Characterization of anti-apoptotic protein family members (Bax inhibitor-1 and Lifeguard) in *Hydra vulgaris*

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Contents

Abstract

Programmed cell death is an important mechanism to regulate development as well as responses to cell and especially DNA damage in fungi, animals, plants and even in unicellular protists. Programmed cell death in animals, also called apoptosis, is well characterized and many of its components are unique to animals. They involve caspases and Bcl-2 proteins and are conserved in very early pre-bilaterian animal phyla, including cnidarians, but not outside animals. The focus of this study was to investigate regulators of programmed cell death, which are conserved in both animal and non-animal phyla, in a representative of early pre-bilaterian metazoans, the cnidarian *Hydra vulgaris*. In this study, three sequences encoding Lfg proteins i.e., Lfg-4 and two homologs of Lfg-1i (Lfg-1ia and Lfg-1ib) were found in *Hydra*. These genes were shown to encode membrane proteins with 7 α-helix transmembrane domain structures. In addition, one gene encoding BI-1, a protein with 6 α-helix transmembrane domains was investigated. Phylogenetic analysis indicated that Lfg and BI-1 proteins form separate protein families. Nevertheless, the data showed at least one member of the Lfg-family, similarly to BI-1, had a very strong effect in protecting HEK 293T cells against campthothecin induced apoptosis. In addition, up-regulation of *Hydra* Lfg-4 transcripts in Benzo[a]pyrene treated animals was observed.

Whilst BI-1 is localized in the ER in *Hydra*, further results showed that the *Hydra* Lfg-4 protein localized at the Golgi complex. The two *Hydra* Lfg-1i proteins were localized mainly at the plasma membrane, but also in Golgi vesicles. In situ hybridization experiments suggested that cell death inhibition exerted by *Hydra* BI-1 and *Hydra* Lfg-4 could play a role in *Hydra* gametogenesis in both, male as well as female gonads. Apoptosis occurs in

Hydra oogenesis and spermatogenesis. This study now shows that *Hydra* Lfg-4 and *Hydra* BI-1 are both expressed in the developing oocyte. BI-1 was also expressed in *Hydra* gonads during spermatogenesis. Therefore, data presented in this work indicate that the Golgi and ER resident proteins of the Lfg and BI-1 protein families may have been involved in the regulation of programmed cells death in very ancient metazoans.

1. Introduction

1.1. Apoptosis

The term programmed cell death was introduced in 1964. It proposed that cell death during development is not of accidental nature but follows a sequence of controlled processes leading to locally and temporally defined self-destruction (Lockshin and Williams, 1964). Kerr et al. (1972) introduced the term apoptosis, which describes the morphological processes leading to controlled cellular self-destruction. The apoptotic mode of cell death is an active process in development of multicellular organisms, and in regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions (Leist and Jaattela, 2001). It plays also an important role during developmental processes of multicellular organisms, particularly during embryogenesis and metamorphosis (Lockshin and Zakeri, 2001), and is involved in differentiation, proliferation/homoeostasis, regulation and function of the immune system as well as in the removal of defect and harmful cells (Fadeel et al., 1999).

1.1.1. Biochemistry and morphological features of apoptosis

1.1.1.1. Morphological features

Changes like deformation and shrinking of the cell, loss of contact to its neighboring cells, chromatin condensation, blebbing or budding of the plasma membrane and finally fragmentation of the cell into compact membrane-enclosed structures, called “apoptotic bodies” are morphological features of an apoptotic cell. In contrast to the apoptosis, during a necrotic mode of cell-death when the cells suffer a major insult, the cellular

contents are released uncontrolled into the cell's environment and this results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Leist and Jaattela, 2001).

1.1.1.2. Biochemistry of apoptosis

Various signals from inside and outside of the cell can induce apoptosis, for example ligation of so called death receptors or cellular stress triggered by drugs, irradiation or oncogenes. There are two major apoptosis signaling pathways in mammalian cells: the extrinsic and the intrinsic apoptosis pathway.

1.1.1.3. The extrinsic apoptosis pathways (Death receptor pathway)

Extrinsic apoptosis signaling is mediated by the activation of “death receptors” that belong to the tumor necrosis factor receptor (TNFR) gene super family, including TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5 (Ashkenazi, 2002). They are located at the cell surface and transmit apoptotic signals after ligation with specific ligands. Subsequent signaling is mediated by the death domain (DD), which is the cytoplasmic part of the death receptor. Adapter molecules such as FADD or TRADD are recruited to the DDs of the activated death receptor and form the so-called death inducing signaling complex (DISC). Procaspase-8 can be recruited to the DISC via the death effector domain (DED) of FADD adaptor and this leads to its autocatalytic activation and release of active caspase-8. The active caspase-8 then processes downstream effector caspases, which subsequently cleave specific substrates resulting in cell death. Cells with the capacity to induce such direct and mainly caspase-

dependent apoptosis pathways were classified into the so called type I cells (Scaffidi et al., 1998).

In contrast, a signal coming from the activated receptor does not generate a strong enough caspase signaling cascade for execution of cell death on its own in type II cells. Therefore, the signal will be amplified via mitochondria-dependent apoptotic pathways. The Bcl-2 family member, Bid provides a link between the caspase signaling cascade and the mitochondria. Bid is cleaved by caspase-8 and translocates to the mitochondria in its truncated form (tBid). In mitochondria it acts in concert with the other proapoptotic Bcl-2 family members (i.e., Bax and Bak) to induce the release of cytochrome c into the cytosol (Luo et al., 1998).

Cytosolic cytochrome c binds to the Apaf-1 and assembles the apoptosome, a complex of wheel-like structure with 7-fold symmetry that triggers the activation of the initiator procaspase-9 (Acehan et al., 2002). Activated caspase-9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase-3, caspase-7, and caspase-6, eventually resulting in cell death (Slee et al., 1999) (see also Fig. 1).

1.1.1.4. The intrinsic apoptosis signaling pathway (Mitochondrial pathway)

Death signals originating from inside the cell such as DNA damage, oxidative stress and starvation propagate through mitochondrial pathway (Wang, 2001). Most apoptosis-inducing conditions involve the disruption of the mitochondrial inner membrane potential as well as the so called permeability transition, resulting in the release of pro-apoptotic proteins from the mitochondrial intermembrane space into the cytoplasm (Bernardi et al., 1999; Loeffler and Kroemer, 2000). Cytochrome c activates the apoptosome and

therefore the caspase cascade. Other pro-apoptotic factors such as the apoptosis-inducing factor (AIF) (Susin et al., 1999), the endonuclease endoG (Li et al., 2001) and Smac/Diablo (Verhagen et al., 2000) are also released from mitochondria. In addition to the release of mitochondrial factors, the biochemical homeostasis of the cell is disrupted, e.g., ATP synthesis is stopped, redox molecules such as NADH, NADPH, and glutathione are oxidized, and reactive oxygen species (ROS) are increasingly generated (Kroemer and Reed, 2000) and increased levels of ROS directly cause the oxidation of lipids, proteins, and nucleic acids (Marchetti et al., 1997) (see also Fig.1).

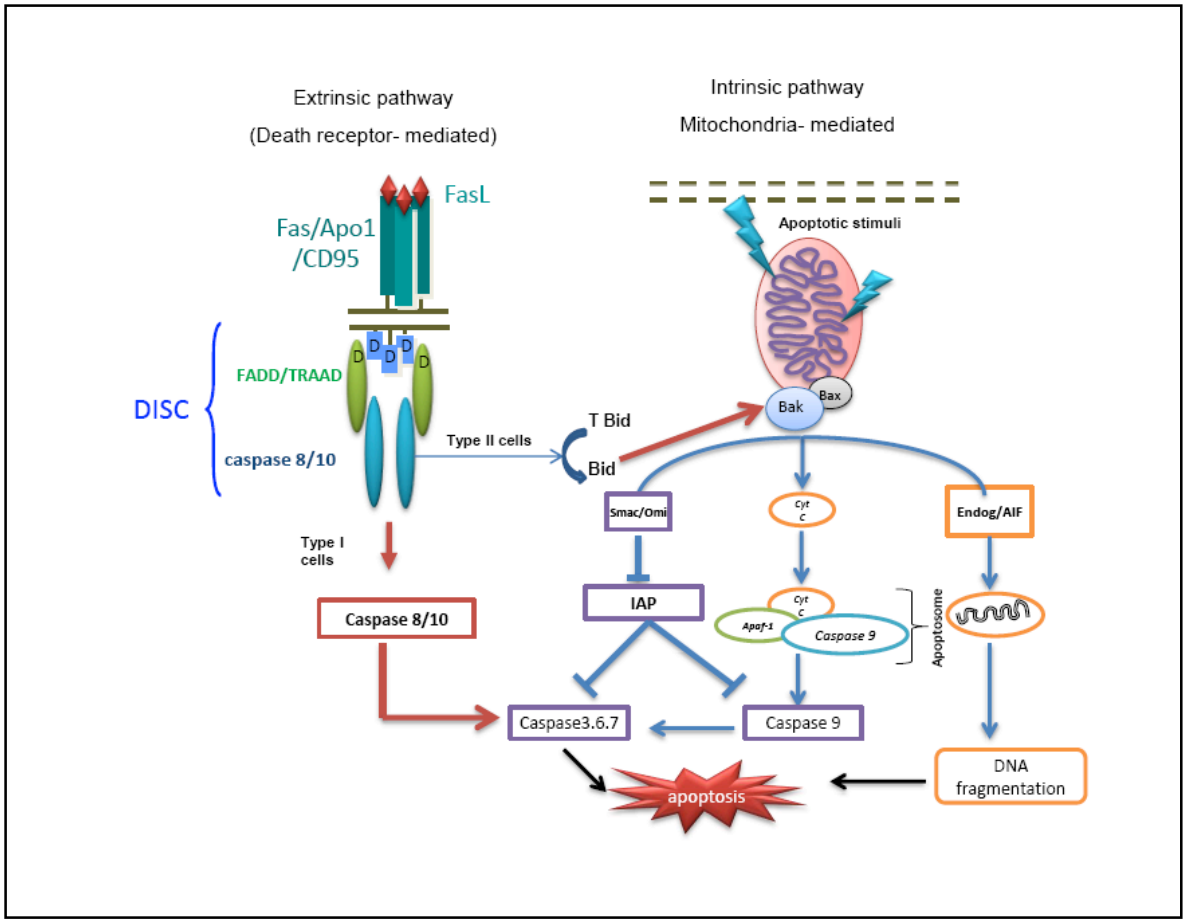

Fig. 1 Apoptosis signaling pathways in mammalian cells (Magdanela et al., 2012).

1.1.2. The apoptosis signaling pathways are evolutionarily conserved

Programmed cell death is a widespread phenomenon, occurring in all kinds of metazoans such as in mammals, insects (Richardson and Kumar, 2002), nematodes (Liu and Hengartner, 1999), and cnidarians (Cikala et al., 1999). In addition, it has become clear that programmed cell death plays a role in plant biology (Solomon et al., 1999), and apoptosis-like cell death mechanisms have been observed and even used as a model system in yeast (Fröhlich and Madeo, 2000). Fascinating insights into the origin and evolution of programmed cell death can be probably explained by the fact that programmed cell death is also an integral part of the life cycle of other unicellular eukaryotes such as the ciliate *Tetrahymena thermophila*, the slime mold *Dictyostelium discoideum* and even prokaryotes such as *Bacillus subtilis, Streptomyces* and *Myxobacteria* (Ameisen, 2002).

1.1.3. Programmed cell death (PCD) in plants

Programmed cell death (PCD) in plants occurs during developmental process, e.g., xylogenesis, embryogenesis, parenchyma formation (Gray, 2004) and is shown to contribute to the formation of female gametes in seed plants and male sexual organs (Greenberg et al., 1994). As in animals, PCD occurs in response to biotic and abiotic stimuli, e.g., plant pathogen interactions, in which PCD serves as a defense mechanism in incompatible interactions and also to promote the propagation of the pathogen in compatible interactions. The PCD in plant cells is mostly similar to the type II or autophagic cell death in animals (Liu et al., 2005). It is associated with cell shrinkage, cytochrome *c* release, chromatin and cytoplasmic condensation and internucleosomal

DNA fragmentation. Vacuole disruption is observed in most cases of plant PCD. The vacuole contains many of the hydrolytic activities that perform PCD i.e., proteases, RNases and DNases that are physically separated from their targets (Lam, 2008).

Moreover, caspase-like enzymatic activities in response to oxidative stress, senescence and seed development have been detected in plant tissues undergoing PCD (2002; Solomon et al., 1999). The Caspase-like activity has been found during chemical and stress induced cell death of tobacco suspension cells and Camptothecin-induced PCD in tomato suspension cells and can be inhibited by caspase-specific peptide inhibitors. This confirms that caspase-like proteases are involved in the death process (De Jong et al., 2000).

The release of cytochrome *c* from mitochondria into the cytosol precedes cell death in various plant systems (Krause and Durner, 2004). Ectopic expression of certain animal anti-apoptosis genes has been demonstrated to provide protection from pathogens and other insults as a result of cell death suppression in transgenic plants (Dickman et al., 2001; Mitsuhara et al., 1999). However, the expression of animal pro-apoptotic proteins such as Bax in plants can induce cell death mechanisms similar to endogenous programs for cell suicide (Yoshinaga et al., 2005). *In vivo*, cytochrome *c* release into the cytosol has been observed during PCD in *Arabidopsis* suspension cells and cucumber (Krause and Durner, 2004; Balk et al., 1999). This observation indicates that mitochondrial function may be shared in a very similar way during cell death in both animals and plants. Moreover, in plant PCD, elevated Ca^{2+} levels have been observed during wheat aleurone differentiation and leaf senescence (Kuo et al., 1996).

1.1.4. Apoptosis in Animals

1.1.4.1. Apoptosis in *Caenorhabditis elegans*

Caenorhabditis elegans is an intensively studied model organism for apoptosis. 131 somatic cells out of 1090 initially formed, undergo programmed cell death during embryonic and post-embryonic development of *C. elegans* (Sulston and Horvitz, 1977). Cell death in *C. elegens* relies on interactions between proteins such as CED-3 (ced: cell death abnormal), CED-4, CED-9 and EGL-1 (egg-laying abnormal 1) (Conradt and Horvitz, 1998; Liu and Hengartner, 1999). CED-3 was found to be related to the mammalian cysteine protease, interleukin-lβ converting enzyme (named ICE or caspase-1) (Yuan et al. 1993). CED-4 is Apaf-1 homologue (mammalian apoptotic protease-activating factor-1) and binds to and activates CED-3 (caspase homologue). The protein EGL-1 is a pro-apoptotic BH3-only-domain protein. It binds to CED-9, displacing CED-4, which in turn activates CED-3 to induce apoptosis (Liu and Hengartner, 1999). However, the CED-4 remains inactive by its association with CED-9 (antiapoptotic Bcl-2 homologue) in the healthy cells. In other organisms, cytochrome *c* binds to WD-40 domains of Apaf-1, while CED-4 does not have this domain and release of cytochrome *c* into the nematode does not play role (see also Fig. 2).

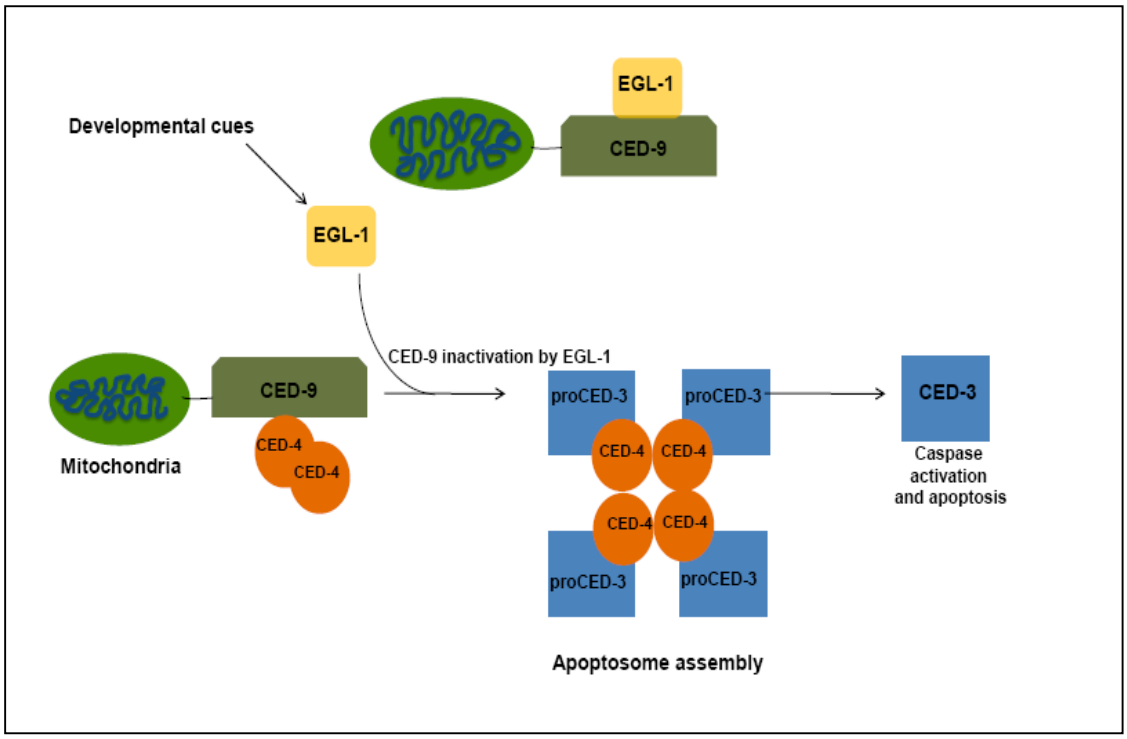

Fig. 2 Programmed cell death in *C. elegans* (modified from Lettre and Hengartner, 2006).

1.1.4.2. Apoptosis in *Drosophila*

The apoptotic machinery is conserved in insects and vertebrates (Riedl and Shi, 2004). Two types of caspases are present in *Drosophila*; i.e. initiator caspases, with autocatalytic activity and effector caspases, which are activated by initiator caspases (Cashio et al., 2005). Seven members of the caspase family, APAF-1-like protein (also called DARK) and two members of the Bcl-2 family (Buffy and Debcl) have been identified in Drosophila. Buffy has an anti-apoptotic function, although it has no obvious BH4 domain (Quinn et al., 2003), and Debcl is a pro-apoptotic protein (Colussi et al, 2000).

Drosophila Inhibitor of Apoptosis Protein-1 (DIAP1) prevents proteolytic activation of caspases. Suppression of DIAP1 is achieved by the pro-apoptotic genes reaper (*rpr*), head involution defective (*hid*) and *grim* products which bind to the DIAP1 to induce its proteolytic degradation (Wing et al., 2002a,b) (see also Fig. 3). Initiation of apoptosis may be induced by a series of upstream factors, such as the p53 gene (Brodsky et al., 2004; Sogame et al., 2003). A protein that activates the apoptotic response after irradiation is DmP53, which is a fly homolog of tumour suppressor gene *p53* (Lu and Abrams, 2006). Dmp53 mutant flies show very little apoptosis response to the X-rays (Lee et al., 2003).

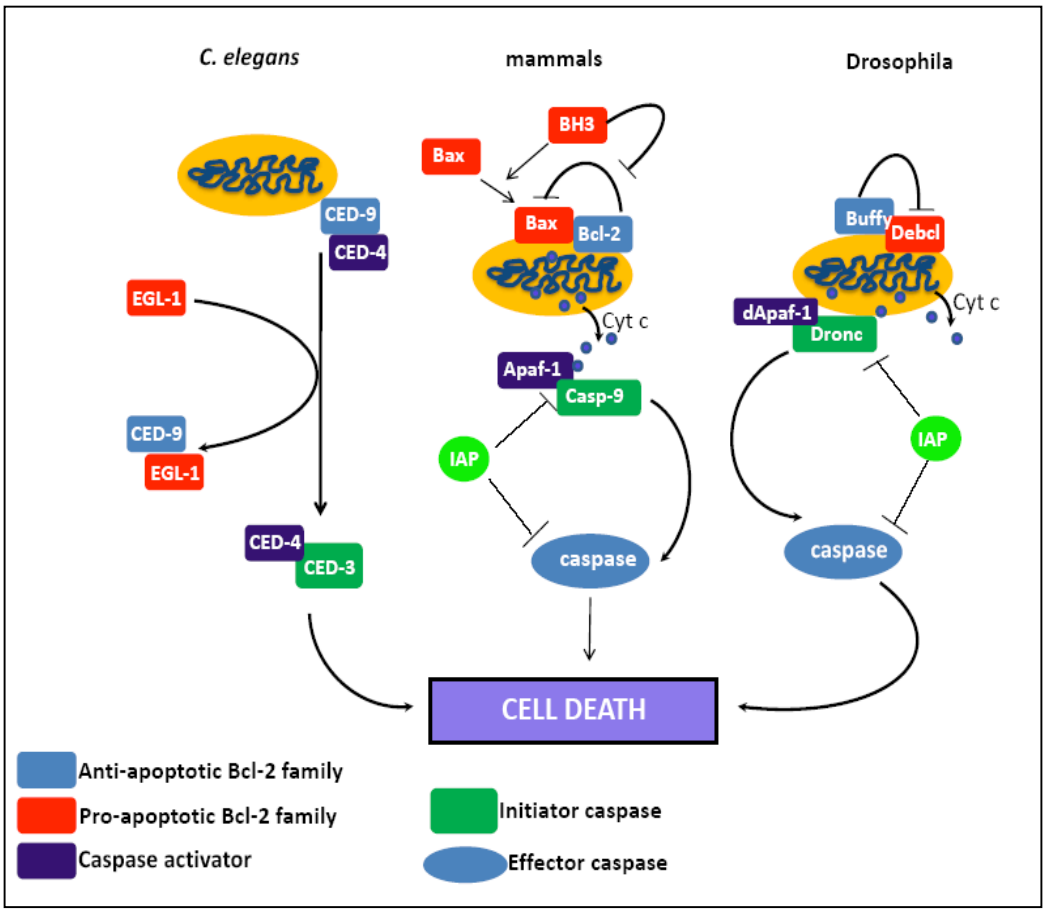

Fig. 3 Apoptosis comparison among nematode, fruit fly and mammals (modified from Colin et al., 2009).

1.2. The transmembrane Bax inhibitor-1 motif containing (TMBIM) protein super family

TMBIM protein family involved in cytoprotection and has highly conserved gene sequences. It is present in all phyla, e.g., animals and even in species where no BCL-2 family members have been identified, including plants, yeast and many viruses (Rojas-Rivera et al., 2012).

Members of this protein family have a hydrophobic nature; therefore they are predicted to be localized in cellular membranes like the endoplasmic reticulum, the Golgi and the plasma membrane. They have six to seven *α*-helix transmembrane domains and no signal peptide was detected at the N-terminus. However, a consensus motif of a protein family UPF0005 is present in the C-terminus of TMBIM protein family members (Reimers et al., 2006). It was reported that seven mammalian proteins/genes belong to this family: GRINA, BI-1/TMBIM6, Lfg/FAIM2, GHITM, RECS1/TMBIM1, GAAP/TMBIM4, and TMBIM1b (Zhou et al., 2008). Based on a detailed sequence and phylogenetic analysis, Hu et al. (2009) suggested that only five of these mentioned proteins form a distinct protein family. Therefore, they have separated the Lifeguard proteins family (Lfg-1_Lfg-5) within the TMBIM super family (Hu et al., 2009). The Lfg family contains these following proteins: GRINA/Lfg-1, FAIM2/Lfg-2, RECS1/Lfg-3, GAAP/Lfg-4, and TMBIM1b/Lfg-5. BI-1 proteins form a distinct group.

Among TMBIM protein members, BI-1/TMBIM6, GRINA/Lfg-1, Lfg-2/FAIM2 and Lfg-4/GAAP have been reported to play a modulatory role in apoptosis (Hu et al., 2009).

In the following the information currently available about Bax inhibitor-1 and Lifeguard proteins reviewed with a focus on their cytoprotective effect and their performance during apoptosis.

1.2.1. Bax inhibitor-1 (BI-1)/TMBIM6 protein family

1.2.1.1. Bax inhibitor-1: Structure and localization

The Bax inhibitor-1 is a small evolutionarily conserved protein (25 to 27 kDa). Proteins similar to BI-1 are present in other eukaryotes, bacteria, and even viruses encode BI-1 like proteins. The human BI-1 contains six α-helix transmembrane domains and both experimental and bioinformatic evidences indicate that its N- and C-terminal are exposed to the cytosol (Xu and Reed, 1998). Subcellular fractionation and immune localization studies showed that BI-1 is located predominantly in the endoplasmic reticulum (ER) and in the nuclear envelope with only a small proportion associated with mitochondrial membranes (Xu and Reed, 1998). The N-terminal parts of BI-1 proteins do not contain any of the characteristic motifs for post-translational modification expected to occur in proteins predicted to reside in the lumen side of ER (Hirokawa et al., 1998), whereas the C-terminal part of BI-1 proteins is highly hydrophilic and well conserved in BI-1 proteins in plants and animals.

The cytoprotective function of BI-1 proteins has been mapped to their C-terminus. This is examined experimentally by deletion or modification of the C-terminal sequences, which lead to the abrogation of the capability of BI-1 to suppress BAX induced cell death in yeast cells (Kawai-Yamada et al., 2004).

Kim et al. (2012) recently demonstrated that the C-terminal deletion of BI-1 does not affect the cellular localization of BI-1 and that both fluorescent images of BI-1 and BI-1 ΔC revealed a similar localization pattern.

1.2.1.2. Mammalian *Bax inhibitor-1*: a testis enhancer gene transcript (TEGT)

The *BI-1* was firstly cloned from adult rat testis and showed high identity (90%) to testis enhanced gene transcript (TEGT) (Walter et al., 1994, 1995) and it was mapped to the human chromosome 12q12–q13. Southern analysis and BLAST searches revealed highly conserved, uncharacterized homologues of the human *BI-1* in all kinds of vertebrates. Two different sizes of *BI-1* transcript have been reported in rat (Chang et al., 1999). They suggested to be resulted from differential initiation of translation and differential splicing and the presence of two alternative polyadenylation sites at the 3□-end.

The *BI-1* expression is assumed to be derived from two alternatives TATA-less promoters resulting in transcripts with different first exons in the 5□-untranslated regions. The distal rat *BI-1* promoter is ubiquitous, whereas the proximal promoter is testis-specific. The most interesting finding relates to the abundance of the short transcript detectable in adult testis of normal adult rats (Chang et al., 1999). Southern analysis represented various stages of spermatogenesis and also the results obtained with the mutant have showed that accumulation of the short transcript occurs mainly in post-meiotic germ cells. Thus, the amount of short transcript is under developmental control in the testis (Chang et al., 1999).

1.2.1.3. The cytoprotective activity of Bax-Inhibitor-1

The BI-1 was originally isolated from a human cDNA library and it is named as Bax-inhibitor because of its ability to suppress cell death induced by the ectopic expression of the mouse Bax gene in yeast (Xu and Reed, 1998). Human BI-1 over expression resulted in tight protection against the intrinsic apoptosis pathway, which is induced by certain types of cell death stimuli, e.g., staurosporine, etoposide and growth factor depletion. Moreover, BI-1 protects certain types of cells against TRAIL, a member of the tumor necrosis factor (TNF) family (Burns and El-Deiry, 2001) but it was not sufficient to protect against Fas (CD95) and TNFα-induced extrinsic apoptotic pathways. Conversely, BI-1 antisense mRNA induced apoptosis in cancer cell lines and suggested that the endogenous BI-1 protein can be important for suppressing apoptosis in some types of tumor cell lines (Xu and Reed, 1998). Moreover, BI-1 antisense RNA transfected into human embryonic kidney 293 cells induced apoptosis, independent of cell stress inducers (Xu and Reed, 1998).

BI-1 cannot physically interact with Bax or Bak but its interaction with Bcl-2 and Bcl-xl through their BH4 domain has been observed by in vivo cross-linking and co-immunoprecipitation experiments. Treating BI-1 overexpressing cells with tunicamycin, an N-linked glycosylation inhibitor (which causes defects in glycoprotein trafficking between ER and Golgi and induced apoptosis by producing ER stress), in the presence of caspase inhibitor zVAD-fmk, did not result in translocation of Bax to the mitochondria (Chae et al., 2004).

In contrast, BI-1 overexpressing cells treated with staurosporine (which is a kinase inhibitor and can induce apoptosis by activation of caspase-3), in the presence of zVAD-

fmk, showed that Bax was translocated to the mitochondria (Chae et al., 2004). These results demonstrated that BI-1 anti-apoptotic activity is caspase-independent and the anti-apoptotic activity of BI-1 is more specific to ER stress inducing agents (Robinson et al., 2011).

Also it is reported that BI-1 plays a key role in the regulation of intracellular ROS, which are highly reactive oxygen containing molecules and cause oxidative stress and induce apoptosis. ROS are produced at the ER by the NADPH-P450 reductase (NPR), phospholipids and the microsomal monooxygenase system composed of cytochrome P450 (CYP) members (Nieto et al., 2002; Robinson et al., 2011). BI-1 is able to regulate ER ROS production in different ways. Firstly, over expression of BI-1 in cells increased activation of the redox-sensitive transcription factor, i.e. Nrf-2 and controls/ increase the expression of different antioxidant enzymes like Heme-Oxygenase-1(see also Fig. 4A). Heme-Oxygenase-1 (HO-1) can block ROS activity and is expressed to counteract ROS accumulation and in this way promoting cell survival (Lee et al., 2007). Also BI-1 can interact through its C-terminus with NPR and destabilization the NPR-CYP2E1 complex and directly inhibit the formation of ROS by blocking electron transfer (Kim et al., 2009) (see also Fig. 4A).

Moreover, BI-1 plays as a role as a regulator of Ca^{2+} in the ER, which is the main cellular store for Ca^{2+}. Release of Ca^{2+} from the ER will determine the sensitivity of cells to apoptosis by determining mitochondrial permeability (Shiraishi et al., 2006) because the inside membrane potential of mitochondria is negative, so they can accumulate Ca^{2+}, which increases their metabolic activity and permeability but excessive Ca^{2+} leads to mitochondrial membrane hyper-permeability and to the influx of cytosolic components

in exchange for mitochondrial proteins, lysis and release of pro-apoptotic proteins (Lebiedzinska et al., 2009). The inositol 3 phosphate receptor (IP3R) and the sarcoplasmic/ER Ca^{2+} ATPase (SERCA) regulate the Cytosolic Ca^{2+} at the ER. Overexpression of BI-1 induced ER Ca^{2+} release and decreased the concentration of Ca^{2+} in the ER (Kim et al., 2008). This approach demonstrated that BI-1 acted as an ER membrane Ca^{2+}/H^{+} antiporter allowing Ca^{2+} release into the cytoplasm (Kim et al., 2008; Ahn et al., 2009). Resting cells have largely monomeric forms of BI-1, which do not act as a Ca^{2+}/H^{+} antiporter but in stressed cell cytosolic acidification is detected by the C-terminus of BI-1 (Ahn et al., 2009), lead to the BI-1 oligomerization and act as a Ca^{2+}/H^{+} antiporter. ER Ca^{2+} release, but also a decrease in cytosolic H^{+} concentration is achieved. This suggests that BI-1 may inhibit apoptosis by decreasing cytosolic (H^{+}) as well as promoting mitochondrial metabolic activity to restore the ATP level and help to restore the neutral pH in cells (Ahn et al., 2010). The binding of Bcl-2 and Bcl-xl to BI-1 brings them in close proximity to IP3R, which blocks the channel, directing the control of the ER Ca^{2+} flux to BI-1 (Rong et al., 2009) and increased the oligomerization and the Ca^{2+}/H^{+} antiporter activity of BI-1 (Ahn et al., 2010) (see also Fig. 4B).

