et al., 2003). *Drosophila* Wengen signalling has been proposed to involve JNK and to function in host defence, tissue growth, pain sensitization and sleep regulation (reviewed in Igaki and Miura, 2014).

As we show here, the cnidarian *Hydra vulgaris* has only one TNF-R homologue. Like eight of the TNF-Rs of the cnidarian *Acropora*, and in contrast to the single *Drosophila* TNF-R Wengen, HyTNF-R has an intracellular DD domain. However, the composition of its extracellular domain, as well as phylogenetic analyses, clearly indicate that it belongs to the EDAR family of TNF-Rs. Phylogenetic analyses also show that all cnidarian TNF-Rs group outside of the mammalian TNF-R, TRAIL and death receptor branch. Furthermore, we only discovered one potential TNF ligand. HyTNF has an extracellular TNF domain followed by a collagen domain, a domain composition that is the same in human EDA, the ligand for EDARs. Of the *Acropora* TNF homologues, two have an extracellular collagen domain, which suggests that they are related to EDA, like HyTNF. This is similar to what has been found for *Nematostella* TNF-homologues where two of four have a collagen domain (Robertson et al., 2006 and Fig. S6). Thus, cnidarian TNF-R superfamily members are related to mammalian EDARs and NGF (p75) receptors. HyTNF and some of the marine cnidarian TNFs also appear to be related to EDA-like ligands.

By immunoprecipitation with anti-TNF-R antibody, we co-precipitated *Hydra* homologues of an IκB kinase subunit and HyTRAF6. We also co-precipitated *Hydra* homologues of cytoskeletal proteins, and thus suggest that HyTNF-R has

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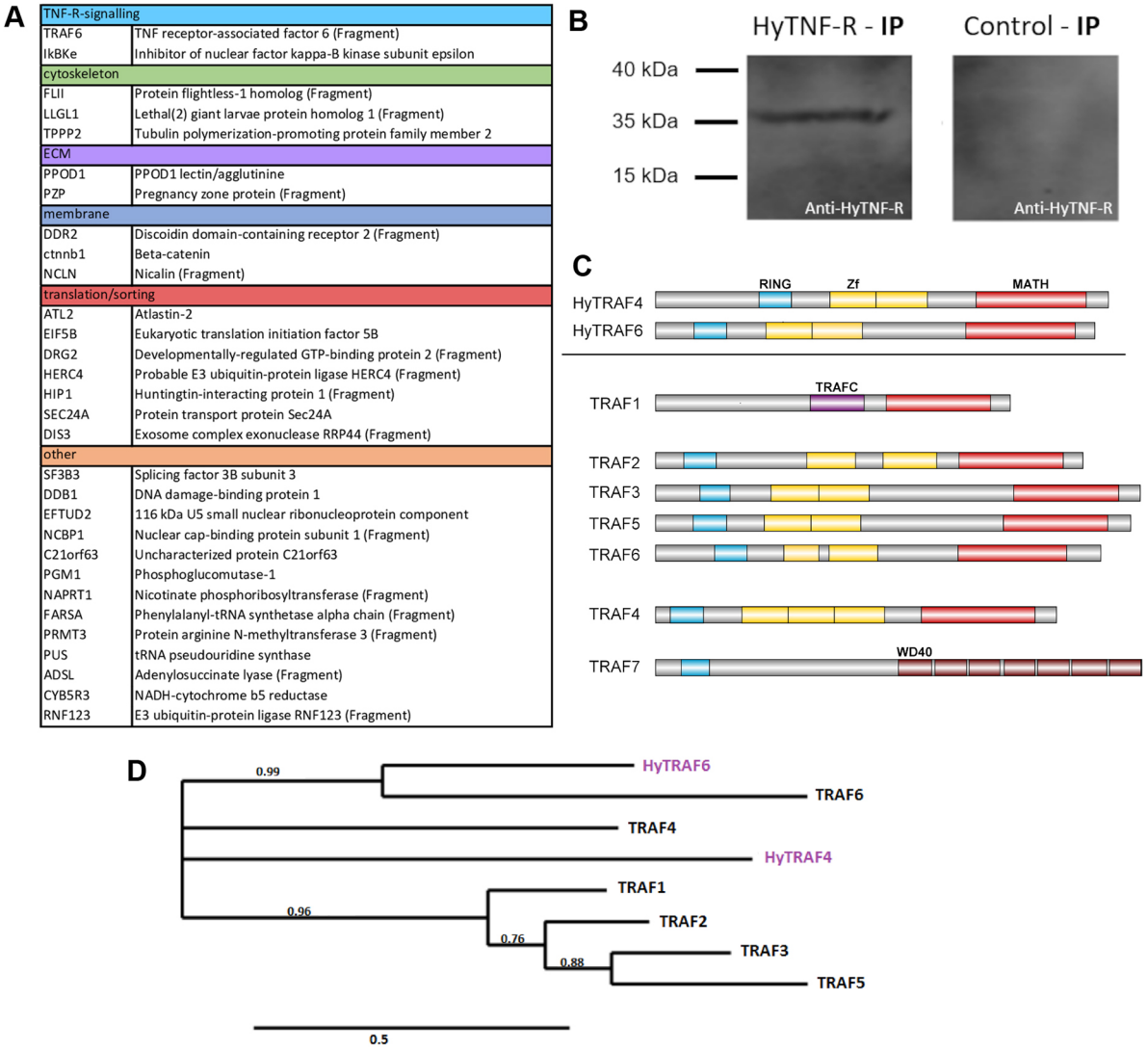

