APOPTOSIS IN HYDRA AND AN ANCIENT FUNCTION OF THE TUMOR NECROSIS FACTOR RECEPTOR FAMILY

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München, den 25.10.2023

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CONTENT

1 Zusammenfassung

Der programmierte Zelltod, auch Apoptose genannt, spielt eine wichtige Rolle bei der Embryonalentwicklung höherer Lebewesen, bei der Regulation des Zellwachstums und der Eliminierung von geschädigten Zellen. Die intrazellulären Signalwege, die zum Tod einer Zelle führen, sind im Fall der Vertebraten sehr gut erforscht. Unklar ist jedoch, wie sich diese Signalwege im Laufe der Evolution entwickelt haben.

In Vertebraten existieren der extrinsische und der intrinsische Signalweg. Beim extrinsischen Signalweg führt ein Signal von außerhalb der Zelle zur Aktivierung von bestimmten Rezeptoren, die zur Familie der Tumor-Nekrose-Faktor Rezeptoren gehören. Die Bindung von Adaptormolekülen führt zur Aktivierung von Caspasen, die im Folgenden für den Abbau der Zelle sorgen. Beim intrinsischen Signalweg bewirken Signale innerhalb der Zelle, wie zum Beispiel zytotoxischer Stress oder Schäden an der DNA, zur Bcl-2 kontrollierten Freisetzung von Cytochrom C aus Mitochondrien, der Ausbildung eines Apoptosoms und damit ebenfalls zur Aktivierung von Caspasen.

Untersuchungen am Modellorganismus Hydra sollten zur Aufklärung der Frage nach der evolutionären Entwicklung dieser Signalwege führen. Im Genom von Hydra lassen sich Mitglieder sowohl des extrinsischen also auch des intrinsischen Signalweges finden. Unsere Untersuchungen haben allerdings gezeigt, dass der einzige Vertreter der TNF Rezeptorfamilie in Hydra nicht mit Apoptose assoziiert ist. Er wird spezifisch in Epithelzellen exprimiert, die eine Nesselzelle beherbergen, sowie in den Batteriezellen in den Tentakeln. In diesen Zellen befinden sich ebenfalls 10 bis 20 Nesselzellen. Wird der Rezeptor durch biolistische Transformation in Hydrazellen überexprimiert, lässt sich keine erhöhte Apoptoserate feststellen. Phylogenetische Analysen haben gezeigt, dass der Hydra Rezeptor die größte Ähnlichkeit mit dem EDAR-Rezeptor der Vertebraten aufweist, der eine wichtige Rolle bei der Entwicklung von ektodermalen Strukturen wie Zähnen und Haaren, spielt. Die ursprüngliche Funktion dieser Rezeptorfamilie dürfte also in der Morphogenese und der Differenzierung, nicht aber in der Regulation von Apoptose liegen.

Dass Hydra Caspasen Apoptose auslösen wurde in früheren Untersuchungen bereits gezeigt. Wird Hydra-FADD, der hypothetische Adaptor für die Akkumulation von DD-Caspasen, in HEK-Zellen exprimiert, lässt sich eine sehr spezifische Lokalisation in sogenannten Death-Effector-Filaments (DEF) feststellen, nicht aber an der Membran, wo der TNF-Rezeptor lokalisiert ist. Hydra DED- und DD-Caspasen zeigen bei einer Coexpression mit HyFADD eine sehr starke Kolokalisierung in den DEFs. Wird HyFADD in Hydrazellen exprimiert, löst es Apoptose aus. Wir gehen daher von einem intrinsisch aktivierten apoptotischen Signalweg zur FADD-und Caspase-Aktivierung in Hydra aus.

Außerdem wurde die evolutionär konservierte Rolle des Membran ständigen, anti-apoptotischen Bcl-2-like 4 bestätigt und in einem physiologischen Kontext überprüft. Experimente mit Bcl-2-like 4 überexprimierenden transgenen Tieren zeigten, dass es die Apoptoserate als Antwort auf Hungerstress oder den PI3 Kinase Inhibitor Wortmannin verringert.

2 Summary

Apoptosis is a form of programmed cell death and occurs in all multicellular animals. The molecular mechanisms inducing and executing apoptosis are very well characterized, but how the signaling pathways governing these processes evolved, is still unclear. In vertebrates there are two pathways, which lead to programmed cell death. In the extrinsic pathway a signal from outside the cell leads to the activation of death receptors, the recruitment of adapter molecules like FADD to these membrane receptors and the activation of caspases, which are the executioners of cell death. In the intrinsic pathway a signal within the cell leads to the release of cytochrome c from mitochondria, which is controlled by members of the Bcl-2-protein family, the building of an apoptosome and, again, the activation of caspases. Studies in a basal multicellular animal, the fresh water polyp Hydra, should help to shed light on the evolution of cell death pathways and the ancestral function of the molecules involved. In the Hydra genome potential members of both, an intrinsic and an extrinsic apoptotic pathway have previously been identified. These include genes encoding 17 caspases, 10 Bcl-2-family members, Apaf-1 and IAP homologs and also one member of the TNF-receptor family, as well as its potential adaptor molecule FADD. In this study another FADD-gene and two TRAF-genes as well as a potential adaptor for the TNF-receptor are described.

We could confirm the antiapoptotic function of Hydra-Bcl-2 in a physiological context using transgenic hydra overexpressing HyBcl-2-like 4. Experiments with Hydra FADD (HyFADD) showed that it induces apoptosis in hydra cells. Expressed in HEK cells, HyFADD forms death-effector-filaments and recruits caspases with DED and DD domains into these filaments. In contrast, the HyTNF-R does not recruit HyFADD in HEK cells. It is expressed in single epithelial cells with incorporated nematocytes and in battery cells of the tentacles. Phylogenetically it is related to the vertebrate EDAR receptor. We therefore suggest an ancestral function of the TNF- receptor family in differentiation and morphogenesis and an intrinsic, receptor-independent activation of apoptosis.

3 Introduction

3.1 The different types of cell death

Cell death is a necessary event in the life of multicellular organisms. It is needed to remove damaged, mutated, infected or old cells and plays a major role in defense and development. Generally two types of cell death are distinguished, a non- programmed or accidental cell death, and a programmed cell death, usually described as necrosis and apoptosis, respectively. Recent studies revealed that there are even more subtypes of cell death, for example necroptosis which is a programmed form of necrosis, and autophagy, which also can lead to cell death.

3.1.1 Necrosis

Necrosis is defined by the accidental nature of cell death caused by extracellular stimuli such as injury or infection and results in the loss of cell membrane integrity and the uncontrolled release of cell components like heat shock proteins, uric acid, ATP, DNA, and nuclear proteins into the extracellular space. This leads to an inflammatory response by inflammasome activation and secretion of proinflammatory cytokines and in the following the elimination of the dead cell by phagocytosis. Often the inflammatory response also results in damage of the surrounding tissue (Festjens et al., 2006).

3.1.2 Necroptosis

Necroptosis is a programmed, caspase independent form of cell death. It is a viral defense mechanism for the case when apoptotic signaling pathways are blocked by the virus. In contrast to necrosis, the permeabilization of the cell membrane is highly regulated under these circumstances. Stimulation of the TNF-R1 by TNFα leads to the recruitment of TRADD and RIPK1 which, together with RIPK3, build the necrosome. The necrosome activates the mixed lineage kinase domain like pseudokinase (MLKL), which inserts into the lipid bilayer-membranes of organelles and the plasma membrane, leading to the release of cellular components into the extracellular space. This activates the immune system and the necrotic cell is taken up by macrophages via pinocytosis (fluid endocytosis) (Galluzzi et al., 2017).

3.1.3 Autophagic cell death

During autophagy cytoplasmic material is delivered to the lysosome for degradation. It can promote cell survival following nutrient limitation by recycling cellular components but sometimes also leads to cell death. Autophagy is executed by autophagy-related (Atg) genes (Denton and Kumar, 2018).

3.1.4 Apoptosis

The term apoptosis was defined 1972 by Kerr et al., and is characterized by morphological processes leading to controlled cellular self-destruction. The morphological features are deformation and shrinking of the cell, the loss of cell-cell contacts, nuclear fragmentation, chromatin condensation, blebbing or budding of the plasma membrane and fragmentation of the cell into so called apoptotic bodies, which are then phagocytosed by neighboring cells. Apoptosis is known to be required for the coordinated differentiation of multicellular organisms. Embryonic development is a complex combination of cell proliferation and cell death, whereby the latter plays very important roles especially in neural development, reduction of egg cells at birth and shaping of extremities in vertebrates. In adult organisms it is needed for cell number regulation and the removal of infected, damaged, mutated or old cells. In vertebrates, there are two signaling pathways, which lead to the programmed death of a cell, the intrinsic and the extrinsic apoptotic pathway.

3.1.4.1 Intrinsic apoptotic pathway

The intrinsic apoptotic pathway is also called mitochondrial pathway. Signals within a cell - induced for example by DNA damage, hypoxia or deprivation of survival factors – lead to the transcriptional activation of pro-apoptotic genes encoding proteins of the Bcl-2 (B-cell lymphoma 2) - family. Those proapoptotic Bcl-2 proteins mediate the release of cytochrome c from mitochondria. Cytochrome c binds to Apaf-1 (apoptotic protease activating factor 1) and, together with Procaspase9, forms a protein complex, the so called apoptosome. Procaspase9 is then cleaved into its active form, which processes and activates a number of effector caspases. The resulting caspase cascade leads to the proteolytic destruction of the cytoskeleton, membrane blebbing, degradation of DNA and chromatin fragmentation.

3.1.4.2 Extrinsic apoptotic pathway

In the extrinsic pathway a signal from outside the cell binds to a so-called death receptor on the outer cell membrane, for instance a member of the tumor necrosis factor receptor (TNF-receptor) family. This leads to the binding of adaptor molecules like TNF-receptor associated death domain protein (TRADD) and Fas associated death domain protein (FADD) to the death domain (DD) of the receptor. The resulting protein complex is called death inducing complex (DISC). Procaspase8 is recruited via the death effector domain (DED) of FADD and autocatalytically activated. Active caspase-8 then leads to the activation of the caspase cascade by processing downstream effector caspases.

3.2 The evolution of apoptosis

Programmed cell death occurs in all eukaryotes including yeast, fungi, plants and animals. Investigations of the cell death machinery of many different model organisms were carried out in order to understand the evolutionary process that resulted in the currently known very complex apoptotic pathways observed in vertebrates. In the following the knowledge about extrinsic and intrinsic apoptotic signaling pathways in different organisms is summarized and subjected to comparison.

3.2.1 PCD in plants

In plants, PCD occurs during developmental processes and is part of the defense program against environmental stress. PCD in plants shares common morphological and biochemical features with animal cell apoptosis, including cell shrinkage, chromatin condensation, DNA fragmentation and the release of cytochrome C from mitochondria. The activation of caspase-like proteases (metacaspases) also seems to play a role in plant PCD as camptothecin induced cell death can be inhibited by protease-specific inhibitors (Reape et al., 2008). Whilst Bcl-2 proteins only evolved in metazoans there are regulators of programmed cell death which are highly conserved and also present in plants, the BI-1 (Bax-inhibitor-1) and LFG (Life guard) – proteins. BI-1 contains six α-helix transmembrane domains and is located in the ER (endoplasmatic reticulum). It was shown to suppress cell death

induced by mammalian Bax when overexpressed in plant cells (Kawai et al., 2001). The LFG-protein family consists of seven-transmembrane proteins. The genome of Arabidopsis was found to encode five LFG proteins. LFGs in plants are also supposed to function in cell death regulation (Weis et al., 2013).

3.2.2 Apoptosis in pre-bilaterian animal organisms

Animals like sponges and corals, which evolved long before the development of bilaterian organisms already possess many genes encoding members of apoptotic pathways, similar to higher animals and even vertebrates. They include members of intrinsic apoptotic pathways like homologs of Bcl-2-family proteins and several caspases (Wiens et al., 2001, 2003), but also potential members of an extrinsic apoptotic pathway. In the genome of the coral *Acropora digitifera* for example, 40 members of the TNF-R superfamily and 13 potential ligands were found (Quistad et al., 2014). In corals, apoptosis was shown to be a response to heat stress (Palumbi et al., 2014).

The basal cnidarian *Hydra* possesses 17 potential caspases, of which 15 have putative active-site sequences with conserved histidine and cysteine residues and 9 have pro-domains including CARD, DED and DD domains. It was shown that *Hydra* caspases functionally participate in apoptosis. *Hydra* also has homologues of APAF-1 and IAP and 9 Bcl-2 family members. HyBak-like 1 and 2 strongly induce apoptosis when they are expressed in mammalian cells. HyBcl-2-like 1, -2, -3, -4, -6 and -7 inhibit camptothecin induced apoptosis in mammalian cells. Bcl-2-like 4 has the strongest protective effect and was shown to interact with HyBak-like 1 in a yeast two-hybrid assay. Furthermore, there are two BH-3-only proteins, one of these interacts with Bcl-2-like 4 and induces apoptosis in mammalian cells (Lasi et al., 2010b). The genome of *Hydra* also codes for one potential TNF-R and one potential TNF-R ligand, as well as two FADD-like proteins.

3.2.3 Apoptosis in *C. elegans*

The role of apoptosis in embryonic development is extensively studied in the nematode *Caenorhabditis elegans*. 131 out of 1090 initially forming somatic cells undergo apoptosis during embryonic and post-embryonic development. The mechanisms leading to apoptotic cell death are similar to vertebrates. The proapoptotic BH3 protein EGL-1 inactivates the antiapoptotic Bcl-2-family member protein CED-9. As a consequence CED-4 and CED-3, homologs of Apaf-1 und Caspase-3, are no longer sequestered by CED-9 and apoptosis takes place (Hengartner MO, 1999). The genome of the nematode *C. elegans* does not code for any members of the extrinsic apoptotic pathway.

3.2.4 Apoptosis in *D. melanogaster*

The small fruit fly *Drosophila melanogaster* belongs to the phylum Arthropoda and has intrinsic apoptotic mechanisms similar to higher animals. Two Bcl-2-family members (Buffy and DebcI), one Apaf-1 homolog (DARK) and 7 caspases have been identified. Normally IAP1 inhibits the proteolytic activation of caspases. When apoptosis is induced, the pro-apoptotic proteins reaper, hid and grim inhibit IAP1, the initiator caspase Dronc gets activated and apoptosis takes place. In addition to components of this intracellular apoptotic pathway, *Drosophila* possesses two potential TNF-receptor homologs called Wengen and Grindelwald. The intracellular domains of these receptors do not have conserved DD-or TRAF-binding domains. Nevertheless, Wengen was shown to interact with Drosophila TRAF2 and to induce apoptosis when overexpressed, as does the possible Wengen-ligand Eiger (Kauppila et al., 2003). Signaling was proposed to function via JNK (N-terminal Jun kinase) mediating several functions besides the induction of apoptosis, including host defence, tissue growth, pain sensitization and even sleep (reviewed in Igaki et al., 2014). Grindelwald seems to play a role in mediating loss of cell polarity and neoplastic cell growth (Andersen et al., 2015). There also exists one FADD homolog in *Drosophila*. It can bind to the *Drosophila* caspase DREDD to induce apoptosis but its upstream stimulation is mediated by IMD and Toll pathways and not by Wengen (Kanda at al., 2002). Recently Igekawa et al identified the first *Drosophila* BH3-only protein sayonara. Sayonara was found to interact with the BCL-2 homologous proteins,

Buffy and Debcl and induces apoptosis in a BH3 motif-dependent manner (Igekawa et al., 2023). Figure 1 compares apoptosis related genes of the different species mentioned above.

	Bcl-2 proteins	Caspases	FADDs	death-receptors	TRAFs	APAF-1	BH3 only
Homo sapiens	Bcl-2, Bcl-XL, BclW, Mcl-1, BclB, Bcl-2A1, Bax, Bak, Bok	Caspases 1-14	FADD1, 2	TNF-R1, 2, CD95, CD40, p75,...	TRAF1-7	APAF-1	Bid, Bmf, NOXA, PUMA, Bad, Bim, Bik, Hrk
Drosophila	Debcl, Buffy	Dredd, Dronc, Dream, Dcp1, DRICE, Decay, Daydream	dFADD	Wengen, Grindelwald	dTRAF1, 3, 6	DARK	sayonara
Caenorhabditis	Ced 9	Ced-3 Csp-1, 2			TRAF	Ced-4	Egl-1
Hydra	HyBcl-2 –like 1-7 HyBak-like 1, 2	HyCasp A-I, L, M HyCARD-1, 2 HyDEDCasp HyDDCasp	HyFADD1, 2	HyTNF-R	HyTRAF4, 6	HyAPAF-1	HyBH3only 1-4

Figure 1. Overview of apoptosis related proteins in different species.

3.3 *Hydra* as a model organism

Hydra vulgaris is an established model organism to study the evolution of early multicellular organisms. As a cnidarian, it is positioned at the base of the animal phylogeny. Cnidarians diverged from the metazoan lineage before the appearance of bilaterians (see figure 2). *Hydra* has served as a model organism since the mid eighteenth century when it was discovered. By now the whole genome of *Hydra* has been sequenced and there exist several transcriptomes, including a single cell transcriptome (Siebert et al., 2019)).

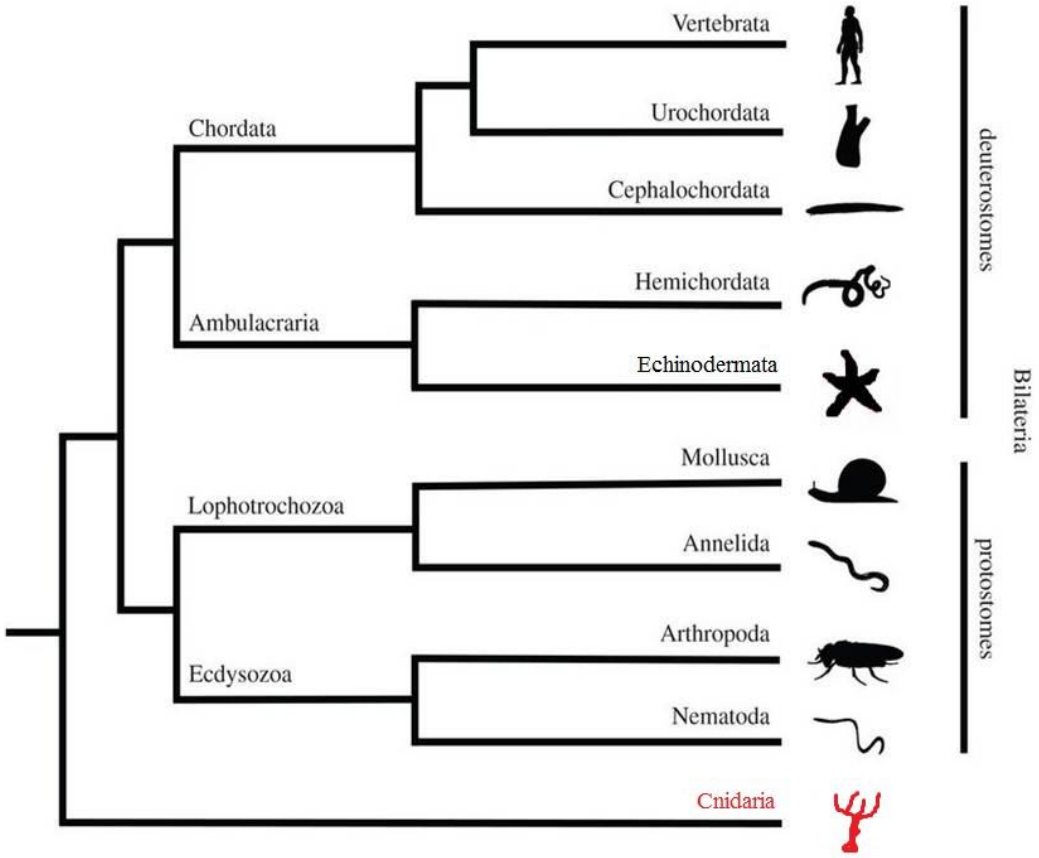

Figure 2. Animal phylogeny. The phylum Cnidaria (shown in red) is basal to the bilateria. (Modified after Semmens et al., 2016).

Hydra has a simple body plan with just one body axis. It has a mouth opening and tentacles to catch prey at the apical end, and a peduncle with a basal disc at the basal end. Despite this simplicity it has high tissue dynamics meaning that mechanisms to maintain homeostasis between different cell types are constantly active during adult life of the animal. All known major developmental signaling pathways like Notch (Käsbauer et al., 2007), Wnt (Hobmayer et al., 2000) and BMP (Reinhardt et al., 2004) have been identified. *Hydra* development follows principles similar to those in higher animals. *Hydra* polyps consist of derivatives of two germ layers, the embryonic ecto- and endoderm, which develop into a single cell layer epidermis and a single cell layer gastrodermis. For these adult epithelia layers the names ectoderm and endoderm are used in *Hydra*.

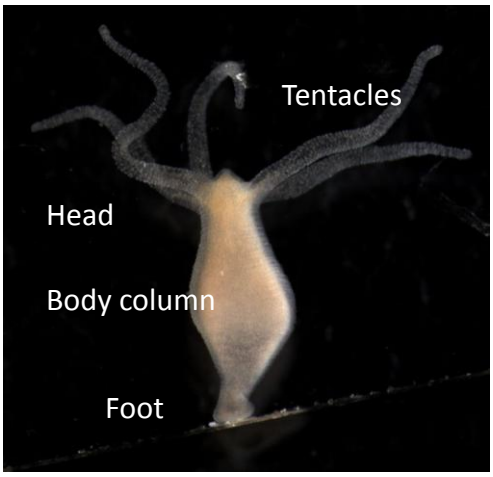

Figure 2. Body pattern of a *Hydra* polyp. Each polyp consists of a foot with a basal disc, a body column, a head with a mouth opening and four to twelve tentacles to catch prey.

All cells of *Hydra* develop from three proliferating cell types: The endodermal epithelial cells, the ectodermal epithelial cells and the interstitial cells. The interstitial cells represent a pluripotent stem cell lineage maintaining itself and supporting differentiation of descendants into gland cells, nerve cells and nematocytes. Epithelial cells combine characteristics of epithelial cells and muscle cells and form the outer epidermis and the inner gastrodermis. Due to constant cell divisions of these cells along the body column, single epithelial cells are moved along the body column and differentiate to tentacle or foot specific cells. Differentiated cells are sloughed off at the tentacle tips and the basal disc. They are replaced through the constant epithelial self-renewing and differentiation process occurring in the body column and at the body ends where foot cells, tentacle battery and hypostome cells are formed. Some cells are always removed by apoptosis to maintain homeostasis of the epithelia. This was discovered, when Bosch and David noticed in 1984 that budding hydra stopped forming buds under conditions of limited food supply, but cell division rates stayed the same.