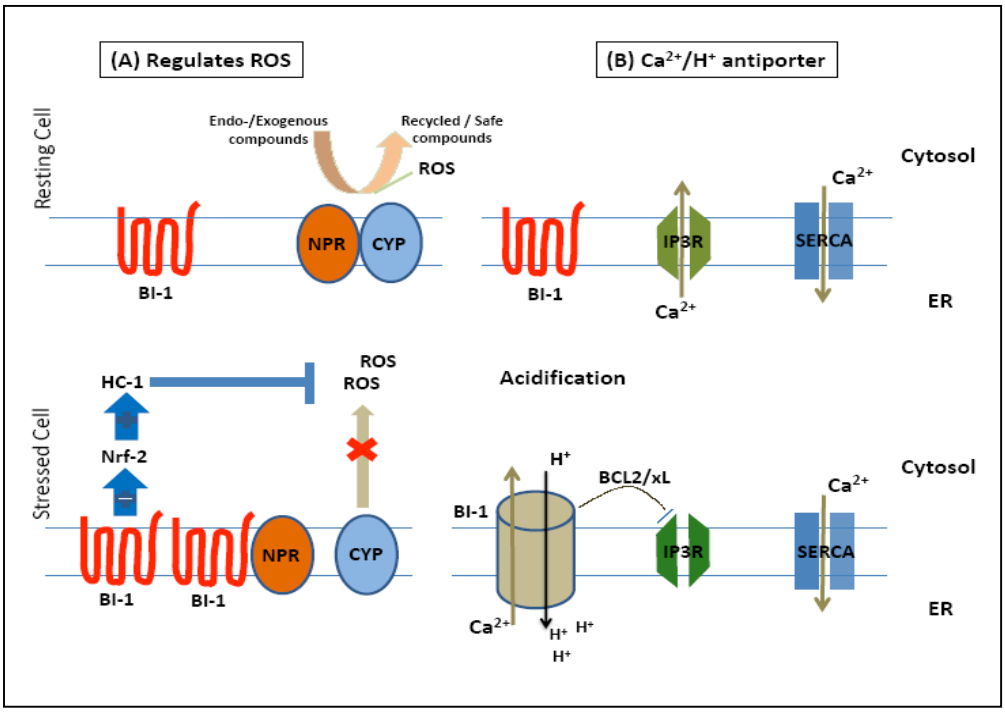

Fig. 4 Comparison of BI-1 functions in the stressed and resting cell. (A) BI-1 function to regulate ROS. (B) BI-1 function in regulation of Ca^{2+} (modified after Robinson et al., 2011).

1.2.1.4. The function of Bax inhibitor-1 in plants

Bax Inhibitor-1 is one of many proteins, which are common to both animals and plants. It is present in several plant species including Arabidopsis, barley, oilseed rape, rice and tobacco (Kawai-Yamada et al., 2001; Bolduc et al. 2003; Watanabe and Lam 2006). Arabidopsis BI-1 (AtBI-1) fused to GFP is observed at the ER and the nuclear envelope (Kawai-Yamada et al., 2004). BI-1 orthologs of rice and Arabidopsis can protect transgenic plants against cell death induced by ectopic expression of mammalian Bax (Kawai-Yamada et al., 1999; Sanchez et al., 2000). BI-1 overexpression increased the resistance to fungal pathogens in barley, probably due to its cell death-suppressive effects (Huckelhoven, 2004) and down regulation of BI-1 in anti sense experiment in tobacco BY-2 cells results in accelerated cell death upon carbon starvation (Bolduc and

Brisson, 2002). Several plant ROS scavenging enzymes such as Fe-SOD, GST, APX and PHGPx were isolated as “Bax Inhibitors” from a cDNA library screen using the yeast-Bax survival screen (Chen et al., 2004). This implies that ROS accumulation is necessary to mediate Bax-dependent cell death pathway in yeast. ROS generated by stresses can act as mediators that function at the early stage in signal transduction, stress response and programmed cell death (Lam, 2008) however, in some cases stress-induced ROS was not significantly changed in BI-1 over expressing cells (Kawai-Yamada et al., 2004). Thus, plant BI-1 may function downstream of the early steps of ROS-dependent cell death pathway. These results suggested that the function of plant homologues of BI-1 seems to be conserved in their human counterparts (Kawai-Yamada et al., 1999; Sanchez et al., 2000). It should be mentioned that plant genes for cell death suppressors such as Bcl-2/ Bcl-xl from humans and ced-9 from *C. elegans* are not found in the Arabidopsis genome (Lam et al., 2001). Therefore, the cell death pathway controlled by BI-1, is evidently Bcl-2/Bax independent in yeast and plants. In conclusion, *BI-1* represents the first endogenous gene to be identified that regulates cell death in both plant and animal cells. Therefore, studying the mechanisms of cell death suppression by BI-1 may help to uncover the ancient ‘core’ program in eukaryotes that is used to determine cell suicide activation.

1.2.2. The Lifeguard (Lfg) protein family

1.2.2.1. Lfg: Structure and function

The Lifeguard protein family belongs to the TMBIM super family and they are defined with seven α-helix transmambrane domain structures with a set of three conserved sequence motifs within their cytoplasmic segments. The distinct Lfg family is very ancient within eukaryotes and widely distributed.

The human Lifeguard protein family has been reported to consist of five members; i.e. GRINA/Lfg-1, FAIM2/Lfg-2, RECS1/Lfg-3, GAAP/Lfg-4, and TMBIM1b/Lfg-5.

Among these members, GRINA/Lfg-1, Lfg-2/FAIM2 and Lfg-4/GAAP have cyto-protective function during apoptosis (Hu et al., 2009). As mentioned before, the Lfg family names were introduced by Hu et al. (2009) and all family members had been reported in previous literature with different names. Figure 5 shows the structure of four members of the TMBIM protein super family; i.e. TMBIM6/BI-1, TMBIM2/FAIM2 and TMBIM4/GAAP (two members of Lifeguard proteins) and GHITM (The Growth-hormone inducible transmembrane protein).

The available information of each Lfg protein family members is reviewed in the following;

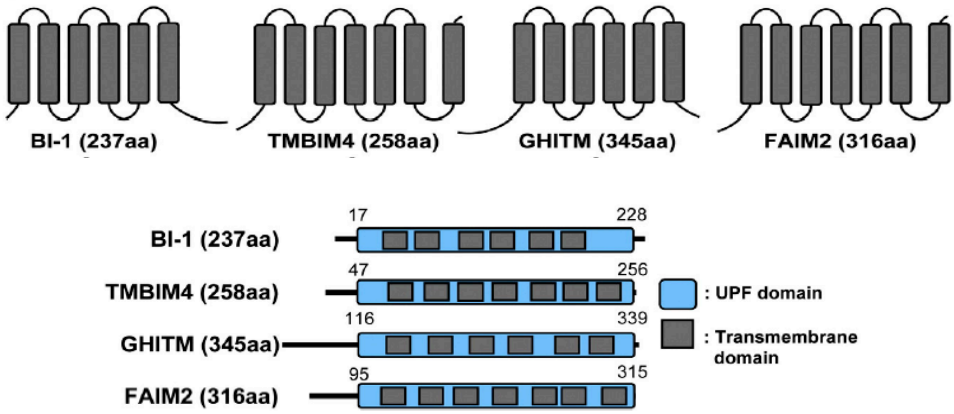

Fig. 5 Comparison of the primary structure of TMBIM6/BI-1, GHITM and two members of Lifeguard proteins (TMBIM2/FAIM2 and TMBIM4/GAAP) (Kim et al., 2012).

1.2.2.2. Lifeguard protein family members

1.2.2.2.1. Lifeguard-1

Lfg-1 is the TMBIM3 member of Bax inhibitor motif containing superfamily and also known as Glutamate binding protein (GBP), Glutamate receptor, ionotropic N-Methyl-D-Aspartate associated protein (GRINA), Putative MAPK-activating protein (PM02) and NMDARP-71 (71 kDa NMDA receptor protein) in the literatures. It was originally identified as a glutamate-binding subunit (GBP) and localized to the neuronal cell bodies and dendrites in brain and spinal cord (Pal et al., 1999). Moreover, chronic exposure to ethanol increased its expression significantly in the cerebellar granule cells and cortical neurons (Bao et al., 2001). It was also reported as a putative MAPK activating protein (PM02) which is a possible protector of retinal damage in ischemia-perfusion injury (Matsuda et al., 2003).

Recently, Rojas-Rivera et al. (2012) revealed the expression pattern of TMBIM3 and demonstrated it was co-localized with ER and Golgi markers. The immunoprecipitation experiments confirmed the physical interaction between TMBIM6/BI-1 and TMBIM3/Lfg-1, indicating that they do form part of a protein complex (Rojas-Rivera et al., 2012). They have shown that targeting Lfg-1 with small hairpin RNA (shRNA) in BI-1 wild-type cells did not cause significant spontaneous cell death but in sharp contrast, knocking down Lfg-1 in BI-1 knockout cells led to the appearance of cell death features. Moreover, it is shown that Lfg1 knocking down increased the susceptibility of BI-1 knock out cells to ER stress-inducing agents. Lfg-1/B1-1 double deficiency could not affect the induction of apoptosis triggered by extrinsic and intrinsic stimuli; i.e. staurosporine, etoposide, TNF-α or nutrient deprivation.

It has been revealed that ER stress triggers the up-regulation of TMBIM3/Lfg1 through a PERK-dependent mechanism (Hetz et al., 2011). The PERK pathway is a branch of unfolded protein response (UPR) signaling and regulates apoptosis-related genes by the induction of the transcription factor ATF4 and play a crucial role in cell survival under ER stress conditions (Hetz et al., 2011). Moreover, TMBIM3/Lfg-1 negatively modulates the release of ER calcium by IP3R (inositol 1,4,5-triphosphate receptor), decreasing the sensibility of cells to ER stress and calcium mediated cell death. Finally they have concluded that, TMBIM3/Lfg-1 and TMBIM6/BI-1 have synergistic function for anti-apoptotic activity and modulate ER calcium homeostasis associated with physical interactions with inositol trisphosphate receptors (see also Fig. 6).

Loss-of-function studies in *Drosophila* and zebrafish revealed that Lfg-1 and BI-1 expression have synergistic anti-apoptotic activities and strong protective effects against ER stress in *Drosophila.*

Similarly, TMBIM3/Lfg-1 deficiency increases the rate of apoptosis during development in zebrafish. Therefore, manipulation of TMBIM3/Lfg-1 levels in zebrafish embryos shows their essential role in the regulation of apoptosis during neuronal development and in experimental models of ER stress. These results indicate that a conserved group of cell death related regulators is present across species beyond the Bcl-2 family and operating at the ER membrane.

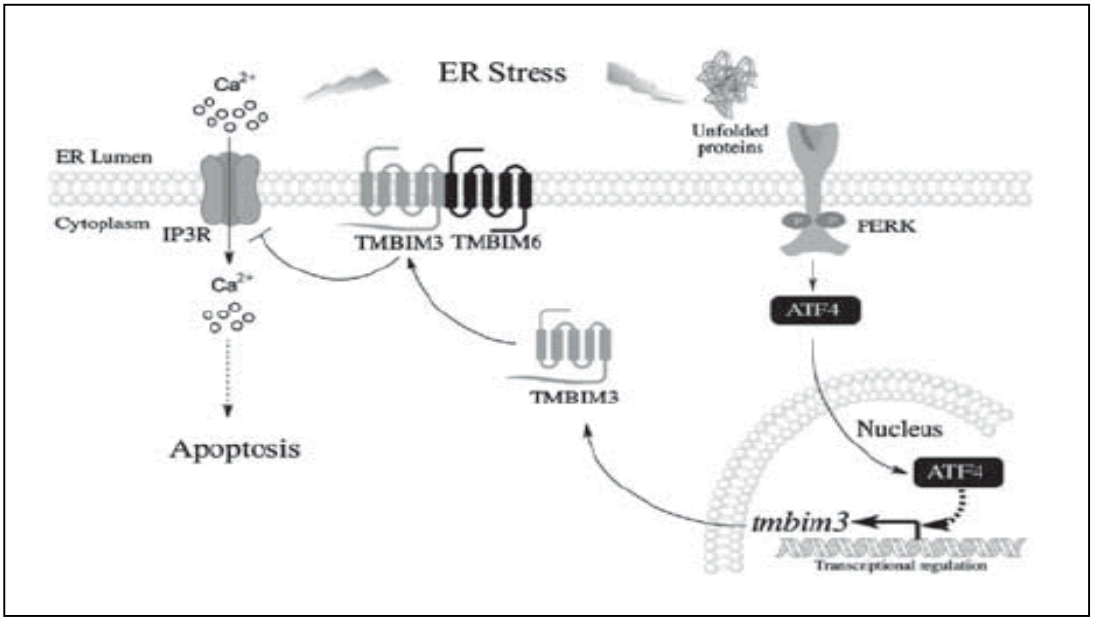

Fig. 6 TMBIM3/Lfg-1and TMBIM6/BI-1 synergistic effects to regulate ER- stress induced apoptosis (Rojas-Rivera et al., 2012).

1.2.2.2.2. Lifeguard-2

Lfg-2 also known as Mammalian Lfg protein and TMBIM2, was first identified as a 35 kD rat neural membrane protein (NMP35) (Schweitzer et al., 1998). It has seven α-helix

transmembrane domains and is expressed in most tissues, except spleen and placenta, being highly abundant in the brain (Somia et al., 1999; Hu et al., 2009).

It has shown that Lfg-2 coimmuno-precipitates with the pro-apoptotic protein Bax. Lfg-2 was identified as a molecule that uniquely inhibits death mediated by Fas, but not other tumour necrosis factor receptor family members (Somia et al., 1999). Therefore, it is characterized as a human Lifeguard protein (Lfg) and Fas apoptotic inhibitory molecule 2 (Faim 2).

Fas and its ligand (FasL), are expressed by the developing cerebral cortex and other differentiating neural cells (Raoul et al., 1999), which suggests that neural cells may actively communicate apoptotic signals to each other. Moreover, Fas is expressed in neurological disease conditions, e.g., Alzheimer's disease, ischemia, multiple sclerosis (Martin-Villalba et al., 1999; Northington et al., 2001; De La Monte et al., 1997; Morishima et al., 2001). Transient expression of Lfg in HeLa cells shows that it is membrane associated and binds to Fas but the molecular mechanism of apoptosis regulation executed by Lfg is not clear, because no down regulation of Fas and no interference with the formation of the Fas/FADD complex were detected (Somia et al., 1999). It is demonstrated that, Lfg protein expression is up-regulated during the maturation and differentiation of CGNs and inhibition of Lfg expression by treatment with a Lfg-siRNA sensitized differentiated CGNs to FasL-induced cell death and caspase-8 activation (Beier et al., 2005).

These observations are related to the other experiment that FasL-mediated apoptosis of CGNs was regulated by phosphatidylinositol 3-kinase-Akt/PKB pathway (Beier et al., 2005). Activation of the phosphatidylinositol 3-kinase-Akt/PKB pathway protects cells

from apoptosis through different pathways such as phosphorylation of proteins like Bax or caspase-9 (Datta et al., 1999), and significantly, expression of Lfg in rat CGN cells (Beier et al., 2005).

1.2.2.2.3. Lifeguard-3

Lfg-3 is also known as TMBIM1 and RECS1 (Responsive to centrifugal force and shear). It has phydrophobic structure of seven α-helix transmambrane domains and localized to endosomal and lysosomal membranes and expressed in all tissues tested except for thymus, spleen and testis (Hu et al., 2009). There were no obvious abnormalities in the young Lfg-3 knockout mice (Zhao et al., 2006a) but in the aged mice the deficiency caused cystic medial degeneration and aortic dilatation. It is hypothesized that Lfg-3 play protective roles in vascular remodeling (Zhao et al., 2006b).

1.2.2.2.4. Lifeguard-4

Lfg-4 is known as a TMBIM4 and identified in humans, as a Golgi anti-apoptotic protein (GAAP) and also identified as a candidate house-keeping gene (Lee et al., 2007; Gubser et al., 2007). It is reported that TMBIM6/BI-1 and TMBIM4/GAAP negatively modulate ER calcium release and have an anti-apoptotic activity. Lfg-4 is an extremely conserved protein and the viral and human counterparts are sharing 73% sequence identity (Hu et al., 2009). It is localized on the Golgi membrane under physiological conditions, but also present in the ER and nuclear membrane when overexpressed (Gubser et al., 2007). GAAPs proteins are predicted to have seven helix transmembrane domains and they are expressed in all 16 human tissues tested, and are essential for cell viability. They show similarity to the Bax inhibitor-1 with whom they share 28%

identity (45% similarity) and also share 34% identity (51% similarity) with the Lfg-2 protein which protect cells from Fas-mediated cell death in the brain and other tissues (Gubser et al., 2007).

Moreover, it was shown that Lfg-4 can inhibit apoptosis induced by intrinsic pathway activators; i.e. Bax, staurosporine, cisplatin and doxorubicin. In addition, it was able to inhibit apoptosis induced by extrinsic stimuli such as Fas and TNF at a step downstream of caspase-8 activation (Vant et al., 2002). The mechanism by which GAAPs inhibit apoptosis remains to be determined. The ability of GAAPs to inhibit cell death induced by various instrinsic apoptotic stimuli suggests strongly that it acts at a step in the mitochondrial pathway. The finding that human-GAAP suppressed TNF-induced apoptosis at a step downstream of caspase-8 activation is consistent with this idea. Extrinsic and siRNA inhibition of human GAAP expression caused apoptosis also the human Lfg-4 gene was up-regulated in some breast tumors (Vant et al., 2002).

A significant increase in the expression of Lfg-4 was shown in marine diatoms when exposed to the benzo(a)pyrene (BP) (Carvalho et al., 2011a,b), which is a pro-carcinogen. PAHs (polycyclic aromatic hydrocarbon) substances and the enzymatic metabolism of BP will produce benzo(a)pyrene diol epoxide, a molecule which intercalates with DNA by covalently bonding to the nucleophilic guanine nucleobases at the N2 position. It has been linked with mutagenic and carcinogenic effects among others in exposed organisms (Carvalho et al., 2011a,b).

1.2.2.2.5. Lifeguard-5

Lfg-5 is found exclusively in eutherian mammals and not in invertebrates (Hu et al., 2009). This protein was reported as Tmbim1b because of its similarity with Tmbim1/Lfg-3 in the sequence. In mouse it is expressed only in adult testis (Hu et al., 2009).

1.2.3. The Phylogeny of Lifeguard protein family

The previous phylogenetic analysis by Hu et al. (2009) showed that duplications of a Lfg-1 ancestor lead to the derivation of Lfg-2 and Lfg-3 lineages in vertebrates but the origin of the mammalian Lfg-5 is not clear. They proposed that Lfg-5 has equally diverged from Lfg-2 or Lfg-3. The Lfg-5 has testis-restricted expression, which indicates that it is likely to be recent and possibly under sexual selection (Hu et al., 2009). The Lfg-1i (invertebrates) gene was originally identified in invertebrates like mollusk, sea anemone, amphioxus, and purple sea urchin and only ray-finned fish have an Lfg1i gene among vertebrates. It was proposed by Hu et al. (2009) that Lfg-1 and Lfg-1i derived genes appear in metazoans but not in all phyla, e.g. not in Platihelminthes, Annelids and Hemichordata and the ancestor of Lfg-4 duplicated to generate the ancestral Lfg-1 and/or Lfg-1i prior to the Metazoan divergence (see also Fig. 7).

Distribution of identified Lfg protein members in different taxonomical representatives are shown in (Table 1). It is proposed that there is correlation between the evolutionary appearance of the animal Lfg genes and their expression profiles and sub-cellular re-localization (Hu et al., 2009). For instance in fruit fly there are five Lfg-1i homologues (lfg-1ia to Lfg-1ie) and their expression profile show increasing specificity (Hu et al., 2009). Furthermore, different localizations are also found in vertebrates. For instance, in

mouse, Lfg-4 is found in Golgi; Lfg-1 and Lfg-2 are on the plasma membrane; and Lfg-3 in endosomes/lysosomes and Lfg-5 is testis but the sub-cellular localization of this protein is unknown in the cells (Hu et al., 2009).

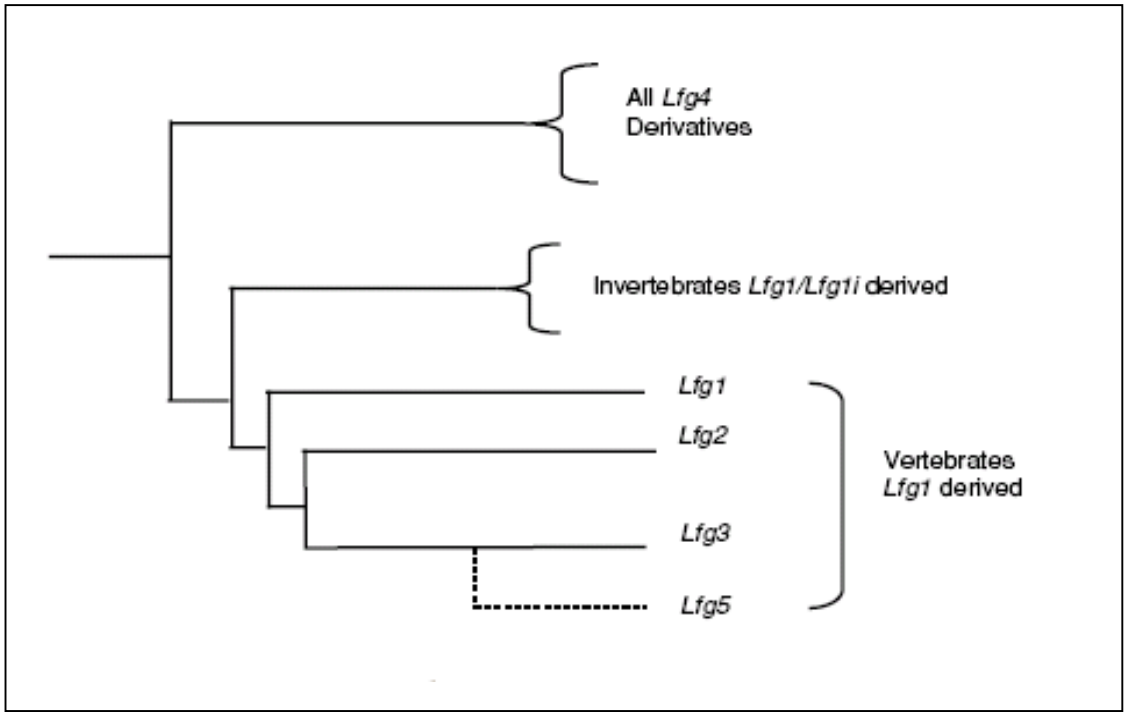

Fig. 7 Phylogenetic analysis of lifeguard protein among vertebrates and invertebrates (Hu et al., 2009).

Table 1. Number of distributed identified lifeguard protein in taxa (Modified after Hu et al., 2009)

Genome	Common name	Lfg4-drived	Lfg1/Lfg1i-drived	Phylum
Mus musculus	Mouse	1	4	Chordata
Gallus gallus	Chicken	1	2	Chordata
Xenopus tropicalis	Frog	1	3	Chordata
Eptatretus burgeri	Hagfish	1	0	Chordata
Ciona intestinalis	Sea squirt	1	1	Chordata
Lottia gigantea	Owl limpet	1	2	Mollusca
Nematostella vectensis	Sea anemone	1	2	Cnidaria
Caenorhabditis elegans	Nematode	1	3	Nematoda
Schmidtea mediterranea[a]	Flatworm	1	1	Platyhelmint hes
Schizosaccharomyces pombe	Fission yeast	1	0	Ascomycota
Chlamydomonas reinhardtii	Green algae	0	2	Chlorophyta
Cyanidioschyzon merolae	Red algae	1	0	Rhodophyta

1.3. *Hydra* as a model organism

1.3.1. Systematic and morphology of *Hydra*

Hydra is a small freshwater Cnidarian polyp with a simple body structure. It belongs to the phylum Cnidaria and is placed at the base of metazoans in the phylogenic tree (Table 2 and Fig. 8A). There are special cells in this taxon, which are used to capture prey and also for defense. They are called nematocytes or cnidocytes and the name of Cnidaria refers to these unique cells. *Hydra* has a hypostome with a mouth opening and tentacles on its apical end and a peduncle with special cells that secrete mucous glue from the foot to attach the animal to the substratum at the basal end of the body column

(Fig. 8B) (Hoffmeister and Schaller, 1985). The body structure consists of two cell layers, endoderm and ectoderm with an acellular mesoglea in between. *Hydra* normally reproduces asexually by budding, during which the cells arising from the proliferation zone on the body column are deposited into the bud and then the bud develops a hypostome and tentacles. Finally with formation of the peduncle the bud detaches from the adult body column. In some environmental conditions, like starvation and cold weather, *Hydra* undergoes sexual reproduction by formation of Gonads.

Table 2. Taxonomical classification of Genus *Hydra* (Colins, 2002).

Kingdom	Animalia
Subkingdom	Eumetazoa
Phylum	Cnidaria
Subphylum	Medusozoa
Class	Hydrozoa
Subclass	Leptolinae
Order	Anthomedusae
Suborder	Capitata
Family	Hydridae
Genus	***Hydra***

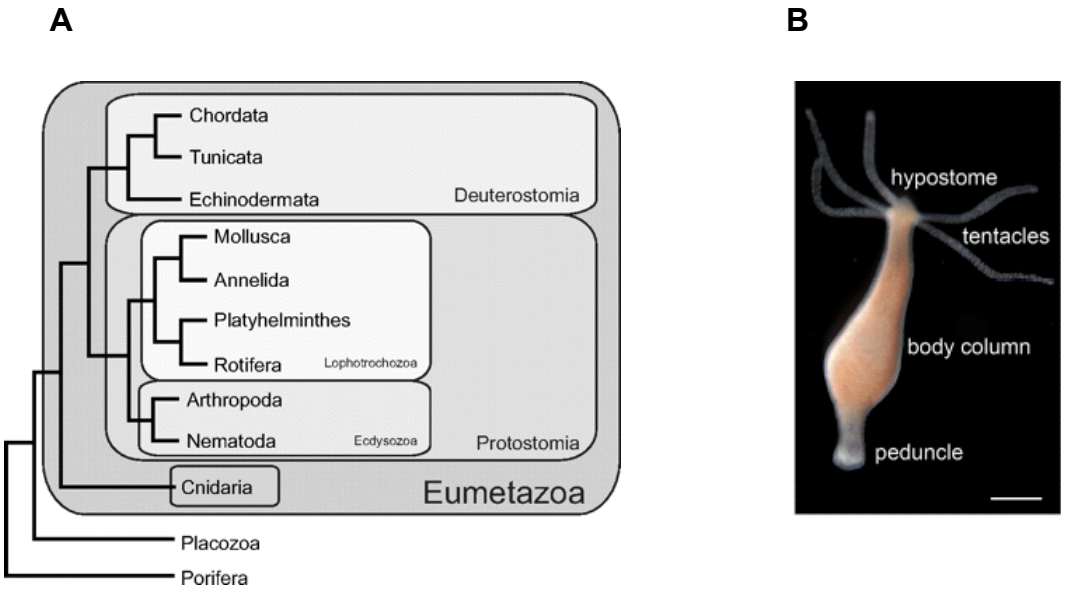

Fig. 8 Position of Phylum Cnidaria in phylogenic tree (Harcet et al., 2010) (A); *Hydra* polyp body pattern (B), Scale bar 100 µm (Böttger and Alexandrova, 2007).

1.3.2. Apoptosis in *Hydra*

1.3.2.1. History and function of programmed cell death in *Hydra*

It is now clear that phagocytosis occurs in *Hydra* and epithelial cells can phagocytose other cells of the animal (Campbell, 1976; Bosch and David, 1984; reviewed in Böttger and Alexandrova, 2007). Moreover, phagocytosis is involved in the regulation of cell numbers in response to the variation of food supply in adult tissue, in eliminating excess cells during embryogenesis; maintain cellular homeostasis and regeneration (Bosch et al., 1984; Cikala et al., 1999; reviewed in Böttger and Alexandrova, 2007). In 1999, phagocytosed cells in *Hydra* were qualified as apoptotic because of the similarity to apoptotic morphology and the expression of two genes with strong homology with caspase-3 family (Cikala et al., 1999). Recently, it was revealed that apoptosis signaling

pathway is very well conserved in hydra and it is comparable with that in higher animals like mammals and it even shows higher complexity in comparison with other invertebrate's model organisms, e.g., *Caenorhabditis* and *Drosophila* (Lasi et al., 2010). The apoptosis pathway plays an important role during development of *Hydra* and is involved in spermatogenesis and oogenesis (Kuznetsov et al., 2001; Technau et al., 2003). When *Hydra* is faced with irradiation or treated with cytostatic agents, cell death will occur mainly in the interstitial cells because they have the highest rate of cycling (David and Gierer, 1974). Moreover, apoptosis was observed in interspecies grafts between two species of *Hydra* which indicates that *Hydra* cells are capable to recognize and remove non-self tissue that can be considered as a primitive immune response (Bosch and David, 1986).

1.3.2.2. The molecular cell death machinery in *Hydra*

By search in the whole *Hydra* genome and ESTs sequences, all components of the molecular cell death machinery have been identified in *Hydra* and Lasi et al. (2010) showed experimentally that Bcl-2 and caspase family genes are expanded in *Hydra* and functionally participate in apoptosis. They have found 17 sequences with homology to caspases and 15 of them have putative active-site sequences with conserved histidine and cysteine residues (Lasi et al., 2009).

Two Bak-like proteins and seven Bcl-2-like have been described in *Hydra* and in all of those genes a conserved intron is present which is splitting the coding region of the BH2 domain. This intron is present in human and invertebrate Bcl-2 genes (Herberg et al., 1998) and indicates that the *Hydra* genes are orthologs of the mammalian Bcl-2 genes.

Hydra Bcl-2 family proteins are localized in the mitochondrial except for HyBcl-2-like 7, which is localized to ER-like structures in the cytoplasm and not to mitochondria.

To test their anti-apoptotic effect, they were expressed in HEK 293T cells and then apoptosis was induced with camptothecin (Topo-I inhibitor). Results indicated that all HyBcl-2-like proteins have some protective effect and HyBcl-2-like 4 showed the strongest inhibition of camptothecin-induced apoptosis. A mutation (L256E) of the conserved leucine residue in the BH3 domain of HyBcl-2-like 4 abolished the protective effect. It has been shown that HyBak-like 1 and 2 strongly induced apoptosis when expressed in mammalian cells. Moreover, IAPs (caspase inhibitors) and also components of an extracellularly regulated apoptotic pathway such as TNF-R and FADD have been found in *Hydra.*

Four *Hydra* gene models encoding novel proteins with conserved BH3 domains described and HyBH3-only 2 interacted with Bcl-2-like 4 in the yeast two-hybrid system and induced apoptosis in mammalian cells. In Figure 9 the *Hydra* apoptosis pathway demonstrated and it shows that this is an evolutionary conserved pathway (Modified from Lasi et al., 2010).