A	
TNF-R-signalling	
TRAF6	TNF receptor-associated factor 6 (Fragment)
IkBKe	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
cytoskeleton	
FLII	Protein flightless-1 homolog (Fragment)
LLGL1	Lethal(2) giant larvae protein homolog 1 (Fragment)
TPPP2	Tubulin polymerization-promoting protein family member 2
ECM	
PPOD1	PPOD1 lectin/agglutinine
PZP	Pregnancy zone protein (Fragment)
membrane	
DDR2	Discoidin domain-containing receptor 2 (Fragment)
ctnnb1	Beta-catenin
NCLN	Nicalin (Fragment)
translation/sorting	
ATL2	Atlastin-2
EIF5B	Eukaryotic translation initiation factor 5B
DRG2	Developmentally-regulated GTP-binding protein 2 (Fragment)
HERC4	Probable E3 ubiquitin-protein ligase HERC4 (Fragment)
HIP1	Huntingtin-interacting protein 1 (Fragment)
SEC24A	Protein transport protein Sec24A
DIS3	Exosome complex exonuclease RRP44 (Fragment)
other	
SF3B3	Splicing factor 3B subunit 3
DDB1	DNA damage-binding protein 1
EFTUD2	116 kDa U5 small nuclear ribonucleoprotein component
NCBP1	Nuclear cap-binding protein subunit 1 (Fragment)
C21orf63	Uncharacterized protein C21orf63
PGM1	Phosphoglucomutase-1
NAPRT1	Nicotinate phosphoribosyltransferase (Fragment)
FARSA	Phenylalanyl-tRNA synthetase alpha chain (Fragment)
PRMT3	Protein arginine N-methyltransferase 3 (Fragment)
PUS	tRNA pseudouridine synthase
ADSL	Adenylosuccinate lyase (Fragment)
CYB5R3	NADH-cytochrome b5 reductase
RNF123	E3 ubiquitin-protein ligase RNF123 (Fragment)

Fig. 6. Immunoprecipitation of HyTNF-R and TRAF-protein identification. (A) Functional classification of *Hydra* proteins co-immunoprecipitated (IP) with HyTNF-R. (B) HyTNF-R in western blot (WB) of precipitated proteins from *Hydra* lysate, for anti-HyTNF-R antibody and control antibody. WB stained with anti-TNF-R antibody and is representative of two repeats. (C) Representation of domain structures for human and *Hydra* TRAF proteins. RING domain, blue; zinc-finger (Zf) domain, yellow; MATH domain, red; TrafC domain, purple; Wd40 repeats, brown. (D) Phylogenetic tree of *Homo sapiens* and *Hydra vulgaris* TRAFs based on MATH domain sequences. Bootstrap support is shown at nods; branch length represents evolutionary distance proportional to the number of substitutions per site (scale bars).

biochemical interactions with components similar to those present in the mammalian EDAR signalling cascade. The adaptor protein EDARADD was not found; the gene was also not present in the *Hydra* genome. However, in mammalian 293T and MCF-7 cells it has been shown that EDAR also interacts with TRAF6 directly (Kumar et al., 2001). HyTNF-R protein was localised very specifically in battery cells and in those epithelial cells of the body column, which have nematocytes incorporated. This suggests that ectodysplasin signalling in *Hydra* functions within in a pathway of epithelial cell differentiation producing nematocyte-enclosing cells. TNF signalling is known to mediate morphogenesis by interacting with components of the cytoskeleton. The process of nematocyte integration into epithelial cells is a very unique differentiation event in *Hydra*. It involves the formation of lateral extensions of epithelial muscle processes, and establishment of specific junctions between nematocytes and epithelial muscle cell (Campbell, 1987). We suggest that TNF-R could mediate the extensive cytoskeletal re-arrangements required for nematocyte integration and ensure specificity of cell–cell junction formation. As potential interactors of HyTNF-R, we found three proteins with UniProt annotations as cytoskeleton-associated proteins including FLII [UniProtKB-T2MD87 (T2MD87_HYDUV)], a protein with a gelsolin-like actin-binding domain involved in actin remodelling, LLGL1 [UniProtKB-T2MCV2 (T2MCV2_HYDVU)], a protein associated with myosin and TPPP2, a tubulin polymerisation factor [UniProtKB-T2MFG9 (T2FG_HYDUV)]. Moreover, our data suggest that TNF-R could be in a complex with β-catenin, which is known to participate in cell–cell contacts. Future loss of function and protein interaction studies should test these hypotheses.