Hydra has an almost unlimited capability to regenerate (reviewed in Bode et al., 2003 and Holstein et al., 2003). When *Hydra* polyps are cut in the middle, head and foot are regenerated completely. A whole *Hydra* can also regenerate out of small pieces when all proliferating cell types are present and even out of dissociated cells when they are allowed to aggregate, e.g by centrifugating them together (Gierer et al. 1972). Fujisawa and David showed 1984 that apoptosis also takes place during regeneration when nematocytes in the regenerating tissue are phagocytosed by epithelial cells.

3.4 Aim of the study

Whereas there are forms of programmed cell death even in prokaryotes, the process of caspase and Bcl-2 dependent programmed cell death, called apoptosis, is restricted to animals. The course of evolution of apoptotic regulatory networks within the animal kingdom, especially of the extrinsically induced apoptotic pathways, is unclear. Apparently not present in *C. elegans*, family members of potential regulators of the extrinsic apoptotic pathway have been discovered in Cnidarians. These include homologs of TNF-receptor- and of FADD- and TRAF-family members. In this study experimental evidence for a functional conservation of these apoptotic proteins was to be acquired. Moreover, the role of Bcl-2-like proteins as major apoptosis regulators, which was previously investigated in a heterologous system, was to be confirmed in a physiological context using transgenic animals overexpressing HyBcl-2-like 4 in ectodermal epithelial cells. Both lines of investigation were designed to connect the molecular evolution of regulators of apoptosis with a functional evaluation of existing apoptosis pathways in the cnidarian *Hydra*.

4 Results

4.1 Apoptosis in *Hydra*: function of HyBcl-2 like 4 and proteins of the transmembrane BAX inhibitor motif (TMBIM) containing family

In this work we investigated components of the intrinsic apoptotic pathway of *Hydra*. Expression patterns of all known *Hydra* genes of the Bcl-2 and TMBIM-families are presented, including HyBax-Inhibitor-1, HyLifeguard-1a, -1b and HyLifeguard-4. It is shown that TMBIM-family proteins are localized to ER and Golgi membranes, whereas HyBcl-2-like 4 is present in the outer mitochondrial membrane. HyBax-inhibitor-1 was able to protect human cells from camptothecin induced apoptosis. Moreover, we could show that *Hydra* cells overexpressing HyBcl-2like 4 in transgenic polyps are protected from apoptosis induced by the PI(3)kinase inhibitor wortmannin or by processes maintaining cellular homeostasis under conditions of limited food supply. This confirms that not only genes encoding proteins involved in intrinsic apoptotic pathways are conserved through evolution from pre-bilaterians to human, but also their functions.

Contribution to the paper

I have participated in the conception and execution of the experiments with the transgenic animals expressing GFP-tagged HyBcl-2-like 4 in the mitochondria of all ectodermal cells and animals that expressed mitochondrial GFP in ectodermal cells as a control. This included apoptosis induction with Wortmannin or starvation, maceration of the animals, quantification of apoptotic epithelial cells and statistical evaluation of the results. I have also planned and supervised the analysis of the expression levels of the TMBIM genes *HyLfg-1a, HyLfg-1b, HyLfg-4, HyBI-1, HyBcl2-4 and HyBcl2-5* via RT-qPCR under several apoptosis inducing conditions, including treatment with benzamidine, camptothecin, UV light, Wortmannin and starvation, as well as primer design and evaluation, generation of standard curves, RNA isolation, reverse transcription, real time qPCR and analysis of the results. I have contributed in writing and editing the paper.

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Apoptosis in Hydra: function of HyBcl-2 like 4 and proteins of the transmembrane BAX inhibitor motif (TMBIM) containing family

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ABSTRACT Mechanisms of programmed cell death differ between animals, plants and fungi. In animals, apoptotic cell death depends on caspases and Bcl-2 family proteins. These protein families are only found in multicellular animals, including cnidarians, insects and mammals. In contrast, members of the TMBIM-family of transmembrane proteins are conserved across all eukaryotes. Sequence comparisons of cell death related proteins between phyla indicate strong conservation of the genes involved. However, often it is not known whether this is paralleled by conservation of function. Here we present the first study to support an anti-apoptotic function of Bcl-2 like proteins in the cnidarian Hydra within a physiological context. We used transgenic Hydra expressing GFP-tagged HyBcl-2-like 4 protein in epithelial cells. The protein was localised to mitochondria and able to protect Hydra epithelial cells from apoptosis induced by either the PI(3) kinase inhibitor wortmannin or by starvation. Moreover, we identified members of the TMBIM-family in Hydra including HyBax-Inhibitor-1, HyLifeguard-1a and -1b and HyLifeguard 4. Expressing these TMBIM-family members in Hydra and human HEK cells, we found HyBax-inhibitor-1 protein localised to ER-membranes and HyLifeguard-family members localised to the plasma membrane and Golgi-vesicles. Moreover, HyBax-inhibitor-1 protected human cells from camptothecin induced apoptosis. This work illustrates that the investigated Bcl-2- and TMBIM-family members represent evolutionarily conserved mitochondrial, ER, Golgi and plasma membrane proteins with anti-apoptotic functions. The participation of ER and Golgi proteins in the regulation of programmed cell death might be a very ancient feature.

KEY WORDS: *Hydra, bcl-2, lifeguard, TMBIM-family, Bax-inhibitor*

Introduction

Programmed cell death has been observed in almost all organisms including protozoans, fungi, plants and animals. It is crucial for removal of damaged cells and functions as an important regulator of development.

The molecular mechanisms governing induction, regulation and execution of programmed cell death differ between animals, plants, fungi and protists. In animals, they involve the activation of cysteine proteases (caspases) through aspartate specific substrate cleavage. In vertebrates, caspase activation proceeds either via extrinsic receptor-based stimuli or through intrinsic pathways, which

Abbreviations used in this paper: APAF-1, apoptotic protease activating factor 1; Bcl-2, B-cell lymphoma 2; BI, Bax inhibitor; ER, endoplasmatic reticulum; FCS, foetal calf serum; HEK, human embryonic kidney; TMBIM, transmembrane bax-inhibitor motif.

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employ members of the "B-cell lymphoma 2" (Bcl-2)- protein family. Programmed cell death that depends on caspases and Bcl-2 proteins is commonly referred to as apoptosis. Most mammalian Bcl-2 proteins are localised at the mitochondrial outer membrane and only a few are associated with ER-membranes. Upon apoptosis-inducing signals, the outer mitochondrial membrane gets permeabilised by inhibitors of anti-apoptotic Bcl-2 family proteins. Thereby several pro-apoptotic factors, including cytochrome C, are released from the intra-membrane space. Cytochrome C then forms an apoptosome with "apoptotic protease activating factor 1" (APAF-1) and initiator caspases, promoting caspase self-activation and subsequent activation of effector caspases, which cleave specific substrates to induce morphological and molecular changes leading to cell death.

The *Bcl-2* gene family is conserved from early invertebrates, including *Caenorhabditis* and *Drosophila,* to humans. However, its function for intrinsic apoptosis induction differs between invertebrates and vertebrates. In *Caenorhabditis* the "Bcl-2 homology domain 3" (BH-3)-only protein "Egg laying deficient" (Egl-1) induces apoptosis via inhibition of ced-9 (the only Bcl-2 homolog found in *Caenorhabditis*), release of ced-4 (APAF-1) and activation of ced-3 (caspase 3) (Conradt and Horvitz, 1998). The function of the *Drosophila* Bcl-2 family members, *debcl* and *buffy*, is unclear. Both genes are not essential for the development of flies (Sevrioukov *et al.,* 2007).

To better understand the evolution of apoptosis we have previously analysed caspases and Bcl-2 family members in the pre-bilaterian cnidarian model organism *Hydra* (Lasi *et al*., 2010a, Lasi *et al*., 2010b) and described seven *Bcl-2-like* and two *Bak-like* genes. Additional members of the *Hydra Bcl-2* family were found through cell type specific transcriptome analyses (Wenger *et al*., 2016). In accordance with these published data a recent search in available sequencing data from *Hydra* has now revealed nine genes encoding Bcl-2-like proteins and two encoding Bak-like proteins. This gene family was also found recently to be present in corals of *Acropora* (Moya *et al.,* 2016). The *Hydra* proteins that we had previously studied were associated with membranes, five with mitochondria and two with ER-membranes. When expressed in HEK (human embryonic kidney) cells three family members induced apoptosis. The other Bcl-2 like proteins protected HEK-cells from camptothecin induced apoptosis. Moreover, the *Hydra* genome encodes several BH-3 only proteins. Of those, a 96 amino acid protein, which we named HyBH3-only 2, induced apoptosis in human cells (Lasi *et al*., 2010b).

Apoptosis in *Hydra* is found at the extremities of the animals, where cells are sloughed off and replaced by new ones. It is also involved in oogenesis and spermatogenesis. Moreover, it is used to regulate cell numbers under conditions of variant nutrient supply (reviewed in (Böttger and Alexandrova, 2007, Reiter *et al*., 2012)). The role of Bcl-2 proteins in these developmental processes in *Hydra* is not understood yet. We therefore investigated a transgenic *Hydra* line overexpressing HyBcl-2-like 4 protein in ectodermal cells. We found that the elevated levels of HyBcl-2-like 4 in this *Hydra* line protected *Hydra* epithelial cells from Wortmannin and starvation induced apoptosis. This work represents the first functional study of a Bcl-2 protein in cnidarians.

In contrast to the Bcl-2 protein family, which has only been detected in animals, the family of "transmembrane bax-inhibitor motif" proteins or TMBIMs, is also conserved in protists, plants and fungi (Hu *et al*., 2009). Members of this membrane protein family are cytoprotective and implicated in the regulation of programmed cell death. The name giving member of the family is Bax-Inhibitor (BI-1 or TMBIM6), (Rojas-Rivera and Hetz, 2015). BI-1 plays a role in protection from ER-stress, including such caused by disturbances in Ca^{2+}-homeostasis and unfolded protein response (reviewed in (Ishikawa *et al*., 2011)). The neuronal membrane protein TMBIM 2 obtained the name "life guard" (Lfg-2) due to its ability to inhibit FasL induced apoptosis (Somia *et al*., 1999). This name was then applied to other TMBIM family members including Lfg-1, -3, -4 and -5. Lfg-4 is a Golgi-protein, which protects cells against both intrinsic and extrinsic apoptosis activation (de Mattia *et al*., 2009, Gubser *et al*., 2007). Whether Lfg-1 and Lfg-5 are also involved in the regulation of apoptosis is not known to date.

Results

Hydra *Bcl-2-like 4 protects from apoptosis induced by Wortmannin or nutrient deprivation*

Some members of the extended Bcl-2-like protein family in *Hydra* are able to protect human cells from camptothecin induced

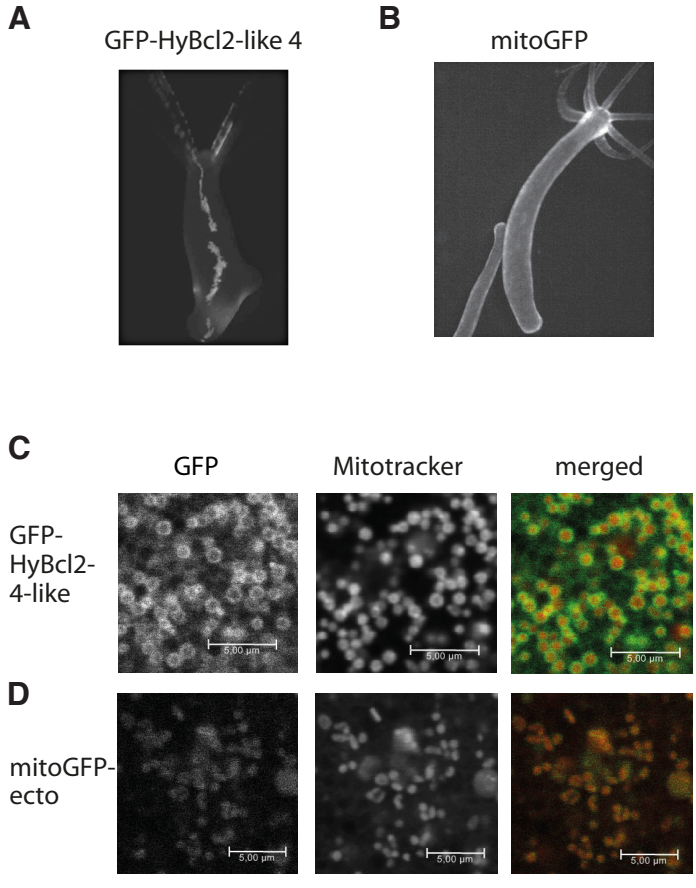

Fig. 1. Confocal microscopic optical section from the ectoderm of the body column of transgenic *Hydra* polyps *(Bcl-2-like 4ecto) expressing GFP-HyBcl-2-like 4 in most ectodermal epithelial cells and of* mitoGFPecto *animals expressing MitoGFP in all ectodermal epithelial cells.* **(A,B)** *Whole animals;* **(C,D)** *co-labelling of GFP-signals with mitochondrial marker Mitotracker RedCMXRos (Invitrogen); scale bars, 5µm.*

apoptosis in heterologous assays (Lasi *et al.*, 2010b). The strongest protective effect was observed with the mitochondrially localised HyBcl-2-like 4. This gene is also expressed most strongly in all cell types of *Hydra* (see later, Fig. 10). To gain insight into the physiological role of HyBcl-2-like 4 we investigated its effect on apoptosis in *Hydra* by analysing transgenic animals expressing GFP-tagged HyBcl-2-like 4 in the mitochondria of all ectodermal cells (HyBcl-2-like 4ecto). For control we used animals that expressed mitochondrial GFP in ectodermal cells (mitoGFPecto). For both transgenic lines we confirmed the presence of the plasmid in the transgenic animals by PCR-amplification of the respective sequences from genomic DNA and sequencing (Fig. S2). Fig. 1A shows an animal expressing GFP-HyBcl-2-like 4 in ectodermal cells. A week signal can be found in almost all ectodermal cells, however there are patches, which express the transgene strongly. In contrast, the expression of mitoGFP in the control cell line is equally strong in all ectodermal cells (Fig. 1B). Fig. 1C shows a live image of the cytoplasm of a *Hydra* ectodermal epithelial cell overexpressing GFP-HyBcl-2-like 4 from a patch of cells that expressed the transgene strongly. The GFP signal clearly localises to the outer part of mitochondria in contrast to the mitochondrial marker MitoTrackerRed CMXRos, which accumulates in the matrix of active mitochondria. This suggests that GFP-HyBcl-2-like 4 is present in the outer mitochondrial membrane. A patch of cells from control animals, as shown in live images in Fig. 1D, indicates that mitoGFP, as expected, co-localises with the mitochondrial marker.

Apoptosis was induced with the phosphoinositide-3-kinase (PI(3)-kinase) inhibitor Wortmannin. For quantification animals were macerated and the percentage of apoptotic epithelial cells amongst all epithelia cells was determined. Apoptotic cells of the interstitial lineage were not considered. Fig. 2A shows that Wortmannin induced apoptosis in *Hydra* epithelial cells in a concentration dependent manner. However, epithelial cell apoptosis was significantly reduced in HyBcl-2-like 4ecto transgenic animals compared with mitoGFPecto animals and non-transgenic animals (*Hydra*-AEP wild type).

We then induced apoptosis in *Hydra* cells by depriving the animals of food for seven days. Regularly fed animals from the *Hydra*-AEP wild type strain usually have less than 1.5 % of apoptotic epithelial cells, this number does not change when the animals are incubated in 1 % DMSO for 4.5 hrs (Fig. S3). It is also evident in our data where we found in all *Hydra* strains ca. 1 % of apoptotic cells in the control group which had been treated with DMSO for 4.5 hrs for the Wortmannin experiment (Fig. 2A, 0 Wortmannin). After starving the animals for seven days, in *Hydra*-AEP wildtype- and mitoGFPecto-control polyps, 5 % of epithelia cells were apoptotic. In the HyBcl-2-like 4ecto strain, this number was reduced to 2% (Fig. 2B) indicating that HyBcl-2-like 4 protects cells from apoptosis that occurs in Hydra during periods of starvation.

We wondered whether this protection from apoptosis in starved HyBcl-2-like 4ecto animals could allow budding that was normally suppressed by food deprivation. This was not the case. When we compared budding rates of HyBcl-2-like 4ecto- and control-polyps, budding rates were the same in all strains (Fig. 3).

Taken together, these results confirm an anti-apoptotic role for HyBcl-2-like 4 in *Hydra.* Moreover, apoptosis was also reduced under physiological conditions of starvation, indicating a generally conserved function of anti-apoptotic Bcl-2 family members in animals from pre-bilaterians to humans.

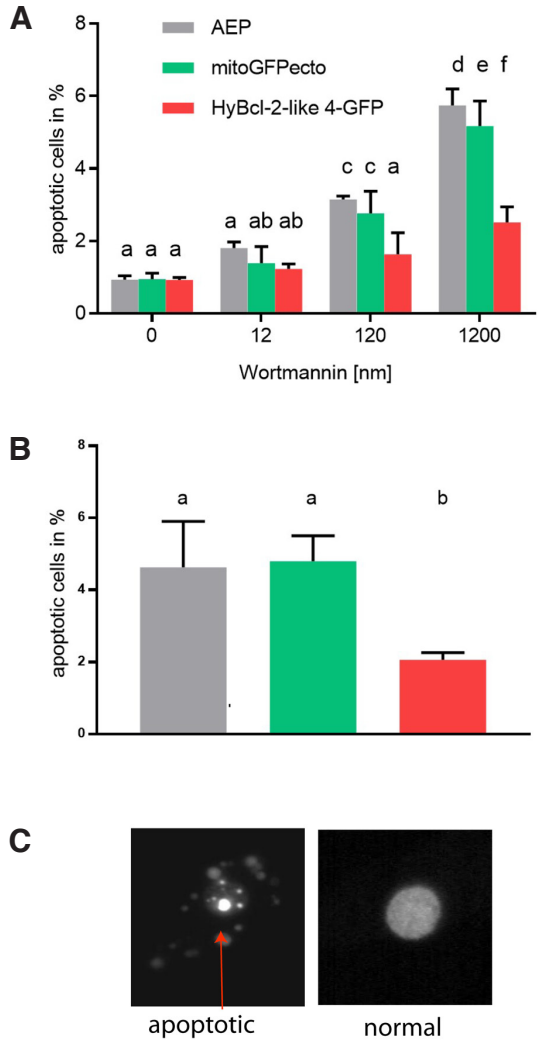

Fig. 2. Apoptosis in transgenic and wild-type Hydra induced by Wortmannin or food deprivation. (A) *Diagram presenting apoptotic* Hydra *cells in % of epithelial cells after treatment of* **Bcl-2-like 4ecto** *polyps with PI(3) kinase inhibitor Wortmannin.* **Hydra-AEP wild type** *founder strain (grey) and* **mitoGFPecto** *(green) animals were used for control. Standard deviation from counts of 4 biological replicates. Two-way ANOVA statistical analysis (Tukey test) was applied. Letters above columns indicate significance for p-values < 0.05. The same letter on two columns indicates no significant difference between values obtained under indicated conditions or for indicated strains, different letters stand for significant differences between such values;* **(B)** *Diagram presenting apoptotic cells in % of epithelial cells from polyps after 7 days without food; Animals of the* **Hydra-AEP wild type** *founder strain (grey), transgenic animals expressing mitochondrially targeted GFP in ectodermal cells (***mitoGFPecto***) green) and animals expressing GFP tagged HyBcl-2-like 4 in ectodermal cells (***HyBcl-2-like 4ecto***, red) were analysed. Standard deviation from counts of four biological replicates. One way ANOVA statistical analysis (Tukey test) was applied and p-values <0.05 were assumed statistically significant. b on red column indicates that* **HyBcl-2-like 4ecto** *line counts differ significantly from counts in control animals (AEP and mitoGFPecto);* **(C)** *epi-fluorescence microscopic images of DAPI-stained epithelial cell nuclei from macerates of Wortmannin treated polyps, arrow points to apoptotic nucleus. Apoptotic and non-apoptotic epithelial cells were counted on macerates by microscopic inspection; in (A,B) percentage of apoptotic cells is given in relation to all of the inspected epithelial cells.*

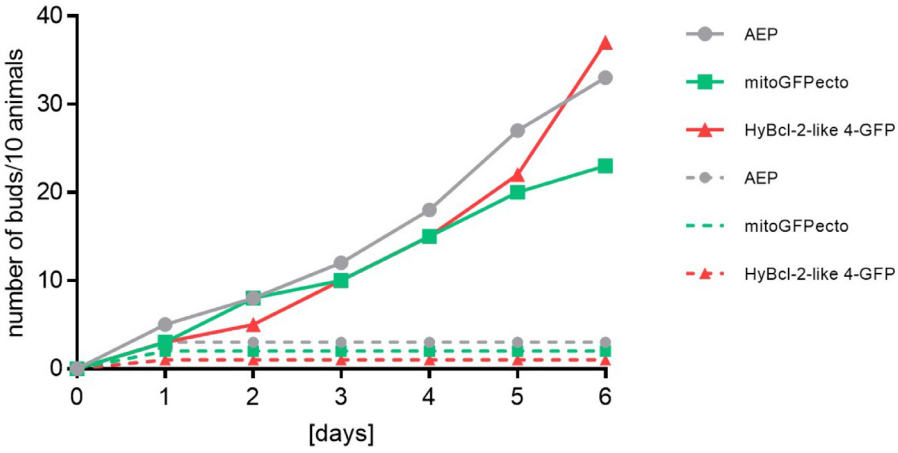

Fig. 3. Hydra budding rates depend on feeding regime. *Budding rates in Hydra polyps of transgenic and control strains in fed and non-fed animals.*

The Hydra *Lifeguard and Bax-Inhibitor genes*

We then investigated *Hydra* genes encoding members of the TMBIM family of cytoprotective proteins that could play a role in regulation of apoptosis independently of HyBcl-2-like proteins. We identified one Bax-Inhibitor homolog (HyBI-1) and three Lifeguard homologs (HyLfg-1a, HyLfg-1b and HyLfg-4). Full-length cDNAs of these four genes were cloned and sequenced. Phylogenetic analysis of HyLfgs, HyBI-1 and their homologs from vertebrates, plants, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Nematostella vectensis* was performed (Fig. 4). All sequences are clearly related and the separation into different groups is not very strongly supported by bootstrap values. Nevertheless, we found two main branches separating Bax-Inhibitor (TMBIM6, blue branch) and Lfg-4- sequences (TMBIM4, green branch for plants; red branch for animals) from all other animal Lfg-sequences, including Lfg-5 (TMBIMB1b, purple branch), Lfg-1 (TMBIMB3, grey branches including invertebrate and vertebrate sequences), Lfg-2 (TMBIM2, pink branch)) and Lfg-3 (TMBIM1, brown branch). BI-1 sequences are found in animals and plants and this is also the case for Lfg-4 sequences, where the plant sequences have expanded. In animals we only found one Lfg-4 sequence in each species that was included in the analysis. Cnidarian genes (*Hydra* and *Nematostella*) are clearly represented in the animal branches of both, BI-1 and Lfg-4 genes. In contrast, Lfg-4 was not found in *Caenorhabditis elegans*.