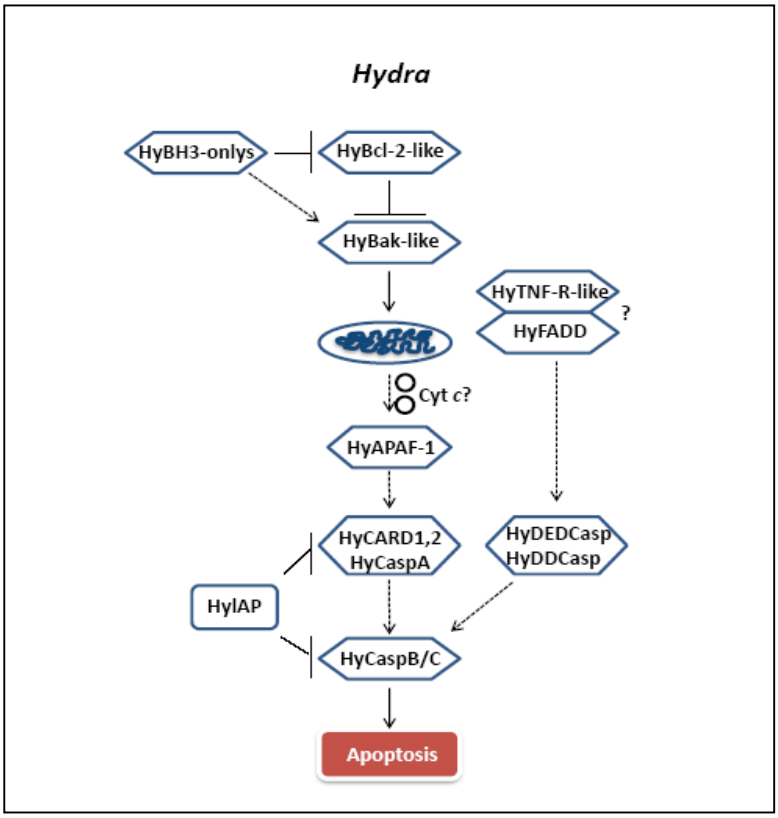

Fig. 9 Apoptotic molecular pathways in *Hydra*, Dotted lines indicate that links have not yet been shown experimentally (modified from Lasi et al., 2010).

1.3.3 Apoptosis is involved in *Hydra* gametogenesis

1.3.3.1. Spermatogenesis

The differentiation pathway and structure of the mature spermatozoa in hydra resemble that of higher metazoans (Kuznetsov et al., 2001). In higher animals germ cell proliferation and differentiation is regulated by programmed cell death and a complex network of endocrine and paracrine signals (Print and Loveland, 2000). Moreover, apoptotic proteins such as caspases are involved during spermatid individualization of *Drosophila.* Caspases inhibitors caused male sterility by preventing the removal of bulk cytoplasm in spermatids and blocked sperm maturation. Loss-of-function experiments in

the *Drosophila* cyt-c gene caused to block of caspase activation and subsequent spermatid terminal differentiation (Arama et al., 2003).

Both sperm and egg differentiation in *Hydra* are triggered by environmental signals such as low temperatures or starvation. Germ cells in *Hydra* derive from multipotent interstitial stem cells (Bosch and David, 1986). In male polyps testis is made of ectodermal epithelial cells and spermatogenesis is initiated by accumulations of interstitial cells within the intercellular spaces of ectodermal epithelial cells (Tardent, 1974; Kuznetsov et al., 2001). Between the mesoglea and the outer epithelial wall, the various cell layers represent the succession of the different stages of spermatogenesis (Brien, 1966; Kuznetsov et al., 2001).

Acridine orange staining of mature male *Hydra vulgaris* polyps indicates a large number of apoptotic cells within the testes and also apoptosis in testes was confirmed by using a TUNEL assay which identified nuclei with clear signs of DNA fragmentation (Fig. 10) (Kuznetsov et al., 2001). TUNEL assay shows that most of the stained nuclei are located in the peripheral parts of the testes and represent early stages of spermatogenesis. However, acridine orange positive cells are not restricted to distinct regions of the testis. This indicates that all cells and cell fragments which are contained in phagocytic vacuoles can be stained with acridine orange while the TUNEL assay specifically labels cells that are undergoing apoptosis, independent of whether the cells are in vacuoles or not. Alternatively, DNA fragmentation is a rapid process and this difference in the staining pattern could indicate that the acridine orange positive phase of apoptosis is longer than the TUNEL positive phase (Kuznetsov et al., 2001).

Ectodermal epitheliomuscular cells are involved in the formation of the outer wall of the testis during spermatogenesis (Tardent, 1985) and it was shown that ectodermal epithelial cells play an active role during spermatogenesis as phagocytes. Recent evidence from genetically modified mice shows that control of male germ cell apoptosis is mediated by signals derived from the Sertoli cells (Print and Loveland, 2000). It is shown that in *Hydra* testis, the epithelio-muscular cells function like Sertoli cells by controlling the microenvironment of the developing interstitial sperm precursor cells and sequestering the sperm precursor populations into environmentally distinct compartments. Moreover, epithelial cells in *Hydra* produce specific factors, which are necessary for interstitial cell differentiation, e.g., epithelial cells directly influence the nerve cell differentiation pathway by secreting PW peptides in *Hydra*. The PW family peptides, which are also termed as an epitheliopeptides, mediate communication between epithelial cells and neurons to maintain a specific density of neurons in *Hydra* (Takahashi et al., 2000).

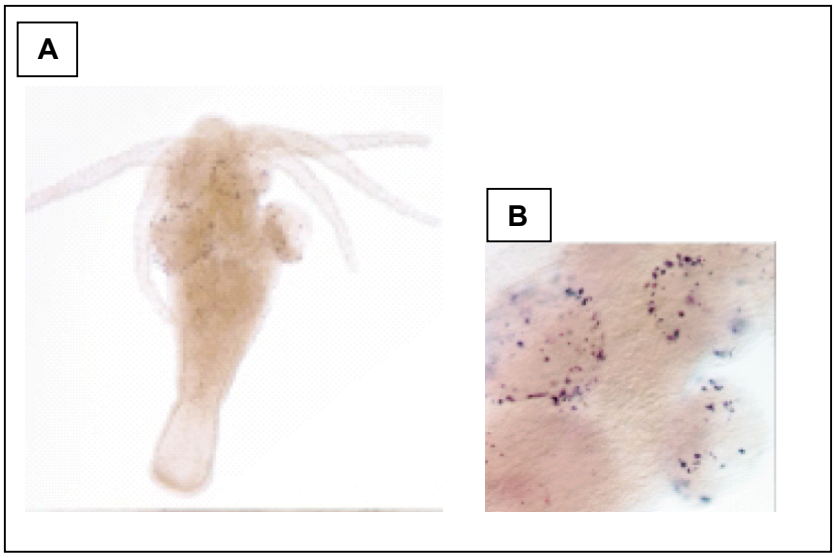

Fig. 10 Whole-mount detection of DNA fragmentation in male *Hydra vulgaris* by the TUNEL method (A); Higher magnification shows numerous TUNEL positive nuclei at the periphery of each testis (B) (Kuznetsov et al., 2001).

1.3.3.2 Oogenesis

Normally during oocyte development, the nurse cells undergo apoptosis and are finally phagocytosed by the oocyte (McCall and Steller, 1998; Technau et al., 2003) for instance in *C. elegans* half of the female germ cells are engulfed by the surrounding sheath cells (Gumienny et al., 1999). Apoptosis is involved in maintenance of germ line homeostasis because full-size oocytes cannot form in the apoptosis-deficient mutant's *ced-3* and *ced-4* for instance in *C. elegans* (Matova and Cooley, 2001; Gumienny et al., 1999). Moreover, in *Drosophila,* the caspase-3-deficient mutant *dcp-1* cannot form fertile oocytes (McCall and Steller, 1998; Technau et al., 2003). Apoptosis of nurse cells in *Drosophila* is involved in regulating the intracellular rearrangements of the actin cytoskeleton, which are important for the transfer of cytoplasmic components into the oocyte (De Cuevas and Spradling, 1998).

Figure 11 shows a schematic comparison of oogenesis among *Hydra*, *C. elegans* and *Drosophila*. The common feature is that multiple germ cells contribute cytoplasm to the oocyte. For instance, in *C. elegans,* nuclei proliferate in a syncytium and their gene products contribute to form a common mass of cytoplasm. Apoptosis occurs in the half of these nuclei and the remaining nuclei enclose units of the common cytoplasm to form oocytes (Gumienny et al., 1999). In Drosophila germ cells in the ovariole transfer cytoplasm through ring canals to the growing oocyte (Mahajan-Miklos and Cooley, 1994) and in the *Hydra* egg patch, 4000 germline cells contribute to the growing oocyte. These cells fuse with the oocyte and transfer cytoplasm to it before undergoing apoptosis. The *Hydra* oocyte has a size of nearly 600 µm, *Drosophila* oocyte is roughly 150 µm and a *C. elegans* oocyte is about 50 µm in diameter. These differences between oocyte sizes correlated with the number of germ cells contributing to each oocyte. Therefore, alimentary oogenesis represents a strategy to rapidly produce egg cytoplasm by utilizing multiple germline nuclei to synthesize it.

In the case of nurse cells differentiation, they are derived from germline cells, which undergo a premeiotic S-phase prior to differentiation. Some of these nurse cells in *Hydra* (GCIV) and two cells in *Drosophila* initiate meiosis (De Cuevas et al., 1997), while all nuclei in *C. elegans* reach meiotic pachytene. At this stage, nurse cells undergo apoptosis and are phagocytosed. At this time point, MAPK pathway activation is involved in C. elegans and mutants in MAPK do not exit pachytene and do not undergo apoptosis (Gumienny et al., 1999). It is not clear yet, if similar signaling pathway regulates apoptosis in *Hydra* and *Drosophila*. The fate of nurse cells is different in these mentioned invertebrates. As shown in Figure 11, *C. elegans* and Drosophila nurse cells are phagocytosed by somatic tissue (sheath or follicle cells) surrounding the oocyte

(Gumienny et al., 1999; Nezis et al., 2000) but in *Hydra* are phagocytosed by the oocyte itself (Alexandrova et al., 2005).

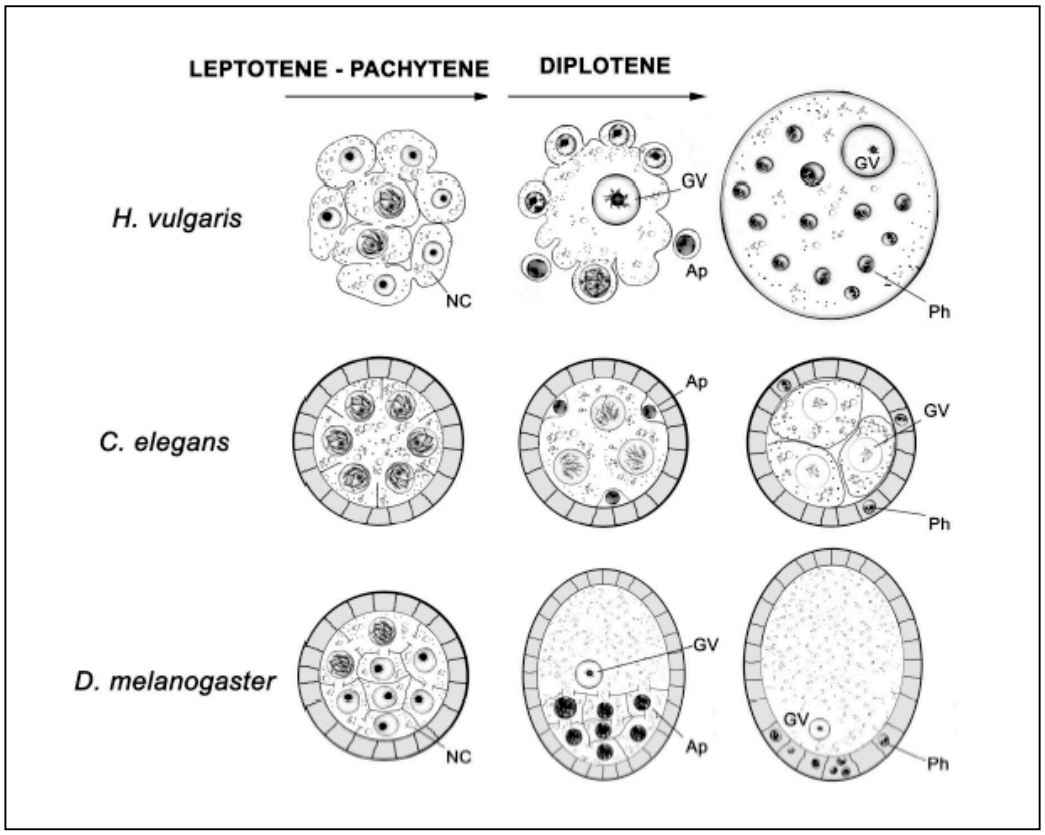

Fig. 11 Comparation of oogenesis among *hydra*, fruit fly and *C. elegans* (Alexandrova et al., 2005).

In *Hydra,* Oocyte precursors are derived from interstitial stem cells (Bosch and David, 1986) and Oocyte development begins in a so-called egg patch in the ectoderm layer of body column and progressively thickens over 6 days and rapidly contracts to form the oocyte (Fig. 12). Every egg patch contains 4000 germ cells but only one oocyte is formed per egg patch and the rest of germ cells differentiate as nurse cells (Miller et al., 2000; Alexandrova et al., 2005). Nurse cells directly transfer cytoplasm through specialized ring canals to the developing oocyte and then undergo apoptosis and are phagocytosed by the oocyte (Nezis et al., 2000; Alexandrova et al., 2005).

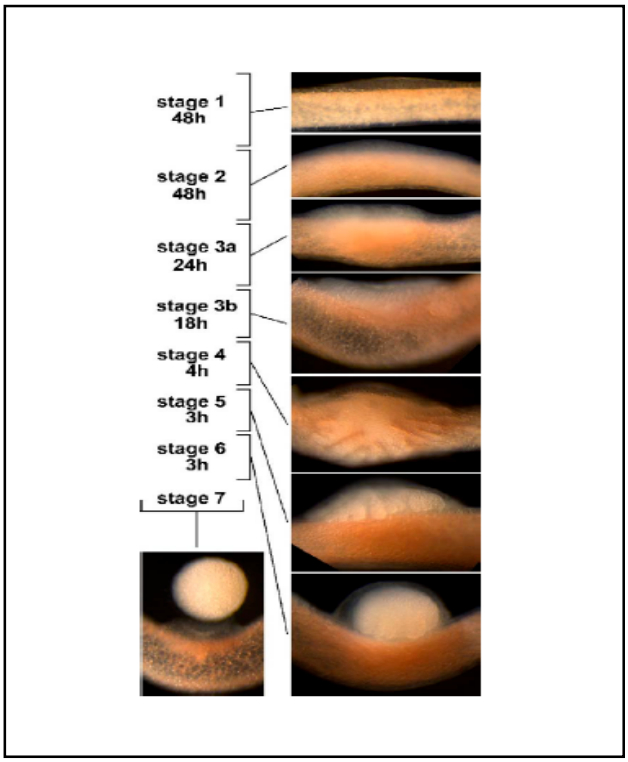

Fig. 12 Stages of oogenesis in *Hydra vulgaris* (from Alexandrova et al., 2005).

Progressive stages of differentiation in *Hydra* germ cells (GC) during oogenesis are shown with GCI, GCII, GCIII and GCIV according to their morphology (Fig. 13) (Alexandrova et al., 2005). GCI cells are in a postmitotic G1 phase and turn into GCII cells when entering a pre-meiotic S-phase and increasing their cytoplasmic and nuclear volume. GCII cells complete DNA replication and turn into GCIII cells, which have 4n DNA content. GCIII cells increase dramatically in size with the synthesis of numerous cytoplasmic vacuoles and granules (Aizenshtadt, 1975; Honegger et al., 1989) there after about 1% of GCIII cells enter prophase I of meiosis (leptotene to pachytene) thus becoming GCIV cells and only Two to three GCIV cells develop into diplotene oocytes. These cells increase dramatically in size from 100 µm to 600 µm due to transfer of cytoplasm from adjacent nurse cells and then they fuse into one cell. The surrounding GCIII and GCIV cells initiate apoptosis and are phagocytosed by the oocyte (Fig.14).

The single remaining oocyte undergoes two meiotic divisions and the egg is ready for fertilization.

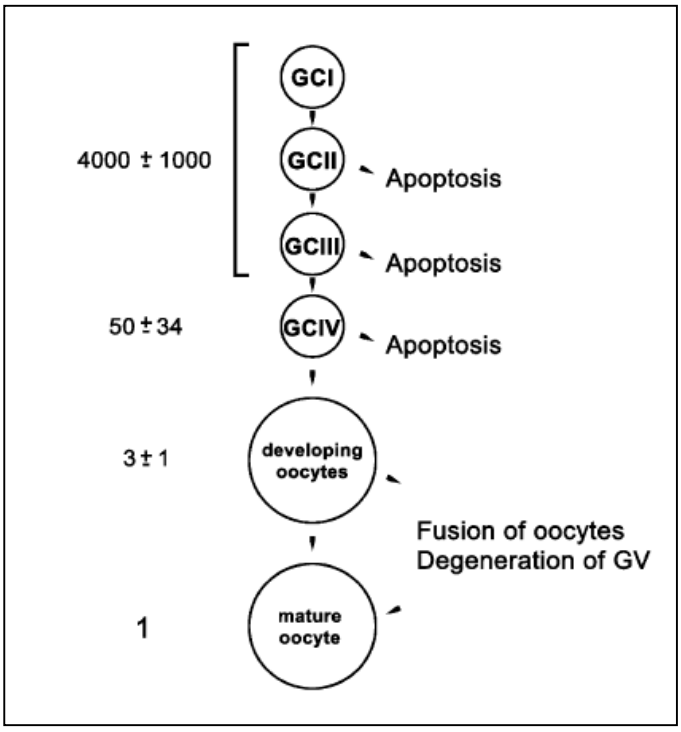

Fig. 13 Germ cell (GC) differentiation scheme in *Hydra* oogenesis (Alexandrova et al., 2005).

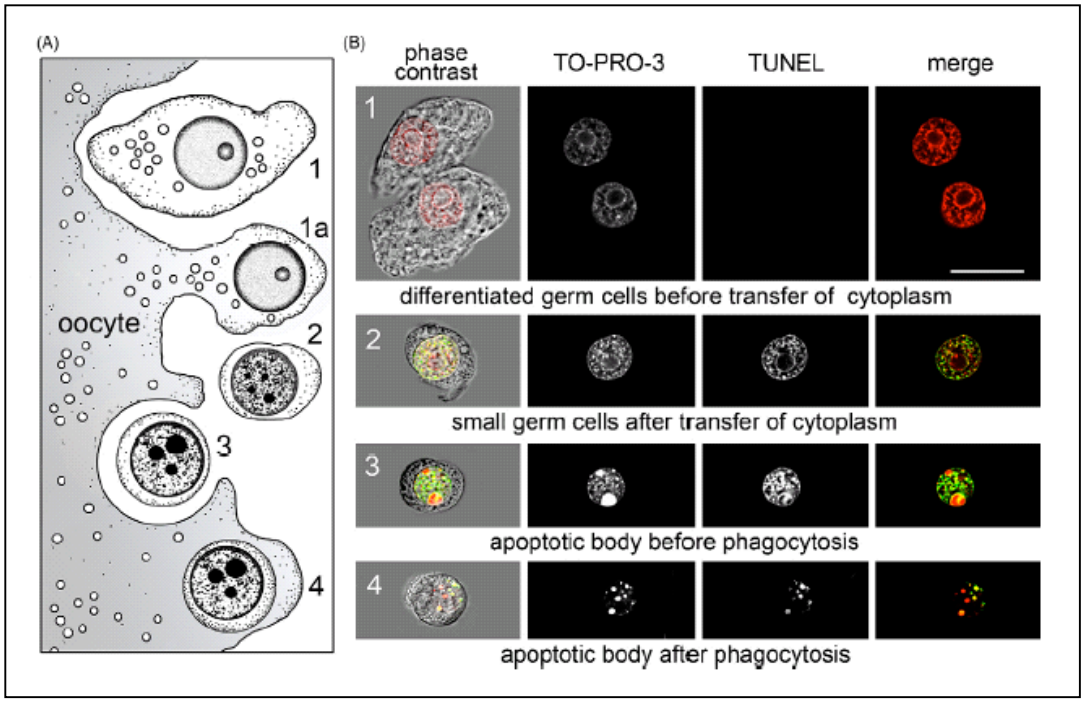

Fig. 14 (A) Schematic representation of germ cells in *Hydra* oogenesis; (B) TUNEL assay on germ cells in macerates (stages indicated in the picture). It is shown that germ cells in stages 2–4 are positive for TUNEL. Scale bar: 20µm (Böttger and Alexandrova, 2007).

As described above, the remains of nurse cells are phagocytosed by the oocyte and the morphological features of their nuclei suggest apoptosis. The fascinating thing is that nurse cells are not further degraded after phagocytosis by the oocyte and instead they are kept by oocyte enclosed to the phagocytic vacuoles even after fertilization, which is termed as an arrested apoptosis (Technau et al., 2003). During embryonic development they are distributed to the endodermal cells of the embryo and only get degraded after the new polyp has hatched. After hatching of the polyp, apoptosis is resumed and the nurse cells are degraded within 3 days. Persistent peroxidase activity in nurse cells detected throughout oogenesis and embryogenesis which have been implicated as anti-apoptotic agents in other systems. Peroxidase is therefore potentially an agent

responsible for preventing nurse cells from completing apoptosis until hatching and high level of peroxidase activity was found in the nurse cells from early stages of oogenesis on throughout embryogenesis (Habetha and Bosch, 2005) (Fig. 15). In newly hatched polyp peroxidase activity decreases within following 1–3 days, this decreasing is started at the apical end of the polyp and scattered over the whole body. This pattern of decrease in peroxidase activity is correlated with the pattern of degradation and precedes the disappearance of nurse cells after hatching, suggesting a role for peroxidase in preventing apoptosis in nurse cells until hatching (Habetha and Bosch, 2005) (Fig. 15).

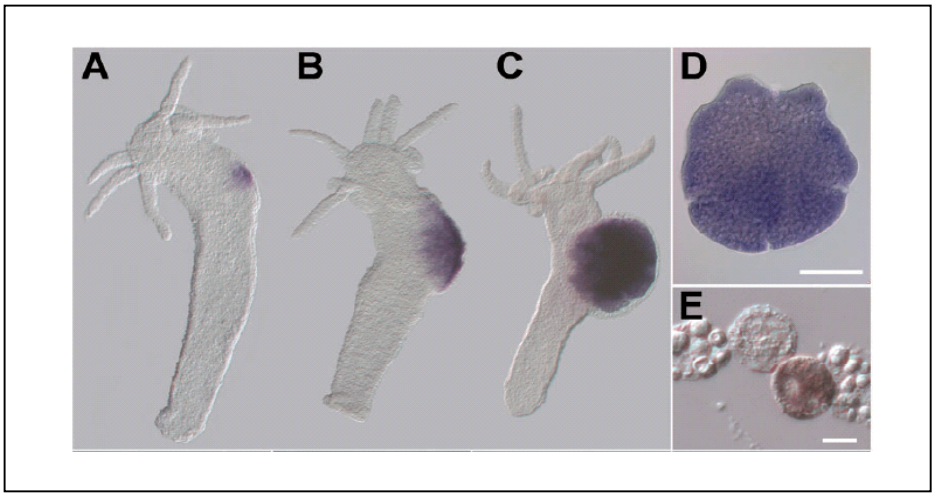

Fig. 15 Expression of HvAPX1 (*Hydra viridis* plant related ascorbate peroxidase) activity in hermaphroditic *H. viridis* (A-C) Whole-mount in situ hybridisation. (D-E) Cellular in situ hybridisation. (D) Early oocyte; (E) I-cells from the ovary region showing HvAPX1 expression in one of the interstitial cells. Scale bars: 100µm (D) and 5µm (E) (Habetha and Bosch, 2005).

1.4 Aims of this study

Transmembrane Bax inhibitor-1 motif (TMBIM) is an evolutionarily conserved protein family with regulatory function in apoptosis. Interestingly, the members of this family, e.g., Bax inhibitor 1 (BI-1) and Lifeguard (Lfg) are not only present in animals, but also exist in other kingdoms like plant, bacteria and even viruses. This ancient protein family is likely to be involved in both, development and pathophysiology, so its understanding has potential medical implications and will shows new insight to the evolutionary aspect of programmed cell death.

The aim of this study was to characterize the members of this protein family and investigate their role during development of our model organism (*Hydra vulgaris*), which is one of the oldest metazoans with a conserved molecular programmed cell death pathway.

2. Results

2.1. Identification of genes encoding members of the Lifeguard protein family in *Hydra*

A search of whole genome and EST (Expressed sequence tag) sequences from *Hydra* (http://hydrazome.metazome.net/cgi-bin/gbrowse/hydra/) has revealed three sequences with homology to the anti-apoptotic protein family, Lifeguard.

Through blast analysis, the Lifeguard-4 (XM_002162893.1) gene with a conserved protein sequence to its Human homolog (Golgi anti-apoptotic protein-GAAP) was identified in the *Hydra* genome.

Moreover, it was revealed that two Lfg-1i (Lifeguard-1 derivatives in invertebrates) protein family homologues are present in *Hydra*. They are named Lfg-1ia and Lfg-1ib in this study. There were two gene predictions for Lfg-1ia protein and also an incomplete sequence of Lfg-1ib in the EST data base.

In order to obtain complete cDNA-sequences for these genes, RACE (Rapid Amplification of cDNA Ends) reactions were carried out. For determination of the unknown 5' end with 5'-RACE, *Hydra* mRNA was ligated to the GeneRacer RNA oligo. Subsequently, reverse transcription was performed by using random primers or a gene specific primer. Finally, PCR was performed and the 5´ end of the template amplified by using a gene-specific 3' primer and a 5' gene racer primer. The complete amplified *Hydra Lfg-1ia* and *Lfg-1ib* nucleotide sequences are in appendix.

The PFAM program assigned the *Hydra* lifeguard members to the protein family UPF0005, whose members are predicted to contain six or seven α-helix transmembrane domains. Another member of this protein family, *Hydra* Bax inhibitor-1 protein, already had been identified in the *Hydra* genome (Hma1.130444), the

cDNA was cloned and the protein was predicted to have six α-helix transmambrane domains structure (Fig. 16). Moreover, it was shown to be localized in the ER of mammalian and *Hydra* cells (Anita Wagner, Evolution von Mechanismen der Apoptose: APAF-1 und Bax-Inhibitor-1 aus *Hydra vulgaris,* diploma thesis, 2009; Lasi et al., 2010).

2.2. Analysis of the putative Lifeguard protein sequences in *Hydra*

The protein sequences of the three lifeguard protein family members in *Hydra* were analyzed. *Hydra* Lfg-4 is compared with human Lfg-4 in Figure 17. Lfg-1ia and Lfg-1ib protein sequences in comparison with human Lfg-1 are shown in Figure 18. *Hydra* Lfg-4 and human Lfg-4 share 50.4% amino acid identity and Lfg1ia and Lfg-1ib have 37.8% and 38.6% amino acid identity with human Lfg-1 respectively.

The TMHMM program predicted seven α-helix transmambrane domain structures for all lifeguard protein members in *Hydra* (Fig. 19). The C-terminal region of all *Hydra* Lifeguard members was predicted to be present extracellularly (see also Figs. 17-18). Sequence comparison of Bax inhibitor-1 from *Hydra* with its human homolog showed 45.6% identity. The TMHMM program predicted a six α-helix transmembrane domains structure. In contrast to the lifeguard protein members, both the C-terminal and the N-terminal regions of BI-1 are predicted to be present intracellularly (see also Fig. 16).

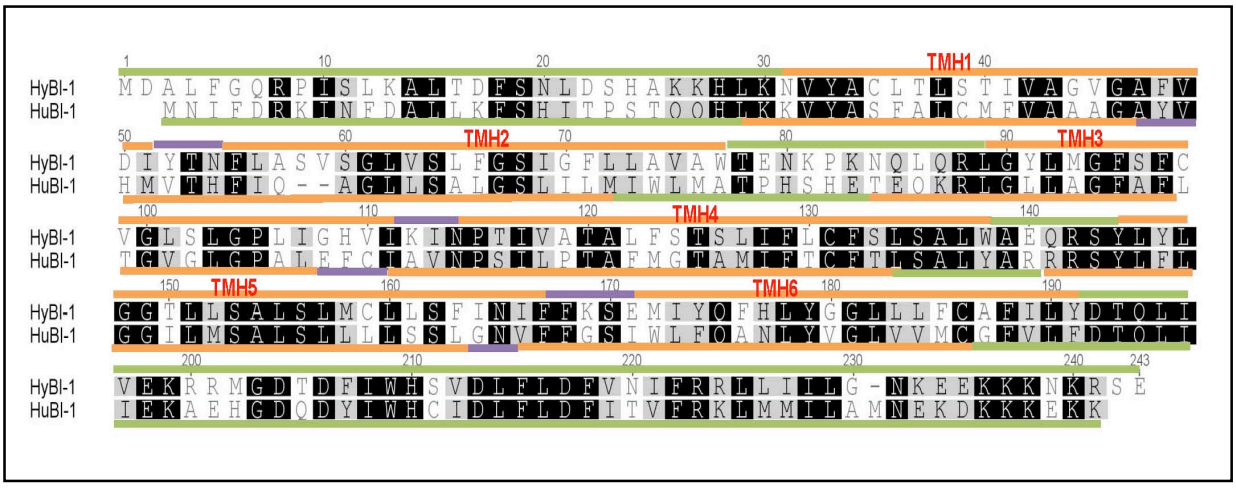

Fig. 16 Sequence comparison of *Hydra* BI-1 (Hma1.130444) with *Homo sapiens* BI-1 (NM_003217.2). Black color shows identical amino acids and dark gray similar amino acids. The predicted helix (Orange), the intracellular loops (green), the extracellular loops (purple).

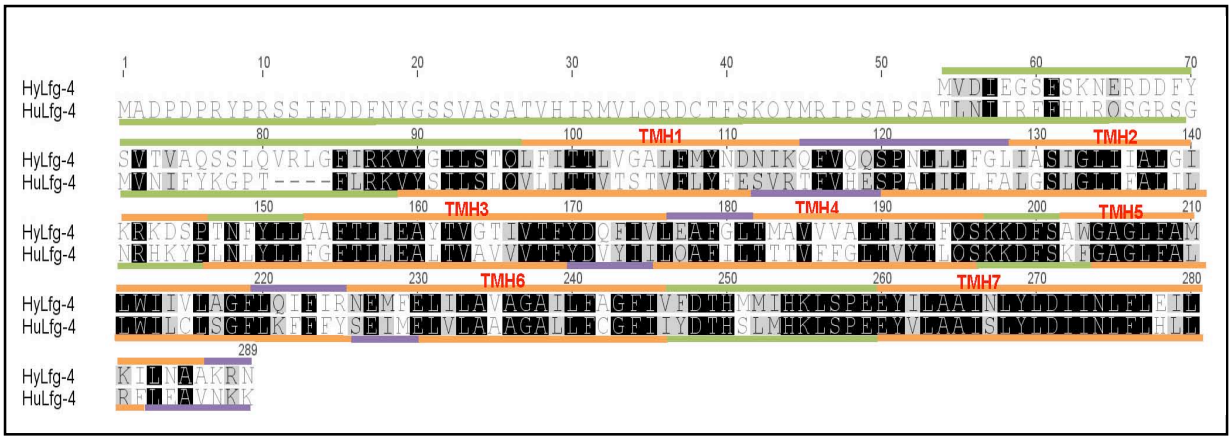

Fig. 17 Sequence comparison of *Hydra* Lfg-4 (XM_002162893.1) with *Homo sapiens* Lfg-4 (NM_016056.2). Black color shows identical amino acids and dark gray similar amino acids. The predicted helix (Orange), the intracellular loops (green), the extracellular loops (purple).