Thus, we suggest that the role of TNF-R signalling in the process of nematocyte integration into epithelial cells in *Hydra* might be comparable with the role of EDAR signalling during the formation of hair follicles and teeth in mammals. Here, EDAR is expressed in an epithelial thickening called a placode, which is the first morphological sign of a developing ectodermal appendage (Mikkola, 2008). The epithelium then grows deeper into the mesenchyme followed by specific epithelial morphogenesis. It is imaginable that the epithelial

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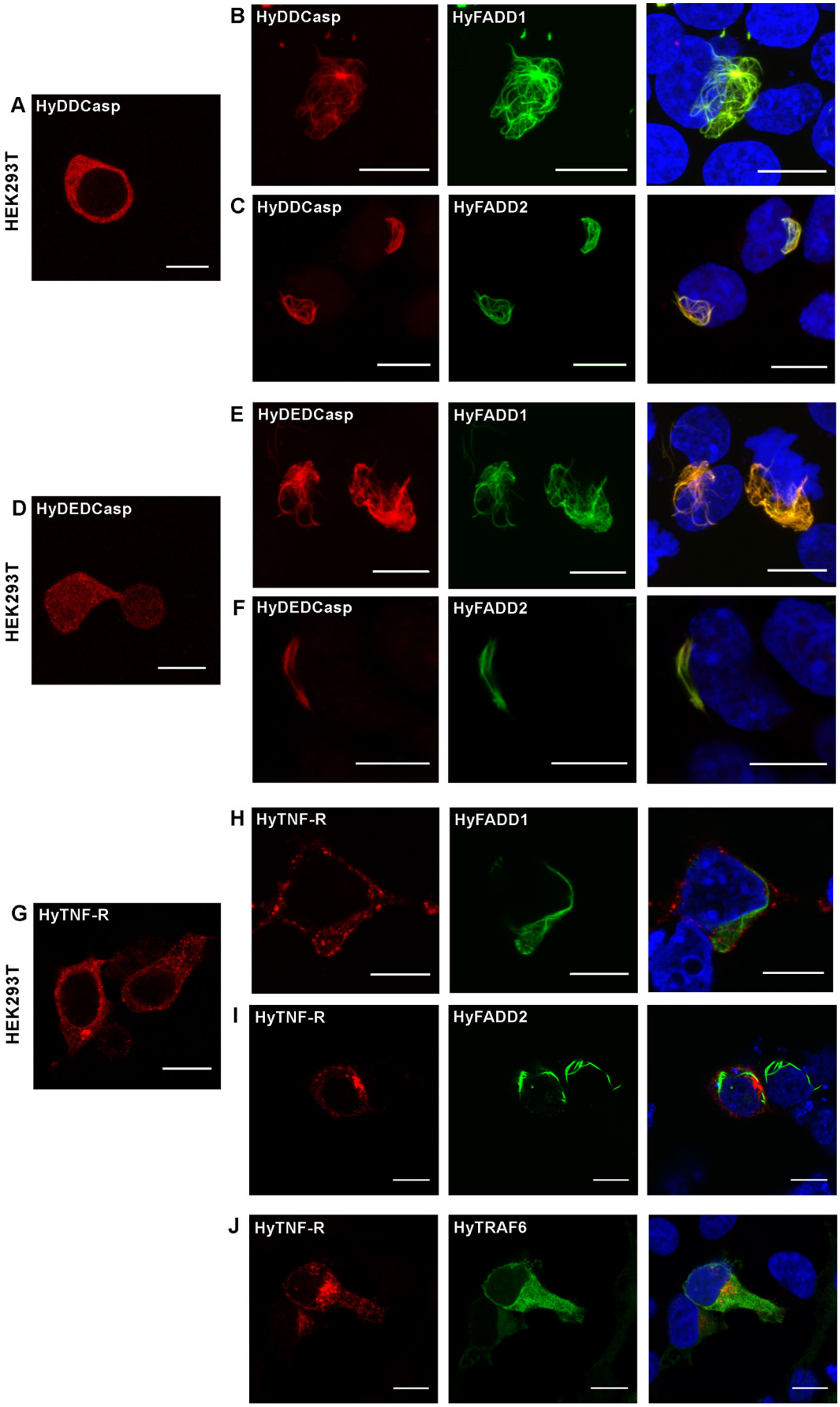

Fig. 7. See next page for legend.