The sequences for Lfg-1, -2, -3 and -5 appear as a sister branch of the Lfg-gene of the single-cell green alga *Chlamydomonas reinhardtii*. The *Hydra* sequences are clustered with *Nematostella* and *Caenorhabditis* sequences and separated from Lfg-1, -2 and -3, which are present in vertebrates. Lfg-5 and Lfg-1 have been considered by other authors to be ancestral to Lfg-2 and Lfg-3 derivatives (Mariotti *et al.*, 2014). *Hydra* Lfg-1a and -1b do not cluster with Lfg-5.

Our phylogenetic analysis and the placement of the *Hydra* sequences is in accordance with the hypothesis that the TMBIM gene family expanded from a single ancestor that arose before the divergences of animals, plants, fungi and protozoa (Hu *et al.,* 2009). It then split into BI-1 and Lfg-4 lineages, the latter expanded in plants. In animals a Lfg-1/5 like sequence evolved and expanded in both, invertebrates and vertebrates. It also got lost from some phyla (e.g. birds and reptiles (Hu *et al.*, 2009, Mariotti *et al.*, 2014)).

Closer analysis of the HyBI-1 and HyLfg protein sequences provides strong confirmation for the grouping of these sequences within the phylogenetic tree. Fig. 5A shows an alignment of HyBI-1 with human BI-1. The Bax-Inhibitor motif (PS01243) is labelled in bold red letters and, although clearly recognisable, it is not completely identical between the *Hydra* and human sequences. For the HyBI-1 sequence the TMHMM program predicts a six α-helix transmembrane domain structure with intracellular C- and N-terminal regions (Fig. 5A), similar to human BI-1. Moreover, the C-terminal region of BI-1 is also conserved between *Hydra* and *Homo sapiens*. It contains a semi-hydrophobic helix with four conserved aspartate residues and a very basic C-terminal region, implicated in tetramerisation of the protein and in pH-sensing (Bultynck *et al.*, 2012, Henke *et al.*, 2011)(Fig. 5A).

Fig. 5B shows an alignment of all three *Hydra* Lfg-proteins with human Lfg-4 (isoform b). The TMHMM program predicts a seven α-helix transmembrane domain structure with an extracellular C-terminal region for all *Hydra* proteins, similar to human Lfg-4. Ten characteristic sequence motifs diagnostic of the Lifeguard family have been described (Hu *et al.,* 2009). Fig. 5B shows that these are conserved in HyLfgs. Motif 1 in the N-terminal and transmembrane region 1 (TM 1) is present in all Lfg-family members in animals and in plants and is also present in the three *Hydra* Lfgs. Motif 2 is only found in Lfg-4 proteins in animals and accordingly we have identified it in *Hydra* Lfg-4. Motifs 3 and 6 are specific for Lfg-2, -3 and -5 family members in animals and they are not found in any of the *Hydra* Lfgs. In contrast, motif 4, which is specific for animal Lfg-1, -2, -3 and -5 is present in HyLfg-1a and HyLfg-1b within the predicted second intracellular loop. Motif 5 in the fourth intracellular loop is conserved in all animal lifeguard members and, accordingly in all *Hydra* family members. Motifs 7 and 8 are very similar and they are distinguished with respect to their difference in animal Lfg-1, -2, -3 and -5 (motif 8) and Lfg-4 (motif 7). They are present in the *Hydra* proteins with motif 8 in Lfg-1a and-1b and motif 7 in Lfg-4. Motifs 9 and 10 belong to the plant Lfgs and have not been identified in animals, thus they were not found in HyLfgs.

In summary, the TMBIM family is highly conserved and in animals appears to be divided in BI-1, Lfg-4 and Lfg-1, 2, 3 and 5 groups, whereby *Hydra* and other earlier derived animals only have BI-1, Lfg-4 and genes related to Lfg-1. The originally assigned BI-1 motif (PS01243) in *Hydra* is specific for BI-1 like family members (see also (Hu *et al.*, 2009). Lfg-family members possess a number of highly conserved motifs that are not present in BI-1 with a specific signature in different Lfg-proteins between animals and plants and between Lfg-4 and animal specific Lfg-1, -2, -3 and -5 proteins. As we show here, specific sequence motifs for animal Lfg-1 related proteins are present in *Hydra* and therefore have evolved before the divergence of bilaterians.

Subcellular localisation of HyBI-1 and HyLfg proteins

In order to get an idea about the function of *Hydra*-TMBIM fam-

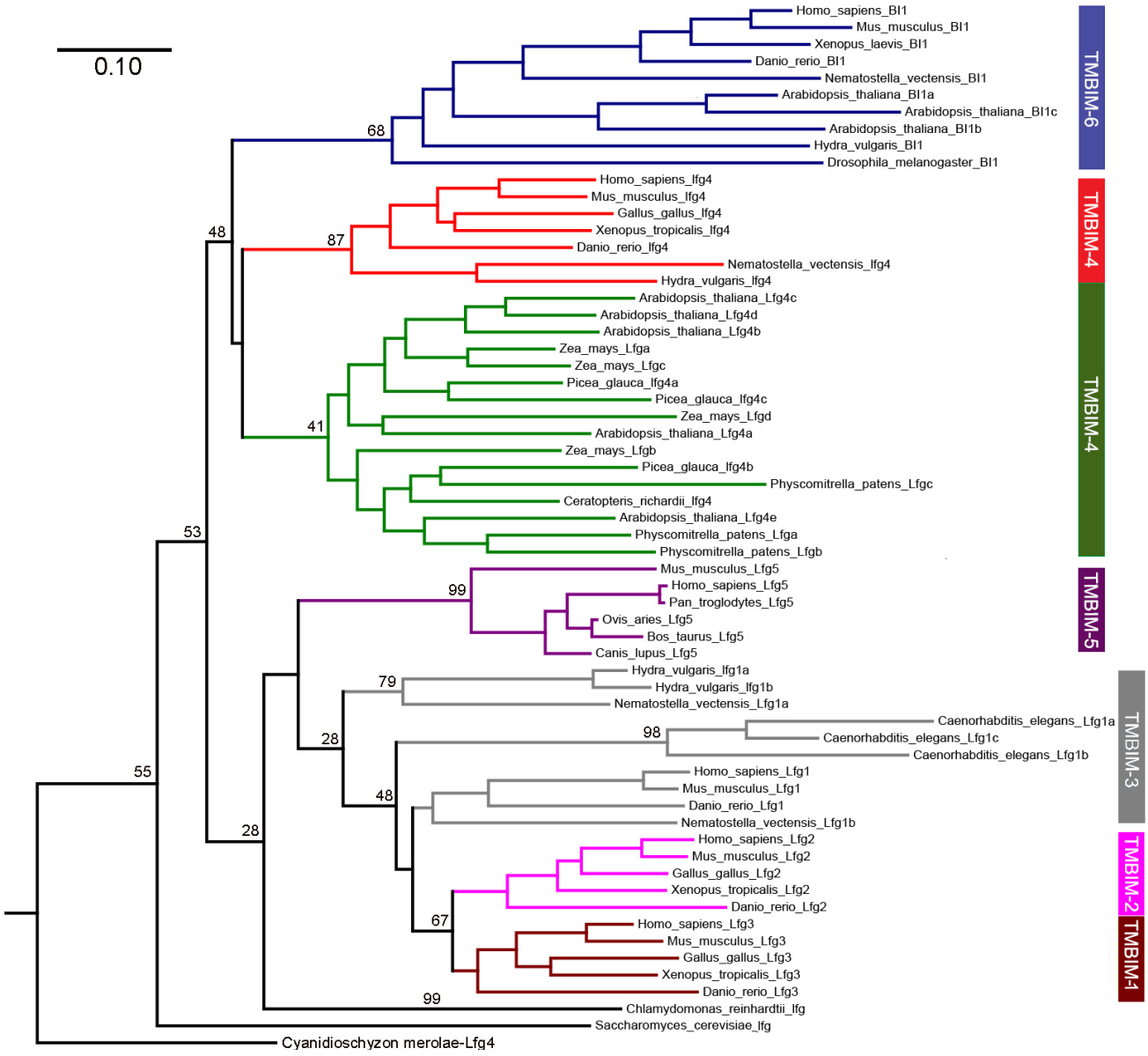

Fig. 4. Phylogenetic tree representing BI-1 and Lfg-genes from plant, fungi, algae and animal species; *BI-1 with* **blue** *lines, Lfg-4,* **red** *lines, plant Lfgs* **green** *lines, Lfg-5* **lila** *lines, Lfg-1* **grey** *lines, Lfg-2* **magenta** *lines, Lfg-3,* **brown** *lines. The TMBIM family annotation related to the Lfgs and BI-1 member indicated on the right side. Bootstrap values from 10000 iterations. Nucleotide accession number list:* Hydra vulgaris *Lfg-4: XM_002162893.3;* Hydra vulgaris *Lfg1b: XM_012709043.1;* Hydra vulgaris *Lfg1a: XM_012704850.1;* Hydra vulgaris *BI-1: XM_002159829.3;* Nematostella vectensis *Lfg-4: XM_001640172.1;* Nematostella vectensis *Lfg1b: XM_001641388.1;* Nematostella vectensis *Lfg1a: XM_001629587.1;* Nematostella vectensis *BI-1: XM_001637805.1;* Xenopus tropicalis *Lfg-4: NM_001004879.1;* Xenopus tropicalis *Lfg3: NM_001030497.1;* Xenopus tropicalis *Lfg2: NM_001078889.1;* Xenopus tropicalis *Lfg1: NM_001016038.2;* Pan troglodytes *Lfg5: XM_016957765.1;* Arabidopsis Thaliana *Lfg-4a: NM_100189.2;* Arabidopsis Thaliana *Lfg-4b: AY735633.2;* Arabidopsis Thaliana *Lfg-4c: NM_116196.2;* Arabidopsis Thaliana *Lfg-4d: NM_117558.2;* Arabidopsis Thaliana *Lfg-4e: NM_117636.2;* Arabidopsis Thaliana *BI-1a: NM_117865.1;* Arabidopsis Thaliana *BI-1b: NM_124084.1;* Arabidopsis Thaliana *BI-1c: NM_117636.2;* Bos taurus *Lfg5: NM_001077068.1;* Homo sapiens *Lfg5: NM_001317905.1;* Homo sapiens *Lfg-4: NM_016056.3;* Homo sapiens *Lfg3: NM_001321429.1;* Homo sapiens *Lfg2: XM_017019040.1;* Homo sapiens *Lfg1: NM_001009184.1;* Homo sapiens *BI-1: AY736129.1;* Ceratopteris richardii *Lfg-4: CV734669;* Danio rerio *Lfg-4: BC057432.1;* Danio rerio *Lfg3: BC083414.1;* Danio rerio *Lfg2: NM_001013518.1;* Danio rerio *Lfg1: BC053253.1;* Danio rerio *BI-1: XM_009305712.2;* Chlamydomonas reinhardtii *Lfg: DS496110.1;* Physcomitrella patens *Lfga: XM_001756985.1;* Physcomitrella patens *Lfgb: XM_001768570.1;* Physcomitrella patens *Lfgc: XM_001757644.1;* Mus musculus *Lfg5: NM_029141.4;* Mus musculus *Lfg-4: NM_026617.3;* Mus musculus *Lfg3: NM_027154.5;* Mus musculus *Lfg2: NM_028224.4;* Mus musculus *Lfg1: XM_006521251.3;* Mus musculus *BI-1: NM_026669.4;* Cyanidioschyzon merolae *Lfg-4: AP006486 chromosome 4: 54288-55103;* Drosophila melanogaster *BI1: NM_139948.4;* Zea mays *Lfga: BT088363.1;* Zea mays *Lfgb: NM_001156405.2;* Zea mays *Lfgc: NM_001137112.1;* Zea mays *Lfgd: NM_001321409.1;* Canis lupus *Lfg5: XM_847212.4;* Xenopus laevis *BI-1: NM_001087329.1;* Picea glauca *Lfg-4a: EX444921.1;* Picea glauca *Lfg-4b: EX425899.1;* Picea glauca *Lfg-4c: EX375038.1;* Gallus gallus *Lfg-4: XP_001235093.1;* Gallus gallus *Lfg3: XM_422067.4;* Gallus gallus *Lfg2: XM_424507.3;* Caenorhabditis elegans *Lfg-4: NM_077142.5;* Caenorhabditis elegans *Lfg1a: NM_068949.3;* Caenorhabditis elegans *Lfg1b: NM_073099.5;* Saccharomyces cerevisiae *Lfg: NM_001183143.1* Ovis aries *Lfg5: XM_012176315.2.*

Fig. 5. Alignments of BI-1 and Lfg sequences from Hydra and human. (A) *Clustal alignment of HyBI-1 and human BI-1 protein sequences; Transmembrane domains from TMHMM predictions are underlayed in gray. Transmembrane domains (TM) and loop regions (Loop) are numbered and indicated above the sequences. The predicted semi-hydrophobic helices are in* italics *with arginine residues in* **bold** *and the C-terminal basic domain is illustrated in green. The consensus amino acids of the Bax-Inhibitor motif including TMs 3 and 4 and the loop 3 sequences are labelled red.* **(B)** *Clustal alignment of HyLfg-1a and b, HyLfg-4 and human Lfg-4 (isoform b). Transmembrane domains from TMHMM predictions are underlayed in gray. Transmembrane domains (TM) and loop regions (loop) are numbered and indicated above the sequences. Conserved motifs in Lfg-proteins according to (Hu* et al., *2009) are indicated by blue, red, orange and green letters.*

ily members we investigated the subcellular localisation of these proteins. We transfected *Hydra* polyps with plasmids encoding GFP-HyBI-1, GFP-HyLfg-4, GFP-HyLfg-1a and GFP-HyLfg-1b proteins under the control of the *Hydra*-actin promoter.

GFP-HyBI-1 was found in a net-like distribution all over the cytoplasm of transfected epithelial cells reminding of an ER-distribution (Fig. 6A). The signal was not co-localised with mitochondrial markers (not shown). As we could not find any specific marker to label the ER in *Hydra* cells we resorted to human HEK cells where we expressed the HyBI-1-protein fused with GFP. Co-staining of transfected cells with anti-Calnexin antibody clearly showed ER-localisation of HyBI-1 in human cells (Fig. 6B).

GFP-HyLfg-1a and GFP-HyLfg-1b were found localised at the plasma membrane and in vesicle-like structures in the cytoplasm of *Hydra* epithelial cells (Fig. 7 A,B). GFP-HyLfg-4 was found localised in globular structures of *Hydra* epithelial cells but not on the plasma membrane. The Golgi- and plasma membrane marker BODYPY-TR ceramide strongly stained plasma membranes of living *Hydra* cells. Golgi vesicle staining appeared weaker. However, comparison of GFP-signals of Lfg-1a, Lfg-1b and Lfg-4 with the Golgi-and plasma membrane marker BODIPY-TR ceramide clearly revealed localisation of all three GFP-fusion proteins with these Golgi-structures and in addition co-localisation of HyLfg-1a and 1b with plasma membranes (Fig. 7 A,B,C).

HyBI-1 protects human cells from apoptosis

The ER-localisation of HyBI-1 as well as the high sequence conservation extending into the C-terminal domain prompted us to ask whether the HyBI-1 protein was involved in mechanisms of programmed cell death. Therefore, we introduced GFP-HyBI-1 protein into HEK-cells by transfecting the respective plasmid and counted the percentage of apoptotic cells in relation to transfected cells that showed a GFP-signal (Fig. 8A). In the absence of the DNA-damaging topoisomerase inhibitor camptothecin there are few apoptotic cells. When camptothecin is added, apoptotic cells appear. They are recognised by shrunken nuclei with condensed chromatin. This is illustrated in Fig. 8A by magnifications of a non-apoptotic nucleus (red asterisk) in comparison with an apoptotic nucleus (white asterisk) in the camptothecin treated GFP control cells. Quantification of apoptotic nuclei found in GFP-positive cells revealed that GFP-HyBI-1 did not induce apoptosis above the level found in control cells. In contrast, HyBI-1 protected cells from camptothecin induced apoptosis.

The percentage of apoptotic cells 24 hours after application of camptothecin was much lower in GFP-HyBI-1 expressing cells in comparison with GFP-expressing control cells (Fig. 8B).

HyBI-1 and HyLfg expression levels

We next estimated the expression levels of TMBIM-genes by RT-qPCR (Fig. 9). This clearly indicated that *HyBI-1* is expressed at the highest level, followed by *HyLfg-4* with an eight times lower expression level. We tested several apoptosis inducing conditions, including benzamidine, camptothecin, UV light, Wortmannin and starvation for their effect on the transcriptional level of these genes. None of these stimuli changed gene expression significantly (not shown).

Expression patterns of TMBIM *genes in comparison with members of* Hy-Bcl-2-like *families*

We next analysed the expression patterns of the genes for the nine *Hydra* Bcl-2-family members and four *Hydra*-TMBIM-family members. To this end we analysed the recently published single cell transcriptome data available at the Single Cell Portal of Broad Institute (https://portals.broadinstitute.org/single_cell/study/stem-cell-differentiation-trajectories-in-hydra-resolved-at-single-cell-resolution) (Siebert *et al.,* 2018). From these data we created an expression matrix (Fig. S1), which was used to generate a heatmap and perform cluster analysis (Fig. 10).

The heatmap shows high expression of *HyBI-1* in all cell types and especially in both, ectodermal and endodermal epithelial stem cells and epithelial and battery cells with incorporated nematocytes. *HyLfg4* is expressed to a lesser extent but also most strongly in the epithelia of both layers. Notably, *HyLfg-4*-expression is absent from the male germ line and some specific neuronal cells (nc1, nc2, nc3). In contrast, *HyLfg-4* is expressed strongly in the female germ line and in gland cells. In accordance with RT-qPCR, *HyLfg-1a* and *b* are expressed at much lower levels. *HyLfg1b* expression is restricted to both epithelial lineages and strongest in ectodermal battery cells. *HyLfg1a* is only expressed in early differentiating nematoblasts just after their exit from mitosis when they have ceased to express PCNA (nb3 and nb4).

In the Bcl-2 family cell type preferences of gene expression are also observed. The most ubiquitarian expressed gene is *HyBcl-2-like 4*. Similar

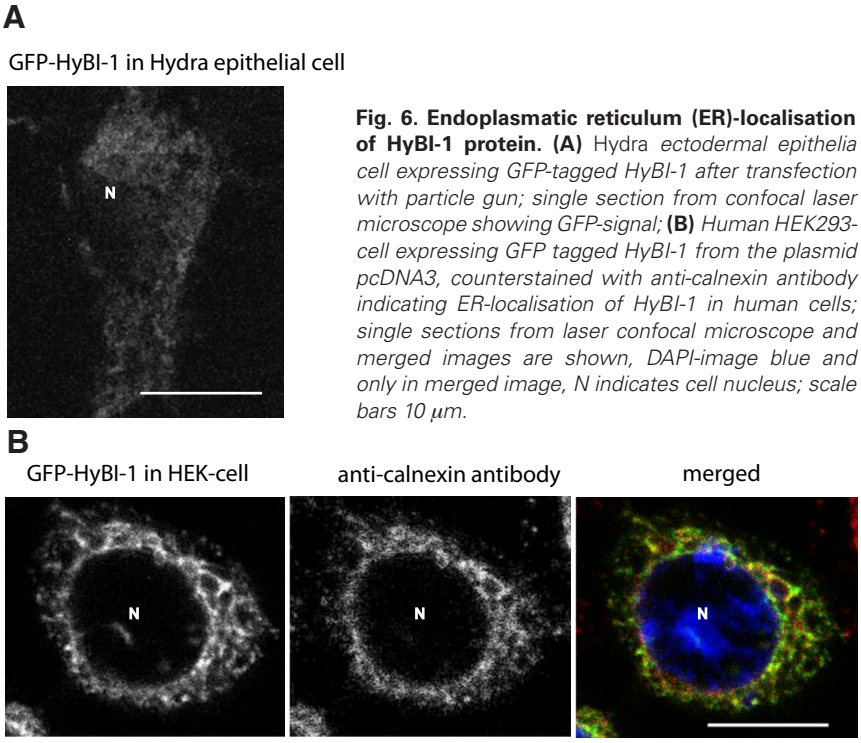

Fig. 6. Endoplasmatic reticulum (ER)-localisation of HyBI-1 protein. (A) Hydra *ectodermal epithelia cell expressing GFP-tagged HyBI-1 after transfection with particle gun; single section from confocal laser microscope showing GFP-signal;* **(B)** *Human HEK293-cell expressing GFP tagged HyBI-1 from the plasmid pcDNA3, counterstained with anti-calnexin antibody indicating ER-localisation of HyBI-1 in human cells; single sections from laser confocal microscope and merged images are shown, DAPI-image blue and only in merged image, N indicates cell nucleus; scale bars 10 μm.*

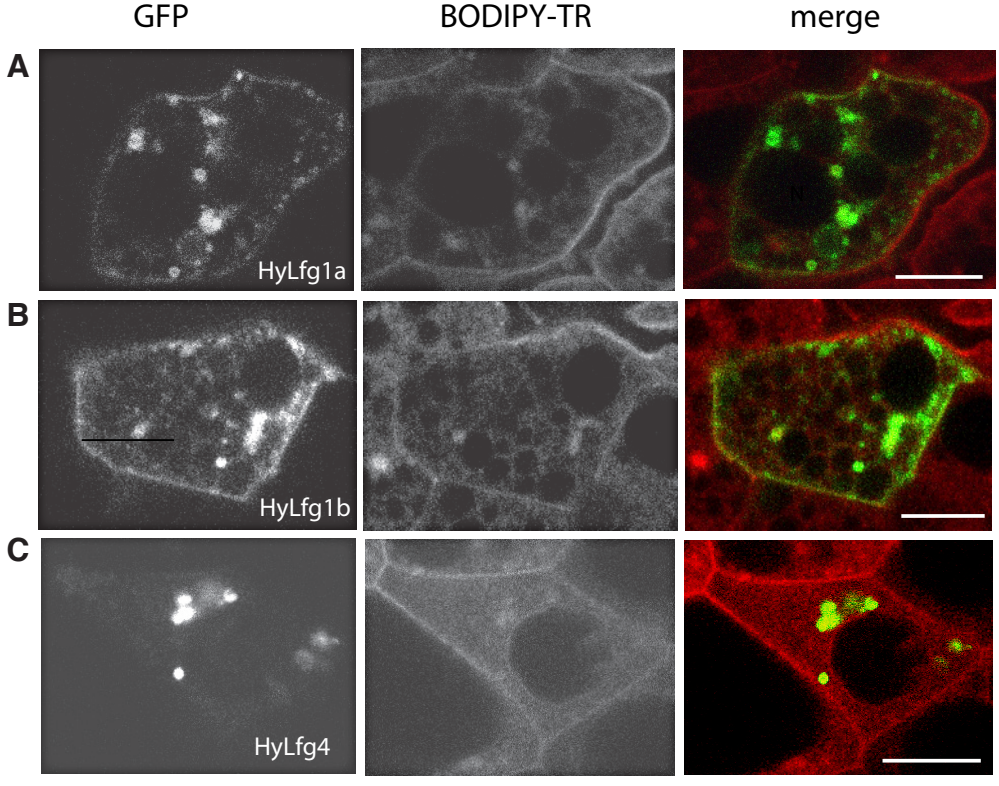

Fig. 7. Golgi- and membrane localisation of HyLfg-proteins. (A-C) *Confocal microscopic single sections of* Hydra *ectodermal epithelial cells of live animals,* **(A)** *expressing GFP-tagged HyLfg-1a;* **(B)** *expressing GFP tagged HyLfg1b;* **(C)** *expressing GFP-tagged HyLfg-4; all are counterstained with BODIPY-TR ceramide to label membranes and Golgi vesicle (middle panels). Merged images in right hand panels, N indicates nuclei; scale bars, 10 μm.*