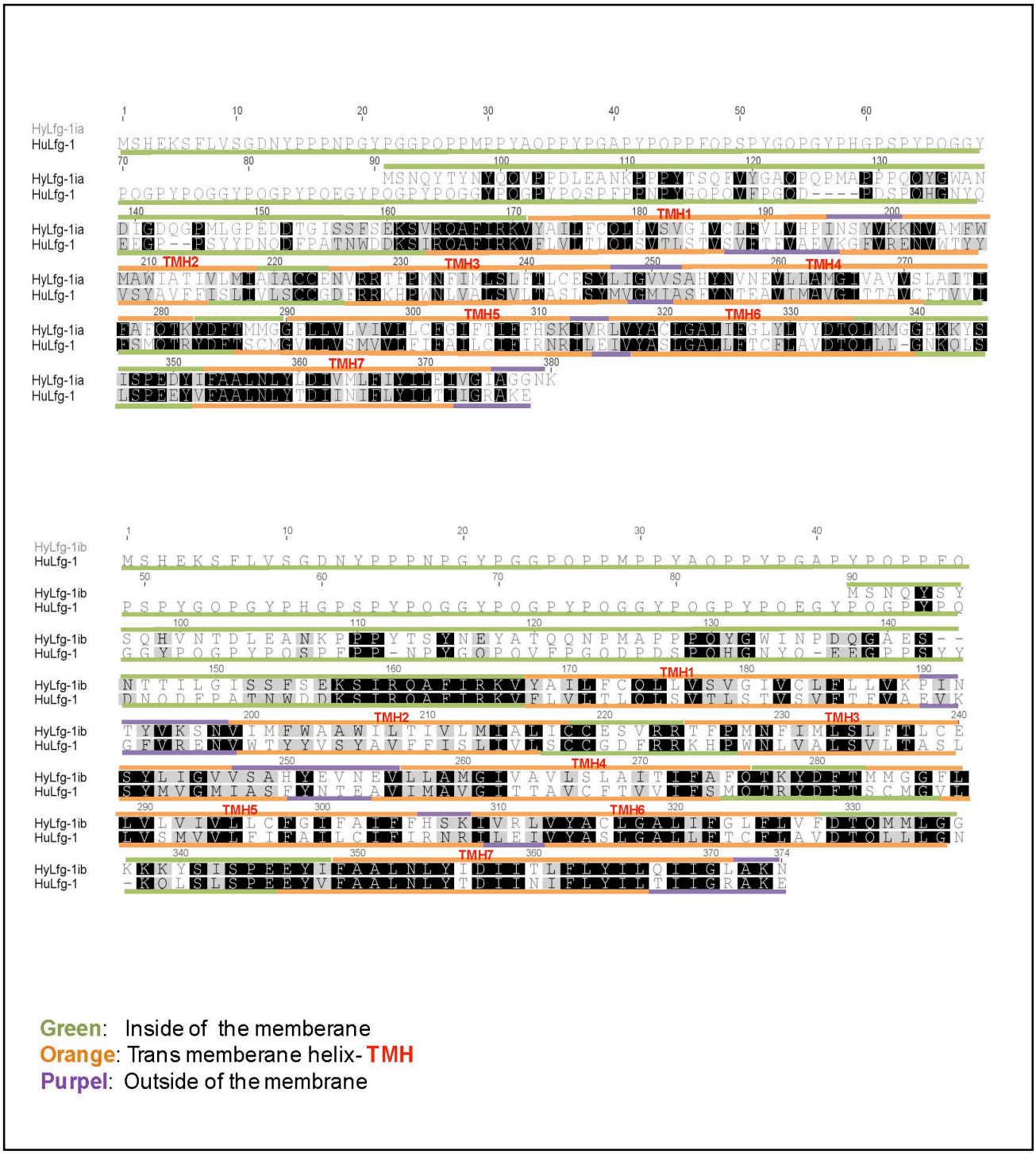

Fig. 18 Sequences comparison of *Hydra* Lfg-1ia (Hma2.214458) and *Hydra* Lfg-1ib (Hma2.205245) with *Homo sapiens* Lfg-1 (NP_000828.1). Black color shows identical amino acids and dark gray similar amino acids.

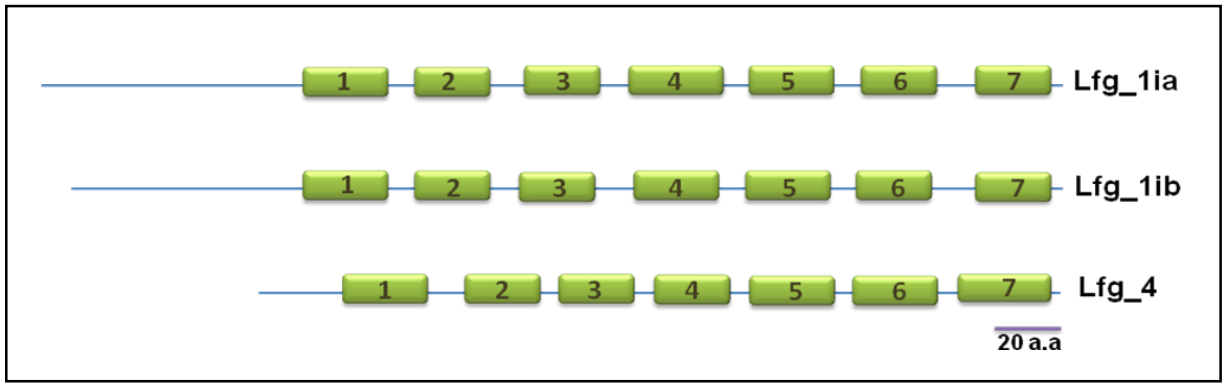

Fig. 19 Schematic α-helix structure comparison of *Hydra* Lifeguard protein members. The N-terminal regions are highly variable. Seven α-transmembrane helices and loops were predicted with the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM). Scale: 20 amino acids.

Hu et al. (2009) identified unique and characteristic sequence motifs diagnostic of the Lifeguard family. This is shown in Figure 20. Ten of the most diagnostic motifs in Lifeguard protein family in animals (mainly vertebrate Lfg proteins) and plants are displayed (Hu et al., 2009). The motifs are listed in N-terminal to C-terminal order. Motifs 7, 8, and 10 are variations of the same region in Lfg proteins.

Motif ID	Consensus sequence motif	Present in proteins	Region	Animals or plants
1	[IV]-R-X(2)-F-[ILM]-X-[KR]-V-[FY]-X(5)-Q	All	N-term + TMH1	Both
2	S-[ILV]-X-[DE]-D-F	*Lfg4*	N-term	Animals
3	W-D-[DN]	*Lfg2,3*	N-term	Animals
4	C-C-X(3)-R-R	*Lfg1–3, 5*	L2	Animals
5	Q-[ST]-[KR]-X(1,2)-D-F-[ST]-X(3)-[AG]	*Lfg1–5*	L4	Animals
6	W-L-H	*Lfg2,3,5*	L5	Animals
7	D-T-H-X-[IL]-M-[HKR]-[KR]-[IV]-S-P-E-[DE]-Y-X(6)-L-Y–X-D-[ILV](2)-N-L-F	*Lfg4*	L6 + TMH7	Animals
8	D-T-Q-X(3)-G-X-[KR]-X(4)-[DNS]-P-E-[DE]-Y-X(6)-[IL]-Y-X-D-[IV](2)-X-[IL]-F	*Lfg1,2,3,5*	L6 + TMH7	Animals
9	[FY]-[FW]-[GA]-X-[HKR]-[KR]-G-X-[DE]-F	*Lfg4*	L4	Plants
10	D-T-X(2)-[IL](2)-[KR](2)-X(2)-Y-[DN]-[EQ]-Y-X(6)-L-Y-L-D-X(2)-N-L-F	*Lfg4*	L6 + TMH7	Plants

Fig. 20 Consensus sequence motifs of Lifeguard family members in animals and plants; Multiple amino acids in brackets, e.g., [IV], mean that either amino acid could appear at that position; X(n) means any amino acid could appear in n consecutive positions; X(m, n) means any amino acid could appear at least m, at most n consecutive positions. transmembrane helices shown with TMH1–7and L1–L6 indicates loops (Table from Hu et al., 2009).

In Figures 21-23, all diagnostic motifs of the lifeguard protein family in Figure 20, were investigated in the alignment between *Hydra* lifeguard protein members and their homologues in human.

The result indicates that these diagnostic motifs are very well conserved and present in *Hydra* lifeguard protein members. Motif 1 is present in the N-terminal and in the TMH1 region of lifeguard proteins in both animals and plants. Comparison indicates that motif 1 is present in all *Hydra* Lfg protein members, where it is found in the N terminal and TMH1 region. Motif 5 is present in the fourth loop of all animal lifeguard members (Hu et al., 2009) and it is found in all *Hydra* Lfg protein family members. Motif 2 and 7 are present in the animals Lfg-4 protein homologous (Hu et al., 2009) and they are also identified in *Hydra* Lfg-4 protein in this study (see also Fig. 21). Motifs 4 and 8 are present in the animals Lfg-1, 2, 3, 5 (Hu et al., 2009) also identified in *Hydra* Lfg-1i homologues (Figs. 22-23). Moreover, motif 3 is identified in

human Lfg-1 protein. Motifs 9 and 10 belong to the plants and they were not identified in animals (Hu et al., 2009) and not in *Hydra.*

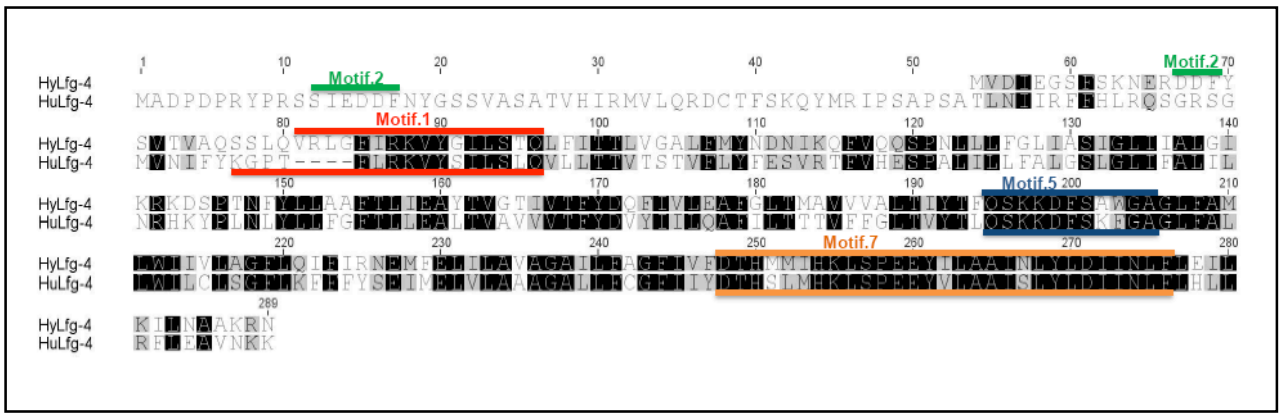

Fig. 21 Investigation of lifeguard-4 diagnostic motifs in alignment between *Hydra* and human; All animal Lfg-4 diagnostic motifs i.e., 1, 2, 5 and 7 are present in *Hydra* Lfg-4 protein.

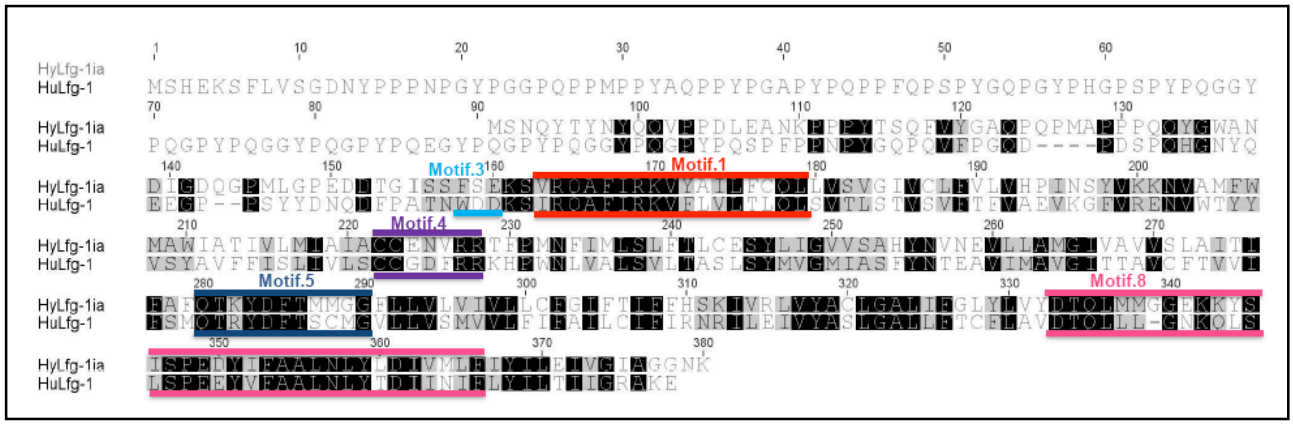

Fig. 22 Investigation of lifeguard-1diagnostic motifs in alignment between *Hydra* and human; All animal Lfg-1 diagnostic motifs i.e., 1, 4, 5, and 8 are present in *Hydra* Lfg-1ia protein.

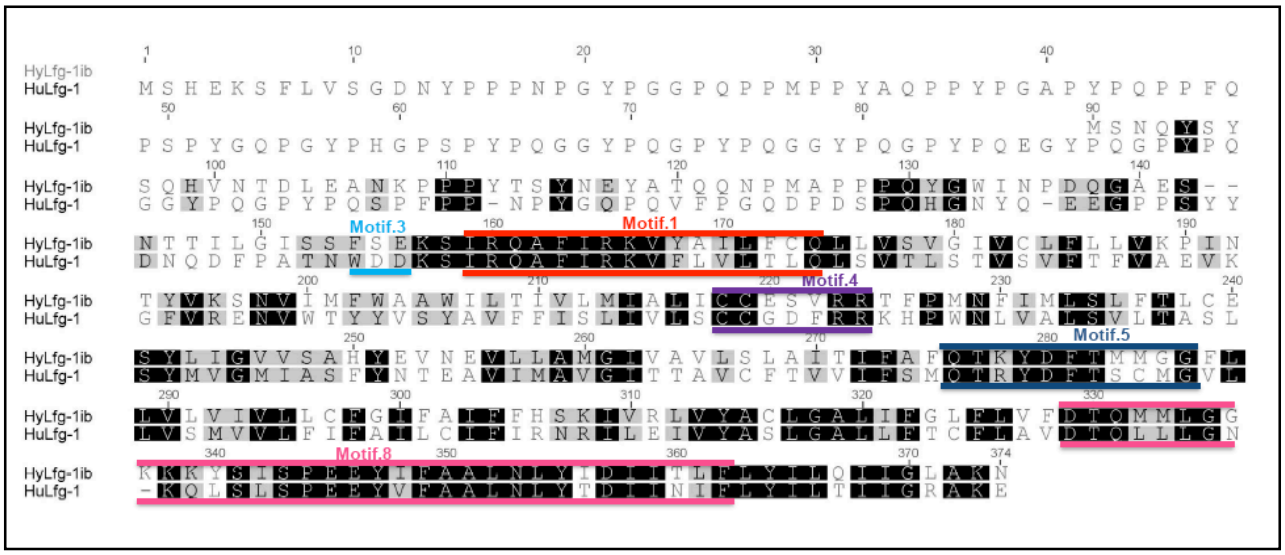

Fig. 23 Investigation of lifeguard-1diagnostic motifs in alignment between *Hydra* and human; All animal Lfg-1 diagnostic motifs i.e., 1, 4, 5, and 8 are present in *Hydra* Lfg-1ib protein.

In Figure 24 the phylogenic relationship between Lifeguard and Bax inhibitor-1 proteins is shown based on Clustal W alignment and Neighbor Joining (NJ) method. The NJ tree shows that the Bax Inhibitor-1 protein family in animals and plants forms a separate clade (yellow box) from the Lifeguard protein family. Moreover, the Lifeguard protein clade has divided in to the six clades: animal Lfg-4 proteins (pink box), plant Lfg-4 proteins (blue box), Lfg-1i derivatives in invertebrates (green box), Lfg-1 derivatives i.e., Lfg-1, Lfg-2, Lfg-3 in vertebrates and invertebrates (orange box). Lfg-5 found inside the eutherian mammals and makes a separate clade (gray box) and green algae-Lfg-1 as a representative of plants Lfg-1 (purple box). The *Hydra* Lfg-1ia and Lfg-1ib homologues are most similar to invertebrates Lfg-1 proteins and *Hydra* Lfg-4 is grouped with vertebrate and invertebrate Lfg-4 family members.

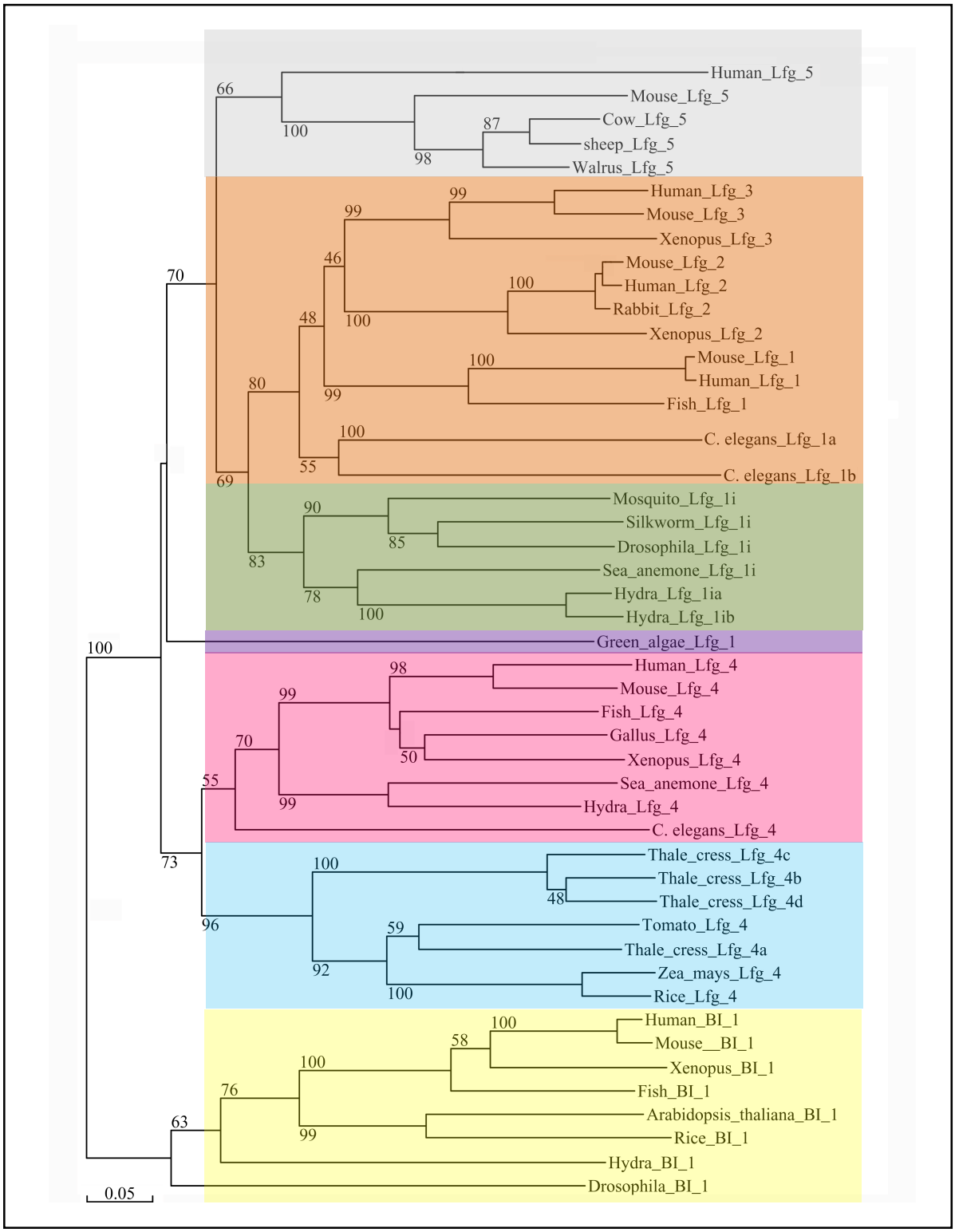

Fig. 24 Lifeguard and Bax inhibitor-1 phylogenic tree; Neighbor joining tree based on a Clustal W alignments of various lifeguard and BI-1 proteins in animals and plants. Proteins accession number list is in the appendix. Numbers indicate percentage values of 10,000 bootstraps. Scale shows the substitution per site.

2.3. Cellular localization of Lifeguard proteins in *Hydra*

In order to investigate the sub cellular localization of the *Hydra* Lfg protein in individual cells, the Lfg genes were cloned into the hoT G vector. This vector was designed for the expression of GFP fusion proteins under the control of the *Hydra* actin promoter (Böttger et al., 2002). After biolistic transformation, Lfg-GFP fusion protein was expressed in *Hydra* epithelial cells. The expression of the proteins was visible after 24-48 hr. Thereafter, animals were selected and fixed. For nuclear staining DAPI/ TO-PRO-3 was used.

2.3.1. Lifeguard-4 cellular localization

Lfg-4 is known as Golgi anti-apoptotic protein (GAAP) and has been reported to be localized in the Golgi apparatus and it was found in the ER, when it was over expressed (Gubser et al., 2007).

Figure 25 illustrates the Lfg-4-GFP expression in a *Hydra* endodermal epithelial cell. The fusion protein was localized in globular structures in the cytoplasm that seemed to be vesicles. To test if *Hydra* Lfg-4 protein is localized in the endoplasmic reticulum, Lfg-4 was co-expressed with the ER marker HyBax inhibitor-1-RFP (see also Fig. 26). BI-1 is an anti-apoptotic protein with six transmembrane domains which is inserted into the ER (Lasi et al., 2010). The co-expression result shows that Lfg-4 is co-localized with BI-1 in the cytoplasm in relatively diffuse structures but not in the vesicular regions.

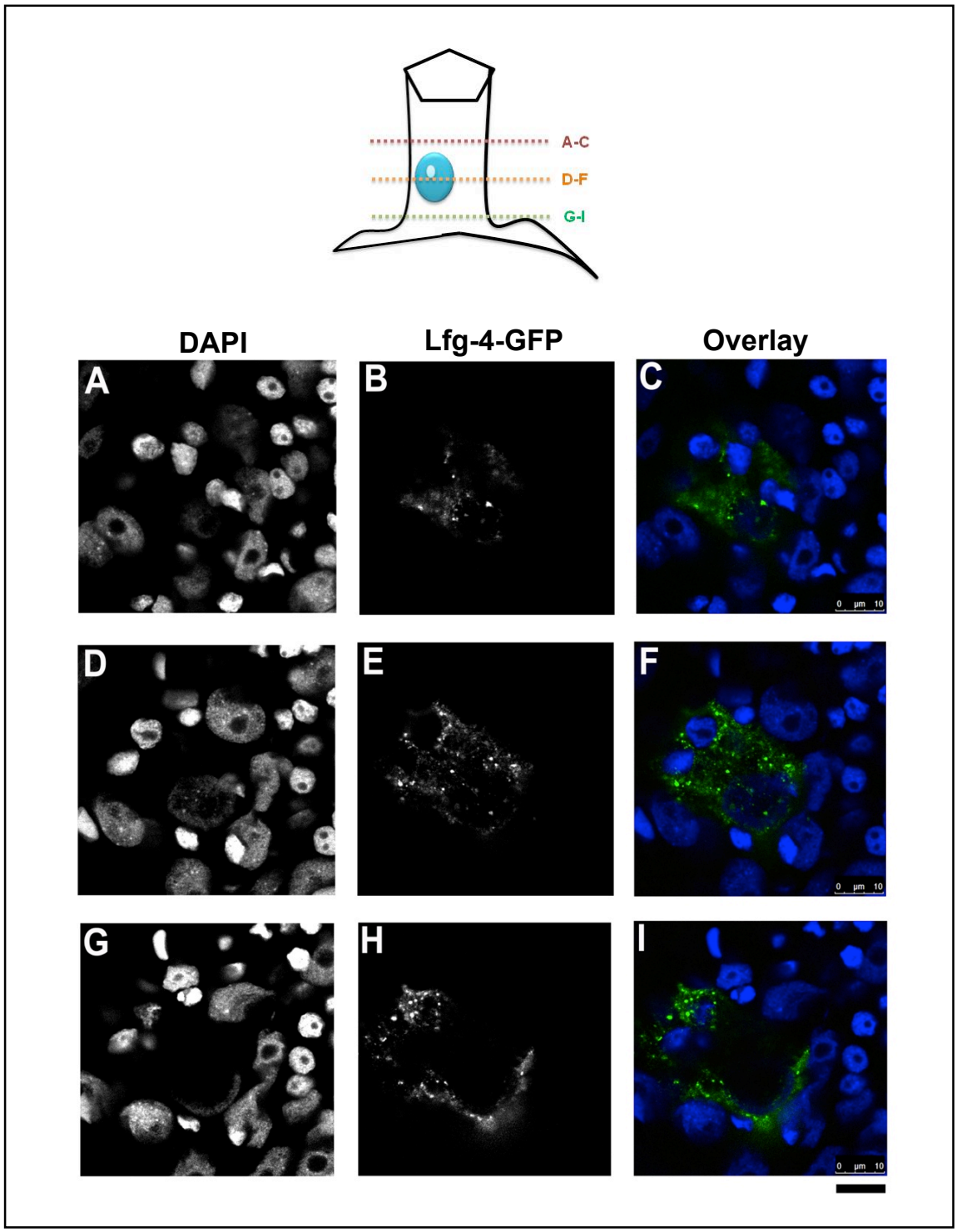

Fig. 25 Expression of Lfg-4 proteins in *Hydra* cell; (A, D, G) DNA staining with DAPI/TO-PRO-3; (B, E, H) Lfg-4-GFP; (C, F, I) overlay of all channels; Single sections; all as indicated in the schematic *Hydra* cell; Scale bar: 10 µm.

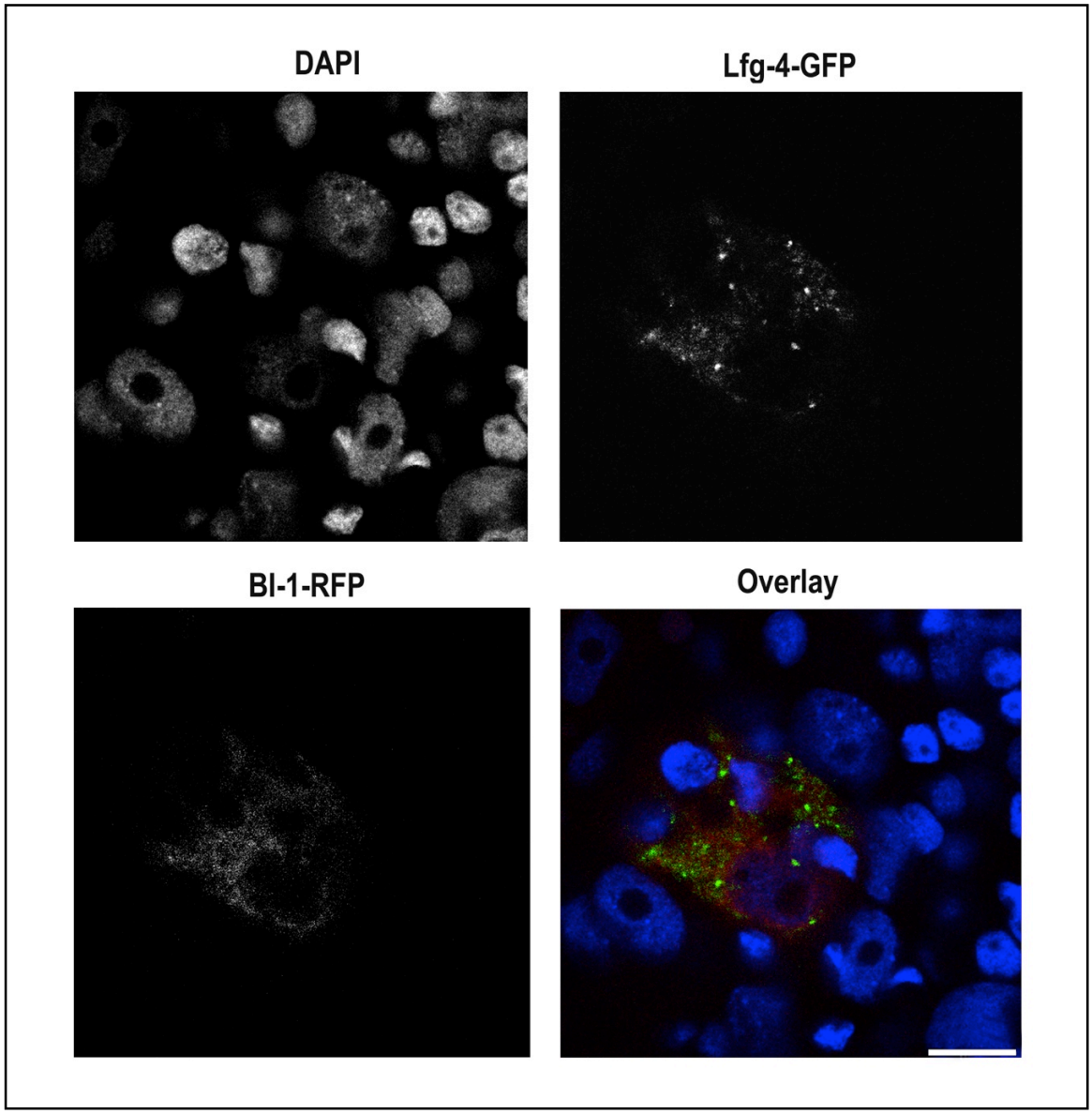

Fig. 26 Co-localization of *Hydra* Lifeguard-4 with *Hydra* Bax-inhibitor-1 (the ER marker); an endodermal epithelial cell in *Hydra* expressing Lfg-4-GFP and BI-1- RFP; Nucleus stained with DAPI/TO-PRO-3; overlay of all channels show co-localization of Lfg-4 with BI-1 in diffuse structures in the cytoplasm; Single section; Scale bar: 10 µm.

To investigate whether the vesicular structures were associated with Golgi-vesicles *Hydra* Lfg-4-GFP protein was over expressed in *Hydra* cells and BODIPY-TR ceramide used as Golgi marker. BODIPY-TR ceramide is a red fluorescent structural marker for the Golgi apparatus and also for the plasma membrane. It has an absorption spectrum peak at 598 nm, and its emission spectrum peaks at 625 nm. 1

µl of 500µM BODIPY TR ceramide were injected into the gastrocavity of *Hydra* polyps and then they were additionally incubated in hydra medium with 50µM BODIPY-TR ceramide for 30 min. Live imaging was performed directly after staining. The result demonstrates that *Hydra* Lfg-4 was localized in the Golgi complex (see Fig. 27).