Fig. 7. Co-expression of *Hydra* caspases HyDDCasp–HA and HyDEDCasp–HA, or HyTNF-R with GFP-tagged HyFADD1 and HyFADD2 in human HEK293T cells. Laser confocal images of: (A) HyDDCasp only, (B) HyDDCasp co-expressed with HyFADD1, (C) HyDDCasp co-expressed with HyFADD2, (D) HyDEDCasp only, (E) HyDEDCasp co-expressed with HyFADD1, (F) HyDEDCasp co-expressed with HyFADD2. Compare A with B plus C, and D with E plus F to see complete change of localisation of HyDEDCasp and HyDDCasp after co-expression with HyFADDs. (G) HyTNF-R only. (H) HyTNF-R co-expressed with HyFADD1. (I) HyTNF-R co-expressed with HyFADD2. The localisation of HyTNF-R in G remains unchanged in H plus I after co-expression with HyFADDs. (J) HyTNF-R co-expressed with HyTRAF6 shows similar localisations. GFP, green; anti-HA antibody, red; DAPI, blue. Right hand panels show merged images. Scale bars: 10 µm.

cells in *Hydra* encapsulate the nematocyte in a similar way as the tooth enamel organ encases the condensed mesenchyme.

We did not find any indication of a pro-apoptotic function of HyTNF-R. Moreover, we did not find evidence for an interaction of the HyTNF-R death domain with the *Hydra* FADD homologues HyFADD1 and HyFADD2. However, expression of HyFADDs in *Hydra* cells seemed to induce cell death. Moreover, when expressed in human HEK293T cells, HyFADD proteins formed DEFs, which recruited HyDDCasp and HyDEDCasp. This suggested that HyDDCasp as well as HyDEDCasp interact with HyFADDs. Occasionally we observed apoptosis in HEK293T cells, when HyDEDCasp was expressed in HEK cells, either alone or together with HyFADD1 or 2 (Fig. S5). Therefore, we propose that a FADD-dependent death pathway might exist in *Hydra*. Future experiments will be needed to reveal how HyFADDs are activated to recruit HyDEDCasp and/or HyDDCasp and induce apoptosis. This could happen through an extrinsic pathway involving an unknown receptor, but also through intrinsic apoptotic triggers.

We propose that HyTNF-R and HyFADD proteins are not part of a common extrinsic death pathway. This might be different in the cnidarian *Acropora*. There, a much larger superfamily of TNF-R homologues was found and at least four of the predicted TNF-R proteins have the classical domain structure with transmembrane and intracellular DD domains as we see for HyTNF-R. A further two have DDs but no transmembrane domains (Quistad et al., 2014; Quistad and Traylor-Knowles, 2016).

HyTNF-R, as we show here, probably is involved in morphogenesis rather than apoptosis. This suggests that non-apoptotic and morphogenetic functions are an ancient trait of TNF-R superfamily members. How extrinsic pathways of apoptosis induction work in invertebrates is still an open question.

MATERIALS AND METHODS

Gene cloning

Searches for *Hydra* genes with sequence homology to human FADD, TNF, TNF-R, TRAF6 and TRAF4 were performed using the *Hydra* genome server (https://metazome.jgi.doe.gov/pz/portal.html) and the NCBI homepage (http://www.ncbi.nlm.nih.gov/). Sequences for HyFADD1 (XP_002166467.1), HyFADD2 (XP_002166848.2), HyTNF-R (XP_004209206.1), HyTNF (XP_012554653.1), HyTRAF6 (XP_004206538.1) and HyTRAF4 (XP_004207529.1) were amplified from *Hydra* cDNA and cloned into the pSC-B-amp/can vector using the StrataClone Blunt PCR Cloning Kit (Stratagene). For expression of GFP-tagged proteins in Hydra cells genes were cloned into the HoTG vector (Böttger et al., 2002). For expression in mammalian cells genes were cloned into the vector pCMV-HA (Clontech) for HA-tagged proteins, and into the vector pEGFP-C1 (Clontech) for GFP-tagged proteins.

Cell culture, transfection and immunostaining

Human embryonic kidney (HEK) 293T cells (ATCC CRL-1573TM) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}) at 37°C, 5% CO_2. For microscopy, HeLa cells (ATCC CCL-2TM) were grown to 50–70% confluence on glass coverslips and transfected with DNA of plasmids pCMV-HA:HyTNF-R, pEGFP-C1:HyFADD1, pEGFP-C1:HyFADD2 and pEGFP-C1:Hy-TRAF6 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 24 h post-transfection, cells were fixed with 4% paraformaldehyde and permeabilised with 1% Triton-X-100 in phosphate-buffered saline (PBS). Anti-HA (1:500, H6908, Sigma) was used as primary antibody, Alexa-Fluor-495-conjugated anti-rabbit-IgG (Invitrogen) was used as secondary antibody.