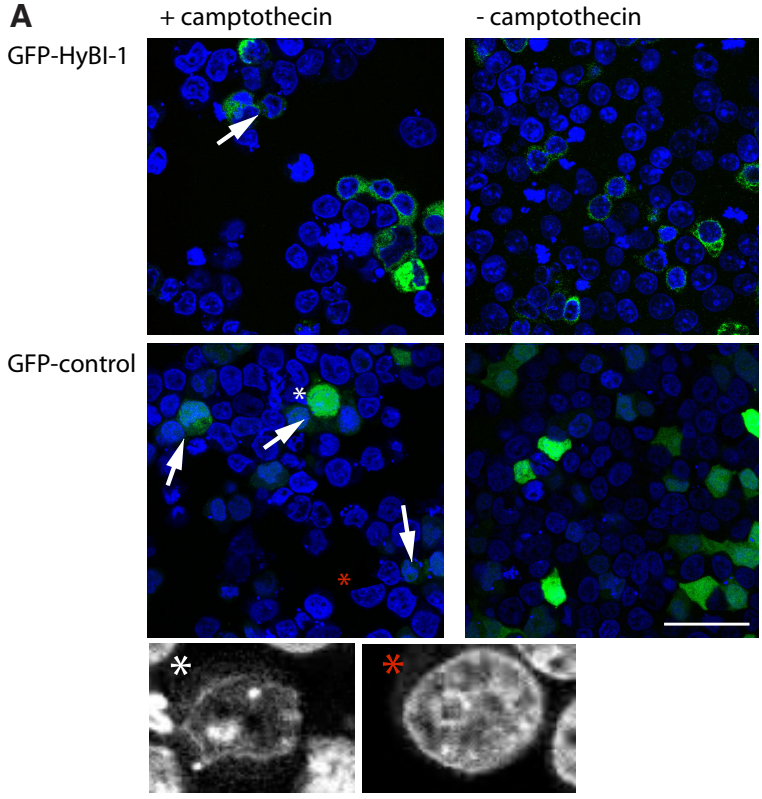

Fig. 8. HyBI-1 protects from camptothecin induced apoptosis. (A) *Confocal microscopic single sections of HEK-cells after transfection with plasmid encoding GFP-HyBI and GFP control. Arrows point out apoptotic cells. To obtain the data shown in (B) apoptotic nuclei were distinguished from non-apoptotic nuclei by visual inspection. Red asterisk shows normal cell nucleus, while white asterisk shows apoptotic nucleus; scale bar 50 μm; enlargements of DAPI stained nuclei with red and white asterisks are provided.* **(B)** *Results of apoptosis assay in HEK-cells; GFP or GFP-tagged HyBI-1 were expressed in HEK-cells. Apoptotic cells as percentage of GFP-positive cells were counted in untreated cells (-camptothecin) and in cells treated with camptothecin (+camptothecin).*

to *HyBI-1* it is expressed in all cell types. Interestingly, its expression is stronger in one type of nerve cells (nc4). The pro-apoptotic *HyBak-like 1* is expressed in all epithelial cells and in female germ cells, these are the same germ cells where the second *HyBak-like* gene *(HyBak-like 2)* is present, while it is not expressed in any other cell type. *HyBcl-2-like*6 is strongest in endodermal epithelial cells and absent from the I-cell lineage. In contrast, *HyBcl-2-like 1/3* is most prominent in nerve cells and gland cells, but it is absent from their precursors and less expressed in epithelial cells. *HydraBcl-2-like 2* is very strongly expressed in female germ cells.

Discussion

The molecular mechanisms that regulate and execute programmed cell death differ between animals, fungi and plants. However, some protein families involved in regulating this process are conserved throughout the whole tree of life. This includes the TMBIM family of membrane proteins. On the other hand, pro- and anti-apoptotic members of the Bcl-2 family are only conserved in animals. Already in cnidarians, one of the very first derived metazoan phyla, they form a large family. This is all the more striking when we consider that cell death in the nematode *Caenorhabditis elegans* only requires one *Bcl-2* related gene, *ced-9*. It was therefore very important to investigate whether members of the elaborated Bcl-2 family in *Hydra* perform similar functions as in mammals. Here we provide experimental evidence for HyBcl-2-like 4 to act as an inhibitor of apoptosis in *Hydra* epithelial cells. This was evident when we induced apoptosis with the PI(3)-kinase inhibitor Wortmannin in transgenic animals that overexpressed GFP-Bcl-2-like 4. Starvation induced apoptosis was also blocked in these animals. It should be noted that our analysis, due to the impossibility to recognise GFP-signals after maceration, included all epithelial cells independently of their expression of GFP-HyBcl-2-like 4. Therefore, the protective effect of GFP-HyBcl-2-like 4 was rather under- than overestimated.

The observed decrease in apoptosis in HyBcl-2-like 4ecto animals did not lead to higher numbers of buds during the starving period. It is possible that this was because HyBcl-2-like 2 was only overexpressed in ectodermal cells and not in endodermal or interstitial cells. On the other hand, the regulatory circuit between cell

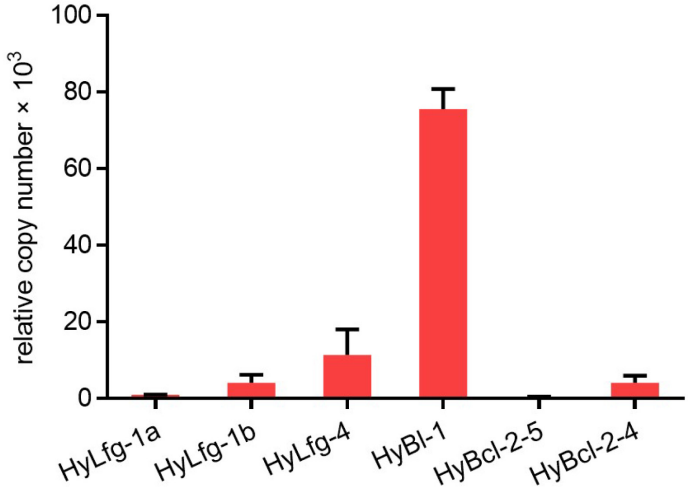

Fig. 9. Relative expression levels of Bcl-2- andTMBIM-family members in *Hydra* *as determined by quantitative RT-qPCR. Relative copy number is an arbitrary unit.*

death, cell numbers, animal size and budding (reproduction) should be investigated further on the level of signalling. It is known that extensively fed *Hydra* polyps can grow much larger than normally fed ones and then both, budding rate and animal size increase. It remains speculative at the moment whether HyBcl-2-like 4 and possibly other members of the *Hydra* Bcl-2 family are involved in this regulation. What we can conclude from our data is that HyBcl-2-like 4 as a mitochondrial protein in *Hydra* fulfils a similar function in protecting cells from apoptosis as it does in mammals.

We then investigated the TMBIM protein family in *Hydra*. Some members of this family had been implicated in cytoprotection in plants and in animals. Phylogenetic analysis revealed three groups comprising Lfg-1 derived proteins, Lfg-4 related proteins and Bax-Inhibitor related proteins. The *Hydra* sequences fit very clearly into these groups.

Lfg-1 is conserved from plants to humans. However, in mammals it splits into four different groups, Lfg-1, 2, 3 and 5. Invertebrate Lfg-1 proteins in *Hydra* and *Caenorhabditis elegans* also have two Lfg-1 homologs each. In a recent study it was shown that human BI-1 and TMBIM 4 and 5 (corresponding to Lfg-4 and-5) were localised in the ER, whereby TMBIM 5 could also be sorted to mitochondria. In contrast, TMBIMs 1-3, including Lfg-1 were found associated with the Golgi (Lisak *et al.,* 2015). Localisation studies by other authors had indicated similar subcellular distributions of TMBIM family proteins, except for Lfg-4, which was found associated with Golgi membranes in human cells and it was therefore called Golgi-associated anti apoptotic protein (GAAP), reviewed in (Carrara *et al.*, 2017). In comparison with *Hydra* and consistent with the studies in human cells, HyBI-1 was localised in the ER and HyLfg-1a and -1b were associated with Golgi-vesicles. HyLfg-4 is found in Golgi vesicles in *Hydra*.

Remarkably, the number of family members in the Bcl-2-family did apparently not gradually increase during evolution but rather we have found that pre-bilaterian phyla already started with extended families. A possible explanation for this could be cell type specificity. By using data from the recent single cell transcriptome analysis by the Juliano lab (UCDavis) (Siebert, 2018), we established the expression patterns for all previously analysed members of the Bcl-2 family in *Hydra* and of TMBIM family members described in this work. Ubiquitarian expression in all cell types was only found

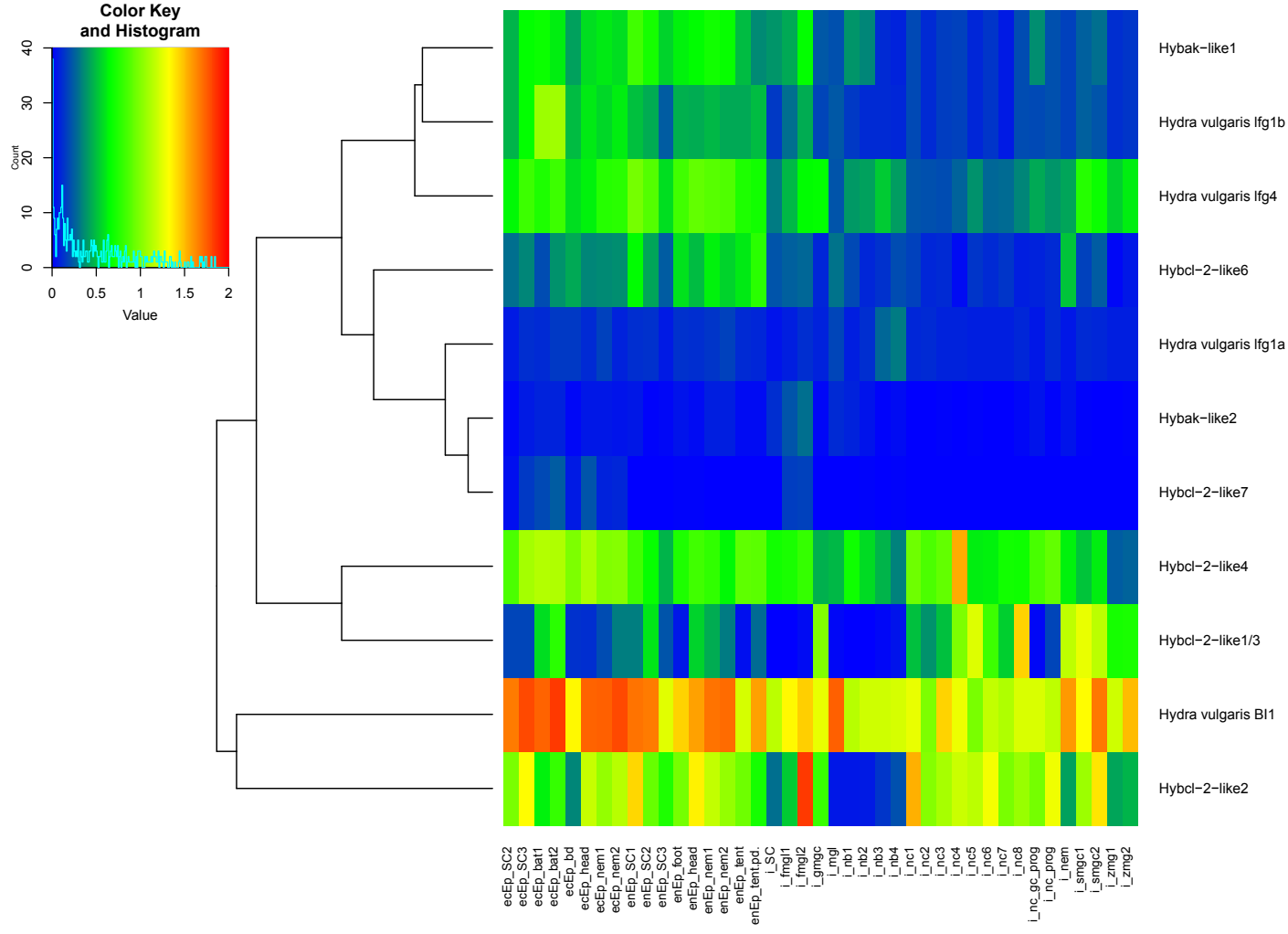

Fig. 10. Cell type specific mRNA-expression of HyBcl-2- and TMBIM-family members *Heat map and cluster analysis from expression matrix on the basis of single cell transcriptome data provided at (https://portals.broadinstitute.org/single_cell/study/stem-cell-differentiation-trajectories-in-hydra-resolved-at-single-cell-resolution) for HyBcl 2- and TMBIM-family genes.*

for *HyBI-1* and *HyBcl-2-like* 4. These genes might have more general cellular functions. All other members of both gene families were expressed at lower levels and not in all cell types. However, some expression patterns can be correlated with apoptotic events in *Hydra*. For instance, high numbers of apoptotic cells are found in the tentacles of the polyps, where cells are continuously dying and this correlates with high expression of *HyBcl-2-like 2, 6, 7*, *HyBak-like 1*, *HyLfg1b, 4* and *HyBI-1* in battery cells. Moreover, a kind of arrested apoptosis occurs during oogenesis (Alexandrova *et al*., 2005, Technau *et al*., 2003). A pronounced expression peak in female germ cells was found for *HyBcl-2-like 2*, *HyBak-like 1* and 2 and *HyLfg-4*. Apart from this a clear association of the expression patterns with specific cell types is not observed. Therefore, the functions of the individual family members might be more complex and it is possible that they have specific cellular tasks outside the regulation of apoptosis.

Conclusion

Here we have shown that anti-apoptotic Bcl-2 family proteins and proteins of the TMBIM-family represent mitochondrial, ER-, Golgi and plasma membrane proteins that fulfil cytoprotective functions in *Hydra.* Therefore, by trying to understand the evolution of molecular mechanisms of programmed cell death the focus on mitochondria could be extended and other membrane bound cellular compartments should also be taken into consideration.

Materials and Methods

Hydra culture

Hydra vulgaris strain Basel was cultured at 18 °C in *Hydra* medium (0.1 mM KCl, 1 mM NaCl, 0.1 mM $MgSO_4$, 1 mM Tris and 1 mM $CaCl_2$). The animals were fed regularly with freshly hatched *Artemia nauplii* from Sanders Brine Shrimp Company.

Gene cloning

A search of whole genome and EST (Expressed sequence tag) sequences from *Hydra* (http://hydrazome.metazome.net/cgi-bin/gbrowse/hydra/) revealed three sequences with homology to human TMBIM-family members. Through Blast analysis, *HyLfg-4* (XM_002162893.1) and two Lfg-1 homologues were found in *Hydra*. They are named *HyLfg-1a* (Hma2.214458) and *HyLfg-1b* (Hma2.205245) in this study. Based on gene models for each homolog of Lifeguard in *Hydra*, pairs of primers were designed and used for PCR amplification from *Hydra* cDNA and cloning of the respective sequences into the HoTG expression vector using the SmaI restriction site (Böttger *et al*., 2002). Another member of the TMBIM family, *HyBI-1*, had previously been identified in the *Hydra* genome (Hma1.130444) (Lasi *et al*., 2010b).

Transfection of Hydra *cells and transgenic animals*

Gold particles (1.0 μm, BioRad) were coated with plasmid DNA and introduced into *Hydra* cells with the Helios gene gun system (BioRad) as previously described (Böttger *et al*., 2002). For transgenic animals HoTG plasmids with the *Hydra* actin promoter encoding GFP tagged HyBcl-2-like 4 and mitochondrially targeted GFP (mitochondrial targeting sequence of *Hydra*AIF in frame with GFP, described in ((Müller-Taubenberger *et al*., 2006) were injected into fertilised eggs of *Hydra* of the AEP strain (Wittlieb *et al*., 2006). After development of the embryos, we obtained two lines of transgenic animals, HyBcl-2-like 4ecto and mitoGFPecto. Transgenic animals were kept in the lab for several years without any obvious effect on their fitness or morphology. However, the expression of GFP-HyBcl-2-like 4, as judged by the strength of GFP-fluorescence, became weaker with time. Therefore, animals with strong GFP-signals in as many cells as possible were chosen, grown up under permanent selection for strong fluorescence and then used for the experiments described in this work. To confirm the presence of plasmid DNA in the respective transgenic strains we amplified these sequences from genomic DNA using primers annealing with the HoTG-vector sequence (5'-primer: CACCATCTAATTCAACAAGAATTG; 3'primer: GCGCTCAAGCGATTCACC). Genomic DNA was obtained from polyps treated with lysis buffer (50μl/polyp of 100mM Tris, 10 mM EDTA, 250 mM NaCl, 200μM Proteinase K) for 30 min at 37°C and subsequently at 95°C for 2min. PCR-products were sequenced.

Immunofluorescence of HEK cells

HEK-cells were grown in DMEM, 5 % FCS on cover slips and transfected with plasmid pEGFP:HyBI-1 encoding GFP-HyBI-1. After 24 hrs they were fixed with 4% paraformaldehyde in PBS. They were permeabilised with 1% Triton X100 in PBS and blocked for 30 min with 10 % FCS, 0.2 % Tween in PBS. Anti-calnexin antibody (mouse, Chemicon) was applied in PBS for 1 hour. Cells were washed with 1% bovine serum albumin (BSA), 0.2 % Tween 20 in PBS before Cy5 anti-mouse antibody (Dianova) was applied for 1 hour. After washing, cells were mounted with Vectashield before imaging.

Hydra *labelling with BODYPY-TR-ceramide and MitotrackerRedCMXRos*

BODIPY-TR ceramide and MitotrackerRed CMXRos (InVitrogen)) labelled animals were imaged alive. 1 μl of 500 μM BODIPY-TR were injected into the gastric cavity of *Hydra* polyps. They were then incubated in 5μM BODIPY-TR in *Hydra* medium for 30 min, relaxed in 2% urethan and imaged on slides with wax-footed coverslips. For MitotrackerRedCMXRos-staining animals were incubated with 750 nM Mitotracker in *Hydra* medium for 15 min before relaxation and imaging.

Confocal laser scanning microscopy

Leica SP5-2 confocal laser-scanning microscope was used for Light optical serial sections. (Plan-Apochromat 100/1.4 NA objective lens). EGFP, FITC, Alexa488 were visualized with an argon laser at 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 at 415 – 465 nm was used for DAPI. Cy3, MitotrackerRedCMXRos and BODIPY-TR were visualized using a Krypton laser excited at a wavelength of 561nm and emission filter at 604-664 nm. Image resolution was 512 × 512 pixel with a pixel size ranging from 195 to 49 nm depending on the selected zoom factor. The axial distance between optical sections was 300 nm. Each section image was averaged from three successive scans. The 8-bit grey scale single channel images were overlaid to an RGB image assigning a false colour to each channel and then assembled into tables using Adobe Photoshop 8.0.

Phylogenetic tree

In addition to the cloned sequences from *Hydra*, Blast analysis at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) retrieved genes encoding TMBIM-family members from vertebrates, plants, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Nematostella vectensis*. All sequences were aligned using Muscle 3.6 (Edgar, 2004) in Geneious R8 (Biomatters, (Kearse *et al*., 2012))under default settings. The phylogenetic tree was created based on Maximum likelihood tree (ML). Reconstructions were performed using PhyML as incorporated in SeaView Version 4 (Gouy *et al*., 2010) under the GTR-model of substitution. 10,000 bootstrap replications were used. Genious.8 phylogeny software was used for tree drawing.

Apoptosis assay in Hydra

Regularly fed animals (Mo, Wed, Fr) were kept aside from the main culture for 2 days, and then incubated with Wortmannin (1167 nM, 116.7 nM, 11.67 nM, 0 nM – each in 1% DMSO) for 4.5 hours at 18 °C. Afterwards, the animals were macerated with 270 μl maceration solution (glycerol, glacial acetic acid, H_2O – 1:1:13 – v/v/v) for 1 hour (RT), incubated for

10 minutes in an equal volume of 8% PFA (paraformaldehyde) in PBS (phosphate buffered saline), RT. The cell suspension was transferred with 50µl 0.1% Tween onto a defined area on a microscope slide, and dried for one hour. The dried slide stained with DAPI [1µg/µL], mounted with Vectashield and sealed (nail polish). Under the fluorescence microscope, 1000 epithelial cells were counted per time point and concentration, and the number of apoptotic cells was determined on the basis of the apoptotic morphology of the DAPI-stained epithelial cell nuclei as shown in Fig. 2C. Apoptotic interstitial cells and phagocytosed apoptotic cells that were found in vacuoles of mostly endodermal cells were not counted. Results are from four biological replicates.

Starvation protocol

Regularly fed animals (Mo, Wed, Fr) were fed again on two consecutive days (Mo, Tue). From then on, they were either starved for the following seven days or fed daily. They were then macerated (see above) at indicated time points and analysed. The experiment was carried out with 10 animals in each group and for each time point. Before maceration buds were counted. Since there was no difference in the budding rate bud counts are only shown for one experiment.

Statistical analysis

The statistical analysis was implemented using the program SigmaPlot 11.0, a Two-Way ANOVA (Tukey test) was applied to the Wortmannin treated *Hydra* (Fig. 2A), a One-Way ANOVA (Tukey test) was applied to the starved *Hydra* (Fig. 2B). The significance level α was set to 0.05, which means that p-values ≤ 0.05 were assumed statistically significant. Letters above the columns express, whether two values are significantly different according to this p-value. These letters allow statistic evaluation of differences in all conditions, including the chosen *Hydra* strains and the particular treatments. For columns with the same letters, the values are not statistically different, columns with different letters display statistically significant differences with p-values ≤ 0.05.