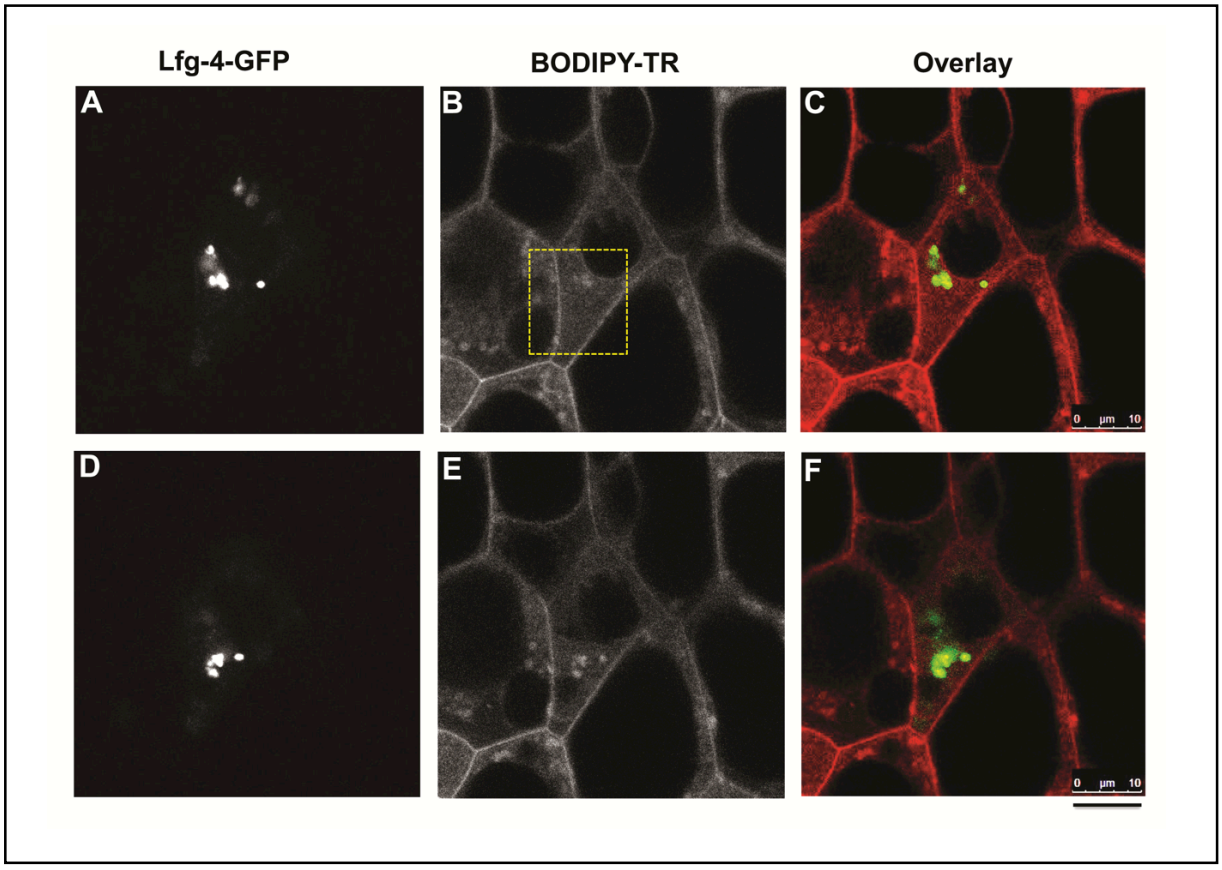

Fig. 27 Live cell imaging in *Hydra* cells; (A, D) *Hydra* Lfg-4- GFP over expression in *Hydra* cells; (B, E) whole mount *Hydra* polyp stained with BODIPY-TR; (C, F) Overlay channel demonstrated the co-localization of *Hydra* Lfg-4 with BODIPY-TR ceramide (Golgi marker). Yellow box shows the Golgi apparatus region. Single sections; Scale bar: 10 µm.

2.3.2. Lifeguard-1i homologues (Lfg-1ia and Lfg-1ib) cellular localization in *Hydra*

When Lfg-1ia- and Lfg-1ib- GFP-fusion proteins were over expressed in *Hydra* cells, they were localized mainly in the plasma membrane and in vesicle-like structures in the cytoplasm (Figs. 28-29). This is consistent with their predicted sub-cellular

localization mainly in the plasma membrane. PSORT II software was used for sub-cellular prediction (http://psort.hgc.jp/cgi-bin/runpsort.pl).

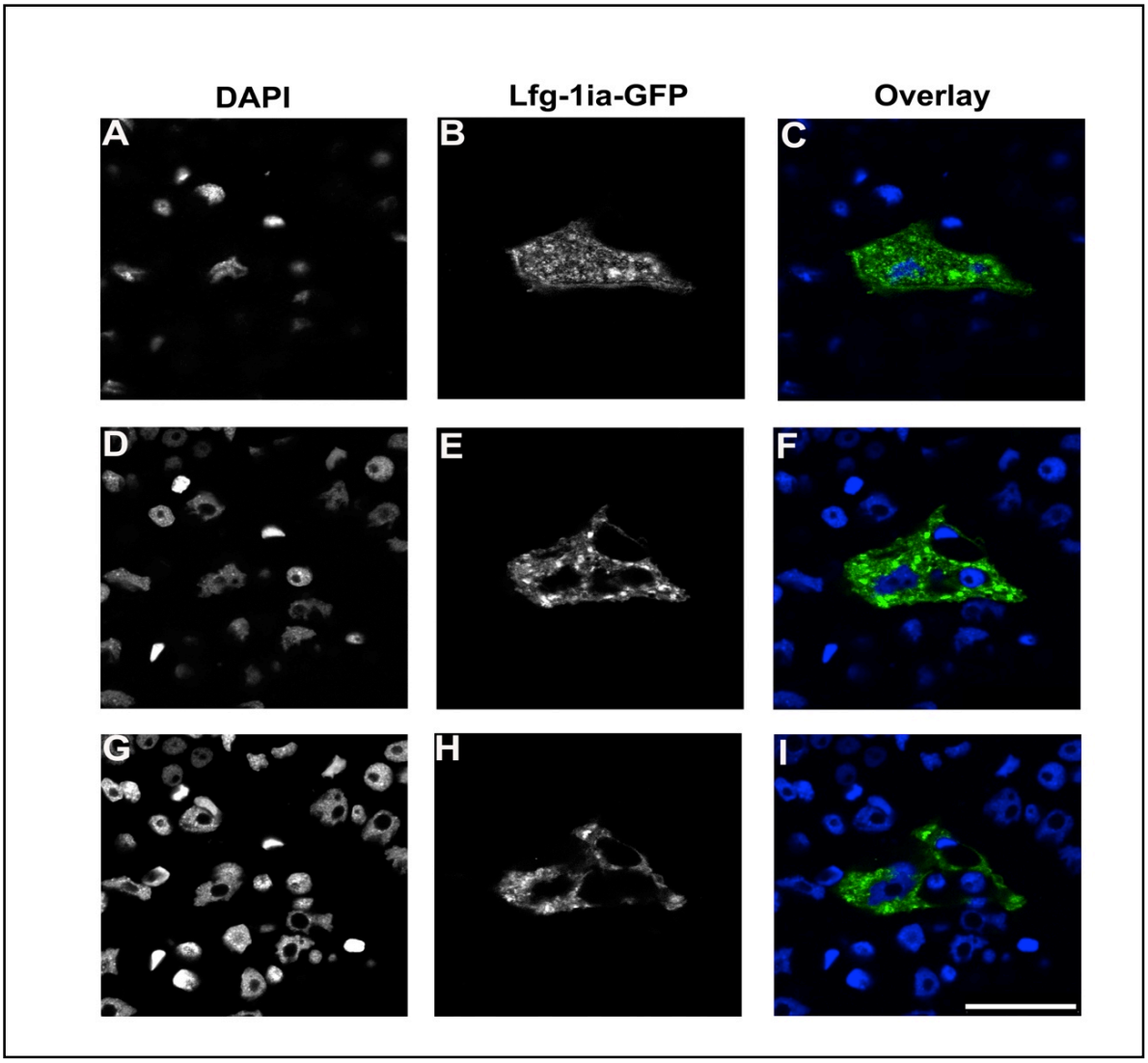

Fig. 28 Expression of *Hydra* Lfg-1ia proteins in *Hydra* cell; (A, D, G) DNA staining with DAPI/TO-PRO-3; (B, E, H) Lfg-1ia-GFP; (C, F, I) overlay of all channels; Single sections; Scale bar: 25 µm.

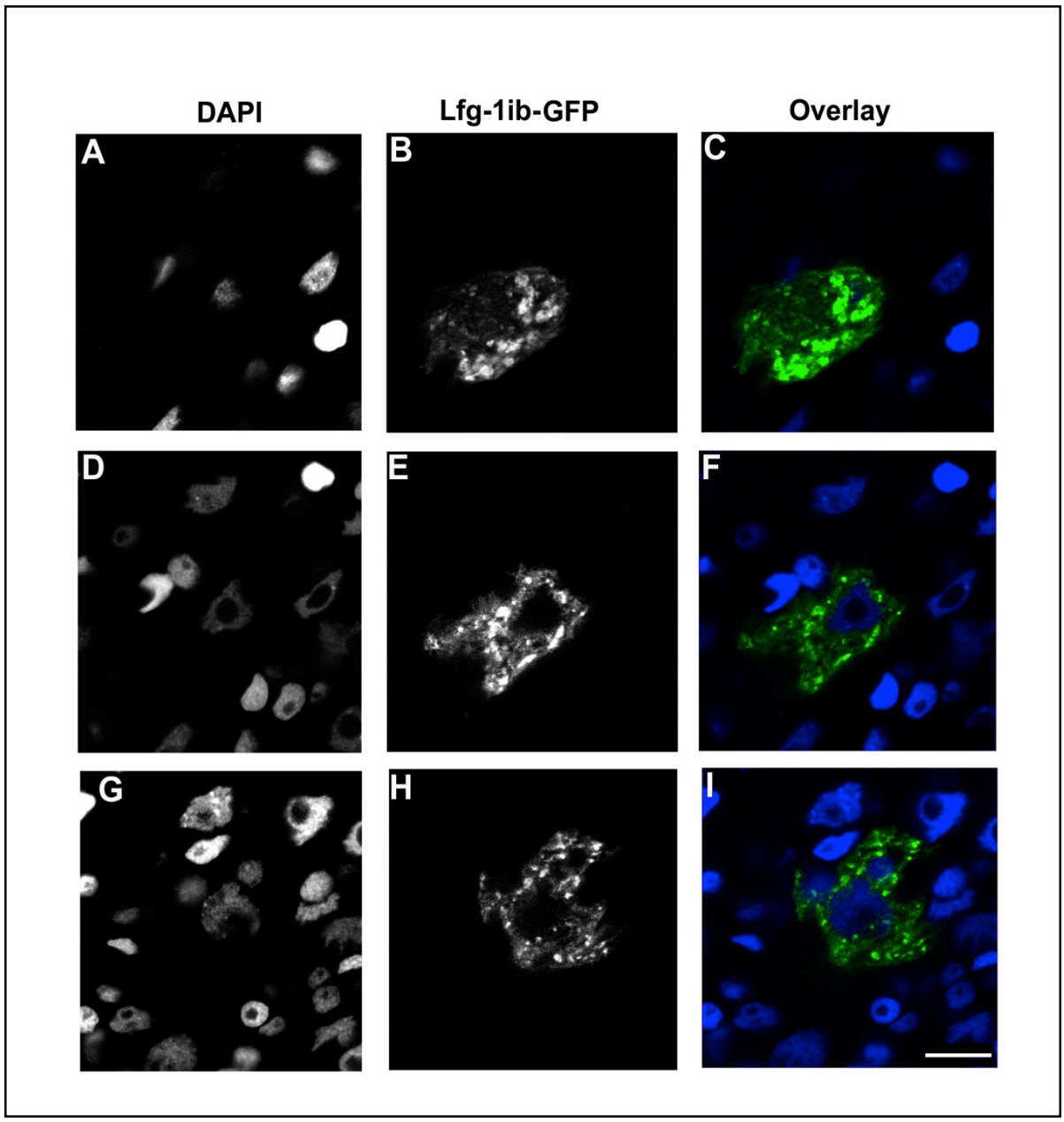

Fig. 29 Expression of *Hydra* Lfg-1ib proteins in *Hydra* cell; (A, D, G) DNA staining with DAPI/TO-PRO-3; (B, E, H) Lfg-1ib-GFP; (C, F, I) overlay of all channels; Single sections; Scale bar: 10 µm.

To investigate their localization precisely, Lfg-1ia and Lfg-1ib expression were tested with the Golgi marker in the live *Hydra* cells. The result demonstrates that Lfg-1ia and Lfg-1ib localized in the plasma membrane, Golgi complex as well as Golgi-vesicles (Figs. 30-31).

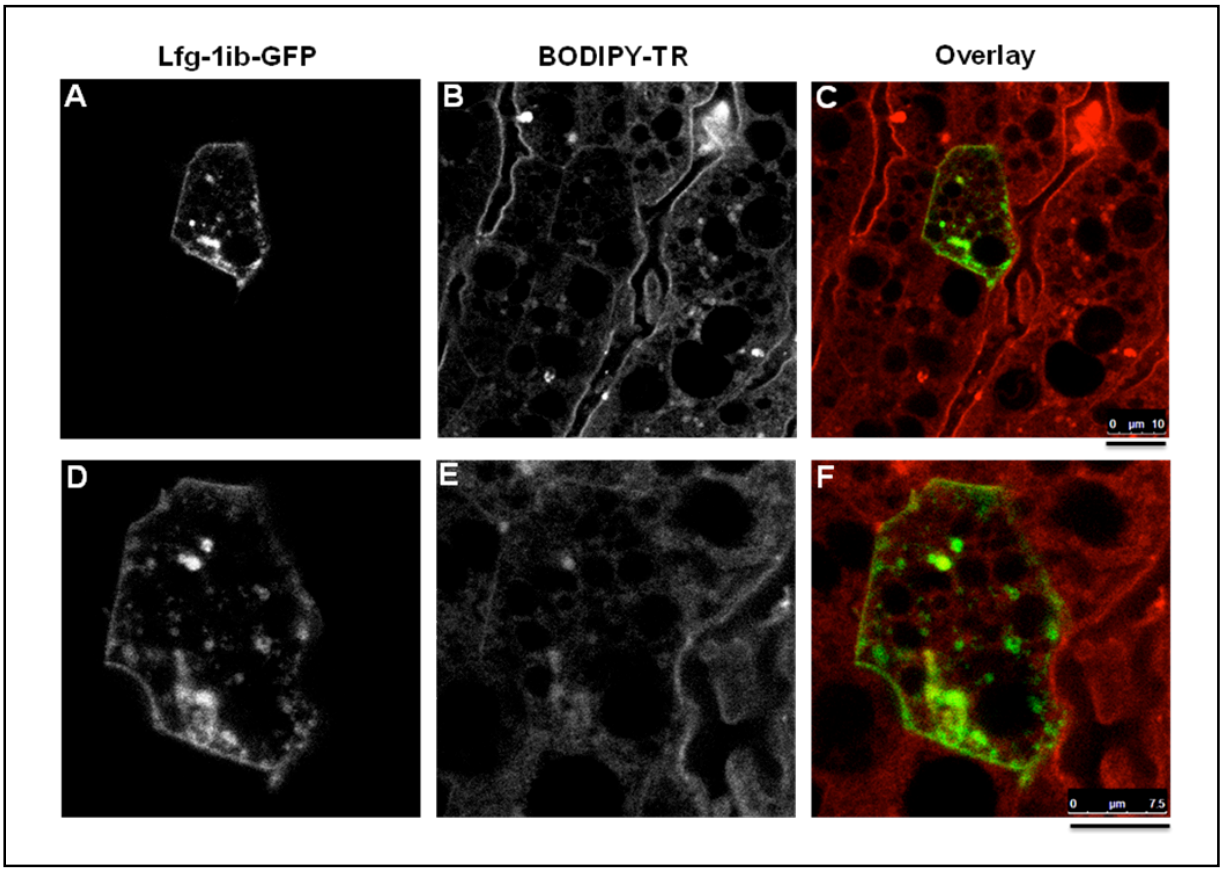

Fig. 30 Live cell imaging in *Hydra* cells; Upper panel (A, D) *Hydra* Lfg-1ib-GFP over expression in *Hydra* cells; (B, E) Whole mount *Hydra* polyp stained with BODIPY-TR; (C, F) Overly channel shows that Lfg-1ib localized in the plasma membrane, Golgi and Golgi vesicles. Single sections; Scale bar: upper panel: 10 µm; Lower panel: 75 µm.

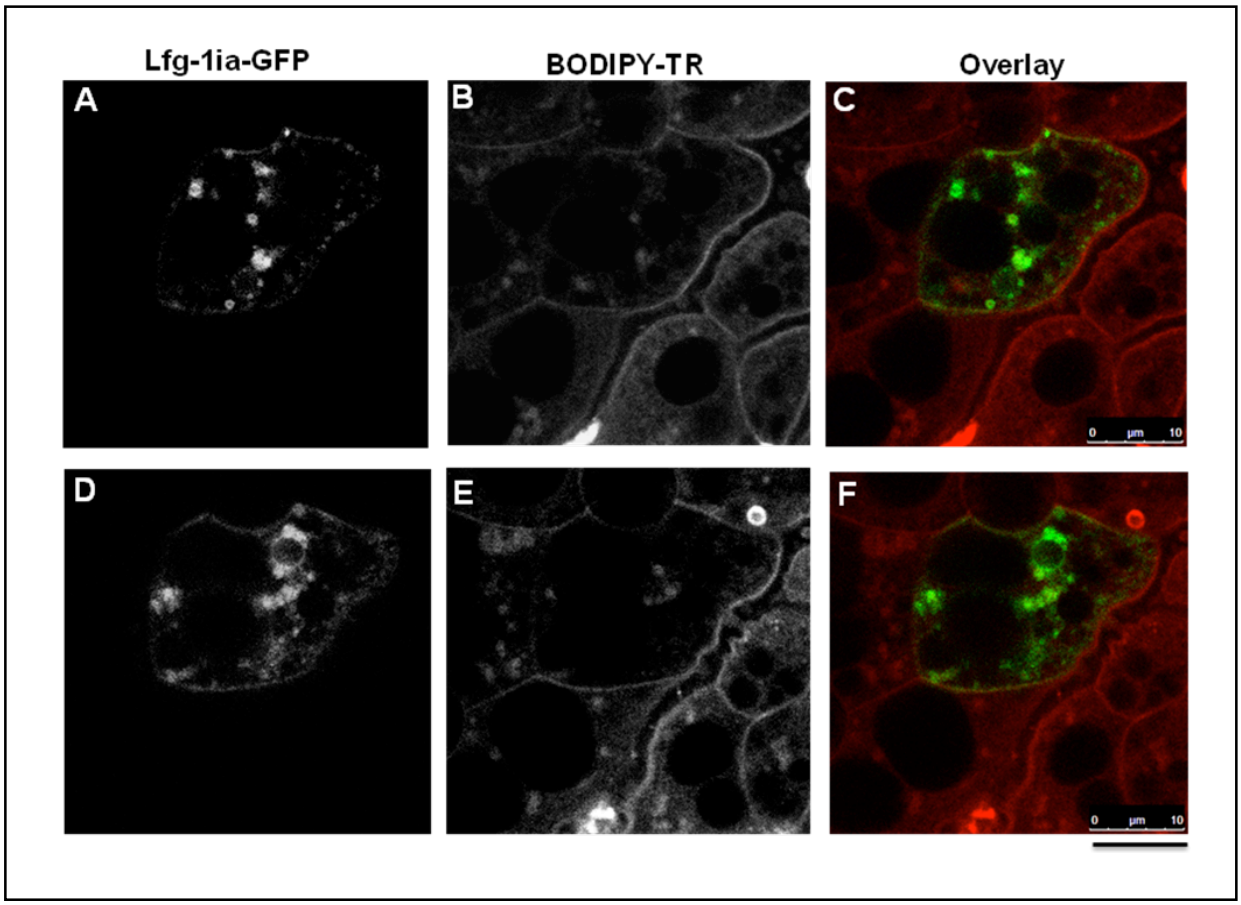

Fig. 31 Live cell imaging in *Hydra* cells; (A, D) *Hydra* Lfg-1ia-GFP over expression in *Hydra* cells; (B, E) Whole mount *Hydra* polyp stained with BODIPY-TR; (C, F) Overly channel shows that Lfg-1ia localized in the plasma membrane, Golgi and Golgi vesicles. Single sections; Scale bar: 10 µm.

2.4. Function of *Hydra* Lifeguard proteins in apoptosis

In the following study possible anti-apoptotic properties of *Hydra* Lifeguard proteins were investigated in human HEK 293T cells. After transfection of HEK 293T cells with plasmids encoding the *Hydra*-Lifeguard GFP-fusion proteins, apoptosis was induced with 10 µM camptothecin. Camptothecin is a topoisomerase-I inhibitor and can induce apoptosis in mammalian cells (El-Assaad et al, 1998). After 24 hr the cells were fixed and the nuclei stained with DAPI/TO-PRO-3. In Figure 32a, the apoptotic feature of Lfg-4-GFP expressing cell is shown in human HEK 293T cells. The

percentage of apoptotic GFP-positive cells in relation to all GFP-positive cells after transfection was calculated and shown in figure 32b. For control, GFP and *Hydra* Bcl-2-like-4 were used. HyBcl-2-like 4 had an especially strong protective effect in apoptosis induced by camptothecin in mammalian cells (Lasi et al., 2010). The percentage of apoptotic cells determined in figure 32b showed that camptothecin treatment caused apoptosis in 82% of GFP-expressing cells (control, red bar in Fig. 32b). On the other hand, HyBcl-2-like 4 –GFP decreased the number of apoptotic cells to only 18% and thus shows a strong anti-apoptotic effect. The expression of *Hydra* Lifeguard proteins suppressed the camptothecin-induced apoptosis to different degrees. *Hydra* Lfg-4 shows 41% suppression effect. *Hydra* Lfg-1ia and Lfg-1ib suppressed apoptosis, but the effect was not so strong (see also Fig. 32b). All experiments were repeated six times independently.

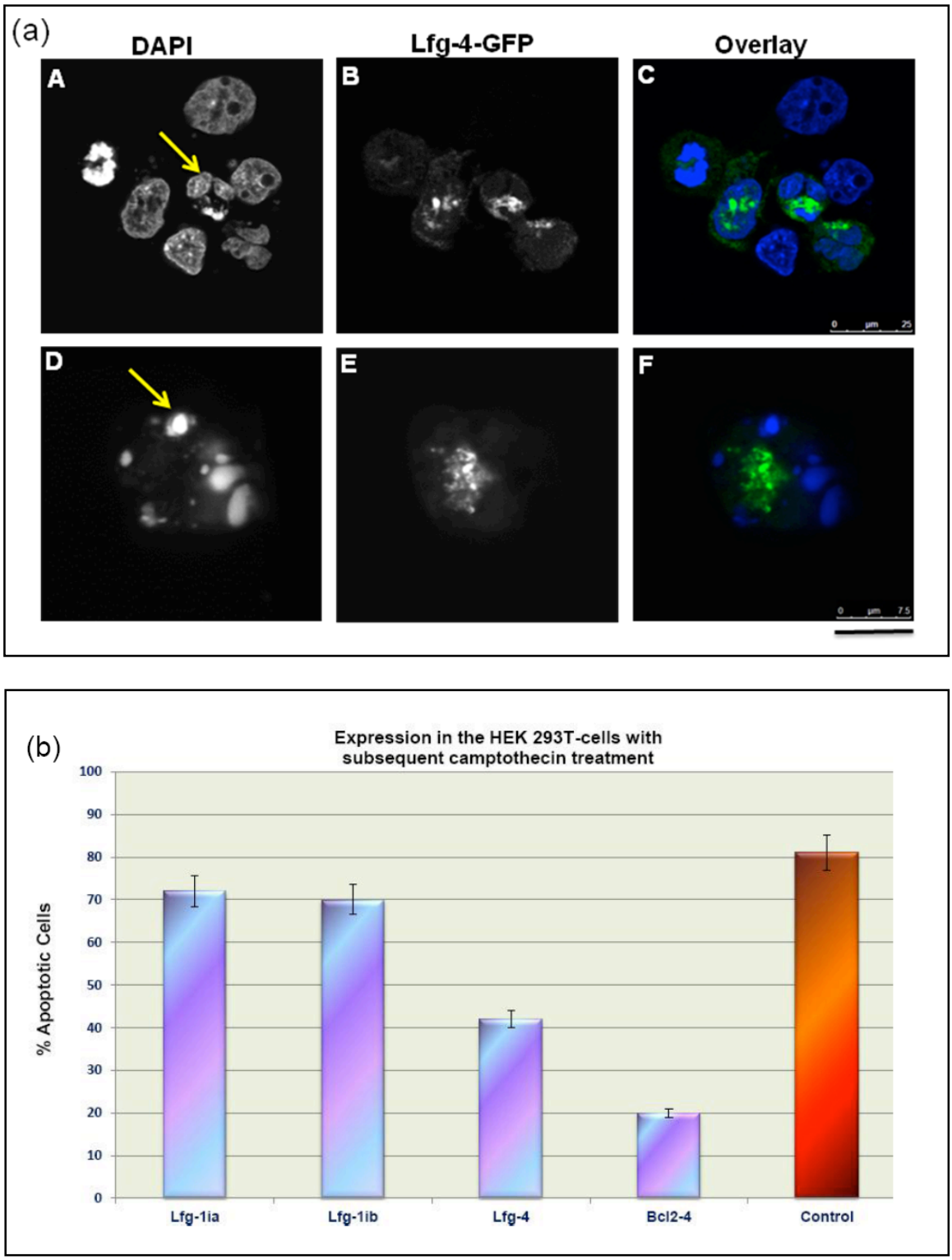

Fig. 32 (a) Expression of *Hydra* Lfg-4-GFP protein in HEK 293T cells treated with camptothecin. (A, D) DNA staining with DAPI/TO-PRO-3; (B, E) Lfg-4-GFP; (C, F) overlay of all channels; yellow arrows show the apoptotic cells; Single section; Scale bar: upper panel, 25 µm; Lower panel, 7.5 µm. (b) Inhibition of apoptosis by *Hydra* Lifeguard proteins in HEK 293T cells with subsequent camptothecin treatment; Diagrams showing percentage of apoptotic GFP-positive cells in relation to all GFP-positive cells after transfection of plasmids encoding the indicated GFP-fusion proteins.

2.5. Expression of *Hydra* Lifeguard proteins in HEK 293T cells

2.5.1. *Hydra* Lfg-4 expression in HEK 293T

Camptothecin is a topoisomerase inhibitior which causes DNA-double strand breaks, which induce the intrinsic apoptotic pathway. As *Hydra* Lfg-4 was localized in the Golgi apparatus and Lfg-1i homologues in the plasma membrane in hydra cells the question arose how the apoptosis protective effect in human cells was mediated. Therefore, at first the sub-cellular localization of these proteins in human cells was investigated. *Hydra* lifeguard proteins with C-terminal GFP-tags were expressed in HEK 293T-cells. Lfg-4-GFP showed signals mainly in the cytoplasm with some globular like structures in nucleus (Fig. 33). This nuclear localization was not found in *Hydra* cells and might be due to the expression of *Hydra* protein in human cells.

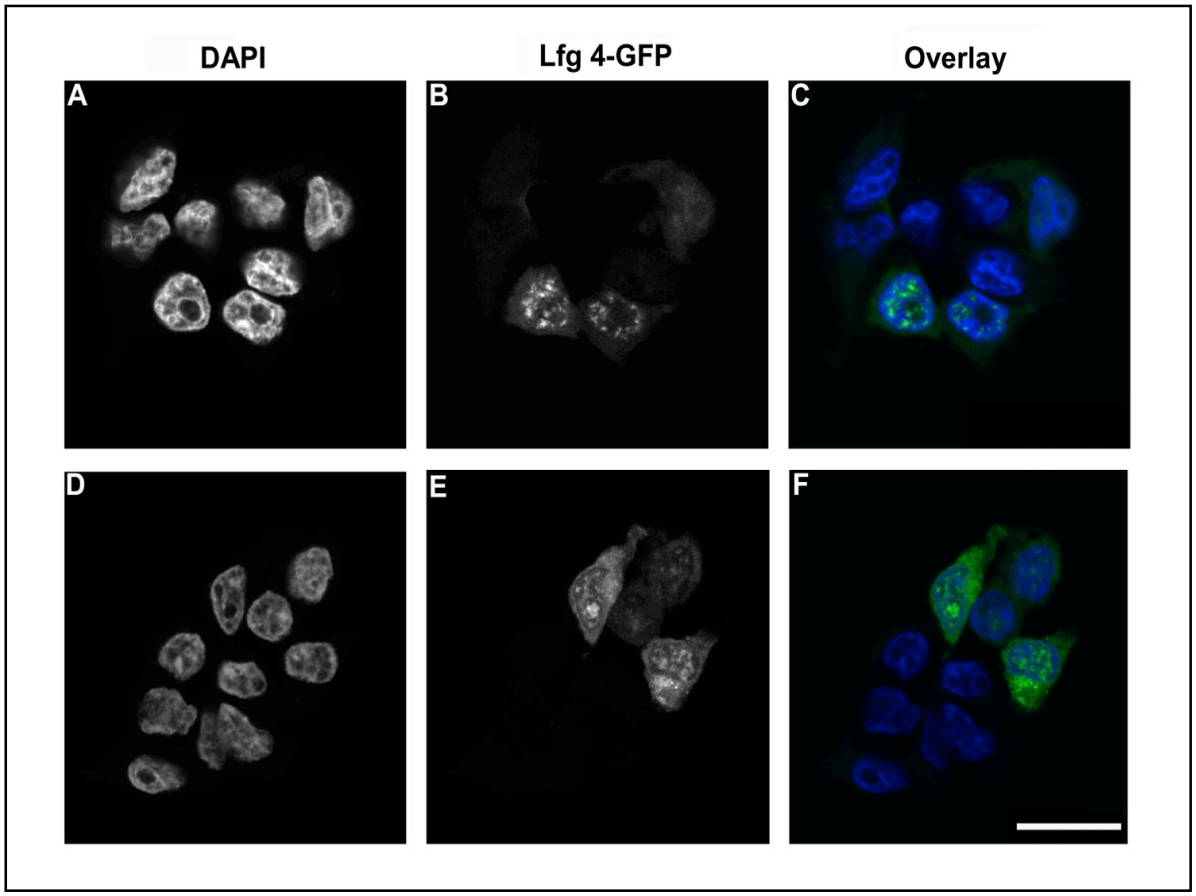

Fig. 33 Expression of Lifeguard-4 protein in HEK 293T cells; (A, D) DNA staining with DAPI/TO-PRO-3; (B, E) Lfg-4-GFP; (C, F) overlay of all channels; Single sections; Scale bar: 10 µm.

In addition, to evaluate the Golgi sub-cellular localization for the *Hydra* Lfg-4 protein, GFP-Lfg-4 protein was over-expressed in human embryonic kidney cells (HEK 293T) and 5 µM BODIPY TR ceramide was used as a Golgi marker (Fig. 34). The result, demonstrates that *Hydra* Lfg-4 co localized with Golgi marker, however GFP signal could also be detected in the nucleus.