Hydra culture

Hydra vulgaris (Basel strain) were cultured in *Hydra* medium [0.1 mM KCl, 1 mM NaCl, 0.1 mM $MgSO_4$, 1 mM Tris-HCl, 1 mM $CaCl_2$ (pH 7.6)] at 18°C. They were fed daily with freshly hatched *Artemia nauplii* larvae and washed after 6–8 h to remove undigested material.

Alsterpaullone treatment

Four sets of 24 *Hydra* polyps were incubated with 5–10 µM GDK-β-inhibitor Alsterpaullone (ALP; AG Scientific) in 0.1% DMSO in *Hydra* medium in the dark. Each animal was treated separately in 1 ml. After 24 h the medium was exchanged with fresh 0.1% DMSO in *Hydra* medium and incubated for another 72 h to induce growth of ectopic tentacles and continue with immunostaining.

Acridin Orange staining

Hydra polyps were treated with 3 µM Acridin Orange (Roth) for 15 min in the dark and washed twice with *Hydra* medium. Each group consisted of five animals with five replicates. Then they were relaxed in 2% urethane for 2 min and examined with the LEICA DM750 microscope at an excitation wavelength of 550 nm.

Generation of antibodies

The peptide (EDIAHFDLSPKTATVDLLHD) was injected into rabbits for immunization, the antibody was produced by Davids Biotechnology, Regensburg and affinity purified on 1 ml matrix with 3 mg immobilised antigen (Epoxy immobilisation). The purified antibody detected GFP-tagged HyTNF-R introduced into human HEK293T-cells after SDS-PAGE/western blotting used at 1:10 (Fig. S3A). It precipitated HyTNF-R from lysates of polyps used at 10 µg per ml. Precipitated protein was visible on western blot after SDS-PAGE at the expected size of 36 kDa (Fig. 7B). Moreover, it specifically stained HyTNF-R that was overexpressed in human HEK-cells in immunofluorescence experiments when used at 1:50 (Fig. S3B).

Immunoprecipitation of endogenous HyTNF-R

500 *Hydra* polyps (~5×10^7 cells) were lysed by pipetting in 500 µl solubilization buffer (50 mM Tris-HCl, 1% Triton X-100, 1 mM PMSF, 0.1% Bacitracin and 0.2% Aprotinin), left at 4°C for 30 min and centrifuged at 186,000 ***g*** for 1 h at 4°C. The clear supernatant was incubated at 4°C for 2 h with 5 µg of anti-HyTNF-R antibody and for control with anti-Hyp53, 50 µl of protein G–Sepharose beads (4 fast flow, GE Healthcare) were added for 2 h at 4°C. After centrifugation, the beads were washed four times with wash buffer 1 (150 mM NaCl, 20 mM Tris-HCl pH 7.5), four times with wash buffer 2 (300 mM NaCl, 20 mM Tris-HCl pH 7.5) and resuspended for mass spectrometric analysis or PAGE/western blotting.

Label-free LC-MS/MS analysis of immunoprecipitation of endogenous HyTNF-R

For mass spectrometry (MS), 10 µg of the samples were digested with a modified filter aided sample preparation (FASP) procedure. Proteins were reduced and alkylated using dithiothreitol and iodoacetamide. Then they were centrifuged through a 30 kDa cut-off filter device (PALL), and washed three times with 8M urea in 0.1 M Tris-HCl pH 8.5 and two times with 50 mM ammonium bicarbonate. Probes were incubated on the filter for 2 h at room temperature with 1 µg Lys-C (Wako Chemicals) and for 16 h at

