Apooptosis 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, bel-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 cytochrome c 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; Bel-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 inner-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 cnidian 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 (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 Ca2+-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 (Somnia 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 apoptosis

![Fig. 1. Confocal microscopic optical section from the ectoderm of the body column of transgenic Hydra polyps](Image)

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 apoptosis.
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 nuclei. 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.
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_117868.1;
- *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: AY736612.1;
- *Ceratopteris richardii* Lfg4: XM_001756985.1;
- *Physcomitrella patens* Lfga: XM_001768570.1;
- *Physcomitrella patens* Lfgb: XM_001757644.1;
- *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;
- *Zea mays* Lfga: BT088363.1;
- *Zea mays* Lfgb: NM_001156405.2;
- *Zea mays* Lfgc: NM_001321409.1;
- *Canis lupus* Lfg5: XM_006669.4;
- *Cyanidioschyzon merolae* chromosome 4: 54288-55103;
- *Drosophila melanogaster* BI1: NM_139948.4;
- *Ovis aries* Lfg5: XM_012176315.2;
studies members 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 reminiscent 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 BODIPY-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 with plasma membranes (Fig. 7 A,B,C).

**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 TM3 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.

**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.
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 ubiquititarian expressed gene is HyBcl-2-like 4. Similar
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. The observed decrease in apoptosis in HyBcl-2-like 4 ecto 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

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.

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).

Fig. 9. Relative expression levels of Bcl-2- and TMBIM-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...
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, *HyLfg*-1b, 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 cytotoxic 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_000126893.1) 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* DNA 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*ALF) 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: CACCATCTAATCCACAAAGATTG; 3’-primer: GCCGTCGAAGCGATTCACCC). Genomic DNA was obtained from polyps treated with lysis buffer (50 μl/poly of 100 mM Tris, 10 mM EDTA, 250 mM NaCl, 200 μM Proteinase K) for 30 min at 37 °C and subsequently at 95 °C for 2 min. 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.

**Confocal laser scanning microscopy**

Leica SP5-2 confocal laser-scanning microscope was used for Light optical serial sections. (Plan-Apochromat 100/1.4 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 455–465 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 x 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$_2$O – 1:1:13 – v/v/v) for 1 hour (RT), incubated for 250 mM NaCl, 200 mOsmol (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_000126893.1) 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* DNA 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).
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/mL], 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 statistical 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 RNA 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.

**References**


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