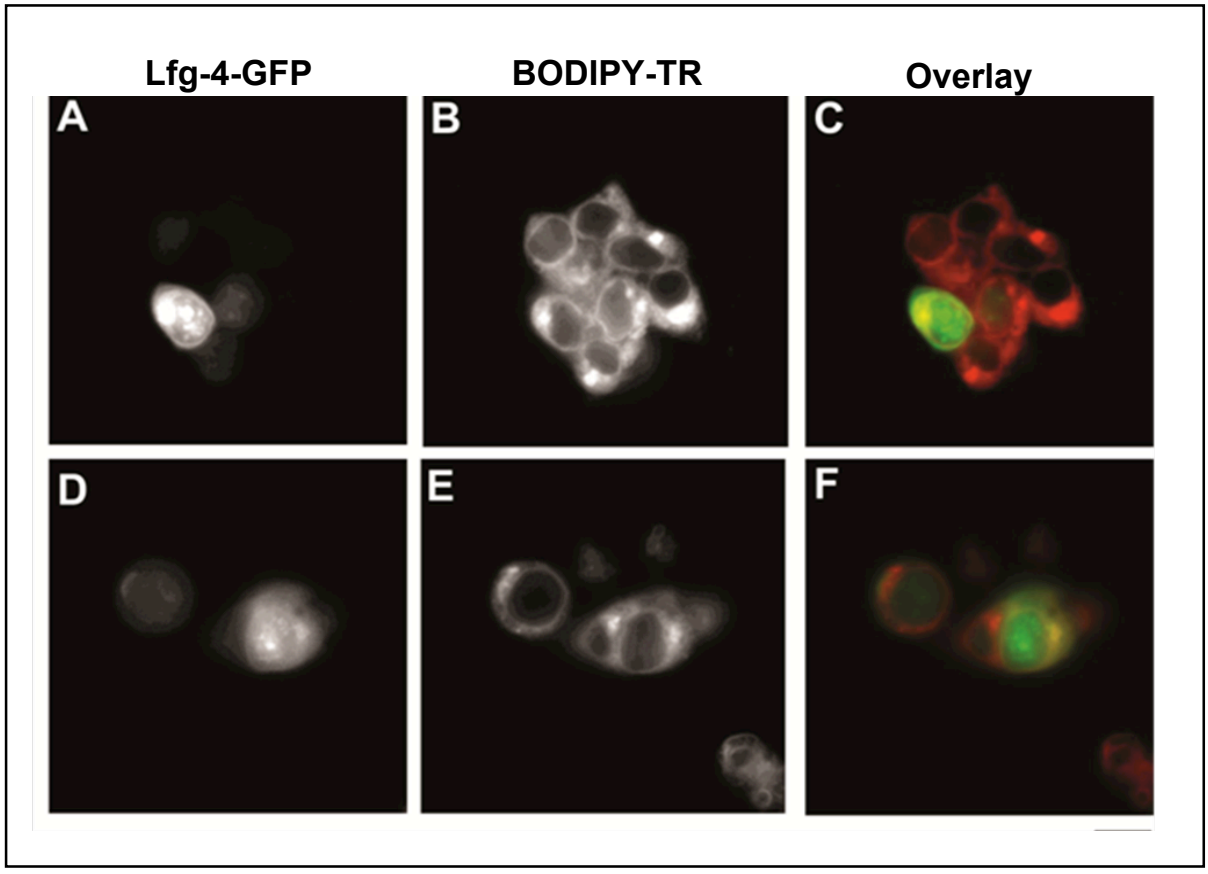

Fig. 34 Live cell imaging in HEK 293T cells; (A, D) *Hydra* Lfg-4- GFP over expression in HEK 293 T cells; (B, E) HEK 293T cells stained with 5 µM BODIPY TR; (C, F) overly channel; The yellow signal demonstrated the co-localization of *Hydra* Lfg-4 with BODIPY-TR ceramide (Golgi marker).

2.5.2. *Hydra* Lfg-1ia and Lfg-1ib expression in HEK 293T

Next, *Hydra* Lfg-1i homologues were expressed as C-terminal GFP-tagged versions in HEK 293T cells. Lfg-1ia- and Lfg-1ib-GFP showed signals in the cytoplasm and nucleus (Figs. 35-36). Similar to the Lfg-4-GFP expression in HEK 293T cells, these nuclear localizations were not found in *Hydra* cells and might be due to the expression of *Hydra* protein in human cells.

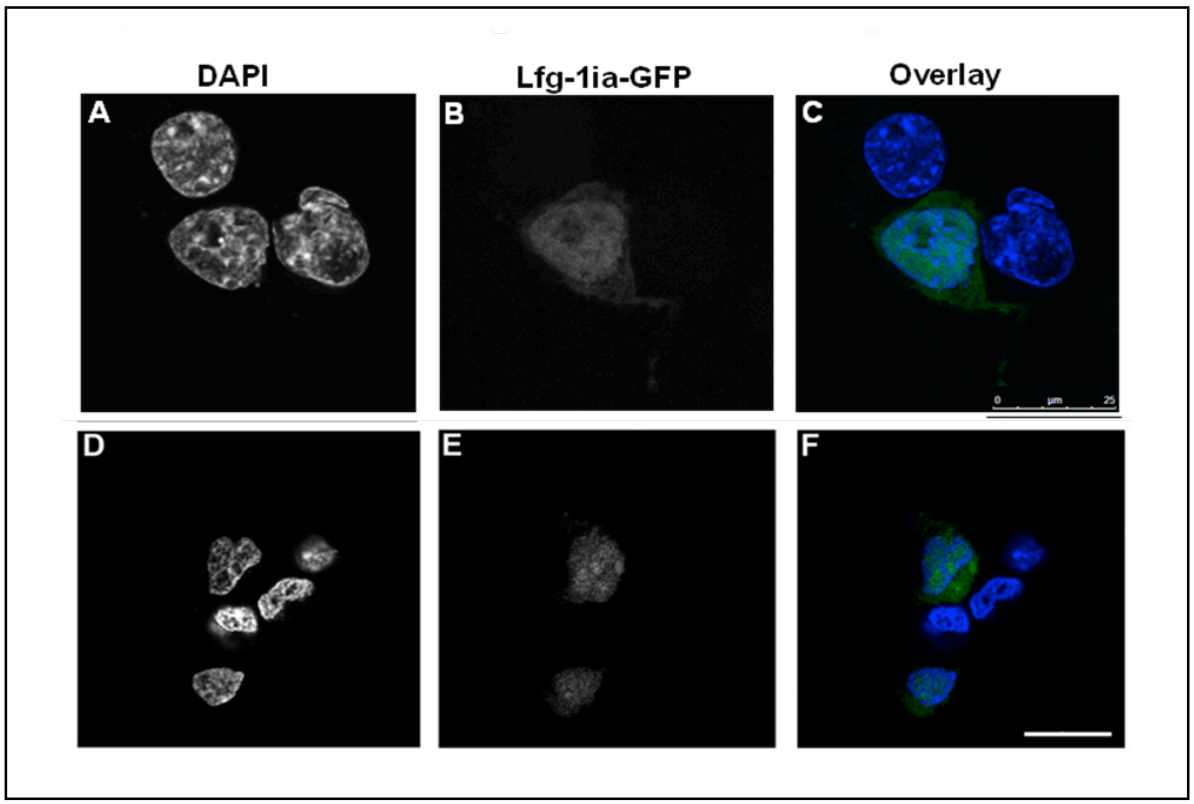

Fig. 35 Expression of *Hydra* Lfg-1i homolog proteins in HEK 293T cells; Lfg-1ia expression; (A, D) DNA staining with DAPI/TO-PRO-3; (B, E) Lfg-1i-GFP; (C, F) overlay of all channels; Single sections; Scale bar: 25 µm.

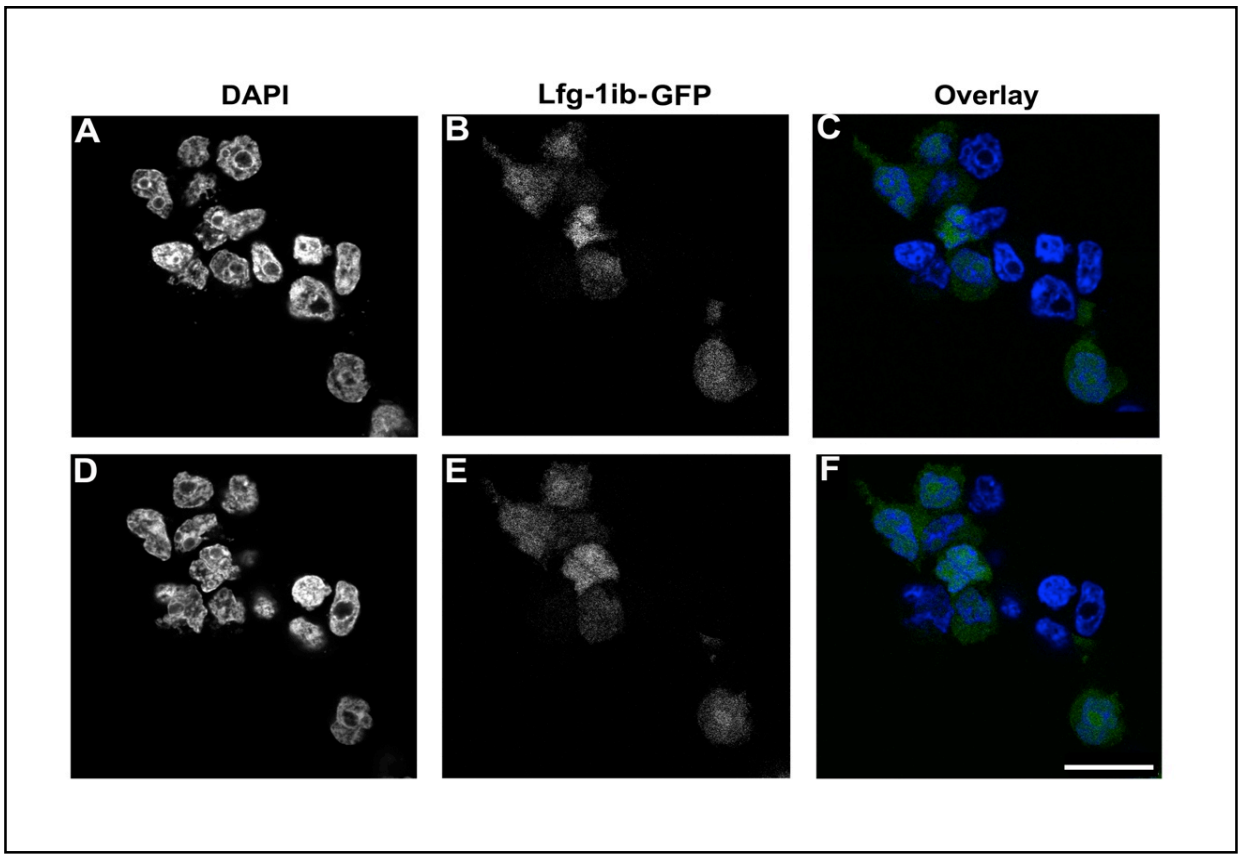

Fig. 36 Expression of *Hydra* Lfg-1i homolog proteins in HEK 293T cells; Lfg-1ib expression; (A, D) DNA staining with DAPI/TO-PRO-3; (B, E) Lfg-1i-GFP; (C, F) overlay of all channels; Single sections; Scale bar: 25 µm.

2.6. Apoptosis in *Hydra* gametogenesis

2.6.1. Spermatogenesis

Acridine orange is an acidophilic dye that stains vesicles with low pH value in living cells (Clerc and Barenholz, 1998). Therefore, phagocytosed apoptotic cells are acridine orange positive. Here we have stained male and female polyps with 3.3 mM acridine orange (AO). In male polyps stained with the acridine orange a little or no signs of apoptotic cell death were found in the body column. In the foot, head and tentacle tissue a slight increase in the number of acridine orange positive cells was

observed. It is consistent with the fact that *Hydra* cells are dying at the extremities and has been described in previous studies (Cikala et al., 1999).

In contrast, in the sexually mature male polyps large numbers of acridine orange positive cells were found within each testis (see also Fig. 37A and C). The cells were not restricted to a certain region of the testis suggesting that cells at various stages of spermatogenesis undergo programmed cell death (see also Fig. 37).

Moreover, a slight acridine orange staining was detected in the immature testis (see Fig. 37B). It is consistent with a previously published TUNEL assay and Acridine orange staining study (Kuznetsov et al., 2001).

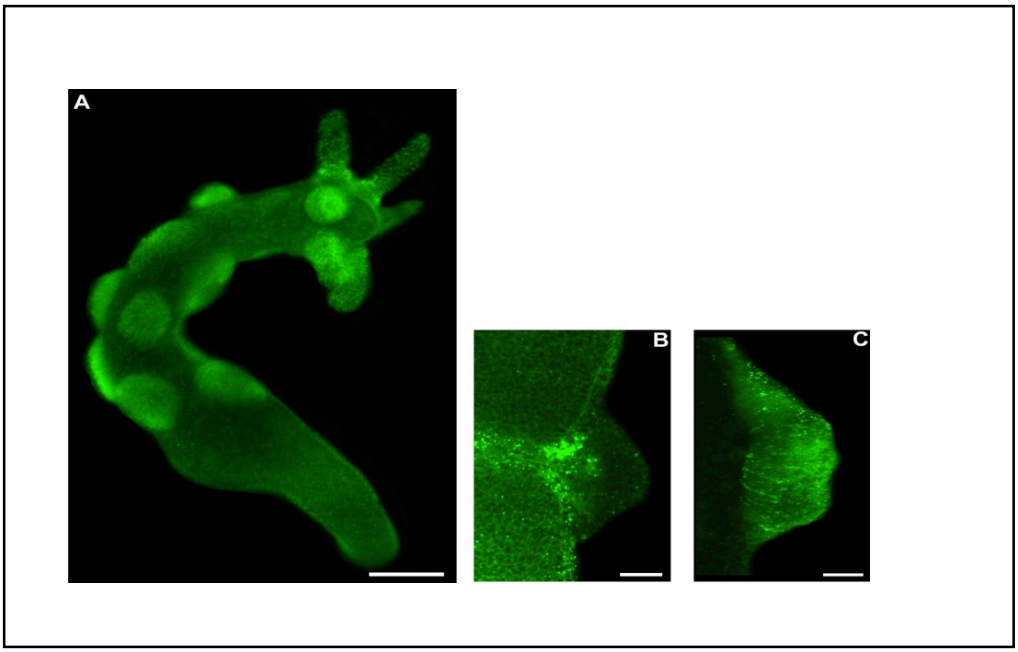

Fig. 37 Acridine orange staining of male polyp *Hydra*; (A) The sexually mature male polyps; (B) immature testis; (C) Mature testis; scale bar: (A) 200 µm; (B and C) 50 µm.

2.6.2. Oogenesis

Next we performed AO-staining of female *Hydra* (Fig. 38A-D). In *Hydra*, the first AO-positive nurse cells can be detected at stage 3 of oogenesis, when the nurse cells are phagocytosed by the growing oocyte (see also Fig. 38B) (Miller et al., 2000; Alexandrova et al., 2005). During later stages the number of AO-positive nurse cells rapidly increases, and results in a brightly stained oocyte with thousands of densely packed green nurse cells inside the oocyte (see also Fig. 38C) (Technau et al., 2003). Moreover, it was shown that *Hydra* caspases-3A mRNA was expressed during stages 2-4 of oogenesis (Technau et al., 2003).

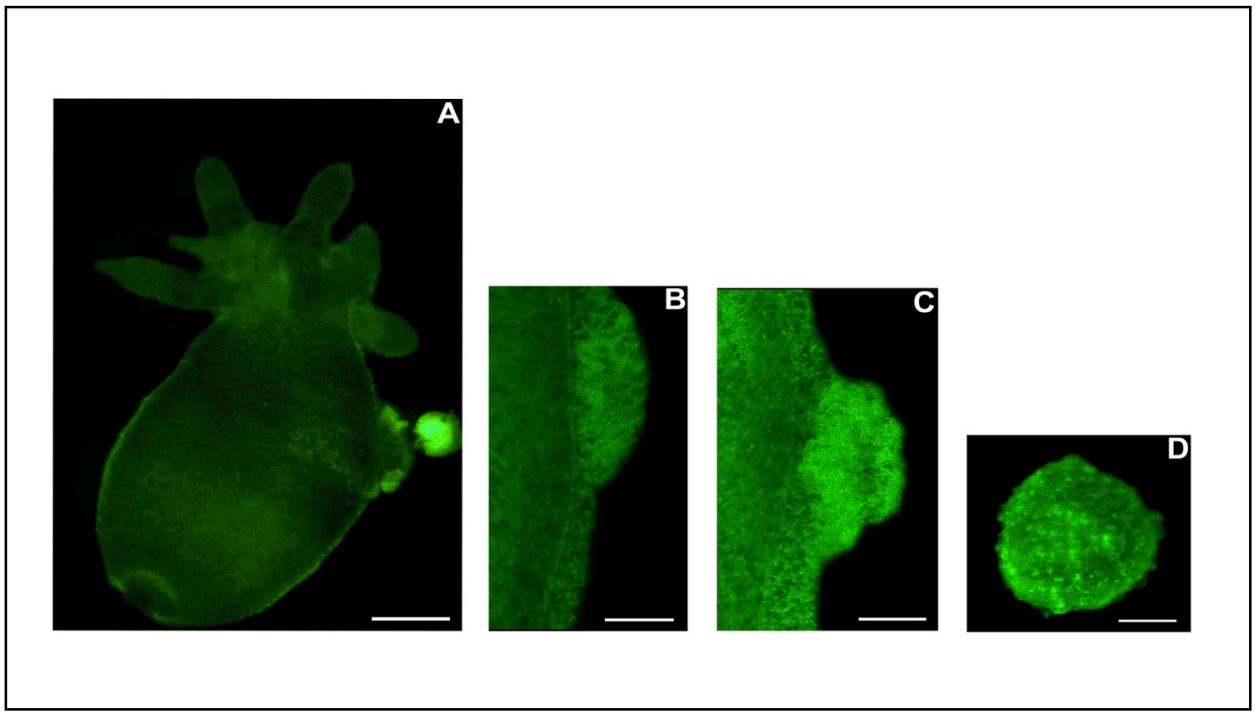

Fig. 38 Acridine orange (AO) staining during *Hydra* oogenesis. (A) AO staining of female *Hydra* polyp with a mature egg. (B) Oogenesis stage 2-3, at this stage the oocyte is determined and nurse cells start differentiating. (C) Showing stage 4-5, this is brightly stained by AO because the oocyte has phagocytosed most of the surrounding nurse cells. (D) Mature oocyte with thousands of incorporated AO-positive nurse cells; Scale bars: (A) 200 µm, (B, C, D) 100 µm.

2.7. Expression pattern of the *Bax inhibitor-1* encoding gene in *Hydra*

BI-1 was originally identified as a testis enhancer gene transcript (TEGT) in mouse. We have examined its expression in *Hydra* male polyp. The result shows that it is expressed in *Hydra* male gonads (see Fig. 39).

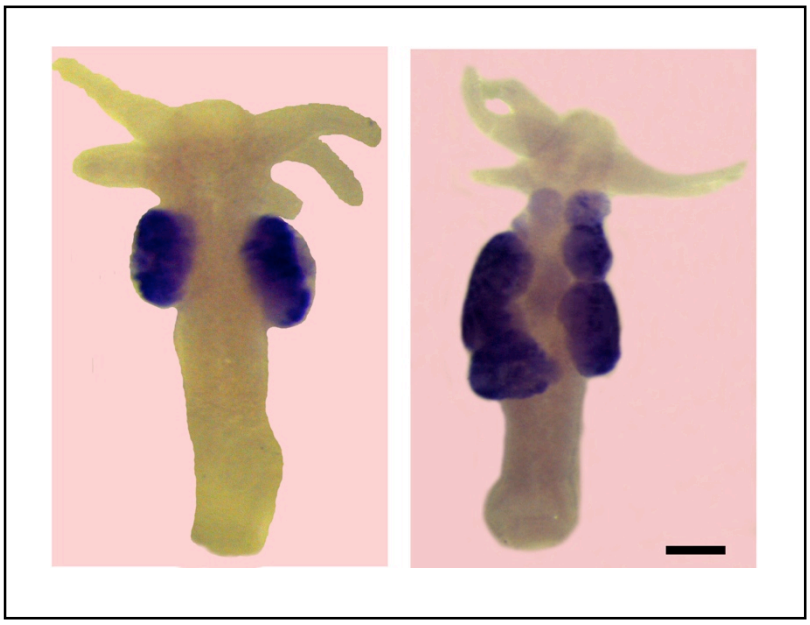

Fig. 39 *Bax-inhibitor-1* in situ hybridization on male polyp *Hydra*. NBT / BCIP used for the staining of *Hydra* BI-1 (blue signals); scale bar: 200 µm.

We also performed in situ hybridization experiments to investigate *Hydra BI-1* expression during *Hydra* oogenesis steps. The result shows that *BI-1* is expressed during all stages of *Hydra* oogenesis (Fig. 40). This is consisting with the oogenesis stages in which apoptosis occurred.

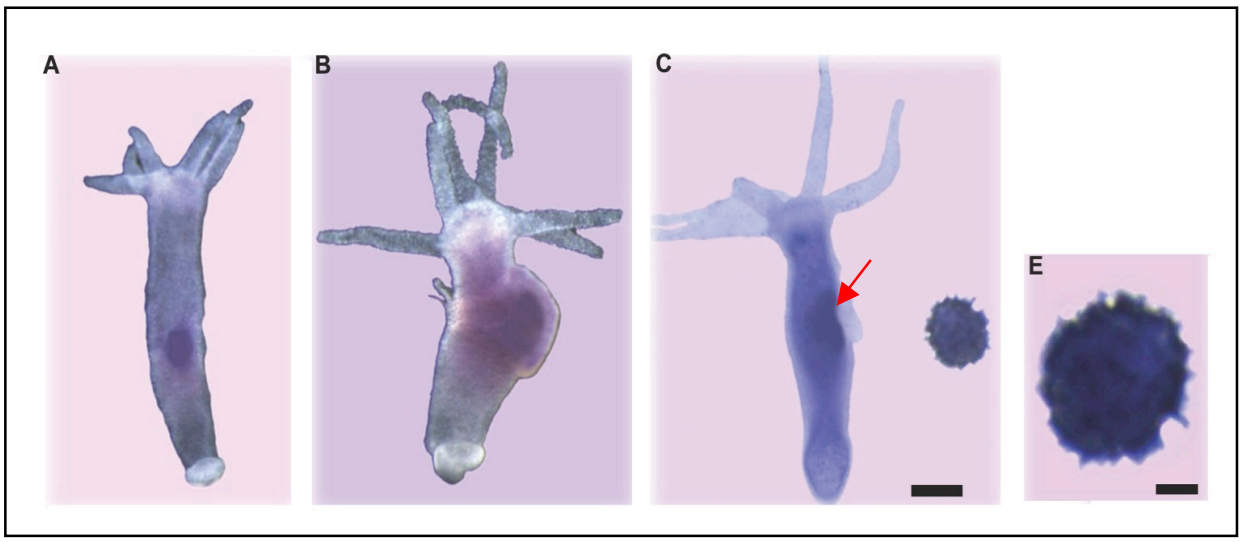

Fig. 40 *Bax-inhibitor-1* in situ hybridizations on female polyp *Hydra*; (A) stages 2-3 of oogenesis. (B) Stages 4-5 of oogenesis. (C) Red arrow shows the expression of *BI-1* at the place of egg cup. (E) Embryo. Scale bars: (A, B, C) 200 µm, (E) 100 µm.

2.8. Expression pattern of genes encoding Lifeguard protein family members in *Hydra*

2.8.1. *Lifeguard- 4*

The expression pattern of *Hydra Lifeguard-4* at the mRNA level was examined by in situ hybridization experiments on asexual and sexually differentiated *Hydra* polyps. In situ hybridization of *Lfg-4* in *Hydra* shows that it is expressed in the endodermal layer of *Hydra*. Examination of *Lfg-4* mRNA expression in sexually differentiated *Hydra* polyps demonstrated that *Lfg-4* is expressed in all *Hydra* oogenesis stages. This can be explained with the anti-apoptotic function of *Lfg-4*. In contrast to *BI-1*, there was not any signal of *Lfg-4* in the testis (see also Figs. 41-42).

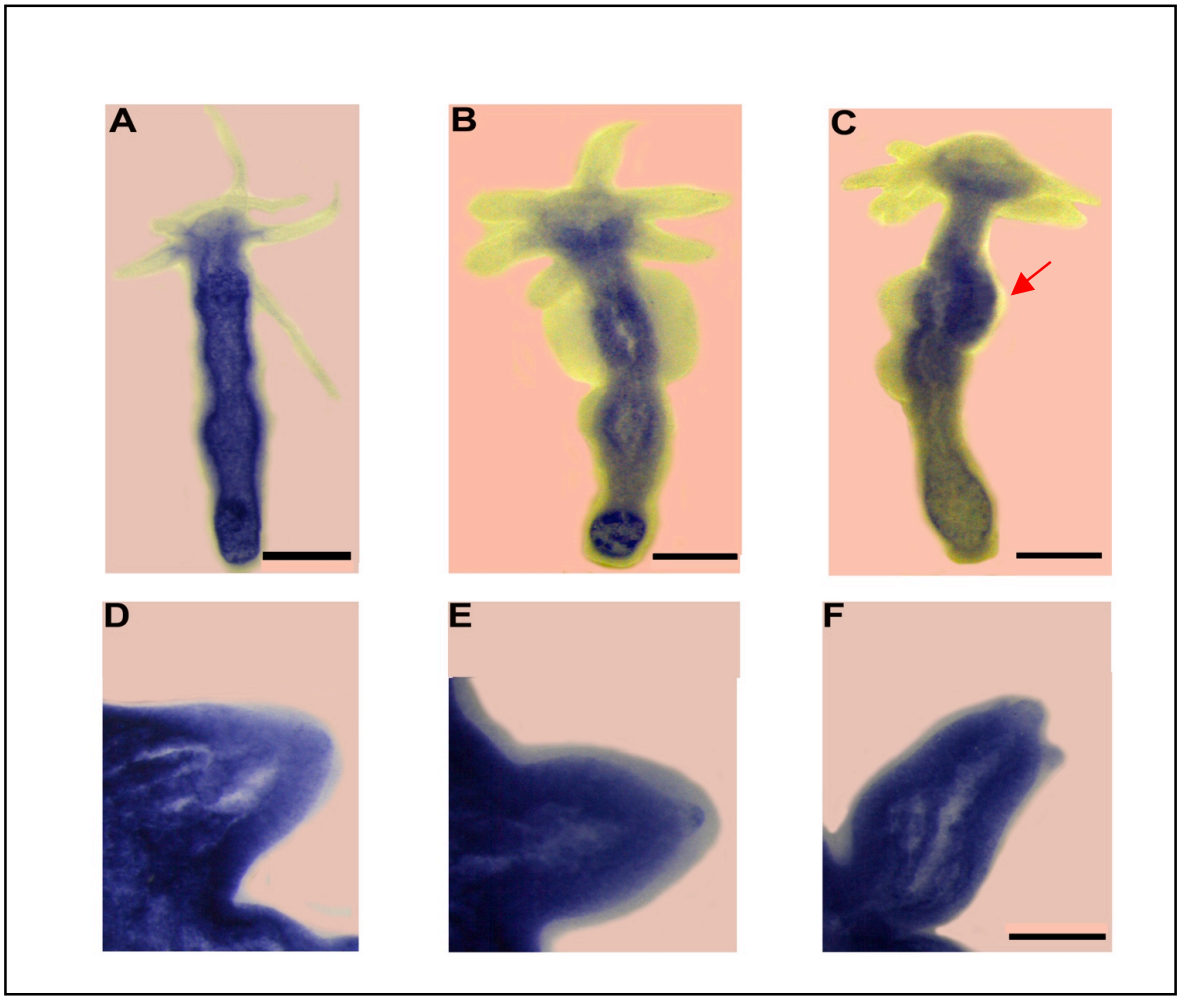

Fig. 41 *Lfg-4* in situ hybridizations; (A) *Lfg-4* expression in whole asexual animal; (B) Male polyp with testis, there is no signal in the testis; (C) Hermaphrodite *Hydra* polyp with testis and oocyte. Red arrow indicates *Lfg-4* expression in the oogenesis stages 3-4 but there is no signal in the testis. (D, E, F) Show budding stages of *Hydra vulgaris* and expression of *Lfg-4* in the endodermal cell layer. Scale bars (A, B, C) 200 µm; (D, E, F) 100 µm.

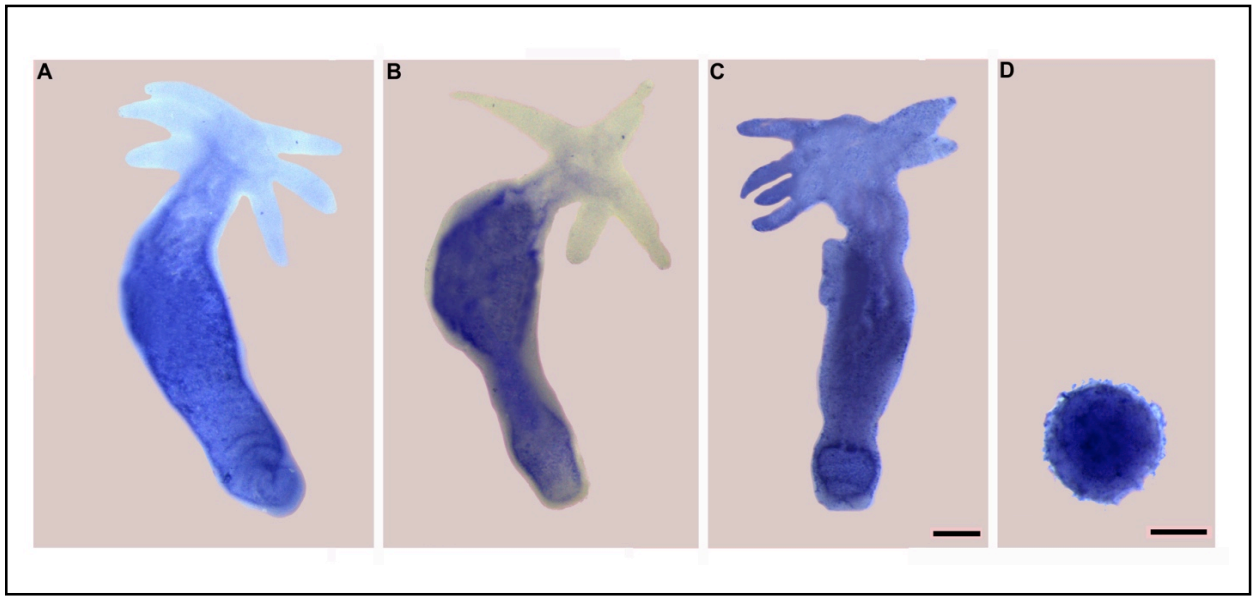

Fig. 42 *Lfg-4* in situ hybridizations on female polyps of *Hydra*; (A) stages 2-3 of oogenesis. (B) Stages 4-5 of oogenesis. (C) A female *Hydra* polyp from which an egg has recently detached. (D) Early embryo. Scale bars (A, B, C) 200 µm and (D) 150 µm.