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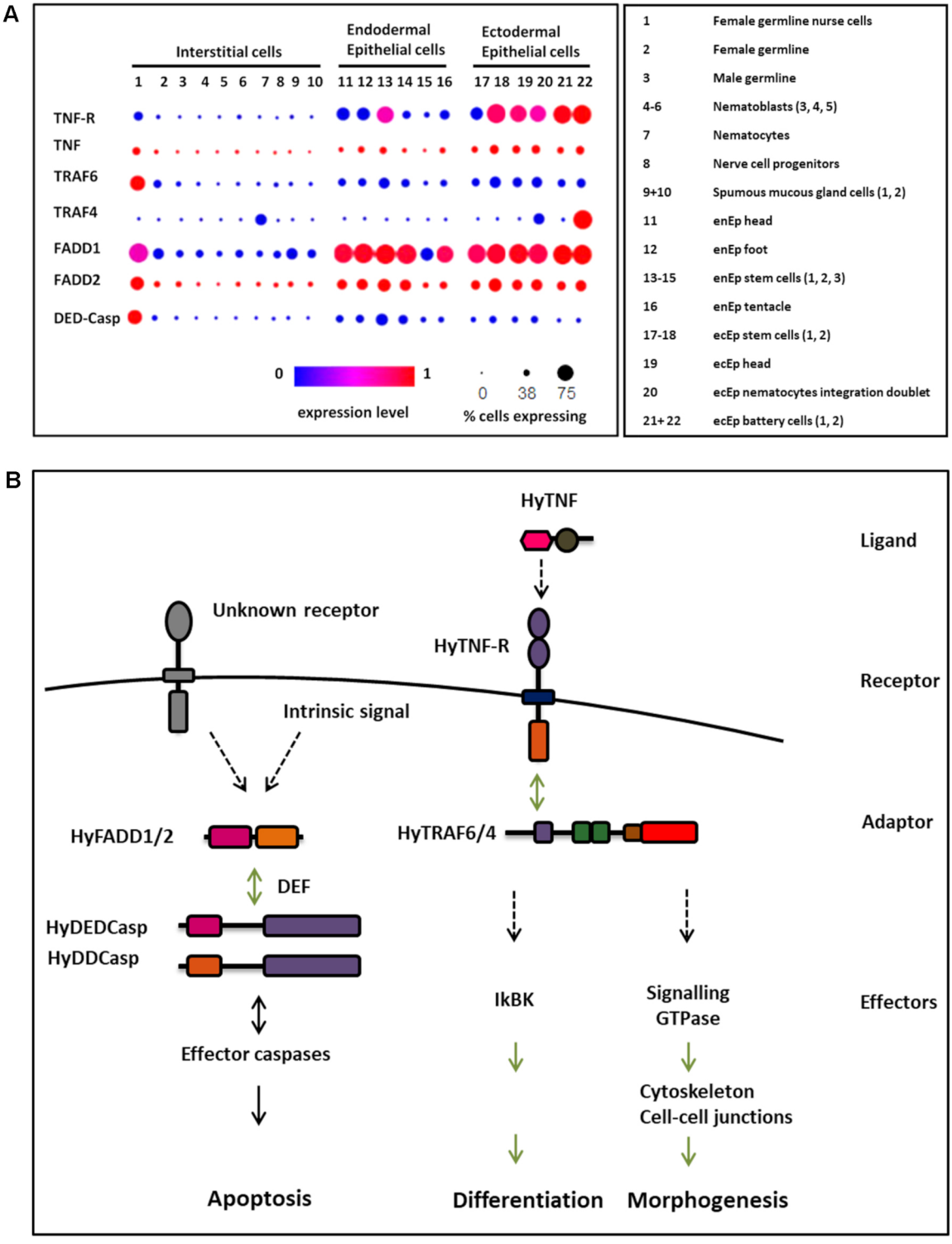

Fig. 8. Expression of TNF-R-pathway genes in *Hydra* and model for TNF, TNF-R and FADD function in *Hydra*. (A) Expression data from single-cell expression atlas. Dot plot grouped in interstitial, ectodermal and endodermal epithelial cells expressing expected TNF-R pathway genes. Colour spectrum represents the relative expression level from 0 (blue) to 1 (red); circle size indicates percentage of cells expressing the gene, cell type subgroup numbers are explained in the legend on the right. (B) Schematic model of HyTNF-R mediating differentiation via TRAF6/4 and possibly NFκB signalling, or mediating cytoskeleton and morphogenesis via TRAF6/4 and signalling GTPases. HyFADD1 and HyFADD2 interact with HyDEDCasp and HyDDCasp, and this might induce apoptosis; the upstream mechanism is unknown.

37°C with 2 µg trypsin (Promega). Peptides were collected by centrifugation for 10 min at 14,000 ***g***. Afterwards samples were acidified with 0.5% trifluoroacetic acid. Liquid chromatography tandem MS (LC-MS/MS) analysis was performed as described previously (Hauck et al., 2010). Samples were loaded on a trap column at a flow rate of 30 µl/min in 3% buffer B [73% acetonitrile (CAN), 3% DMSO and 0.1% formic acid in HPLC-grade water] and 97% buffer A (2% CAN, 3% DMSO and 0.1% formic acid in HPLC-grade water) and incubated for 5 min. The eluate was

loaded on an analytical column (140 min gradient from 3 to 35% of buffer B at 300 nl/min flow rate, 5 min gradient from 35 to 95% buffer B). The 10 most abundant peptide ions which had at least 200 counts were selected for fragmentation in the linear ion trap. A high-resolution MS spectrum with a mass range from 200 to 1500 Da was acquired in the Orbitrap. Acquired spectra were loaded to the Progenesis LC-MS software (version 2.5, nonlinear) for label-free quantification and analysed as described previously (Hauck et al., 2010). All MS/MS spectra were exported as Mascot generic file and used for peptide identification with Mascot (Matrix Science, London, UK; version 2.5.1). Mascot was set up to search the Uniprot_Hydra-vulgaris database (6657 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 ppm. Carbamidomethylation of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine, and oxidation of methionine were specified in Mascot as variable modifications.