Apoptosis assay in HEK293T cells

Apoptosis assays in HEK293T-cells were performed as described by Lasi *et al.,* (Lasi *et al*., 2010b). Briefly, HEK293T-cells were transfected with plasmids encoding HyBI-1 or HyLfg-4. After 24 hours apoptosis was induced with 10µM camptothecin. Cells were fixed in 4% paraformaldehyde and labelled with the DNA-dye DAPI. Amongst GFP-positive cells the percentage of apoptotic cells was estimated on the basis of changes in cell and nuclear morphology based on the DAPI-signal showing fragmentation and condensation of chromatin.

Expression studies using quantitative RT-PCR

Total RNA was isolated from *Hydra magnipapillata* using the Qiagen RNeasy® Plus Mini kit. The concentration and quality of the tRNA was determined using Agilent´s 2100 Bioanalyzer. Total RNA with an RNA Integrity Number > 8 was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). The validation experiments were performed using SYBR® Select Master Mix for CFX (Life Technologies) on a CFX96™ thermocycler (Bio-Rad) with white plastics for enhanced detection.

Expression pattern analysis

Sequences of *HyBcl-2-like*-, *TMBIM*-, and *HyBak*-family members were blasted against the AEP transcriptome (Siebert, 2018). For *HyBcl-2-like 5* no AEP reference was found. *HyBcl-2-like 1* and *3* aligned to the same AEP reference. Distribution plots were generated for each sequence using the Single Cell Portal Broad Institute. For this, the cluster wide study of the whole transcriptome clustering was used. The mean expression values were read by hand from the box plots for each cell cluster and an expression matrix was generated and used for the Heatmap (Fig. S1). The heatmap was generated using the gplots::heatmap.2 function in R version 3.4.3 (2017-11-30) with the methods “maximum” and “complete” for the functions dist and hclust respectively.

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AEP reference	ecEp_SC2	ecEp_SC3	ecEp_bat1	ecEp_bat2	ecEp_bd	ecEp_head	ecEp_nem1	ecEp_nem2	enEp_SC1	enEp_SC2	enEp_SC3	enEp_foot	enEp_head	enEp_nem1	enEp_nem2	enEp_tent	enEp_tent(pd)	i_SC	i_fmgl1	i_fmgl2	i_gmgc	i_mgl	i_nb1	i_nb2	i_nb3	i_nb4	i_nc1	i_nc2	i_nc3	i_nc4	i_nc5	i_nc6	i_nc7	i_nc8	i_nc_gc_prog	i_nc_prog	i_nem	i_smgc1	i_smgc2	i_zmg1	i_zmg2
Hydra vulgartis2 0B2l181aep	1.68	1.81	1.74	1.85	1.35	1.74	1.75	1.81	1.7	1.68	1.26	1.45	1.58	1.7	1.72	1.23	1.58	1.16	1.35	1.46	1.28	1.75	1.15	1.21	1.21	1.23	1.28	1	1.45	1.29	0.99	1.18	1.13	1.24	1.24	1.17	1.6	1.34	1.69	1.21	1.51
Hydra vulgartis9 4lf3g71aaep	0.08	0.13	0.12	0.16	0.16	0.12	0.18	0.1	0.13	0.14	0.08	0.14	0.13	0.15	0.18	0.12	0.11	0.06	0.09	0.13	0.1	0.19	0.09	0.13	0.28	0.33	0.11	0.12	0.1	0.1	0.09	0.09	0.09	0.11	0.07	0.12	0.07	0.12	0.11	0.09	0.09
Hydra vulgartis6 8lf2g71abep	0.48	0.68	1.08	1.09	0.5	0.62	0.57	0.63	0.51	0.46	0.25	0.47	0.46	0.49	0.45	0.45	0.5	0.16	0.3	0.46	0.19	0.24	0.16	0.12	0.12	0.11	0.2	0.12	0.17	0.18	0.11	0.15	0.08	0.21	0.2	0.22	0.18	0.26	0.23	0.13	0.15
Hydra vulgartis1 4lf6g044aep	0.66	0.83	0.75	0.83	0.59	0.64	0.77	0.78	0.98	0.89	0.59	0.84	0.93	0.9	0.88	0.75	0.72	0.33	0.49	0.67	0.68	0.23	0.41	0.43	0.54	0.42	0.23	0.22	0.21	0.27	0.39	0.28	0.29	0.36	0.45	0.4	0.44	0.77	0.7	0.55	0.63
Hybak-like1 t22354aep	0.48	0.68	0.7	0.63	0.43	0.59	0.62	0.59	0.84	0.73	0.56	0.64	0.67	0.72	0.69	0.5	0.37	0.4	0.45	0.68	0.24	0.22	0.4	0.36	0.12	0.1	0.2	0.12	0.17	0.18	0.11	0.15	0.08	0.21	0.38	0.22	0.18	0.26	0.3	0.13	0.15
Hybak-like2 t12101aep	0.03	0.08	0.1	0.1	0.03	0.07	0.07	0.06	0.07	0.02	0.03	0.06	0.04	0.09	0.09	0.04	0.04	0.13	0.23	0.3	0.03	0.12	0.1	0.06	0.02	0.04	0.01	0.01	0.02	0.01	0.02	0.01	0	0.03	0.06	0.02	0.06	0.02	0.01	0.01	0.02
Hybcl-2-like1t/137510aep	0.19	0.19	0.59	0.78	0.14	0.13	0.21	0.34	0.34	0.61	0.23	0.07	0.56	0.46	0.33	0.05	0.29	0	0	0.04	1.01	0.03	0	0.01	0.03	0.06	0.51	0.39	0.51	1	1.25	0.83	0.55	1.45	0.03	0.19	1.17	1.28	1.16	0.73	0.74
Hybcl-2-like-2t14554aep	0.99	1.34	0.64	0.81	0.36	1.19	1.06	1.15	1.44	0.94	0.75	0.98	1.37	1.18	1.06	0.96	0.73	0.3	0.54	1.85	0.82	0.07	0.07	0.08	0.15	0.22	1.54	1.02	1.11	1.2	1.16	1.31	0.99	1.07	0.98	1.3	0.43	1.08	1.4	0.44	0.48
Hybcl-2-like-4t22752aep	0.87	1.1	1.14	1.13	1	1.12	1.01	1.02	0.84	0.69	0.48	0.75	0.84	0.8	0.66	0.92	0.91	0.72	0.74	0.81	0.47	0.49	0.71	0.58	0.48	0.35	0.96	0.87	0.93	1.55	0.63	0.64	0.72	0.71	0.86	0.93	0.64	0.52	0.63	0.25	0.27
Hybcl-2-like-6t14232aep	0.3	0.36	0.21	0.39	0.45	0.36	0.37	0.38	0.67	0.44	0.25	0.6	0.5	0.64	0.56	0.62	0.78	0.23	0.26	0.28	0.11	0.31	0.2	0.11	0.14	0.21	0.18	0.11	0.12	0.04	0.15	0.12	0.15	0.09	0.1	0.12	0.52	0.18	0.25	0.03	0.07
Hybcl-2-like-7t22293aep	0.05	0.16	0.2	0.25	0.08	0.23	0.1	0.11	0	0	0	0.02	0.02	0.01	0.01	0.01	0	0.01	0.17	0.18	0.01	0	0.01	0.02	0.01	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01

Fig. S1. Expression matrix from which heat map in Fig. 10 was created.

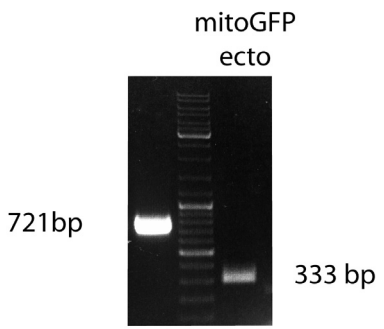

Fig. S2. Agarose gel electrophoresis of PCR-products obtained from genomic DNA of Bcl-2-like 4ecto and mitoGFPecto animals using primers for HoTG-sequences. *Sizes of PCR-products are indicated.*

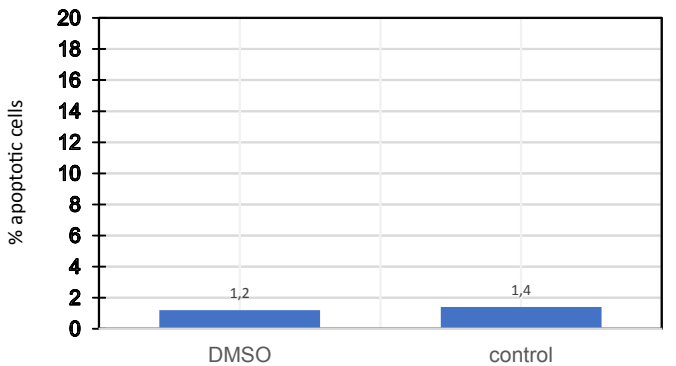

Fig. S3. *Comparison of apoptotic index for epithelial cells in* Hydra *polyps of the AEP-founder strain* **Hydra-AEP wild type** *after 4.5 hrs in* Hydra*-medium with 1% DMSO (DMSO) or without DMSO (control), 1000 epithelial cells from two experiments were counted.*

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

Here we have investigated *Hydra* genes encoding homologs of members of the extrinsic apoptosis induction pathway in vertebrates, including TNF-R, TNF, FADD- and TRAF-family members. We show that HyFADD 1 and 2 form death effector filaments and interact with pro-domains of DED-caspases, but not with the intracellular domain of TNF-R. Immunoprecipitation of HyTNF-R from *Hydra* lysate revealed an interaction with HyTRAF6. Phylogenetic analyses showed that HyTNF-R is closest related to human EDA (ectodysplasin) receptor EDAR, thus belonging to the only TNF-R superfamily member which is not involved in inducing apoptosis. Human EDAR has been functionally related to epithelial cell differentiation, e.g. the formation of hair and tooth cells. Consistent with this, we show that HyTNF-R is expressed very specifically in epithelial cells of the body column which incorporate nematocytes.

Contribution to the paper

I have planned and performed all experiments except the co-staining of hydra whole mounts with anti-HyTNF-R and anti-N-CRD antibodies which was done by Lara Sauermann. I did all bioinformatical analyses (domain structure analyses, phylogenetic trees), gene cloning of *HyFADD1*, *HyFADD2, HyTNF-R, HyTNF, HyTRAF4* and *-6*, cell culture, transfection and immunostaining of HEK cells expressing GFP- respectively HA-tagged HyDD-Caspase, HyDED-Caspase, HyFADD1 and HyFADD2, HyTNF-R, HyTNF, HyTRAF4 and -6, hydra culture, biolistic transformation of hydra cells with *HyFADD1, HyFADD2* and *HyTNF-R* and immunostaining of hydra whole mounts with the anti-HyTNF-R antibody, confocal laser scanning microscopy and immunoprecipitation of endogenous HyTNF-R. Ann-Christine König and Stefanie Hauck supported us with the analysis of the results of the mass spectrometry. I wrote the first manuscript myself and contributed in editing the accepted version of the paper.

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RESEARCH ARTICLE

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

Mona Steichele, Lara S. Sauermann, Ann-Christine König*, Stefanie Hauck* and Angelika Böttger‡

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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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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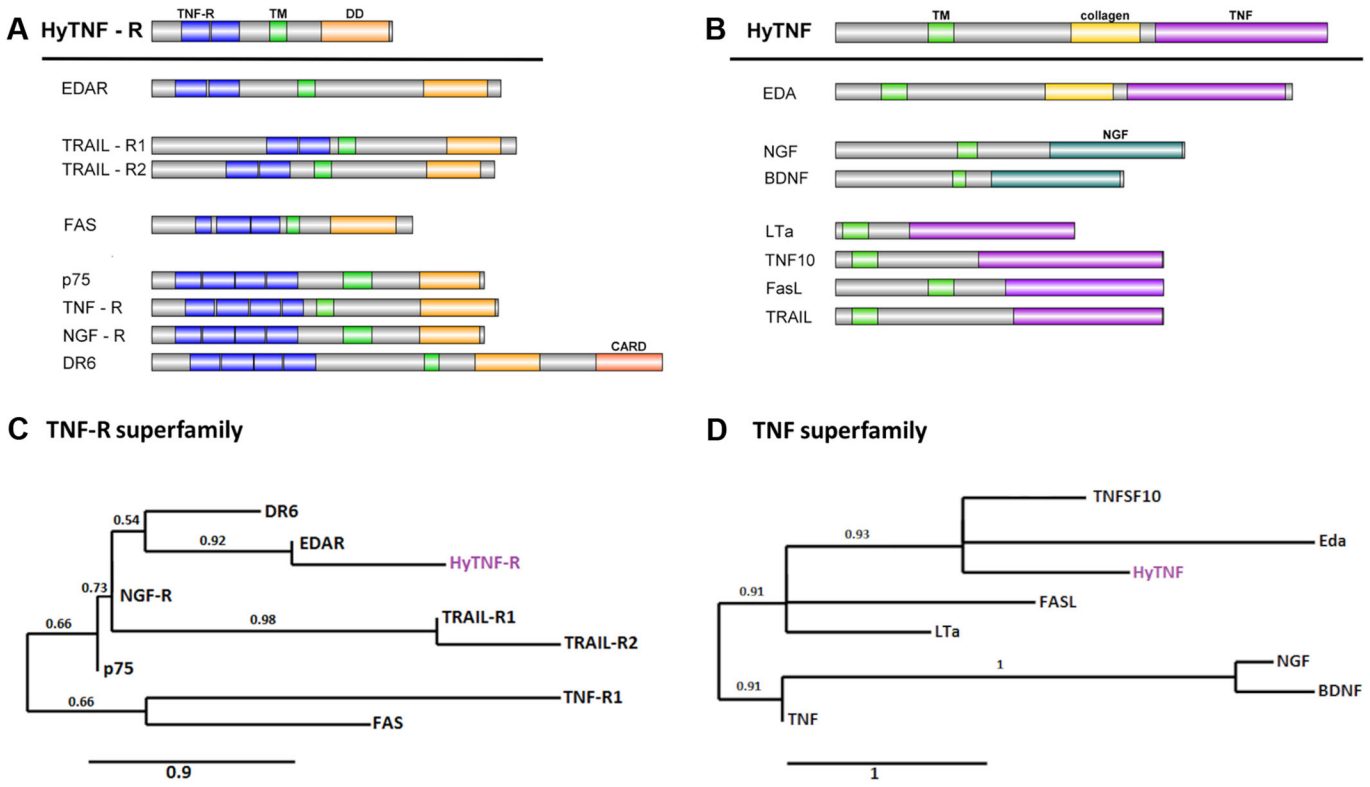

Fig. 1. Comparison of Hydra TNF-R and TNF with human homologues. (A) Comparison of domain structures of human DD-domain-containing TNF-R superfamily members with HyTNF-R. (B) Comparison of domain structures of human TNF superfamily members with HyTNF. TNF-R domains, blue; transmembrane domains (TM), 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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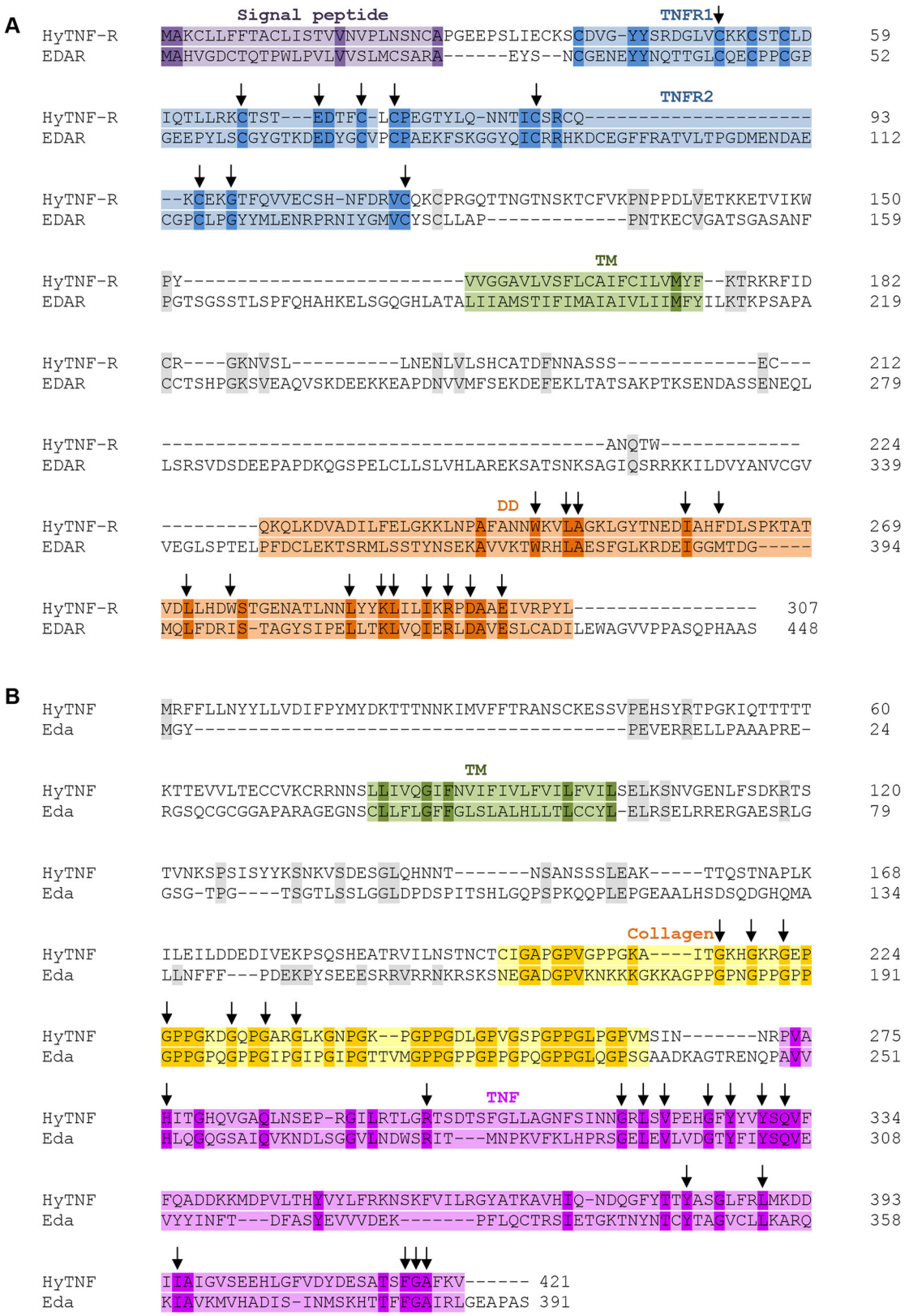

Fig. 2. Sequence alignments of *Hydra* and human TNF-R and TNF sequences. (A) Alignment of HyTNF-R and human EDAR sequences. Signal peptides, purple; TNF-R repeats, blue; transmembrane (TM) domains, green; DD domains, orange; identical amino acids outside conserved domains (grey). Evolutionary conserved 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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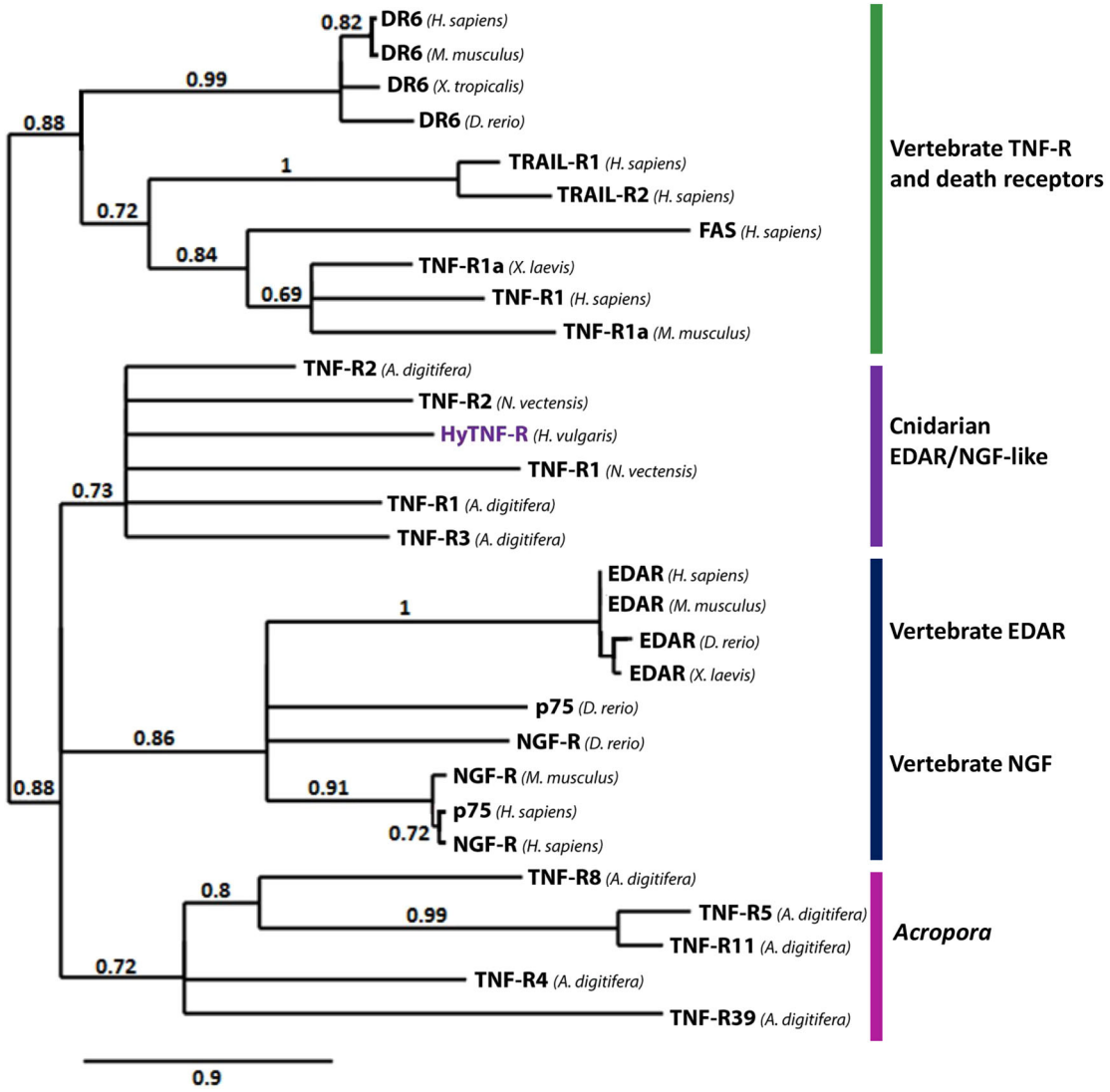