2.8.2. *Lfg-1ia* and *Lfg-1ib*

In situ hybridization experiments were performed for *Hydra Lfg-1i* homologues in both sexual and asexual *Hydra* polyps several times. There were some signals in the foot and head region of *Hydra* polyps but they seemed to be unspecific. Moreover, there were not any specific signals in male polyp and testis. In situ hybridization of female polyps in all oogenesis steps did not show any *Lfg-1i* expression (see Figs. 43-44).

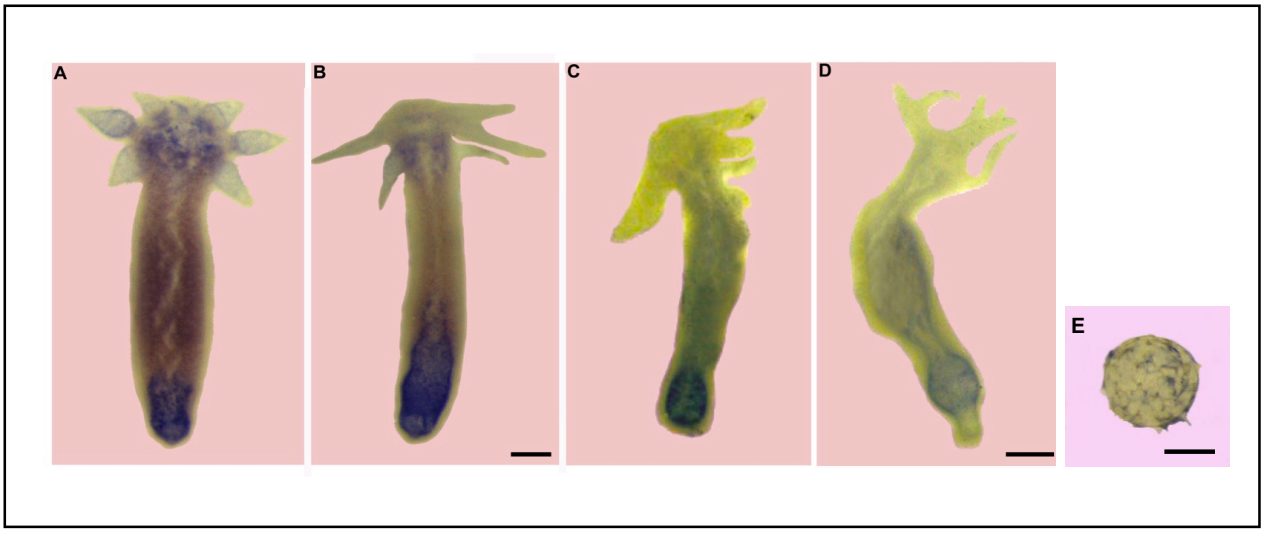

Fig. 43 *Lfg-1ia* in situ hybridization in *Hydra*; (A, B) Asexual *Hydra vulgaris* (C) male polyp with testis; (D) female polyp; (E) Early embryo; Scale bars: (A,B) 200 µm, (C,D) 150 µm and (E) 200 µm.

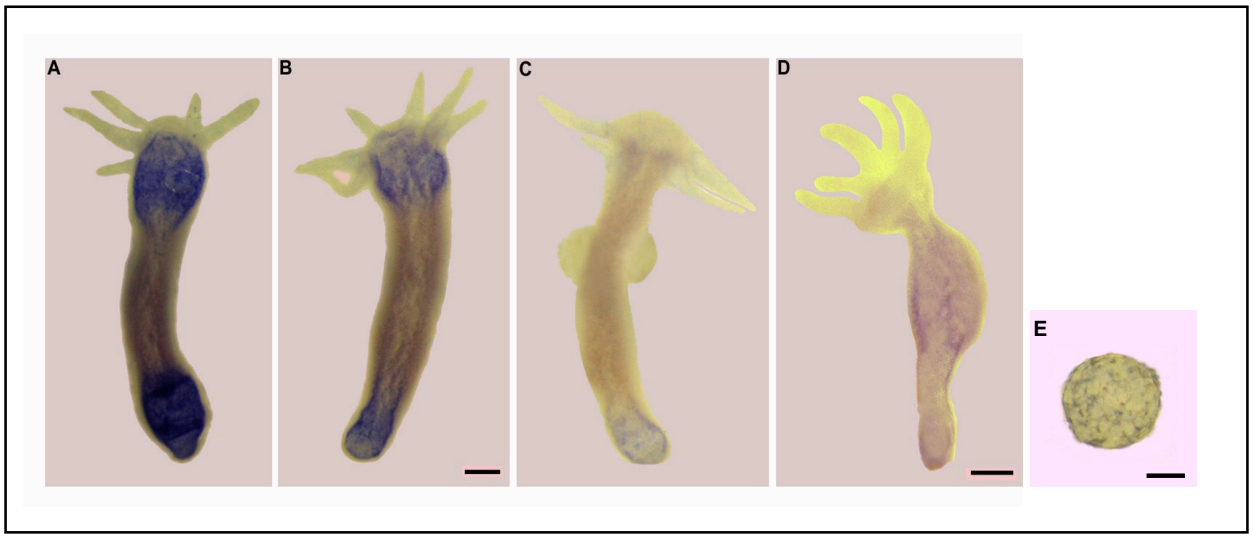

Fig. 44 *Lfg-1ib* in situ hybridization in *Hydra*; (A, B) Asexual *Hydra vulgaris* (C) male polyp; (D) Female polyp; (E) early embryo; Scale bars: (A,B) 200 µm, (C,D) 150 µm and (E) 200 µm.

2.9. Investigation of *Hydra Lfg-4* regulation in Benzo[*a*]pyrene treated polyps

Benzo[*a*]pyrene (BP) is a polycyclic aromatic hydrocarbon found in coal tar. It is a pro-carcinogen component, meaning that the mechanism of carcinogenesis of

benzo[*a*]pyrene depends on enzymatic conversion of benzo[*a*]pyrene to the ultimate mutagen, benzo[*a*]pyrene diol epoxide.

Figure 45 shows three enzymatic metabolic intermediates of BP to produce Benzo[*a*]pyrene-7, 8-dihydrodiol-9, 10-epoxide. This is the carcinogenic product of three enzymatic reactions:

(i) the BP is oxidized by cytochrome P450 1A1 and produces a variety of products, including (+)benzo[*a*]pyrene-7,8-epoxide. (ii) This product is metabolized by epoxide hydrolase, opening up the epoxide ring to yield (-)benzo[*a*]pyrene-7,8-dihydrodiol. (iii) The ultimate carcinogen is formed after another reaction with cytochrome P4501A1 to yield the (+)benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (Jiang et al., 2007) (Fig. 45).

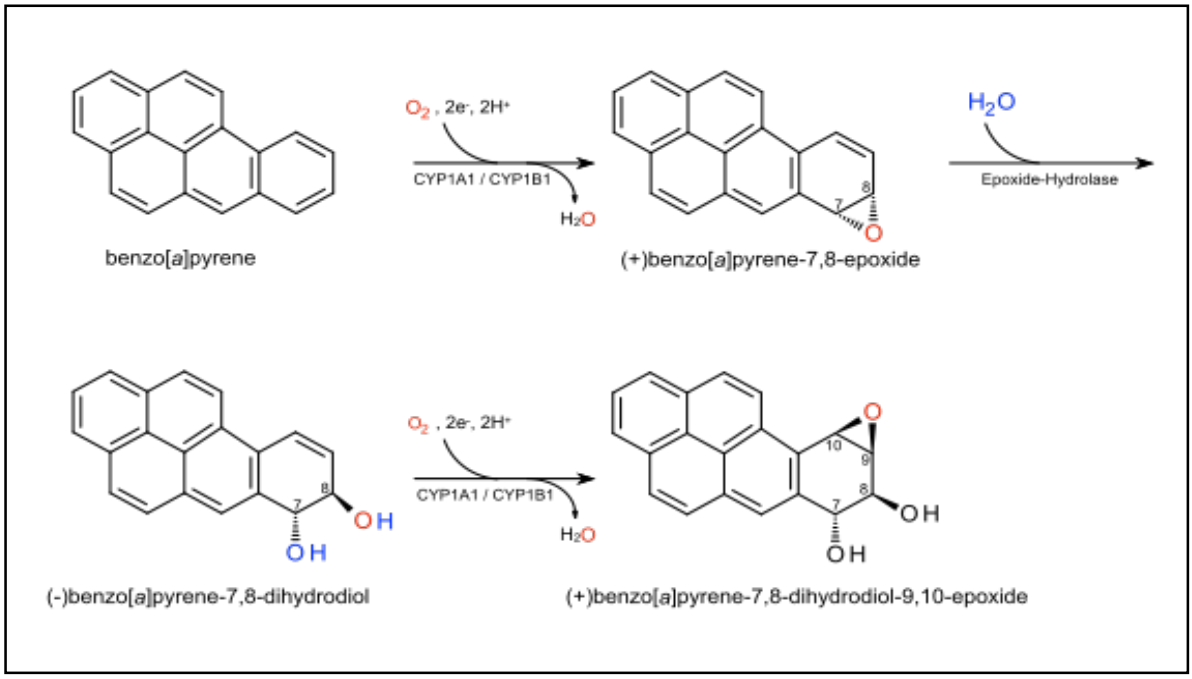

Fig. 45 Three enzymatic metabolic intermediates of benzo[*a*]pyrene to produce carcinogenic Benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (modified from Elie et al., 2012).

Benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide covalently binds to the nucleophilic guanine bases of DNA . This binding distorts the DNA and induces mutations by perturbing the double-helical DNA structure (Volk et al., 2003). This disrupts the normal process of copying DNA and induces mutations and occurrence of cancer.

Here in this study, we performed an experiment to evaluate the *Hydra* Lfg-4 mRNA up regulation in Benzo[*a*]pyrene treated polyps by semi-quantitative PCR (SQ-RT PCR). The RT-PCR method can be used not only to detect specific mRNAs but also to semi-quantify their levels. Therefore, one can compare levels of transcripts in different samples. This can be done by quantitation against levels of transcripts from a control, house-keeping gene (such as Actin and GAPDH). Transcription of house-keeping genes is believed to be unaffected by almost all experimental conditions.

In this study, about 100 *Hydra* polyps were treated with 45 µg/L Benzo[*a*]pyrene for 48 hours. 45 µg/L Benzo[*a*]pyrene solutions were prepared from stock solution of 1000 µg/mL Benzo[*a*]pyrene in acetone. Thereafter, mRNA was extracted and cDNA prepared. The cDNA of polyps treated with 45 µg/L acetone was used as standard and actin-cDNA was amplified as housekeeping gene for comparison.

Semi-quantitative RT-PCR was performed and the products were analyzed after different numbers of amplification cycles. The exponential amplification usually occurs only during the middle cycles, and this depends on the concentration of template, therefore comparison can be done only during this phase. After PCR, reaction products are separated in agarose gel electrophoresis. Images of PCR products were analyzed by visual comparison (see also Fig. 46).

In both 45 µg/L Benzo[*a*]pyrene treated-cDNA and standard cDNA (45 µl/L Acetone treated), *actin* amplified after 25 cycles. This indicates that *actin* transcriptions are unaffected in the Benzo[*a*]pyrene (BP) treated cDNA. Moreover, it shows that there

are the same amounts of initial template concentration in PCR reactions. In 45 µg/L Bp treated cDNA, *Lfg-4* amplified after 26 cycles, however in the standard cDNA (45µl/L Acetone treated-cDNA) only after 28 cycles. This indicates that ca. four times up regulation occurred in *Hydra Lfg-4* transcripts in BP treated cDNA in compare to the standard cDNA. The up-regulation was not detected for the two other *Lfg-1i* homologous in BP treated c-DNA. *Lfg-1ia* cDNA was amplified after 29 cycles in the standard cDNA and after 30 cycles in 45 µg/L Bp treated cDNA (see also Fig. 46). *Lfg-1ib* cDNA was amplified after 30 cycles in both the 45 µg/L BP treated cDNA and standard cDNA (Fig. 46).

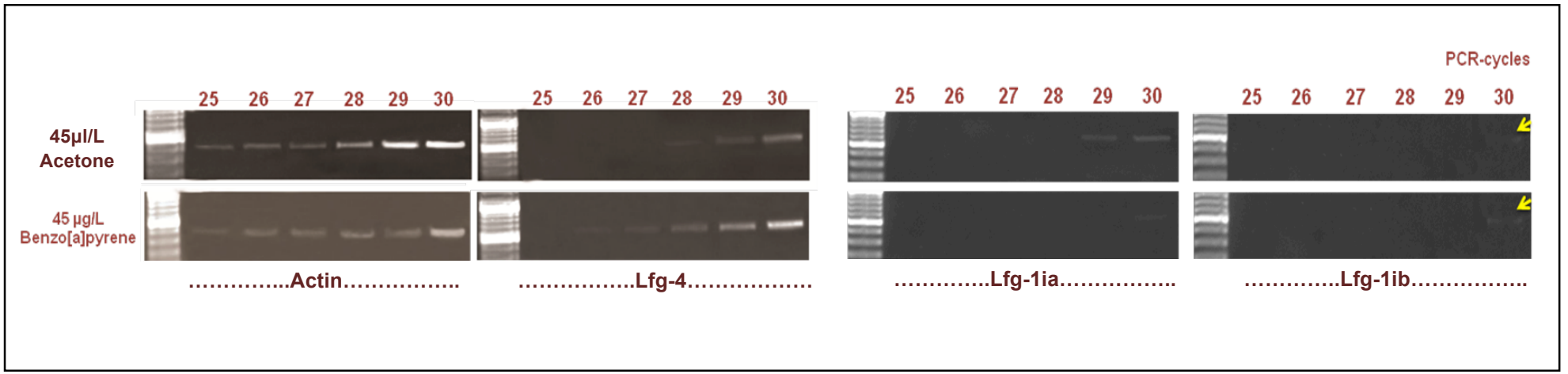

Fig. 46 *Lfg-4*, *Lfg-1ia* and *Lfg-1ib* semi quantitative RT-PCR (Sq-RT-PCR) in cDNA prepared from Benzo[*a*]pyrene treated *Hydra* cDNA. It is performed with normal PCR in parallel with 25, 26, 27, 28, 29, and 30 cycles. Yellow arrows indicate the PCR bands in *Lfg-1ib* transcript after 30 cycles. 45µl/L acetone treated cDNA used for control and *Actin* used as housekeeping gene for comparison.

3. Discussion

3.1. The TMBIM (Transmembrane Bax inhibitor-1 motif containing) super family in *Hydra*

Historically, the main attention in the field of programmed cell death regulation has been focused on apoptosis in animals. Especially the role of the Bcl-2 protein family with its function in mitochondria has been addressed (Luo et al., 1998). Although Bcl-2-related proteins are essential in the regulation of cell death in animal models, most members are poorly conserved in non-animal species where programmed cell death also has an active role in development.

In contrast to the Bcl-2 family, the TMBIM super family members are highly conserved in evolution, with a possible common ancestor in yeast (Rojas-Rivera et al., 2012). TMBIM is an apoptotic protein related family and it is present in all phyla and even in species where no BCL-2 family members have been identified, like plants and viruses (Rojas-Rivera et al., 2012).

The molecular apoptosis signaling pathway is very well conserved in *Hydra.* Two Bak-like and seven Bcl-2-like proteins have been described. It was demonstrated that the caspase family and Bcl-2 genes are expanded in *Hydra* and functionally participate in apoptosis (Lasi et al., 2010).

Moreover, one member of the TMBIM super family was already described in *Hydra* i.e., Bax-inhibitor-1. The Bax inhibitor-1 (BI-1) is a small evolutionary conserved protein (25 to 27 kDa) and located predominantly in the endoplasmic reticulum (ER) (Xu and Reed, 1998). In *Hydra* cells, BI-1 inserted into the ER and was used as an ER marker (Lasi et al., 2010). The BI-1 plays as a role as a regulator of Ca^{2+} in the ER, which is the main cellular store for Ca^{2+}. The anti-apoptotic activity of BI-1 is related to ER stress inducing agents (Robinson et al., 2011).

In addition to the Bax-inhibitor-1, the lifeguard protein family also belongs to the TMBIM super family. The distinct Lifeguard family is very ancient within eukaryotes and widely distributed in this group (Hu et al., 2009). In this study, the Lifeguard family was examined in *Hydra.* A search of whole genome sequences from *Hydra* revealed sequences with homology to the Lifeguard family; these included three members, namely Lfg-4, Lfg-1ia and Lfg-1ib. The PFAM program assigned all of these members to the UPF0005 (Uncharacterized protein family), whose members are predicted to contain six or seven transmembrane domains. Recently the UPF0005 protein family renamed to Bax1-I (Inhibitor of apoptosis-promoting Bax1) (http://pfam.sanger.ac.uk/family/PF01027).

The TMHMM program predicted seven α-helix transmembrane domains structure for all lifeguard protein members in *Hydra.* They are predicted to be localized in cellular membranes of the endoplasmic reticulum, the Golgi and in the plasma membrane.

There were not any signal peptides at the N-terminal end of the predicted protein sequences; however, a consensus motif for the protein family UPF0005 is present in the C-terminal of TMBIM protein family members (Reimers et al., 2006). The cytoprotective function of BI-1/TMBIM6 proteins has been mapped to their C-terminus. This part of the protein sequence is highly hydrophilic and well conserved in the BI-1 proteins in plants and animals (Kawai-Yamada et al., 2004).

Sequence analysis revealed that *Hydra* Lfg-4/TMBIM4 is a very well conserved protein. It is also called Golgi-anti-apoptotic protein because of its function and sub-cellular location. In comparison to the BI-1, the C-terminal region of all *Hydra* Lfg proteins is predicted to be extracellular. In addition to Lfg-4, two homologues of Lfg-1i are present in *Hydra*. Lfg-1i is a derivative from a common Lfg-1/1i ancestor occurring prior to metazoan radiation i.e., about 600 MYa. Among the vertebrates,

only ray finned fishes seem to have an Lfg-1i gene (Hu et al., 2009). The Lfg-1i gene was originally identified in invertebrates, and is found in mollusk, sea anemone, amphioxus as well as purple sea urchin (Hu et al., 2009). The biological role of Lfg-1i still remains undefined.

In contrast to Lfg-1i, a physical interaction between the vertebrate Lfg-1/TMBIM3 homologs and BI-1/ TMBIM6 was shown in 293T cells, indicating that they do form part of a protein complex. Therefore, they have synergistic function for anti-apoptotic activity and modulate ER calcium homeostasis (Rojas-Rivera et al., 2012).

3.2. The evolution of the Lifeguard protein family

The phylogenetic relationship between Lifeguard and Bax inhibitor-1 proteins in animals and plants shows that the Bax Inhibitor-1 protein family forms a separate clade from the Lifeguard protein family members (see also Fig. 24). This is consistent with previous findings showing that only five members of TMBIM super family constitute a unique protein family i.e., Lifeguard protein family (Hu et al., 2009). The *Hydra* Lfg-4 is grouped with vertebrate and invertebrate Lfg-4 family members and *Hydra* Lfg-1ia and Lfg-1ib homologues are most similar to invertebrate Lfg-1 proteins (see Fig. 24).

Hu et al. (2009) proposed that Lfg-5 is only present in eutherian mammals and that Lfg-5 could have arisen from Lfg-2 or Lfg-3. However, the phylogenetic tree in this study indicates that Lfg-5 is a separate clade which was diverged earlier than the Lfg-1 derivatives i.e., Lfg-1i, Lfg-1, Lfg-2 and Lfg-3 (see also Fig. 24). In addition a unique and characteristic sequence motifs diagnostic of the Lifeguard family has proposed by Hu et al, (2009) (see Fig. 20). All of these motifs have been found in *Hydra* Lfg protein members as well as their human homologues (see Figs. 21-23) but not in the

BI-1 homologues. This indicated that these diagnostic motifs are very well conserved in the TMBIM super family and lifeguard protein family members form a separate family from the BI-1 protein family.

Hu et al. (2009) proposed a single common ancestral gene for all Lfg genes extant in modern phyla, a prototypic Lfg-4. The reasons are: it's most pleiotropic expression profile, the conservation of Lfg-4 lineage proteins among animals, plants and fungi, and the conservation of intron/exon gene structure in metazoan. All of these reasons point to a Lfg-4-like ancestor prior to the eukaryotic radiation of plants, animals, fungi, and protozoa i.e., about 2,000 MYa (Hu et al., 2009). *Hydra* Lfg-4 derived from the Lfg-4-like ancestor and is sister to sea anemone Lfg-4. Within the animal's clade, the Lfg-4-like ancestor was duplicated to produce the precursor to Lfg-1 and Lfg-1i. A common Lfg-1/1i ancestor generated with this subsequent duplication prior to metazoan radiation i.e., about 600 MYa. All metazoan phyla contain *Lfg-1i* and/or *Lfg-1* derived genes. In *Hydra*, two Lfg-1i homologues (i.e. *Lfg-1ia* and *Lfg-1ib*) have been found, which may be derived from Lfg-1/1i ancestor. The evolution of the Lfg family appears to have proceeded via selection on sub-cellular localization, tissue distribution, and morphogenic development (Hu et al., 2009).

3.3. Sub-cellular localization of Lifeguard proteins

To investigate the sub-cellular localization of *Hydra* Lfg proteins, the Lfg genes were fused with the GFP-gene sequence and the resulting GFP fusion proteins were over expressed in *Hydra* and HEK 293T cells. In addition, BODIPY-TR ceramide was used as Golgi marker The BODIPY-TR ceramide is a red fluorescent structural marker for the Golgi apparatus and also can outline the plasma membrane. Furthermore, *Hydra* Bax inhibitor-1 fused to RFP fusion protein (Müller-Taubenberger et al., 2006) was used as an endoplasmic reticulum (ER) marker in *Hydra* cells to investigate *Hydra* Lfg protein localization in the ER (see below).

3.3.1. *Hydra*-Lifeguard-4

The result of Lfg-4 over expression with Golgi marker in *Hydra* cells demonstrates that *Hydra* Lfg-4 localized at the Golgi complex (see also Fig. 27). Lfg-4 is known as a Golgi anti-apoptotic protein (GAAP), which is a hydrophobic protein that is resident in the Golgi (Gubser et al., 2007). It has been found that stable expression of both viral GAAP (v-GAAP) and human GAAP (h-GAAP) inhibited apoptosis induced by intrinsic and extrinsic apoptotic stimuli (Gubser et al., 2007). This was considered as evidence for the contribution of the Golgi in the regulation of apoptosis.

3.3.2. *Hydra* Lfg-1ia and Lfg-1ib

In this study, the Lfg-1ia and Lfg-1ib were over expressed in *Hydra* and their localization investigated in *Hydra* cells. Co-staining with the Golgi marker in living *Hydra* cells showed that both *Hydra* Lfg-1i homologous localized mainly at the plasma membrane, Golgi complex and in Golgi vesicles (Figs. 30-31). This is

consistent with the PSORT II program prediction for their sub-cellular localization, i.e. mainly at the plasma membrane.This is the first description of the sub-cellular localization of an invertebrate Lfg-1i protein. The biological role and sub-celullar localization of Lfg-1i in other invertebrates remains to be identified.

In contrast to the Lfg-1i, it was shown that Lfg-1/TMBIM3 is co-localized with ER and Golgi markers, and also it is expressed in the plasma membrane (Rojas-Rivera et al., 2012; Hu et al., 2009).

In addition to the *Hydra* cells, the expression patterns of all lifeguard protein members were tested in mammalian cells (see also Figs. 33-36). *Hydra* Lfg-4-GFP co-localized with the Golgi marker but GFP signal could also be detected in the nucleus. Nuclear GFP signals were also detected for the *Hydra* Lfg-1i homologues.

The most likely explanation for the nuclear localization of *Hydra* Lfg-GFP is that it is an artifact due to the expression of *Hydra* protein in human cells. For instance, the GFP-tag could have been cleaved of, it would then localize in the nucleus, as GFP usually does on its own.

3.4. *Hydra* Lfg-4 protein protects mammalian cells from campthothecin induced apoptosis

The anti-apoptotic properties of *Hydra* Lifeguard proteins were investigated in HEK 293T cells. The *Hydra* Lifeguard protein members were over-expressed in mammalian cells and apoptosis was induced with camptothecin, a topoisomerase-I inhibitor which causes DNA-double strand breaks. *Hydra* Bcl-2-4 was used for comparison, because it had already been shown that HyBcl-2-4 has a strong protective effect in apoptosis induced by camptothecin in mammalian cells (Lasi et al., 2010).

In this study, HyBcl-2-4 decreased the apoptosis from 82% of control to 18% and thus showed a strong anti-apoptotic effect as well. The expression of the *Hydra* Lifeguard proteins suppressed camptothecin induced apoptosis to different degrees. The *Hydra* Lfg-4 had a 41% suppression effect and demonstrated the strongest protective effect within the lifeguard protein familiy members. Moreover, two *Hydra* Lfg-1i homologues showed a very slight apoptosis suppression effect (see Fig. 32).

The anti apoptotic effect of *Hydra* lifeguard-4 is consistent with other studies (e.g. Gubser et al., 2007). It was revealed that the human Lfg-4 protein (h-GAAP) is essential for cell viability, and in its absence cells die by apoptosis (Gubser et al., 2007). Both human and viral GAAPs inhibit apoptosis induced by a wide range of pro-apoptotic stimuli from both the extrinsic and intrinsic pathways. However, the mechanism by which GAAPs inhibit apoptosis is not clear so far. Gubser et al. (2007) have also suggested that GAAPs act at a step in the mitochondrial pathway because of their strong ability to inhibit cell death induced by various intrinsic apoptotic stimuli. The finding that h-GAAP suppressed TNF-induced apoptosis at a step downstream of caspase-8 activation, also supports this suggestion (Gubser et al., 2007). Moreover, the protein sequences of lifeguard-4 are very well conserved among species and it can therefore be hypothesized that lifeguard-4 has a conserved function.

3.5. *Lfg-4* gene expression is up-regulated in Benzo[a]pyrene treated *Hydra* polyps

Benzo[*a*]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH). PAHs are ubiquitous environmental pollutants showing carcinogenic potency after undergoing metabolic activation. Cytochrome P450 and epoxide hydrolase are known to catalyse

the conversion of BaP into epoxides (Jiang et al., 2007), which can bind chemically to DNA and exert potent mutagenic and carcinogenic effects (Volk et al., 2003).

In this study, we performed an experiment to evaluate the *Hydra* Lfg-4 protein regulation in Benzo[*a*]pyrene treated polyps by semi-quantitative RT-PCR (Sq-RT-PCR). The Sq-RT-PCR in *Hydra* indicates that there is a greater than four fold up-regulation of Lfg-4 in Benzo[*a*]pyrene treated *Hydra* (see Fig. 46). Such an effect had also been shown in the diatom (*Thalassiosira pseudonana)* where BP exposure (at 36.45µg/L) causes a 29-fold up-regulation of transcription of the anti-apoptotic TMBIM4/Lfg-4 gene (Carvalho et al., 2011). It was already revealed that the formation of ROS is a general outcome of BP metabolism and this oxidative stress can affect cells in different ways including protein oxidative damage, DNA damage and lipid peroxidation (Carvalho et al., 2011). Therefore, these findings suggest that up-regulation of expression of anti-apoptotic genes i.e., TMBIM-4/Lfg-4 is a cyto-protective response to the accumulation of ROS and oxidative stress. This is consistent with a BP induced stress scenario, in which there is some level of cellular damage triggering repair mechanisms while apparently not being acute enough to induce cell death. So, there is significant increase in the expression of a putative transmembrane BAX inhibitor gene, an inhibitor of Bcl-2-induced cell death (Watanabe and Lam, 2009). Sq-RT-PCR in *Hydra Lfg-1ib* does not show any changes but slight down regulation of *Hydra Lfg-1ia* was detected in Benzo[*a*]pyrene treated cDNA (see also Fig. 46). Down regulation of some apoptotic related genes such as PopA and Fas apoptotic inhibitory molecule 2 (*Faim2/Lfg-2*) were detected in a marine benthic polychaete, *Perinereis nuntia* (Zheng et al., 2011). However, *Lfg-2/Faim2* evolved in bony fishes (Hu et al., 2009) and this gene might have been misidentified in *p. nuntia* and probably is a *Lfg-1* homolog. The apoptotic response at

a transcriptional level in BP-exposed *P. nuntia* is very complex because some of positive apoptosis regulators were found up-regulated i.e., caspase8 (Casp8) and programmed cell death 10 (PDCD10). This indicates that the normal apoptotic response in *P. nuntia* was disturbed by BP (Zheng et al., 2011).

3.6. TMBIM protein family member genes are expressed during *Hydra* gametogenesis

3.6.1. *Hydra BI-1* in *Hydra* spermatogenesis

In mammalian spermatogenesis, apoptosis occurs to remove defective sperm precursor cells (Yin et al., 1998) and to maintain a critical cell number ratio in the testis (Rodriguez et al., 1997). Apoptotic proteins such as caspases are involved in the process of spermatid individualization in *Drosophila* (Arama et al., 2003).

In *Hydra,* both sperm and egg differentiation are triggered by environmental signals and germ cells in *Hydra* derive from multipotent interstitial stem cells (Bosch and David, 1986). Apoptosis in testis has already been detected in *Hydra* with acridine orange staining and TUNEL assay (Kuznetsov et al., 2001). In this study, acridine orange staining on *Hydr*a male polyp was repeated to show that apoptosis occurs during *Hydra* spermatogenesis. In addition, the expression pattern of apoptosis regulatory genes i.e., *BI-1* was investigated in male polyps. There was no considerable expression signal in an asexual *Hydra* polyp, whereas a drastic *BI-1* expression signal was detected in the mature *Hydra* male polyp testis (see Fig. 39). The *BI-1* has been originally cloned from adult rat testis and has shown high identity (90%) to the testis enhanced gene transcript (TEGT) (Walter et al., 1994).

The acridine orange staining demonstrated a large number of sperm precursor cells, which apparently undergo apoptosis in testis (see also Fig. 37). In addition, it had

been shown that epithelio-muscular cells in *Hydra* testis act as Sertoli cells by controlling the microenvironment of the developing interstitial sperm precursor cells and sequestering the sperm precursor populations into environmentally distinct compartments (Kuznetsov et al., 2001). Therefore, the differentiation pathway and structure of the mature spermatozoa in *Hydra* resembles that of higher metazoans and it can be concluded that apoptosis is involved in *Hydra* spermatogensis.

However, in contrast to the growing oocytes, sperm precursors do not feed on adjacent cells. Therefore, the function of programmed cell death and the presence of apoptosis regulatory proteins in testis do not seem to be related to the nutrition. Apoptosis may contribute to maintain a critical cell number ratio between differentiating spermatogonia and epithelio-muscular cells as well as between sperm precursors at different stages of spermatogenesis (Print and Loveland, 2000). Therefore, three major roles for cell death during spermatogenesis can be proposed. Firstly, apoptosis of sperm precursors and their removal by epithelial cells may be used as “quality control” to eliminate defective sperm. Secondly, programmed cell death may also be used to achieve the precise homeostasis of each germ cell type in the mature *Hydra* testis and finally apoptosis may involve in the spermatozoa maturation and individualization.