Scaffold (version Scaffold_4.4.6, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Mascot identifications required at least ion scores must be greater than both the associated identity scores and 30. Protein identifications were accepted if they contained at least one identified peptide. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Immunostaining of *Hydra* whole mounts

A total of 10–20 polyps were relaxed for 2 min in 2% urethane, fixed with 2% paraformaldehyde for 1 h at room temperature, washed with PBS, permeabilized (0.5% Triton X-100 in PBS) and blocked (0.1% Triton X-100, 1% BSA in PBS). Incubation with the primary antibody in blocking solution was carried out overnight at 4° C. The secondary antibody was incubated for 2 h at room temperature followed by washing and DAPI staining. The animals were mounted on slides with Vectashield mounting medium (Alexis Biochemicals). The staining was repeated at least 50 times by different researchers.

Biolistic transformation

Gold particles (1.0 µm Bio-Rad) were coated with DNA of plasmids HoTG-TNF-R, HoTG-FADD1 or HoTG-FADD2 according to the instructions of the manufacturer. They were introduced into *Hydra* cells with the helios gene gun system (Bio-Rad) as previously described (Böttger et al., 2002)

Confocal laser scanning microscopy

A Leica SP5-2 confocal laser-scanning microscope was used for acquiring light optical serial sections and was equipped with an oil immersion Plan-Apochromat 100/1.4 NA objective lens. EGFP and Alexa Fluor 488 were visualized with an argon laser at an excitation wavelength of 488 nm and emission filter at 520–540 nm, a UV laser diode with excitation wavelength of 405 nm and emission filter of 415–465 nm was used for DAPI and a Krypton laser was used to visualize Cy3 with an excitation wavelength of 550 nm and emission filter at 564 nm and Alexa Fluor 495 excited at a wavelength of 561 nm and emission filter at 604–664 nm.

Domain structure analysis

Domain structures of protein sequences were analysed using the SMART database (http://smart.embl-heidelberg.de/). Signal peptides were predicted using the SignalP-5.0 server (http://www.cbs.dtu.dk/services/SignalP/). Protein structures were visualized with DOG 2.0 (Ren et al., 2009). AdTNF1, aug_v2a.02274.t1; AdTNF2, aug_v2a.01701.t1; AdTNF3, aug_v2a.15174.t1; AdTNF4, aug_v2a.19174.t1; AdTNF5, aug_v2a.19173.t1; AdTNF6, aug_v2a.19172.t1; AdTNF7, aug_v2a.21762.t1; AdTNF8, aug_v2a.24713.t1; AdTNF9, aug_v2a.21776.t1; AdTNF10, aug_v2a.17595.t1; AdTNF11, aug_v2a.14625.t1; AdTNF12, aug_v2a.06643.t1; AdTNF13, aug_v2a.01699.t1; AdTNFR1, aug_v2a.12827.t1 315; AdTNFR2, aug_v2a.07010.t1; AdTNFR3, aug_v2a.11053.t1; AdTNFR5, aug_v2a.14243.t1; AdTNFR8, aug_v2a.02522.t1; AdTNFR11, aug_v2a.09194.t1; AdTNFR39, aug_v2a.08700.t1. (Oist marine genomics acropora gene sequencing project: https://marinegenomics.oist.jp/coral/viewer/info?project_id=3).

Phylogenetic trees

For phylogenetic analyses, conserved DD, TNF and MATH domain sequences of proteins from included species were used. Small trees were calculated from *Hydra* and human sequences. For extensive analysis of the phylogenetic relationships in the TNF-R superfamily, vertebrate DD sequences (*Mus musculus*, *Danio rerio*, *Xenopus tropicalis* and *Xenopus laevis*) and available cnidarian sequences (*Acropora digitifera* and *Nematostella vectensis*) were included. Unrooted neighbour joining trees were calculated using the http://www.phylogeny.fr/ server with MUSCLE (MUltiple Sequence Comparison by Log-Expectation) for alignments, PhyML 3.0 for Phylogeny, aLRT (approximate Likelihood-Ratio Test) as statistical test for branch support and TreeDyn for tree drawing. Similar results were obtained by using ClustalX alignments and 10 000 bootstrapping trials as statistical tests for branch supports. Branches with branch support values below 50% have been collapsed. Sequence accessions (GenBank) are as given below.