Fig. 3. Phylogenetic tree of the TNF-R superfamily in cnidarian and vertebrate species. A MUSCLE alignment of DD domains of TNF-R superfamily members from *Homo sapiens* (*H. sapiens*), *Mus musculus* (*M. musculus*), *Danio rerio* (*D. rerio*), *Xenopus laevis* (*X. laevis*), *Xenopus tropicalis* (*X. 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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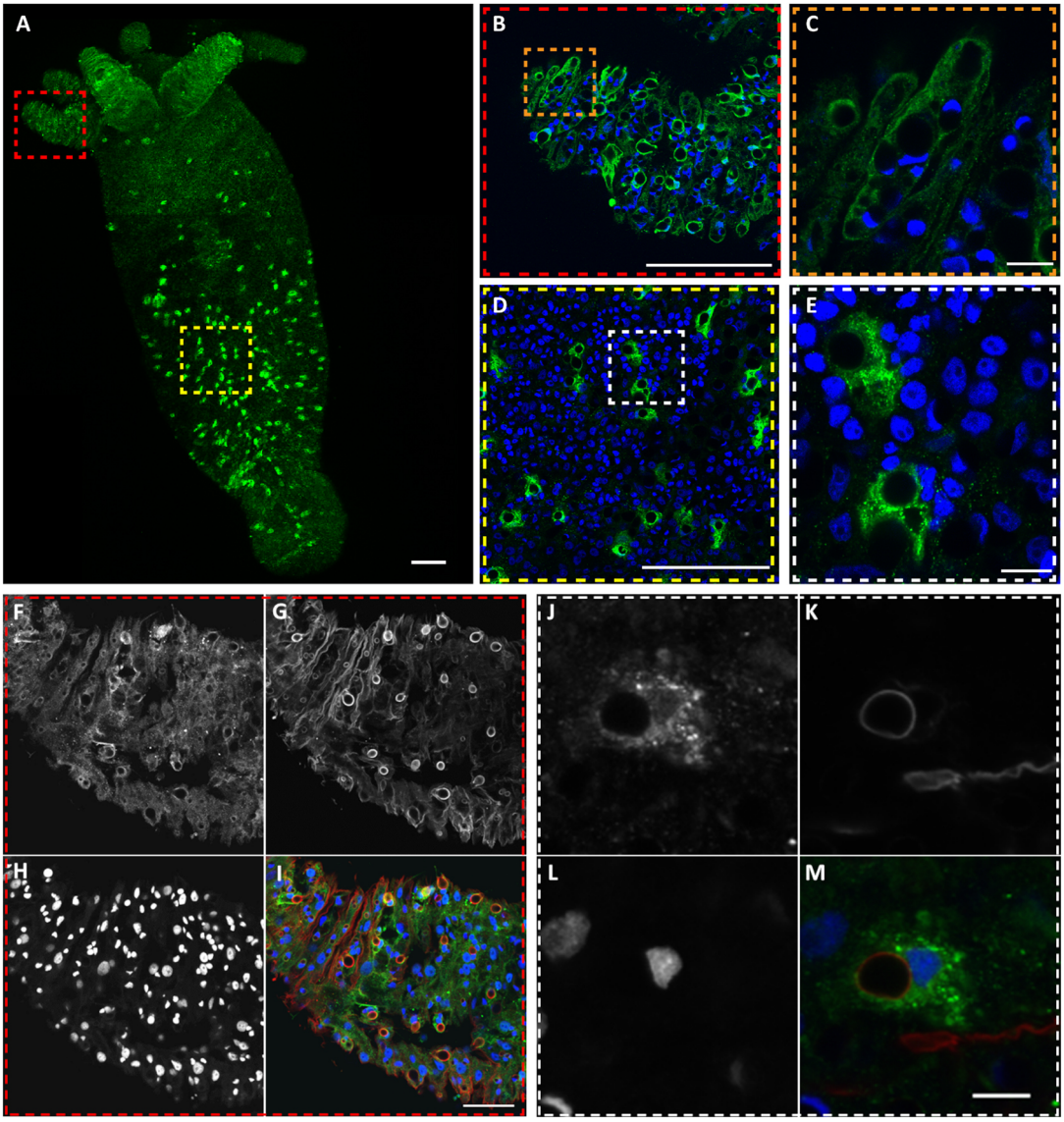

Fig. 4. Localisation of HyTNF-R. Laser confocal microscopy images of *Hydra* whole mounts stained with anti-HyTNF-R antibody (green), DNA-stain DAPI (blue) and anti-minicollagen antibody (N-CRD, Tursch et al., 2016, red). (A) Whole polyp, anti-TNF-R. (B–E) anti-TNF-R and DAPI [(B) tentacle, (C) battery 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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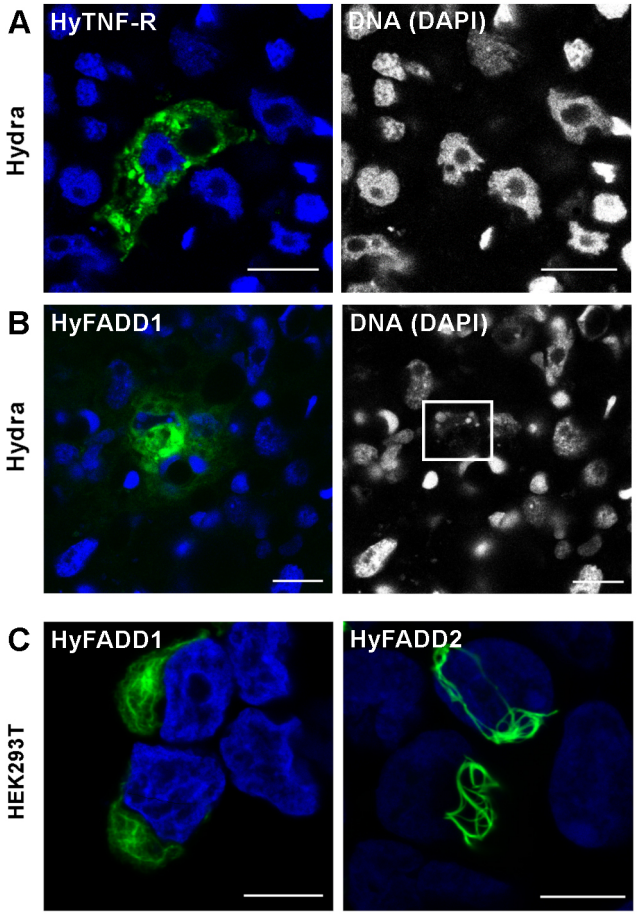

Fig. 5. Overexpression of proteins in *Hydra* and in human HEK293T cells. Laser confocal microscopy images of (A) GFP-tagged HyTNF-R and respective DAPI stained nuclei, (B) GFP-tagged HyFADD1 in *Hydra* cells after biolistic transformation and associated DAPI-stained nuclei (a HyFADD1-positive cell with 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)

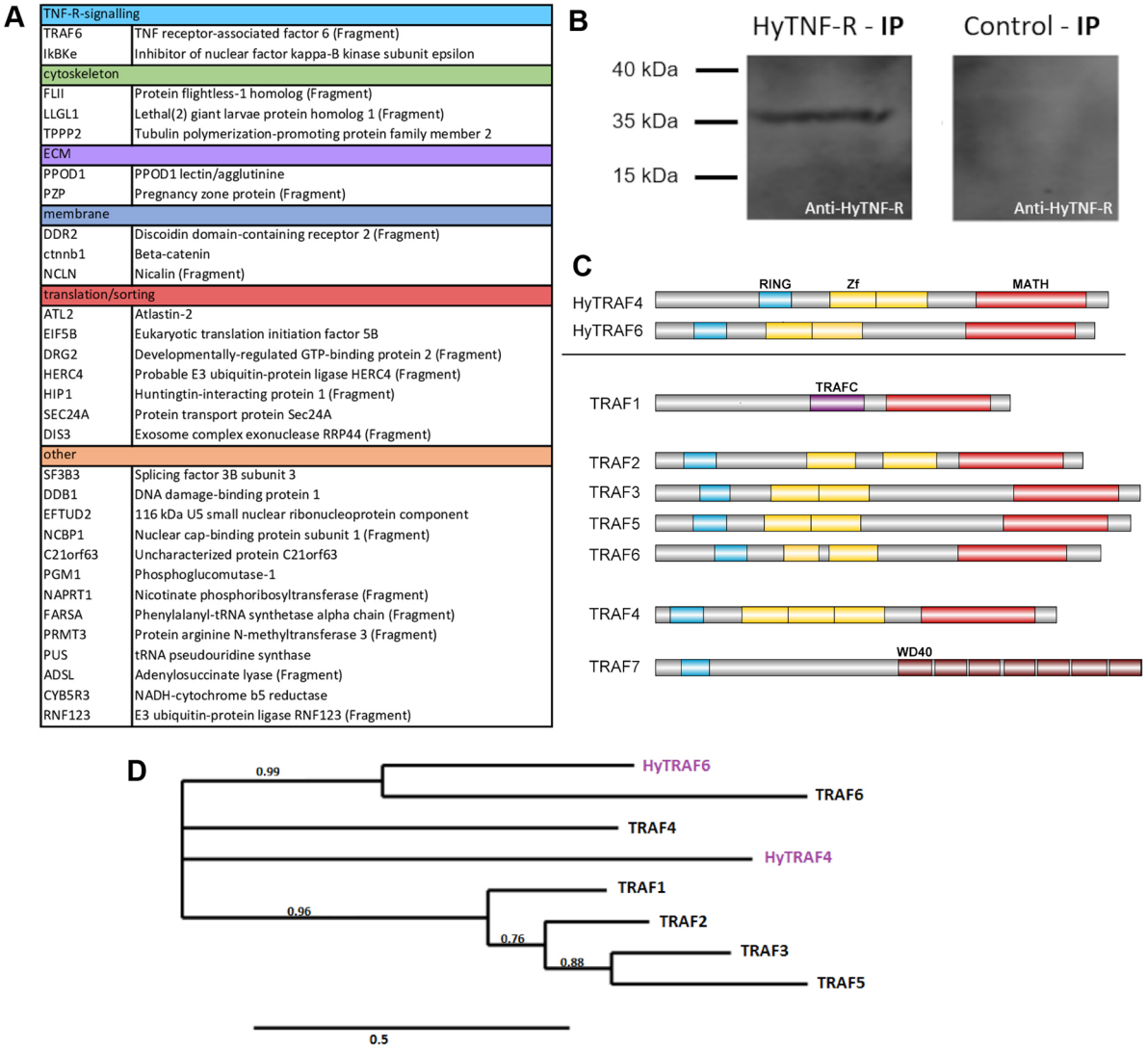

Fig. 6. Immunoprecipitation of HyTNF-R and TRAF-protein identification. (A) Functional classification of *Hydra* proteins co-immunoprecipitated (IP) with HyTNF-R. (B) HyTNF-R in western blot (WB) of precipitated proteins from *Hydra* lysate, for anti-HyTNF-R antibody and control antibody. WB stained 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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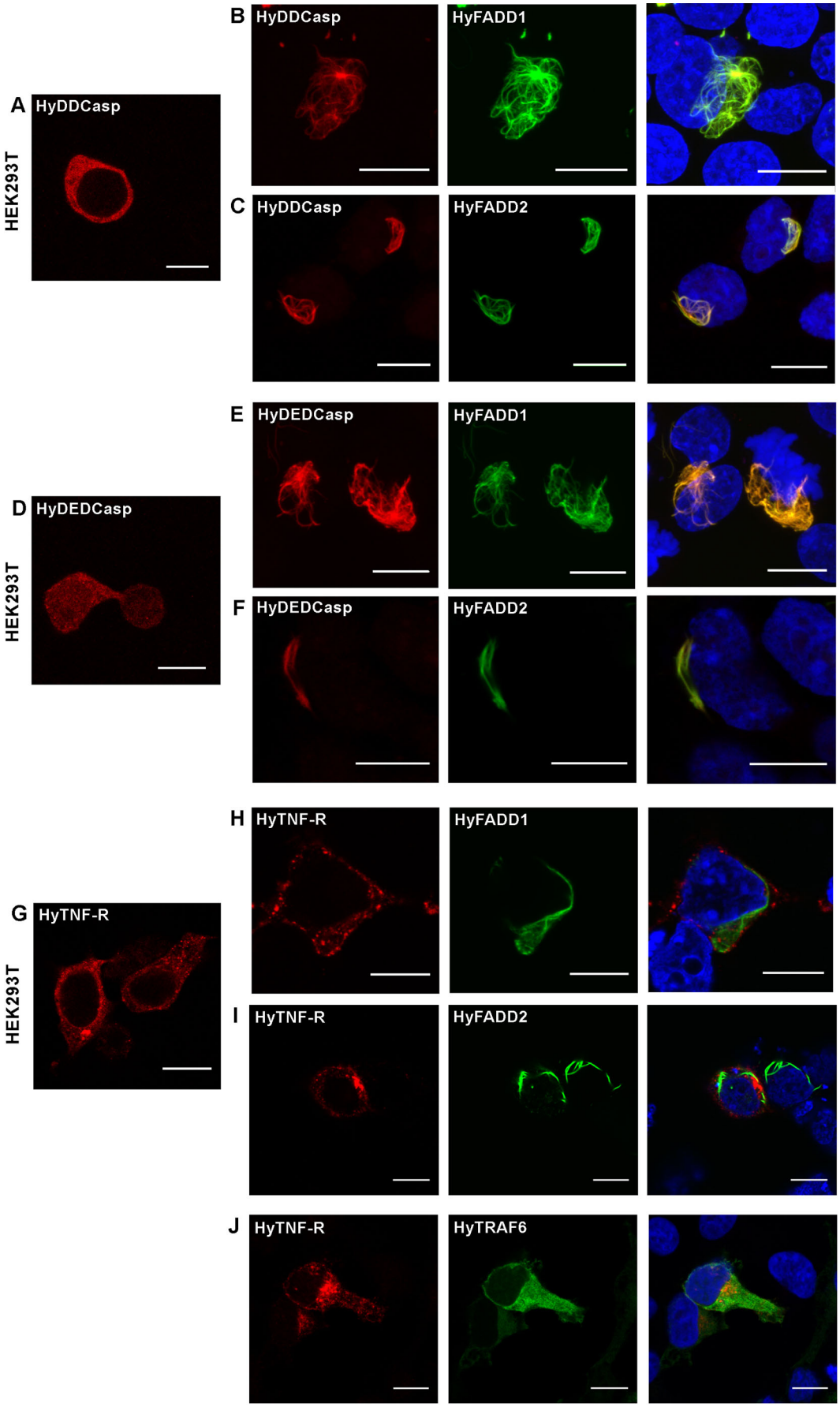

Fig. 7. See next page for legend.

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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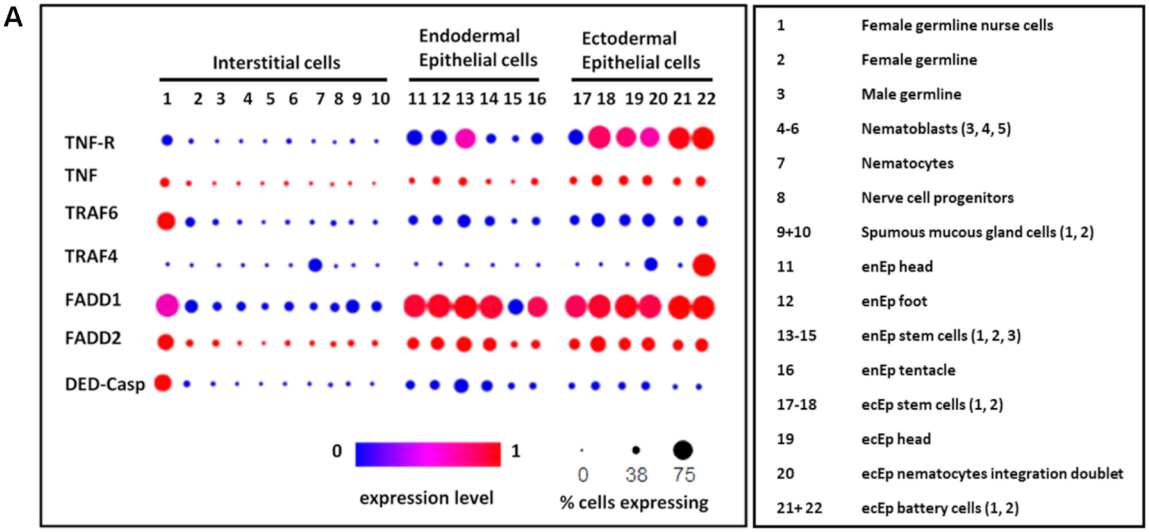

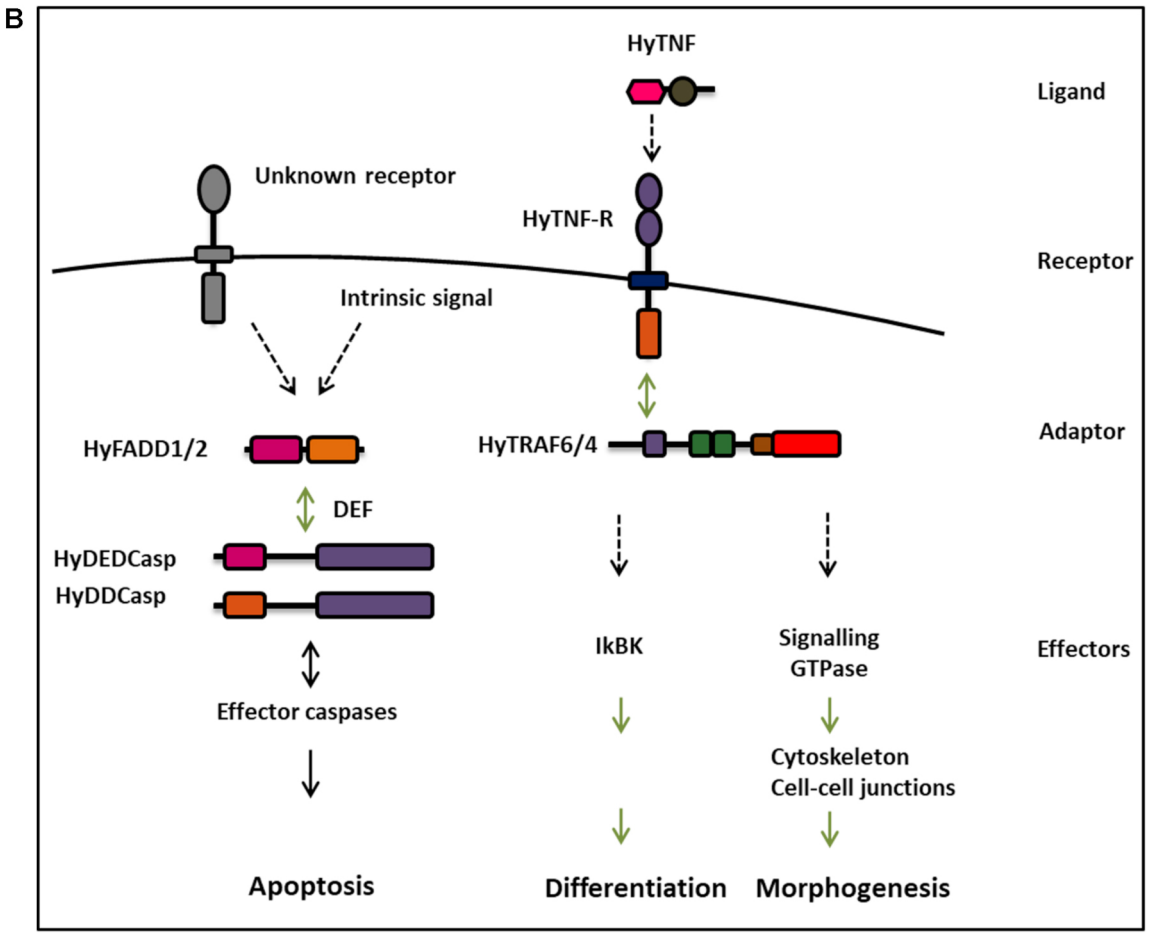

Fig. 8. Expression of TNF-R-pathway genes in *Hydra* and model for TNF, TNF-R and FADD function in *Hydra*. (A) Expression data from single-cell expression atlas. Dot plot grouped in interstitial, ectodermal and endodermal epithelial cells expressing expected TNF-R pathway genes. Colour spectrum represents 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/).

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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.

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Supplementary material

Fig. S1

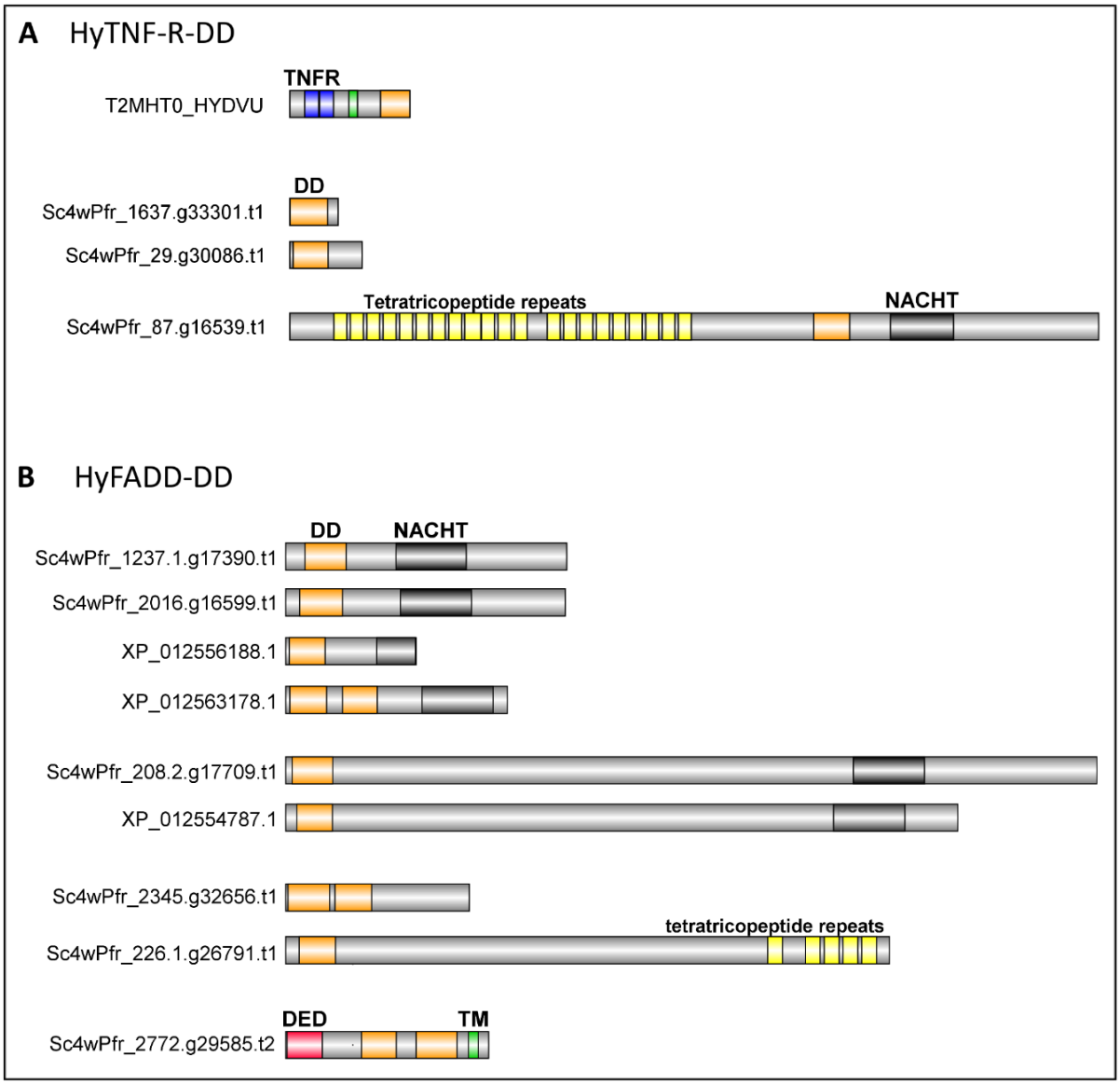

Fig. S1. BLAST search for receptors. (A) HyTNF-R DD-domain BLAST presented proteins with different domain structures than HyTNF-R, no similar protein with TNF-R-repeats or transmembrane domains was found. **(B)** HyFADD-DD-domain BLAST resulted in DD-domain and NACHT-domain containing proteins. One protein with a transmembrane domain and DD-domains was found but uncharacterized. TNF-R-domains (blue, DD-domain (orange), DED-domain (red), NACHT-domain (black), Transmembrane domain (green), Tetratricopeptide repeats (yellow); Accession numbers are from NCBI or Hydra 2.0 genome.

Fig. S2

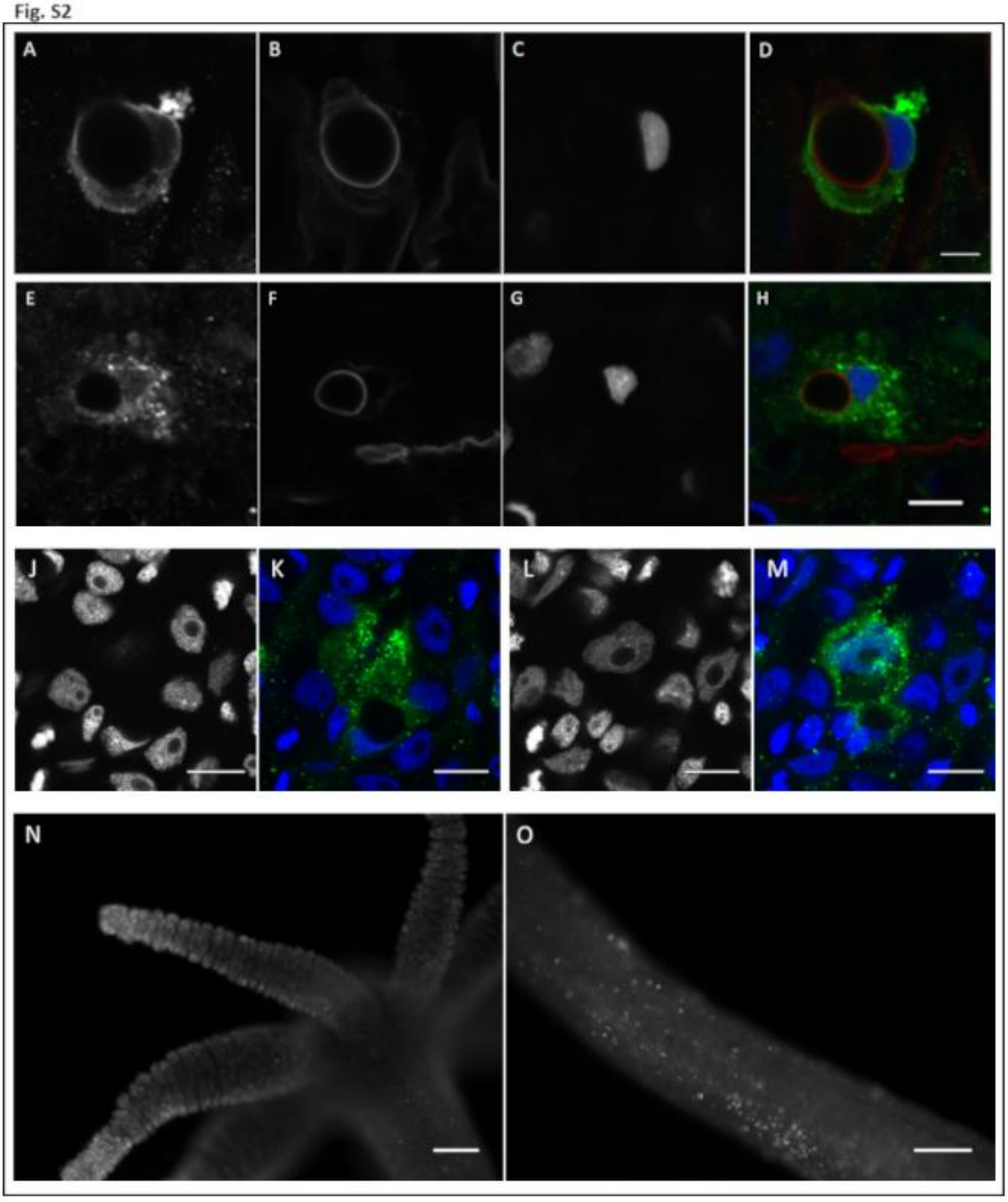

Fig. S2. **HyTNF-R nematocyte staining and localisation of apoptotic cells**. **(A-H)** Laser confocal images of anti-HyTNF-R staining of a nematocyte capsule with crescent-moon shaped nucleus. **(A/E)** anti-HyTNF-R **(B/F)** anti-N-CRD **(C/G)** DAPI **(D/H)** merge. Scale bar: 10 µm. **(J+K)** Anti-HyTNF-R stained epithelial cell on the body column with crescent shaped nematocyte nucleus. **(L+M)** Epithelial cell from (J+K) in lower stack with epithelial, non-apoptotic nucleus. GFP (green), DAPI (blue). **(N+O)** Phase contrast images of apoptotic cells stained with Acridinorange (AO) in living animals. (**N**) tentacles. (**O**) body column. Scale bars: 10 µm (A-H), 100 µm (J+K).

Fig. S3

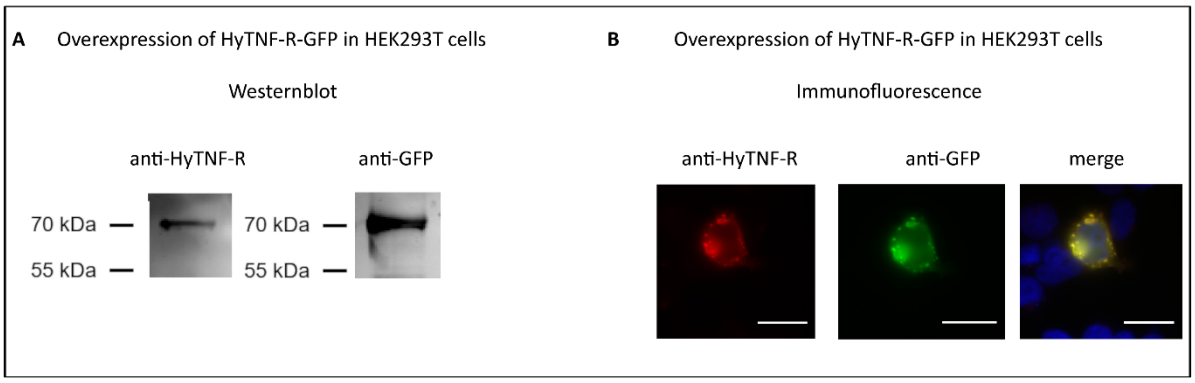

Fig. S3. Characterisation of anti-HyTNF-R antibody. **(A)** Western Blot after PAGE of HEK293T cell lysate overexpressing GFP-tagged HyTNF-R stained with anti-HyTNF-R and anti-GFP antibodies. **(B)** Immunofluorescence staining of HEK293T cells expressing GFP-tagged HyTNF-R stained with anti-HyTNF-R and anti-GFP antibodies. Scale bars: 10 µm.

Fig. S4

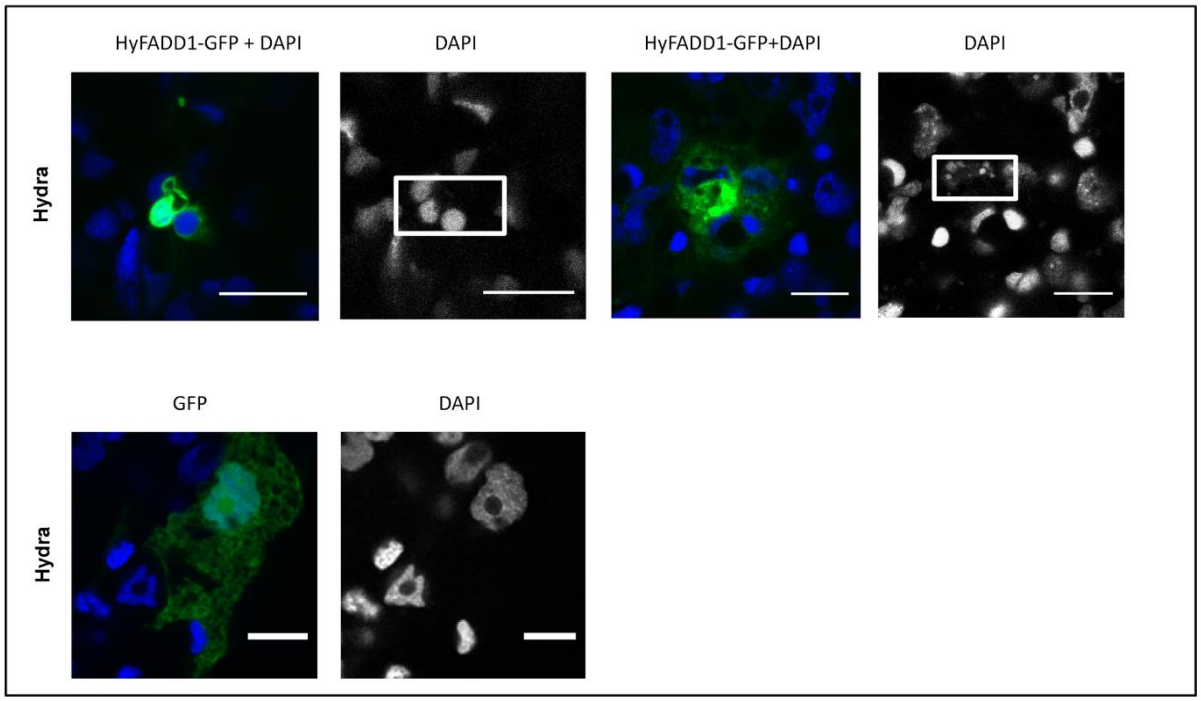

Fig. S4. HyFADD1-GFP in *Hydra* cells. Apoptotic cells were observed after expressing HyFADD1 in *Hydra* (top row), but not after expression of GFP (bottom row). White boxes indicate apoptotic nuclei. GFP (green), DAPI (blue) in merged image, grey in single image. HyFADD1: 2/2 cells were apoptotic, GFP: 0/100 cells were apoptotic. Scale bars: 10 µm.

Fig. S5

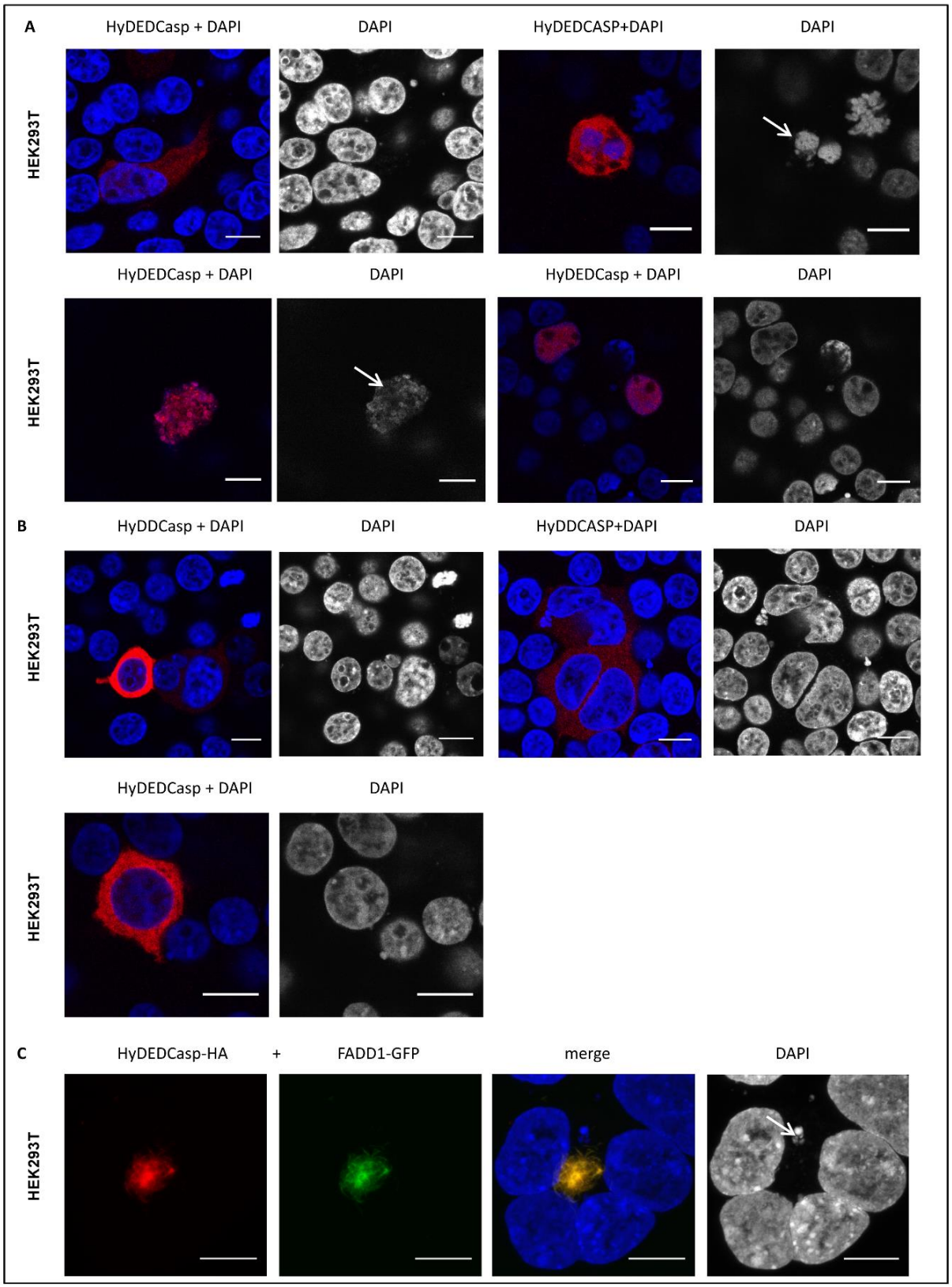

Fig. S5. Expression of *Hydra* caspases in HEK293T cells. **(A)** HyDED-Caspase. **(B)** HyDD-Caspase. **(C)** HyDED-caspase co-expressed with GFP-tagged HyFADD1. White arrows indicate apoptotic nuclei. Green: GFP (green), DAPI (blue) in merged image, grey in single image, HA-tag (red). Scale bars: 10 µm.

S6

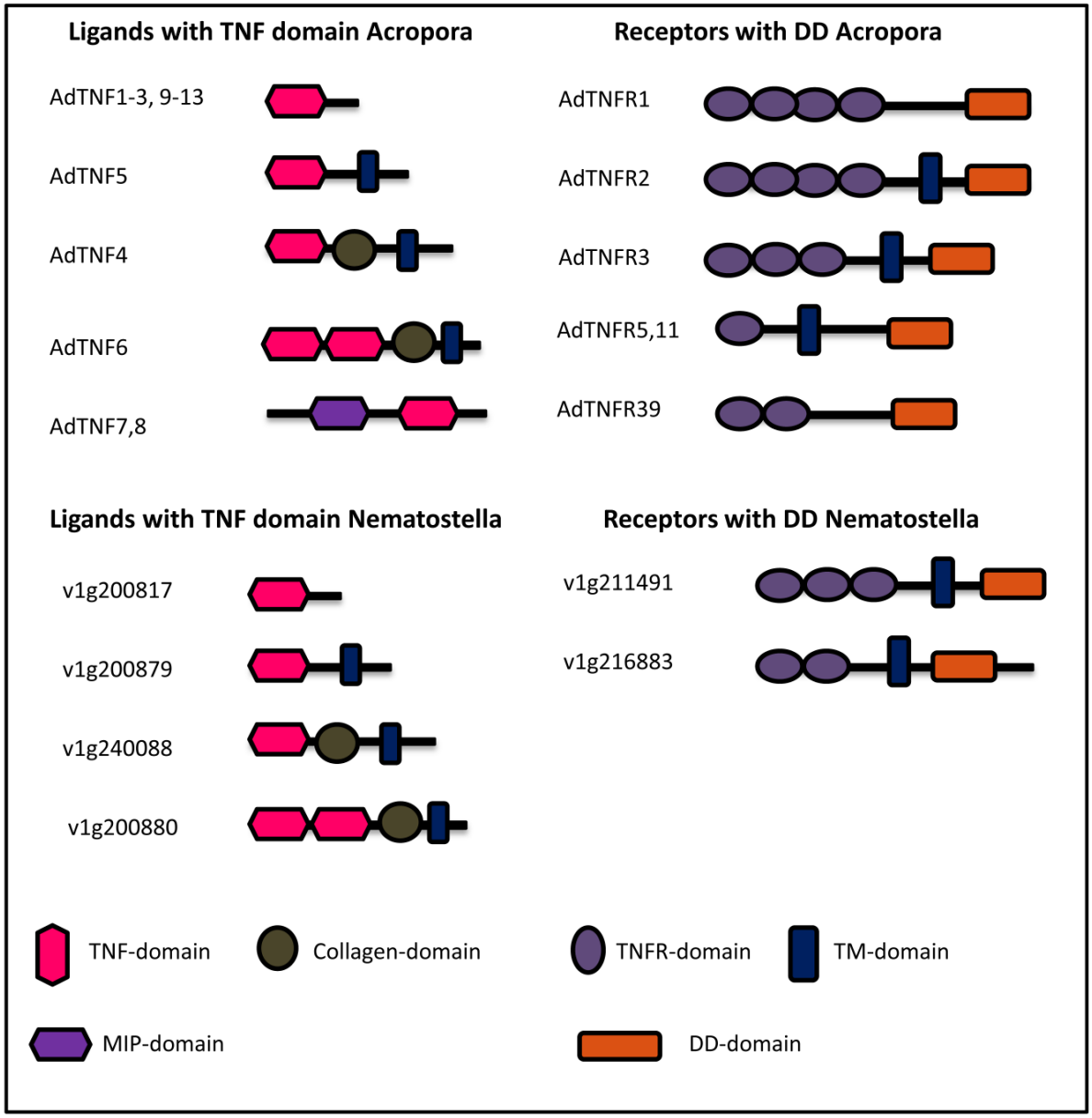

Fig. S6. Comparison of *Acropora* and *Nematostella* TNF-Rs and TNFs. Schematic representation of domain structures of TNF-Rs and TNFs. TNF-domain (pink), collagen-domain (green), TNF-R-domain (purple), Transmembrane domain (blue), MIP-domain (light purple), DD-domain (orange).

Fig. S7

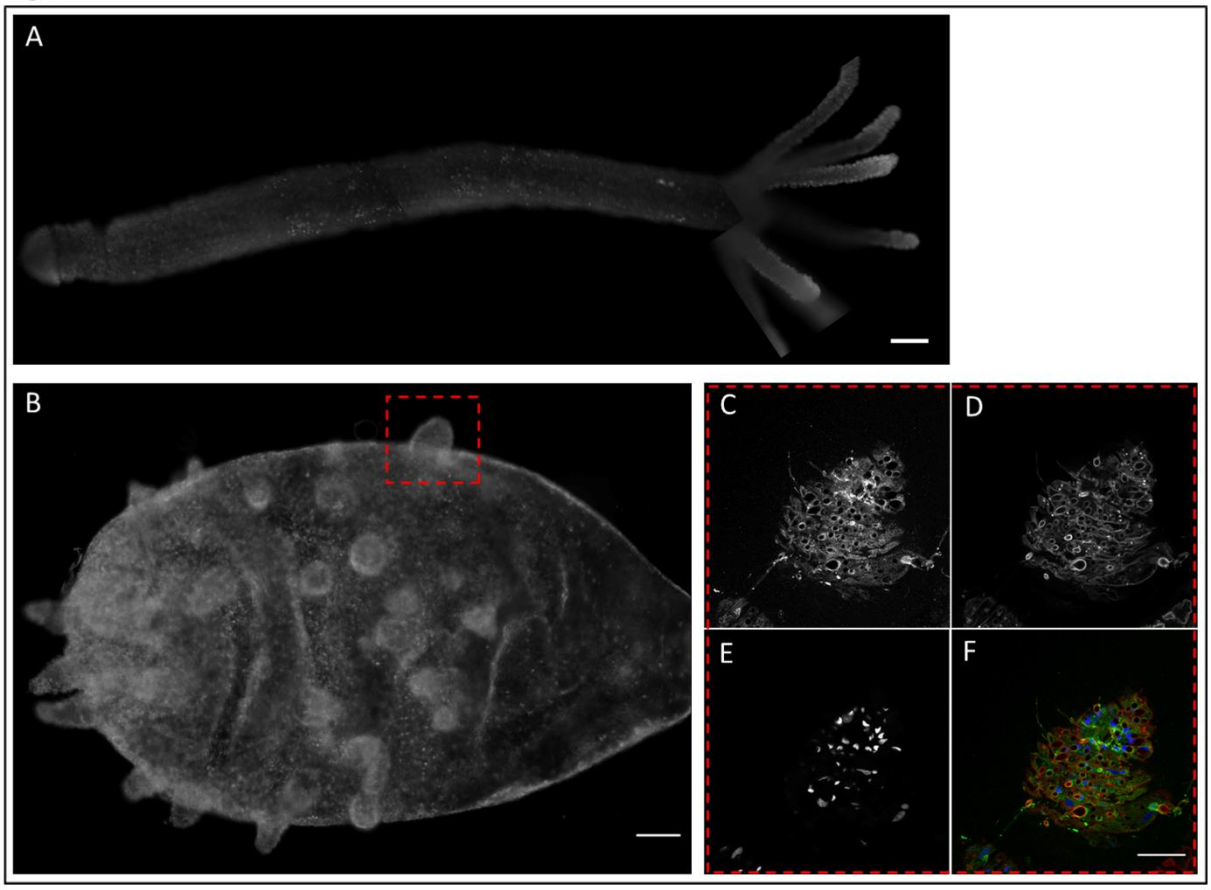

Fig. S7. **HyTNF-R in ectopic tentacles and comparison with apoptotic cell localisation.** **(A)** Phase contrast images of whole *Hydra* with apoptotic cells, stained with Acridinorange (AO) in living animals, three images aligned. **(B)** Phase contrast images of Alsterpaullone (ALP) induced ectopic tentacles on whole *Hydra* and **(C-F)** Laser confocal images of a magnified new tentacle bud. **(C)** anti-HyTNF-R. **(D)** anti-N-CRD. **(E)** anti-DAPI. **(F)** merge. Scale bar: (A) 100 µm; (B-F) 10 µm.