TNF-Rs: TNF-R (*H. sapiens*), NP_001333021.1; p75 (*H. sapiens*), NP_002498.1; NGF-R (*H. sapiens*), AAB59544.1; EDAR (*H. sapiens*), AAD50077.1; FAS (*H. sapiens*), AKB11528.1; TRAIL-R1 (*H. sapiens*), NP_003835.3; TRAIL-R2 (*H. sapiens*), AAC51778.1; DR6 (*H. sapiens*), NP_055267.1; DR6 (*M. musculus*), AAK74193.1; EDAR (*M. musculus*), XP_006513267.1; TNF-R1a (*M. musculus*), AAH52675.1; NGF-R (*M. musculus*), AAD17943.1; EDAR (*D. rerio*), ABP03881.1; p75 (*D. rerio*), XP_700985.3; NGF-R (*D. rerio*), NP_001185589.1; DR6 (*D. rerio*), ABG91568.1; EDAR (*X. laevis*), NP_001080516.1; DR6 (*X. tropicalis*), ABQ51095.1; AdTNFR1 (*A. digitifera*), 12827Acd; AdTNFR2 (*A. digitifera*), 07010Acd; AdTNFR3 (*A. digitifera*), 11053Acd; AdTNFR4 (*A. digitifera*), 14243Acd; AdTNFR5 (*A. digitifera*), 14243Acd; AdTNFR8 (*A. digitifera*), 02522Acd; AdTNFR11 (*A. digitifera*), 09194Acd; AdTNFR39 (*A. digitifera*), 08700Acd; TNF-R1 (*N. vectensis*), v1g211491; TNF-R2 (*N. vectensis*), v1g216883; HyTNF-R (*H. vulgaris*), XP_004209206.1.

TNFs: TNF, NP_665802.1; TNFSF10, NP_003801.1 Eda, AAI26144.1; NGF, AAH32517.2; FasL, AAO43991.1; TRAIL, NP_003801.1; LTa, XP_011512918.1; BDNF, CAA62632.1; HyTNF, XP_012554653.1.

TRAF MATH domains; TRAF2, ADQ89802.1; TRAF3, NP_663777.1; TRAF4, NP_004286.2; TRAF5, NP_001029082.1; TRAF6, NP_665802.1; HyTRAF4, XP_004207529.1; HyTRAF6, XP_004206538.1; TRAF7, EAW85549.1.

Gene expression analysis

FASTA protein sequences were Blasted on the Hydra 2.0 Genome Project Portal (https://research.nhgri.nih.gov/hydra/sequenceserver/) against the transcriptome reference 'Juliano aepLRv2'. Obtained IDs were entered in the Single Cell Portal (https://singlecell.broadinstitute.org/single_cell/study/SCP260/stem-cell-differentiation-trajectories-in-hydra-resolved-at-single-cell-resolution#study-visualize), (Siebert et al., 2019). Dotplots for the analysed genes were extracted, aligned and grouped according to cell type into interstitial cells and ecto- and endodermal epithelial cells. HyTNF-R, t27001aep; HyTNF, t16693aep; HyTRAF4, t25400aep; HyTRAF6, t28162aep; HyFADD1, t25400aep; HyFADD2, t4925aep; HyDEDCasp, t8898aep (https://research.nhgri.nih.gov/hydra/sequenceserver/).

Acknowledgements

We are grateful to Charles David for helpful discussions about this work. We also want to thank Suat Özbek (Centre for Organismal Studies, Heidelberg, Ruprecht-Karls-Universität Heidelberg, Germany) for providing the antibody for staining of nematocyte capsules.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.B., M.S.; Methodology: A.B., M.S., L.S.S., A.-C.K., S.H.; Validation: A.B., M.S., L.S.S. M.S.; Formal analysis: M.S., A.B., S.H., A.-C.K., L.S.S.; Investigation: M.S., L.S.S.; Data curation: M.S., A.-C.K., S.H.; Writing - original draft:

M.S.; Writing - review & editing: A.B.,M.S. L.S.S.; Supervision: A.B., S.H.; Funding acquisition: A.B.

Funding
This work was funded by Deutsche Forschungsgemeinschaft grant BO-1748-7 awarded to A.B.

Supplementary information
Supplementary information available online at
https://jcs.biologists.org/lookup/doi/10.1242/jcs.255422.supplemental

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Summary: The single TNF-R homologue in the cnidarian *Hydra* is related to human EDAR, involved in cell differentiation and not part of a FADD-dependent death pathway.

Funding details

S.No.	Funder name	Funder ID	Grant ID
1	Deutsche Forschungsgemeinschaft	http://dx.doi.org/10.13039/501100001659	BO-1748-7