Identified Proteins (932)	Accession Number	Molecular Weight	antiTNF-R	control antibody	exclusive unique peptides	cellular localisation	manual functional assignment
Phosphoglucomutase-1 OS=Hydra vulgaris GN=PGM1 PE=2 SV=:	T2MG15_HYDVU	60 kDa	100%	0	1	cytoplasm	carbohydrate metabolism
Eukaryotic translation initiation factor 5B OS=Hydra vulgaris GN	T2MHU6_HYDVU	145 kDa	100%	0	1	cytoplasm	translation
Phenylalanyl-tRNA synthetase alpha chain (Fragment) OS=Hydr	T2M2Z6_HYDVU	58 kDa	100%	0	1	cytoplasm	protein biosynthesis
Nicotinate phosphoribosyltransferase (Fragment) OS=Hydra vul	T2MBK0_HYDVU	70 kDa	100%	0	1	cytoplasm, Golgi, nucle	biosynthesis
116 kDa U5 small nuclear ribonucleoprotein component OS=Hyd	T2MDI8_HYDVU	110 kDa	100%	0	2	cytoplasm, nucleus	splicing
Probable E3 ubiquitin-protein ligase HERC4 (Fragment) OS=Hydı	T2MC32_HYDVU	57 kDa	100%	0	1	cytoplasm, nucleus	ubiquitinylation
DNA damage-binding protein 1 OS=Hydra vulgaris GN=DDB1 PE	T2MD81_HYDVU	127 kDa	100%	0	1	cytoplasm, nucleus	ubiquitinylation
Developmentally-regulated GTP-binding protein 2 (Fragment) O	T2MEM0_HYDVU	52 kDa	100%	0	2	cytoplasm, nucleus	signalling GTPase
Protein arginine N-methyltransferase 3 (Fragment) OS=Hydra vı	T2MHW2_HYDVU	56 kDa	100%	0	1	cytoplasm, nucleus	protein modification, methylation
Nuclear cap-binding protein subunit 1 (Fragment) OS=Hydra vul	T2MEB2_HYDVU	97 kDa	100%	0	2	cytoplasm, nucleus	RNA-Cap-binding
Exosome complex exonuclease RRP44 (Fragment) OS=Hydra vul	T2M316_HYDVU	109 kDa	100%	0	1	cytoplasm, nucleus	mRNA decay
E3 ubiquitin-protein ligase RNF123 (Fragment) OS=Hydra vulgai	T2MCA6_HYDVU	135 kDa	100%	0	1	cytoplasm, nucleus, me	ubiquitinylation
Lethal(2) giant larvae protein homolog 1 (Fragment) OS=Hydra '	T2MCV2_HYDVU	165 kDa	100%	0	1	cytoskeleton	cell polarity, epithelial integrity
Tubulin polymerization-promoting protein family member 2 OS:	T2MFG9_HYDVU	19 kDa	100%	0	1	cytoskeleton	microtubule dynamics
Protein flightless-1 homolog (Fragment) OS=Hydra vulgaris GN:	T2MD87_HYDVU	115 kDa	100%	0	1	cytoskeleton, nucleus	actin filament binding
PPOD1 peroxidase OS=Hydra vulgaris PE=2 SV=1	Q2FBK4_HYDVU	32 kDa	100%	0	7	ECM	lectin/agglutinin
Pregnancy zone protein (Fragment) OS=Hydra vulgaris GN=PZP	T2MGN1_HYDVU	171 kDa	100%	0	1	ECM, secreted	signalling, growth factor binding
Nicalin (Fragment) OS=Hydra vulgaris GN=NCLN PE=2 SV=1	T2M7J4_HYDVU	63 kDa	100%	0	2	ER, membrane	protease, signalling
Atlastin-2 OS=Hydra vulgaris GN=ATL2 PE=2 SV=1	T2M979_HYDVU	61 kDa	100%	0	1	ER, membrane	GTPase
Protein transport protein Sec24A OS=Hydra vulgaris GN=SEC24A	T2MH92_HYDVU	113 kDa	100%	0	3	ER/Golgi	vesicletransport
Discoidin domain-containing receptor 2 (Fragment) OS=Hydra vı	T2MCH3_HYDVU	129 kDa	100%	0	1	membrane	signalling, RTK
TNF receptor-associated factor 6 (Fragment) OS=Hydra vulgaris	T2M357_HYDVU	59 kDa	100%	0	1	membrane	signalling, TNF-R
NADH-cytochrome b5 reductase OS=Hydra vulgaris GN=CYB5R3	T2MHI6_HYDVU	34 kDa	100%	0	1	membrane	fatty acid metabolism
Huntingtin-interacting protein 1 (Fragment) OS=Hydra vulgaris (	T2MJV4_HYDVU	39 kDa	100%	0	1	membrane, vesicles	endocytosis, trafficking
Inhibitor of nuclear factor kappa-B kinase subunit epsilon OS=Hy	T2MDT7_HYDVU	80 kDa	100%	0	2	membrane, cytoplasm	NFkB activation
Adenylosuccinate lyase (Fragment) OS=Hydra vulgaris GN=ADSI	T2MGT4_HYDVU	61 kDa	100%	0	1	membrane, cytoplasm	purin biosynthesisi
Beta-catenin OS=Hydra vulgaris PE=2 SV=1	Q25100_HYDVU	90 kDa	100%	0	1	membrane, nucleus	cell contact, transcription
tRNA pseudouridine synthase OS=Hydra vulgaris GN=PUS1 PE=2	T2M305_HYDVU	50 kDa	100%	0	1	mitochondrion, nucleus	t-RNA processing
Splicing factor 3B subunit 3 OS=Hydra vulgaris GN=SF3B3 PE=2	T2MD09_HYDVU	136 kDa	100%	0	2	nucleus	mRNA processing
Uncharacterized protein C21orf63 OS=Hydra vulgaris GN=C21orf	T2MFB8_HYDVU	43 kDa	100%	0	1	unknnown	unknown

T**able S1. Complete list of *Hydra* proteins co-immunoprecipitated with HyTNF-R.** Probability scores, protein lengths, exclusive peptides and functional assignments are shown.

5 Discussion

5.1 The antiapoptotic function of Bcl-2 is conserved from pre-bilaterians to humans

The intrinsic apoptotic pathway is initiated by intracellular stimuli and coordinated by Bcl-2 family proteins. There are pro-apoptotic Bcl-2 proteins including Bax and Bak, and anti-apoptotic Bcl-2 proteins. When the equilibrium between those proteins gets changed, e.g by DNA damage or absence of survival signals, the pro-apoptotic BCL-2 family proteins in the mitochondrial membrane are no longer inhibited by anti-apoptotic Bcl-2 proteins. This leads to the permeabilization of the outer mitochondrial membrane and the release of cytochrome C. Cytochrome C is needed for the formation of the apoptosome, a protein complex forming from cytochrome C, APAF-1 and pro-caspases with CARD domains. Pro-caspases are activated within the apoptosome, and start a caspase activation cascade, which activates effector caspases and leads to the death of the cell.

Hydra possesses seven Bcl-2-like and two Bak-like proteins. When expressed in mammalian cells, HyBak-like 1 and 2 strongly induced apoptosis and six of the Bcl-2 like proteins inhibited camthotecin induced apoptosis with HyBcl-2-like 4 showing the strongest anti-apoptotic effect (Lasi et al., 2010). This is why we chose HyBcl-2-like 4 for the generation of transgenic *Hydra* (done by Alexander Klimovich, Margherita Kemper and Marcell Jenewein). Those animals expressed N-terminally GFP-tagged HyBcl-2-like 4 in all ectodermal cells. When these animals were treated with the phosphoinositide-3-kinase (PI(3)-kinase) inhibitor Wortmannin, the amount of apoptotic epithelial cells was significantly reduced compared to control animals. This was also the case for starving animals. The role of HyBcl-2-like 4 as an inhibitor of apoptosis was thus confirmed in *Hydra* and it can be concluded that the anti-apoptotic function of Bcl-2 proteins seems to be conserved from pre-bilaterian animals to vertebrates.

5.2 FADDs and caspases are involved in a cytoplasmic nonreceptor mechanism of apoptosis

FADD proteins function mainly as adaptors for receptors of the TNFR- and Fas-families and Caspases with DED-domains, such as Caspase 8. When the receptors are activated by ligands, they cluster and recruit FADD proteins via homotypic interaction of the DD-domains. The DED-domains of FADD become unmasked and recruit Caspase 8, again by a homotypic interaction between DED-domains. Procaspase activation is attributed to a mechanism of "induced proximity", which allows proteolytic processing of caspases and assembling their active heterotetrameric structure. However, pathways exist, where FADD activates apoptosis in a receptor independent manner. An example is the triggering of apoptosis by the interferon-induced protein kinase (PKR), through FADD-mediated activation of caspase 8 independently of Fas and TNF-alpha receptors (Gil et al., 2000).

Hydra possesses two FADD proteins with a conserved structure. Both have N-terminal DED and C-terminal DDs. When *Hydra* FADD proteins were produced in mammalian HEK cells, we discovered a very particular pattern of subcellular localization of FADD protein in long cytoplasmic fibres, which are also observed with human FADDs when apoptosis is blocked (Siegel et al., 1998). They are termed death-effector-filaments (DEFs).

There are two caspases in *Hydra* which we considered for a possible interaction with *Hydra* FADD, the HyDEDCaspase with a DED-domain, and the HyDDCaspase with a DD-domain. When these *Hydra* caspases are expressed in HEK cells, they appear equally distributed throughout the cytoplasm. Co-expression with HyFADD1 or 2 leads to a dramatic change in the cytoplasmic distribution, as now they are forming very pronounced DEFs. Both *Hydra* FADDs can recruit both *Hydra* caspases. We therefore suggest that the HyDDcaspase is recruited to the DD-domain, and the HyDEDcaspase to the DED-domain of HyFADD.

When HyFADD1 and 2 and the HyDED- and DD-caspase genes are biolistically transfected into *Hydra* cells, they induce cell death, in contrast to HyTNF-R, which can be expressed in *Hydra* cells and shows a diffuse cytoplasmic localisation of the receptor protein. HyTNF-R and HyFADDs also do not colocalize when their genes are co-expressed in mammalian cells.

We therefore suggest a receptor independent cytoplasmic apoptosis induction for *Hydra* where HyFADD gets activated by an unknown intrinsic signal, resulting in DEF formation, the recruitment of the initiator caspases HyDEDCasp and HyDDCasp and the activation of effector-caspases, followed by apoptosis.

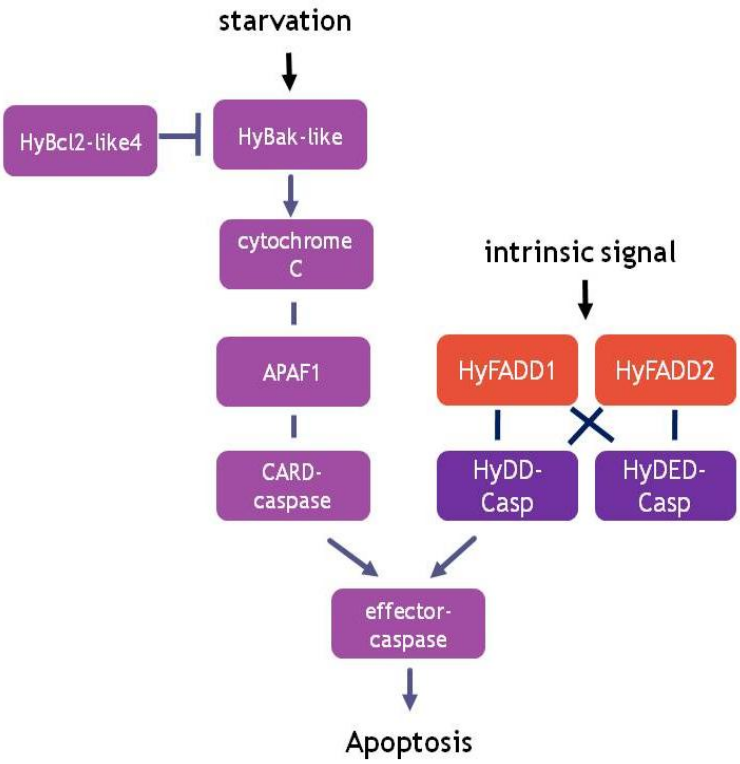

Figure 3. Intrinsic apoptosic pathway in Hydra. Left hand side: Anti-apoptotic HyBcl-2-like4 proteins block pro-apoptotic HyBak-like proteins, which normally are induced by intrinsic signals, for example nutrient deprivation or DNA damage, and lead to the release of cytochrome C, the building of an apoptosome, the activation of caspases and apoptosis. Right hand side: an unknown intrinsic signal activates HyFADD1 and 2, which form DEFs and recruit caspases, this then leads to apoptosis.

5.3 The *Hydra* TNF-R superfamily member belongs to the EDAR subgroup

The vertebrate TNF-R superfamily has numerous members, including TNF-R-, FasR (CD95), TRAIL-R (tumour necrosis factor related apoptosis inducing ligand receptor) and EDAR (ectodermal dysplasia A-receptor). This diversity is reflected in a multiplicity of functions of these receptor and ligand proteins, which are not only in the regulation of apoptosis, but also in promoting cell survival, inflammation and morphogenesis (reviewed in Mathew et al., 2009). The genome of *Hydra* codes for just one member of the TNF-R-superfamily. HyTNF-R consists of a signal peptide, two TNF-R domains with 6 characteristic cysteine residues, a transmembrane domain and a C-terminal death domain. The composition of its extracellular domain as well as phylogenetic analyses clearly indicated that it belongs to the EDAR-family of TNF-Rs. We also discovered only one potential TNF-ligand. It has an extracellular TNF-domain followed by a collagen domain, which is only found in one human TNF member, the EDAR ligand EDA (Ectodysplasin).

In vertebrates, binding of Eda to EDAR leads to the recruitment of the adaptor molecule Edaradd to the receptor via its DD. Edaradd can bind to several TRAFs in vitro, but mostly signals through TRAF6 (Mikkola et al., 2003). Tab2 (Tak1-binding protein) bridges TRAF6 to Tak1 (TGFb-activated kinase). In the following the IKK complex is activated, IkB is phosphorylated and NF-kB is released. NF-kB is translocated to the nucleus where it can activate the transcription of many different target genes which 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).

When we carried out an immunoprecipitation experiment with anti HyTNF-R antibody and *Hydra* membrane proteins, we detected HyTRAF6 and the *Hydra* IkB-kinase in the precipitate, suggesting that they are involved in *Hydra* TNF-R signaling. Figure 4 shows a model of HyTNF-R signaling in *Hydra.*

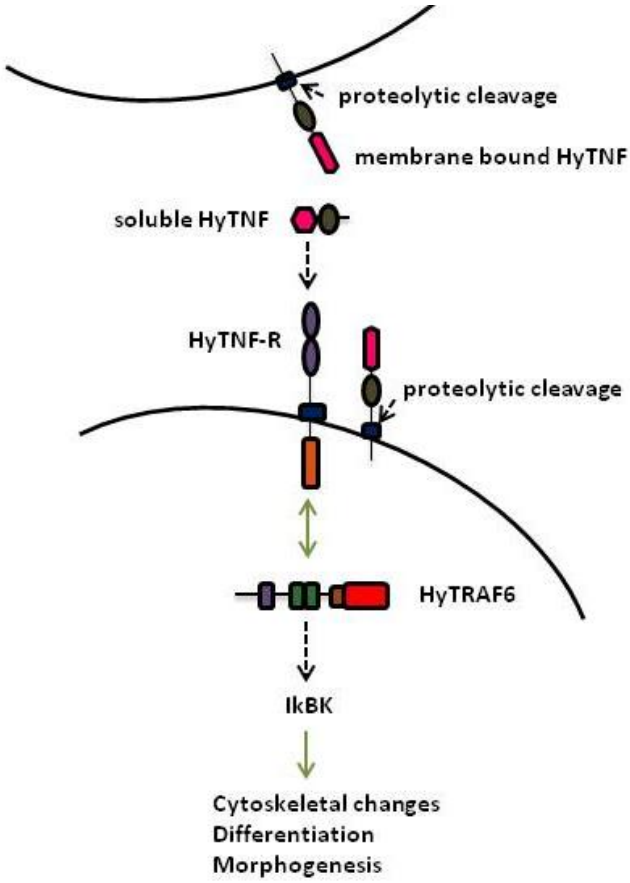

Figure 4. Model of an extrinsic apoptotic pathway in *Hydra*. Soluble HyTNF is released by the membrane by proteolytic processing. It leads to the activation of membrane bound HyTNF-R, the recruitment of HyTRAF6, the activation of IkBK, the release of NF-kB and the transcription of genes that in turn lead to cytoskeletal changes, differentiation and morphogenesis.

5.4 The HyTNF-R is expressed in epithelial cells with integrated nematocytes

Immunofluorescence staining of *Hydra* whole mounts with an antibody against HyTNF-R revealed a very specific localisation of the protein in specialized epithelial cells in the body column, which have incorporated nematocytes, and in battery cells of the tentacles. Battery cells are epithelial cells, which differentiate after reaching the tentacle base and integrate 10 to 20 nematocytes into each cell. The differentiation of normal epithelial cells into battery cells is accompanied by drastic changes in cell shape and adhesive contacts. Little is known about the mechanisms controlling this differentiation process. This work indicated that HyTNF-R signaling could be involved in promoting the execution of a program of terminal differentiation involving cytoskeletal changes and incorporation of nematocytes.

5.5 EDAR signaling is involved in nematocyte integration in *Hydra* as well as in the formation of ectodermal appendages like teeth and hair in vertebrates

The ectodysplasin pathway in higher animals is very well studied and directs the development of ectoderm derived structures like skin, teeth, hair in mammals, feathers in chicken and scales in fish. The role of TNF-R signaling in the process of nematocyte integration into epithelial cells in *Hydra* might be comparable with the role of EDAR signaling during the formation of hair follicles and teeth in mammals. Here EDAR is expressed at low levels in the embryonic ectoderm and gets localized to placodes later in development. A placode is an epithelial thickening and the first morphological sign of a developing ectodermal appendage (Mikkola, 2008). It grows into the mesenchyme to produce a bud followed by epithelial cell specific morphogenesis. The ligand Eda is expressed in the surrounding mesenchyme. In relation to *Hydra* it is feasible that HyTNF-R is expressed in an epithelial cell close to a nematocyte, and the ligand HyTNF is expressed in the surrounding cells. This assumption is supported by the results of gene expression studies using data from the single cell transcriptome analysis. HyTNF-R is expressed very strong in one fraction of ectodermal epithelial cells, whereas HyTNF is expressed in all cell types. HyTNF-R signaling

could then lead to the terminal differentiation of these specialized epithelial cells as well as cytoskeletal changes and morphogenesis. Probably these TNFR expressing epithelial cells in *Hydra* encase the nematocyte in a similar way as the cells of the tooth enamel organ encase the condensed mesenchyme.

5.6 Other cnidarian TNF-Rs also belong to the EDAR subfamily

Phylogenetic analyses with members of the TNF-R superfamily of cnidarian and vertebrate species show, that all cnidarian TNF-Rs either group with human EDAR or outside of mammalian TNF-R-, TRAIL- and NGF-R/p75-families. It seems as if two subgroups of TNF-receptors evolved in cnidarians. One is related to mammalian EDARs and involved in morphogenesis rather than apoptosis. The other group could functionally be related to mammalian TNF-Rs as Quistad et al. showed 2014 that at least one *Acropora* TNF can induce apoptosis in human T cells.

5.7 Outlook

This study reveals an ancient origin of the TNF-R superfamily in regulating morphogenesis and differentiation. In the future the function of other members of the invertebrate TNF-R subfamily in cnidarians should be investigated more closely to confirm that they are involved in extrinsic apoptotic pathways, as it was suggested in Quistad et al., 2014. Members of the intrinsic apoptotic pathway, concluding Bcl-2 family proteins, FADDs and caspases seem to be highly conserved in *Hydra*, both in sequence as well as in function. The mechanism of induction of the apoptotic pathway including HyFADD1 and 2 and HyDD- and DED-Caspases is still an open question. A TNF-R mediated induction as in vertebrates seems unlikely. Immunoprecipitation experiments using HyFADD as bait could detect relevant interaction partners and disclose the mechanism of intrinsic apoptosis induction in ancient organisms.

6 Appendix

6.1 Abbreviations

Apaf-1	Apoptotic protease activating factor 1
Atg	autophagy-related genes
ATP	Adenosine triphosphate
Bak	Bcl-2 homologous antagonist / killer
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 Homology domain 3
BI	Bax-Inhibitor
BLAST	Basic local Alignment and Search Tool
BMP	Bone Morphogenetic Protein
CARD	Caspase-associated recruitment domains
CED	Cell Death protein
DISC	Death Inducing Complex
DEF	Death-Effector-Filaments
DED	Death Effector Domain
DD	Death Domain
DNA	Desoxyribonucleic Acid
EDAR	Ectodysplasin A Receptor
EGL	Egg Laying Defective

FADD	Fas Associated Death Domain protein
HEK	Human Embryonic Kidney
Hv	Hydra vulgaris
Hy	Hydra
IAP	Inhibitor of Apoptosis
JNK	N-terminal Jun Kinase
LGF	Life Guard
MLKL	Mixed lineage kinase domain like pseudokinase
PI3	Phosphatidylinositol-3
qPCR	quantitive Polymerase Chain Reaction
RIPK	Receptor-Interacting serine/threonine-Protein Kinase
TMBIM	The TransMembrane Bax Inhibitor Motif
TNF	Tumor Necrosis Factor
TNF-R	Tumor Necrosis Factor Receptor
TRADD	TNF-Receptor Associated Death Domain protein
TRAF	Tumor Necrosis Factor Receptor Associated protein
Wnt	Wingless / integrated

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