3.6.2. *Hydra Bl-1* and *Lfg-4* in *Hydra* oogenesis

Presence of apoptosis during oocyte development has been confirmed in animals (McCall and Steller, 1998). The common feature is that multiple germ cells contribute their cytoplasm to the oocyte. In *Hydra*, after the cytoplasm contribution, the remains of nurse cells are phagocytosed by the oocyte (Honegger, 1989; Miller et al., 2000; Alexandrova et al., 2005) but the nurse cells are not further degraded and instead

they are kept by oocyte enclosed to the phagocytic vacuoles, which is termed as an arrested apoptosis (Technau et al., 2003). Apoptosis during *Hydra* oogenesis has already been detected in *Hydra* with acridine orange staining (Technau et al., 2003). In this study, acridine orange staining on *Hydr*a female polyp was repeated to show that apoptosis occurs during *Hydra* oogenesis. In addition, the expression pattern of apoptotis regulatory genes i.e., *BI-1* and *Lfg-4* was investigated during oogenesis steps. First acridine orange positive nurse cells are detected at stage 2-3 of oogenesis (see Fig. 38B). This is a time when the nurse cells are phagocytosed by the growing oocyte (Miller et al., 2000; Alexandrova et al., 2005). Later stages showed that the number of AO-positive nurse cells rapidly increases, and results in a brightly stained oocyte with thousands of densely packed green nurse cells inside the oocyte (see Fig. 38C). The *BI-1* in situ hybridization showed expression during all stages of *Hydra* oogenesis, and also in the embryo (Fig. 40). This is in consistent with the oogenesis stages in which apoptosis occurred. In situ hybridization of *Lfg-4* revealed its expression during *Hydra* oogenesis stages and also in the endodermal epithelia cell layer of *Hydra.* Both Bax-inhibitor-1 and Lifeguard-4 have been implicated as anti-apoptotic proteins in other systems (e.g. Xu and Reed, 1998; Gubser et al., 2007). Therefore, these protective proteins might be responsible for preventing nurse cells from completing apoptosis until hatching. In addition, some other anti apoptotic agent such as peroxidase has been found in the nurse cells from early stages of oogenesis on throughout embryogenesis (Habetha and Bosch, 2005). Therefore, these proteins may be involved in arresting apoptosis during *Hydra* oogenesis.

3.7. Expression pattern of Lifeguard protein family members in *Hydra*

Analysis with in situ hybridization in asexual and sexually differentiated *Hydra* polyp, demonstrated the expression of Lfg-4 in the whole endodermal layer of the polyp (Fig. 41). The analysis has additionally shown Lfg-4 expression in the endodermal layer during all budding stages (see also Fig. 41). In contrast to the oogenesis procedure, there was no signal of Lfg-4 in the testis (see Figs. 41-42). Lfg-4 known as a Golgi anti-apoptotic protein and has been shown to be expressed in all human tissues. It can inhibit apoptosis induced by intrinsic and extrinsic pathway activators and was identified as a candidate house-keeping gene (Hu et al., 2009).

Lfg-1i homologues expression patterns in both asexual and sexually differentiated *Hydra* polyps were examined several times. Expression signals were detected in the foot and head region of *Hydra* polyps but they seemed to be unspecific and their expression pattern remains undetermined. Moreover, *Lfg-1i* expression was not detected in oocytes and testes (see Figs. 43-44). In addition, the biological role of Lfg-1i still remains undefined but a correlation between the evolutionary appearance of the animal Lfg genes and their expression profiles and sub-cellular localization has been proposed by Hu et al. (2009). For example, five Lfg-1i homologues (i.e. Lfg-1ia-e) are present in fruit fly. The Lfg-1ia expression is pleiomorphic; Lfg-1ib is expressed in the midgut, tubule and hindgut; Lfg-1ic and Lfg-1id are mainly expressed in testis; and the expression profile of Lfg-1ie is undetermined. As suggested by Hu et al. (2009), their expression profile shows increasing in specificity.

3.8. TMBIM super family is involved in *Hydra* development

In conclusion, this research study revealed that in addition to the conserved apoptosis signaling pathway, which was already described in *Hydra* (see Lasi et al., 2010), an ancient and ubiquitarily distributed apoptosis related gene family is also present in *Hydra*. The transmembrane Bax inhibitor 1 (BI-1) motif containing super family has four members in *Hydra* i.e. Bax inhibitor-1 and three Lifeguard members. The Lfg and BI-1 are closely related cytoprotective proteins and they are evolutionarily conserved within many different species including plants. The existence of highly conserved homologues indicates functional conservation for these proteins as well. A cytoprotective and anti-apoptotic function of *Hydra* BI-1 and Lfg-4 could be confirmed in this study. These two proteins are apparently involved in *Hydra* gametogenesis and thus expressed in a place where apoptosis occurs during development.

4. Zusammenfassung

Der programmierte Zelltod stellt einen wichtigen Mechanismus zur Regulation der Embryonalentwicklung sowie der Antwort auf Zell- und besonders DNA-Schädigung dar. Dies gilt für Pilze, Pflanzen und sogar einzellige Protisten. Der programmierte Zelltod in Tieren (als Apoptose bezeichnet) ist sehr gut charakterisiert und viele seiner molekularen Komponenten kommen nur in Tieren vor. Dazu gehören Caspasen und Bcl-2-Proteine, die innerhalb der Metazoen in einfachen Pre-Bilateria einschließlich der Cnidaria konserviert sind, jedoch nicht außerhalb der Metazoa. Der Fokus dieser Studie liegt auf Regulatoren des programmierten Zelltodes, die sowohl in tierischen als auch Pflanzen, Pilzen und Protisten vorkommen. Sie werden in dem Nesseltier *Hydra vulgaris* untersucht. Im Rahmen dieser Arbeit wurden drei Gene in Hydra identifiziert, die für sogenannte „Life guard"-Proteine (Lfg) kodieren. Es handelt sich um Lfg-1ia, Lfg-1ib und Lfg-4. Diese Proteine haben 7 α-helikale Transmembrandomänen. Zusätzlich wurde ein Gen untersucht, welches für Bax-Inhibitor kodiert, ein Protein mit 6 α-helikalen Transmembrandomänen. Phylogenetische Analyse zeigte, dass Lfg und Bax-Inhibitor Proteine getrennte Proteinfamilien bilden die bereits vor der Trennung aller eukaryotischen Organismenstämme separiert waren. Die Ergebnisse zeigen, dass mindestens ein Mitglied der Lfg-Familie sowie Bax-Inhibitor aus Hydra einen sehr starken schützenden Effekt vor Campthothecin induzierter Apoptose in menschlichen HEK 293T-Zellen zeigten. Außerdem wurde eine Aufregulierung von Lfg-4 in Benz(a)pyren behandelten Hydren beobachtet. Während Bax-Inhibitor im ER von Hydrazellen lokalisiert war, fand sich Hydra-Lfg-4 im Golgi-Komplex, Hydra Lfg-1ia und Lfg-1ib zusätzlich zu Golgi-Vesikeln in der Plasma-Membran. In situ-Hybridisierungen zeigten, dass sowohl Hydra Lfg-4 als auch Hydra Bax-Inhibitor in

der Gametogenese von Hydra eine Rolle spielen könnten. Während der Oogenese und auch der Spermatogenese in Hydra kommt es zu massiver Apoptose. Diese Ergebnisse stützen die Hypothese, dass Golgi-, ER- und Plasmamembranproteine der Bax-Inhibitor und Lfg-Proteinfamilien in sehr frühen Metazoa bereits in die Regulation des programmierten Zelltods involviert waren.

5. Materials and methods

5.1. Materials

5.1.1. Chemicals and companies

Acetanhydrid	Sigma
Acrylamid-Bisacrylamid (29:1)	Biorad
AG501-X8 20-50m (Resin)	Biorad
Agarose PeqGold Universal agarose	PeqLab
Agarose	Biozym
Ammonium acetat	Sigma
Ampicillin	Serva
Atermia nauplii	Sanders Brine Shrimp
Bacto Agar	BD
BactoTM Pepton	Difco
Benzo[*a*]pyrene	Sigma
BCIP	Roche
Blocking reagent	Roche
BM-Purple AP Substrate	Roche
Bromphenol blue	Fluka
BODIPY TR ceramide	Life technology
BSA	Roth
Calcium chloride	Roth
CHAPS	Sigma
Chloramphenicol	Serva
DAPI	Sigma
DAPT	Calbiochem
DEPC	Sigma, Roth
DIG RNA labeling mix	Roche
DMSO	Sigma
dNTP mix	PeqLab
DTT	Sigma
EDTA	Roth
acetic acid p.a.	Roth
Ethanol p.a.	Roth
Ethidium bromid	Sigma
Evans blue	Sigma
Ficoll Typ 400	Sigma
Fluorescein RNA labeling mix	Roche
Formaldehyde	Fluka
Formamid	Fluka
Glucose	Merck
Glycerin	Roth
Glycin	Roth
Guanidinium-HCl	Sigma
Yeast extract	Oxoid
Heparin Sodium	Serva
Imidazol	Roth

Isopropanol p.a.	Roth
di-Kalium hydrogen phosphat-Trihydrat	Roth
Kanamycin	Roth
Lithium chlorid	Merck
Lipofectamine 2000	Invitrogen
Magnesium chlorid-Hexahydrat	Merck
Magnesium sulfat-Heptahydrat	Fluka
Maleic acid	Sigma
Manganchlorid	Sigma
2-Mercaptoethanol	Sigma
MOPS	Roth
Methanol p.a.	Roth
Natriumacetat	Roth
Natriumchlorid	Roth
Tri-Natriumcitrat-Dihydrat	Merck
Natrium dihydrogen phosphat-Dihydrat	Dihydrat Roth
Natrium dihydrogen phosphat-Mono hydrat	Merck
TBT	Roche
Ni Sepharose High Performance	Amersham Biosciences
Paraformaldehyd	Sigma
Potassium acetat	Roth
Potassium chlorid	Fluka
Potassium dihydrogen phosphat	Merck
RNase-Inhibitor	Fermentas
Saccharose	Roth
Hydrochloric acid	Roth
SDS	Roth
Spermidin	Fluka
TO-PRO 3	Molecular Probes
Torula yeast RNA	Sigma
Triethanolamin	Sigma
Tris	Roth
Triton X-100	Roth
Trypton	Oxoid
Tween 20	Roth
Urethan	Fluka
Vectashield	Vector Laboratories

5.1.2. Bacterial strains

XL1-Blue MRF´	D(mcrA)183 D (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F'proAB $lacI^qZDM15$ Tn10 (Tet^r)]
BL21 (DE3)-RIL	E.coli B F-dcm ompT hsdS(rB-mB-)gal λ(DE3)
One Shot® TOP10	F- mcrA ∆(mrr-hsdRMS-mcrBC)Φ80lacZ ∆M15 ∆lacX74 recA1 araD139 ∆(araleu) 7697 galU galK rpsL (StrR) endA1 nupG
SoloPack Competent Cells	StrataClone TM Blunt PCR Cloning Kit /Catalog #240207

5.1.3. Enzymes

Restrictions enzyme with Reactions buffer	New England Biolabs
DNase I (RNase free)	Roche
DNase I	Sigma
Proteinase K	Sigma
RNase A Quiagen	Quiagen
Shrimp alkaline Phosphatase	GE Healthcare, NEB
Taq DNA-Polymerase and Reactions buffer	PeqLab
Phusion Flash High-Fidelity PCR Master Mix	NEB
T4-DNA-Ligase and Reactions buffer	NEB
T3-RNA-Polymerase	Ambion
T7-RNA-Polymerase	Ambion

5.1.4. Equipments

Bandelin Sonoplus SD9	Bandelin
Branson Digital Sonifier Model 450-D G	G.Heinemann Ultraschall- und Labortechnik
Primus 25 advanced thermal cycler	PeqLab
Gene Pulser	BioRad
Gene Pulser	BioRad
Gene Pulser Capacitance Extender	BioRad
GeneQuant RNA/DNA Calculator	Amersham Pharmacia Biotech
Leica MZ16FA	Leica Microsystems
Leica TCS SP5 Confocal laser-scanning	Leica Microsystems
Nikon Eclipse 80i	Nikon GmbH
Odyssey Infrared Imager	Licor
Olympus SZX10	Olympus
PDS-1000/He Particle Delivery System	BioRad
UV/Vis Spectrophotometer DU730	Beckmann Coulter
Ultra centrifuge	Beckmann
Robocycler Gradient 96	Stratagene
UV-Stratalinker 1800	Stratagene
UV table	MWG Biotech, INTAS

5.1.5. Kits

First-Strand cDNA Synthesis Kit	GE Healthcare
RNeasy Mini Kit	Quiagen
GeneRacer Kit	Invitrogen
NucleoBond PC500	Macherey-Nagel
PeqGOLD Gel extraction kit	PeqLab
StrataClone Blunt PCR Cloning Kit	Stratagene
QuickPrep Micro mRNA Purification Kit	GE Healthcare
QIAquick Gel Extraction Kit	Quiagen
Zero Blunt TOPO PCR Cloning kit	Invitrogen
Quickchange site directed mutagenesis kit	Stratagene
GeneJET™ Genomic DNA Purification Kit	Fermentas

5.1.6. Other materials

Macro carrier…….. BioRad

Rupture disks …… BioRad

Gold balls 1.0 µm……BioRad

Faltenfilter Sartorius……...Stedim Biotech

Hybond ECL Nitro cellulose Membrane…….Amersham Biosciences

5.1.7. Softwares

Adobe Photoshop

Adobe Illustrator

Leica Application Suite 3.4.1

Leica Confocal Imaging Software

ApE	http://biologylabs.utah.edu/jorgensen/wayned/ape/
Genomes	http://hydrazome.metazome.net/cgi-bin/gbrowse/hydra/
	http://www.arabidopsis.org/
	http://www.wormbase.org/
NCBI	http://www.ncbi.nlm.nih.gov/
PROMO 3.0	http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi
SignalP 3.0	http://www.cbs.dtu.dk/services/SignalP/
TMHMM 2.0	http://www.cbs.dtu.dk/services/TMHMM
Geneious 6.1	http://www.geneious.com/

5.1.8. Buffers and solutions

DNA loading buffer	30% (v/v) 0.25 mg/ml 0.25 mg/ml	Glycerin Bromphenolblue Xylen blue
Ethidium bromide staining	3.3 mg/ml 0.7x	Ethidium bromide MOPS-solution
10x MOPS-solution	440 mM 84 mM 10 mM pH 7.0 with NaOH, autoclave	MOPS Natriumacetat EDTA
50x TAE-buffer	2 M 50 mM pH 8.3 with Acetic acid	Tris EDTA

Blocking solution (whole mounts)	1% (w/v)	BSA
	0.1% (v/v) in 1x PBS	Triton X-100
DAPI/TOPRO staining solution	1 µg/ml DAPI	DAPI
	1 µg/ml in 1x PBS	TO-PRO 3
Lavdowsky-Fixative	3.6% (v/v) 3.8% (v/v 48% (v/v)	Formaldehyd Acetic acid Ethanol
25x PBS	3 M 1 M 0.3 M pH 7.2 – 7.4	NaCl K_2HPO_4 x 3 H_2O NaH_2PO_4 x H_2O
Permeabilization solution	0.5% (v/v) in 1x PBS	Triton X-100
2% PFA/HM 2% PFA/PBS 2% Urethan	2% (w/v) 2% (w/v) 2% (w/v)	PFA in Hydra medium PFA in 1x PBS Urethan in Hydra medium

10% Glycerin	10% (v/v) sterilized by filtration	Glycerin in H_2O
LB-Agar	20 g/l Autoclave	Bacto Agar in LB-Medium
LB-Medium	1% (w/v) 0.5% (w/v) 1% (w/v) pH 7.0 Autoclave	BactoTM Pepton Hefe extract NaCl
LB-Ampicillin-Medium	100 µg/ml	Ampicillin in LB-Medium
LB-Chloramphenicol-Medium	100 µg/ml	Chloramphenicol in LB-Medium
LB-Tetracyclin-Medium	5 µg/ml	Tetracyclin in LB-Medium
SOC-Medium	2% (w/v) 0.5% (w/v) 8.6 mM 2.5 mM pH 7.0 autoclave 10 mM 20 mM	Trypton Hefe extract NaCl KCl $MgCl_2$ x 6 H_2O Glucose (sterilized by filtration)

RNase A-solution	5µg/ml	RNase A
2.5M $CaCl_2$	2.5 M	$CaCl_2$
50% Glycerin/Hydra medium	50% (v/v)	Glycerin in Hydra medium
Hydra medium	0.1 mM 0.1 mM 1 mM 1 mM 1 mM	KCl $MgSO_4$ x $7H_2O$ NaCl Tris $CaCl_2$
Anti-DIG-Antibody solution	1:2000	Anti-DIG-AP in Blocking solution
Anti-FITC-Antibody solution	1:1000	Anti-FITC-AP in Blocking solution
0.25% Acetanhydrid	0.25% (v/v)	Acetanhydid in Triethanolamin
0.5% Acetanhydid	0.5% (v/v)	Acetanhydrid in Triethanolamin
10x Block reagent	100 mg/ml Store in -20°C	Block reagent in MAP
Blocking solution 1% CHAPS 0.1% CHAPS/2x SSC	1x 1% (w/v) 0.1% (v/v)	Block reagent in MAB-BSA CHAPS in DEPC-ddH_2O CHAPS in 2x SSC
50x Denhardt's	28 mM 25 mM 1% (w/v) in DEPC-ddH_2O Store in -20°C	Polyvinylpyrrolidon Ficoll Typ 400 BSA V
DEPC treating	0.2% (v/v) Shake very well, keep over night / autoclave	DEPC in solution
DEPC-ddH_2O	0.2% (v/v) Shake very well, keep over night/ autoclave	DEPC in ddH_2O
DEPC-Hydra medium	0.2% (v/v) Shake very well, keep over night/ autoclave	DEPC in Hydra medium
0.5M EDTA	0.5 M EDTA pH 8.0 with NaOH-DEPC	in DEPC-ddH_2O
Formamid,deionisiert 2.5g/ml	2.5 g/ml Incubate in falcon for 1hr with shake and filter and store in -20°C	BioRad AG 501-X8 20-50m in Formamid

Glycin-PBT solution	40 mg/ml	Glycin in PBT
10x Glycin-Stock solution	40 mg/ml Store in -20°C	Glycin in DEPC-ddH_2O
Glycin/HCl	100 mM 0.1% pH 2.2 with HCl	Glycin Tween 20
Heparin	10 mg/ml Store in -20°C	Heparin Sodium in DEPCddH_2O
100% Hybridisation solution	50% (v/v) 5x 0.1% (v/v) 0.1% (v/v) 1x 100 µg/ml 200 µg/ml in DEPC-ddH_2O	Deionisiertes Formamid 20x SSC Tween 20 1% CHAPS 50x Denhardt's 10mg/ml Heparin 10mg/ml tRNA Store in 4°C
50% Hybmix/PBT 75% Hybmix/2x SSC 50% Hybmix/2x SSC 25% Hybmix/2x SSC	50% (v/v) 75% (v/v) 50% (v/v) 25% (v/v)	HybMix in PBT HybMix in 2xSSC HybMix in 2xSSC HybMix in 2xSSC
4M LiCl	4 M	LiCl in DEPC-ddH_2O
MAB	100 mM 150 mM pH 7.5 with NaOH	Maleic acid NaCl DEPC- treating
MABB MAB-BSA	2% (w/v) 1% (w/v)	BSA in MAB BSA in MAB
75% MeOH/PBT 50% MeOH/PBT 25% MeOH/PBT	75% (v/v) 50% (v/v) 25% (v/v)	Methanol in PBT Methanol in PBT Methanol in PBT
1M $MgCl_2$ 5M NaCl 5M NaOH	1 M $MgCl_2$ x $6H_2O$ 5 M NaCl 5 M NaOH	in ddH_2O DEPC-treating in ddH_2O DEPC-treating in DEPC-ddH_2O
NBT-BCIP-dye solution	0.64% (v/v) 0.35%	NBT BCIP in NTMT
NTMT	0.1 M 0.1 M 50 mM 0.1% (v/v) in DEPC-ddH_2O	NaCl Tris/HCl pH 9.5 $MgCl_2$ Tween 20 pH 9.5
4% PFA in Hydra medium	4% (w/v) in DEPC-Hydra medium	Paraformaldehyd Store in -20°C

4% PFA in PBT	4% (w/v)	Paraformaldehyd in PBT
1x PBS (High salt)	0.15 M 80 mM 21 mM in ddH_2O DEPC-treating	NaCl Na_2HPO_4 x 2 H_2O NaH_2PO_4 x H_2O pH 7.34 with NaOH
PBS-Glycerin (1:2)	66.7% (v/v) 0.3x	Glycerin PBS
PBT	0.1% (v/v)	Tween 20 in 1x PBS
Proteinase K	10 µg/ml	Proteinase K-solution in PBT
1000xProteinase K-solution	10 mg/ml Store in –80°C	Proteinase K in DEPC-ddH_2O
Sample solution	0.005-0.2 ng/µl	Sampel in HybMix
20x SSC	3 M 0.3 M in ddH_2O	NaCl Na-Citrat x $2H_2O$
10x TE-buffer,	100 mM Tris 10 mM pH 8.0 Autoclave	pH 7.5 EDTA
tRNA	0.5% (w/v) 1.13 mM 0.13 mM in DEPC-ddH_2O Store in -20°C	Torula yeast RNA KH_2PO_4 K_2HPO_4 x $3H_2O$ 1hr in 60°C
0.1M Triethanolamin	0,1 M in DEPCddH_2O pH 8.0 with NaOH-DEPC	Triethanolamin
1M Tris/HCl	1 M Tris DEPC-treating	pH 9.5 in ddH_2O
2% Urethan	2% (w/v)	Urethan in Hydra medium

solution I	50 mM 25 mM 10 mM autoklavieren Store in 4°C	Glucose Tris, pH 8.0 EDTA, pH 8.0
solution II	0.2 N 1% (w/v)	NaOH SDS
solution III	5 M 11.5% (v/v) Store in 4°C	KAc Acetic acid
RNase A-solution	20 µg/ml	RNase A

5.1.9. Primers

Primers for cloning specific gene

Gene	Vector	Primers name	Sequence
HyLfg-4	hot G	Lfg4-SmaI .F	TCCCCCGGGATGGTTGATATAGAAGG
		Lfg4-SmaI .R	TCCCCCGGGGTTTCTTTTTGCTGC
HyLfg-4-Mut	hot G	Mut.Lfg4/HotG.F	GCATTCGTAGAATTCGCCCTTTTAAAAATG GTTGATATAG
		Mut.Lfg4/HotG.R	CTATATCAACCATTTTTAAAAGGGCGAATT CTACGAATGC
HyLfg-4	pEGFP	3-EcoRI-Lfg4	CCGGAATTCGTTTCTTTTTGCTGCATT
		5-XhoI-Lfg4	CCGCTCGAGATGGTTGATATAGAAG
HyLfg-4	pSC-B-amp/kan	n. Lfg4.F	GAAGGAAGTTTTTCTAAAAACGAACGAG
		n. Lfg4.R	CTGGTGATAACTTATGAATCATCATATGTG
HyLfg-1ia	hot G	Sma I-LFG1ia.For	TCCCCCGGGATGTCAAATCAATATAC
		Sma I-LFG1ia.Rev	TCCCCCGGGTTTGTTACCC
HyLfg-1ia	hot G	EcoRI-LFG1ia.For	CCGGAATTCATGTCAAATCAATATA
		EcoRI-LFG1ia.Rev	CCGGAATTCTTTGTTACCC
HyLfg-1ia	pEGFP	EcoRI-LFG1ia.For	CCGGAATTCATGTCAAATCAATATA
		SmaI-LFG1ia. Rev	TCCCCCGGGTTTGTTACCC
HyLfg-1ia	pSC-B-amp/kan	RACE-Lfg1ia.Rev	TTTGTTACCCCCTGCTATACCCACTAT
		GeneRacerTM 5´RNA Oligo	CGACUGGAGCACGAGGACACUGA
HyLfg-1ib	hot G	Sma I-LFG1ib.For	TCCCCCGGGATGTCAAATCAGTAT
		Sma I-LFG1ib.Rev	TCCCCCGGGATTTTTTGCGAGTCC
HyLfg-1ib	hot G	EcoRI-LFG1ib.For	CCGGAATTCATGTCAAATCAGTAT
		EcoRI-LFG1ib.Rev	CCGGAATTCATTTTTTGCGAGTCC
HyLfg-1ib	pEGFP	EcoRI-LFG1ib.For	CCGGAATTCATGTCAAATCAGTA
		Sma I-LFG1ib.Rev	TCCCCCGGGATTTTTTGCGAGTCC
HyLfg-1ib	pSC-B-amp/kan	RACE-Lfg1ib.Rev	TGCGAGTCCAATAATTTGCAAAATATACA
		GeneRacerTM 5´RNA Oligo	CGACUGGAGCACGAGGACACUGA

Primers used in Sq-RT-PCR

Primer	Primer sequence
Lfg-4- For. internal	GAA GGAAGT TTTTCTAAAAACGAACGAG
Lfg-4- Rev.internal	CTGGTGATAACTTATGAATCATCATATGTG
Lfg-1ia. For. internal	GTGTACGCAATTCTTTTTTGTC
Lfg-1ia. Rev. internal	CTC AAGAATGTAAATAAATAACAGAC
Lfg-1ib. For. internal	TTATAATGTTTTGGGCAGCATG
Lfg-1ib. Rev. internal	TTATATCAATGTACAAGTTCAATGC
Actin 34	AAGCTCTTCC CTCGAGAAATC
Actin 35	CCAAAATAGA TCCTCCGATCC

Other Primers

Primer	Primer sequence
GFPi SeqPr 5'	CACCATCTAAT TCAACAAGA ATTG
GFPi SeqPr 3'	GACCACATGG TCCTTCTTG
T3_long	CAATTAACCC TCACTAAAGG GAACAAAAGC
T7_long	GTAATACGAC TCACTATAGG GCGAATTGGA GC

5.1.10. Plasmids

hot G	AG Böttger
hot Red pBluescipt II SK	AG Böttger, M. Lasi, München
pBluescipt II KS	Fermentas
pCR-BluntII-TOPO	Invitrogen
pPCR-Script Amp SK	Invitrogen
pEGFP C1/N1	Clonetech

Used existing plasmids

Gene	Vektor	Expression in	From
HyBI-1	hoT G	*Hydra*	Lasi-Diploma thesis
HyBI-1	hoT Red	*Hydra*	Lasi-Diploma thesis
HyBI-1	pEGFP	HEK 293T	Wagner-Diploma thesis
HyBcl2-like-4	pEGFP	HEK 293T	Lasi-Ph.D thesis

Gene	Vektor	Expression in/for
HyBI-1	pSC-B-amp/kan	*Hydra*/In situ hybridization
HyLfg-4	pSC-B-amp/kan	*Hydra*/In situ hybridization
HyLfg-1ia	pSC-B-amp/kan	*Hydra*/In situ hybridization
HyLfg-1ib	pSC-B-amp/kan	*Hydra*/In situ hybridization
HyBI-1	hoT G	*Hydra*/subcellular localization
HyLfg-4	hoT G	*Hydra*/subcellular localization
HyLfg-1ia	hoT G	*Hydra*/subcellular localization
HyLfg-1ib	hoT G	*Hydra*/subcellular localization
HyBI-1	pEGFP	HEK 293T/anti apoptotic effect assay
HyLfg-4	pEGFP	HEK 293T/anti apoptotic effect assay
HyLfg-1ia	pEGFP	HEK 293T/anti apoptotic effect assay
HyLfg-1ib	pEGFP	HEK 293T/anti apoptotic effect assay

5.2. Molecular biological standard methods

5.2.1. Agarose gel

Agarose gel electrophoresis used to separate DNA according to size. 1 % agarose in 1x TAE buffer prepared and 7µl Ethidium bromide added to per 70 ml TAE. Samples mixed with 5x loading buffer. Additionally, 5µl marker applied. Separation was carried out at a constant voltage of 80 V in 1x TAE. Subsequently, the bands visualized under UV light and photographed.

Table 3. 50µl- Standard-PCR reaction mixture

Component	Volumen [µl]	Final concentration
DNA	variabel	~100ng/µl
Forward primer	2,5µl	0,5pmol/µl
Reverse primer	2,5µl	0,5pmol/µl
10xTaq-Reaction buffer	5 µl	1x
dNTPs	1 µl	200µM
Taq-DNA-Polymerase	0,2µl	1U
dd H2O	ad 50µl	

5.2.2. DNA concentration determination and sequencing

Concentration of DNA was determined at a wave length of 260 nm in the DNA calculator and for sequencing at MWG-Biotech AG, 0.1 µg / µl DNA in total volume of 15 µl was shipped.

5.2.3. PCR

Table 4. PCR-Programe for amplification of specific DNA fragment

Phase	Cycle number	Temperature	Time
First denaturation	1	95°C	4 min
Denaturation	30	95°C	0,5 min
Annealing		Specific annealing tempereture	1min
Elongation		72°C	1min/kb
Finale elongation	1	72°C	7min

Touchdown PCR:

Touchdown PCR is modification of conventional PCR which result in a reduction of nonspecific amplification. It involves the use of an annealing temperature that is higher than the target optimum in early PCR cycles. The annealing temperature was decreased by 2°C every second cycle until a specified or 'touchdown' annealing temperature was reached and followed with 4°C lower than specified annealing temperature. The touchdown temperature was then used for the remaining number of cycles. This allows for the enrichment of the correct product over any non-specific product (Table 5). Touchdown PCR mixture consist of 100ng/µl of DNA to, 0.5 pmol of specific primers and Phusion Flash High-Fidelity PCR Master Mix in total volume of 20µl.

Table 5. Diagram of a touchdown PCR

Phase	Cycle number	Temperature	Time
1	1	95°C	5min
2	2	95°C	0,5min
		Annealing temperature + 4°C	1min
		72°C	1min/kb
3	2	95°C	0,5min
		Annealing tempereture. + 2°C	1min
		72°C	1min/kb
4	2	95°C	0,5min
		Annealing tempereture	1min
		72°C	1min/kb
5	2	95°C	0,5min
		Annealing tempereture - 2°C	1min
		72°C	1min/kb
6	2	95°C	0,5min
		Annealing tempereture. - 4°C	1min
		72°C	1min/kb
7	25	95°C	0,5min
		Annealing tempereture	1min
		72°C	1min/kb
8	1	72°C	7min

Mutagenesis PCR:

The mutagenesis reaction was carried out according to the Herstellers QuikChange ® Site-Directed Mutagenesis Kit from Stratagene. 50 ng of double-stranded DNA used to be mutated (Table 6).

Table 6. PCR program for mutagenesis reaction

Phase	Cycle number	Temperature	Time
1	1	95°C	5min
2	12-18	95°C	0,5min
		55°C	1min
		68°C	2min/kb

5.2.4. Restriction enzyme cleavage

For restriction enzyme cleavage 1-2 µg DNA was used and incubated with the appropriate restriction enzymes in 1x reaction buffer and 1xBSA for 2 h at 37°C or at 25 °C (Sma I).

5.2.5. Dephosphorylation and ligation

The vector was dephosphorylated prior to ligation with the shrimp alkaline phosphatase (SAP). Ligation was performed with T4 ligase overnight at 16°C.The ligation mixture inactivated for 10 minute at 65°C and used directly for transformation.

5.2.6. Gel extraction

The gel extraction was performed with the peq-GOLD Gel Extraction Kit according to the company Peqlab manufacturers. The DNA was eluted in 20-40 µl ddH2O.

5.2.7. RACE amplification

Amersham kit was used for isolation of mRNA from a Hydra. About 100 hydras polyp dissolved in 400µl extraction buffer by help of mechanical pressure. 600µl elution buffer added and centrifuged. The further procedure was carried out according to the manufacturer. For amplification of cDNA, 1µg mRNA was incubated with a Not I-(dT) 18 primer. The reaction was carried out according to manufacturer's instructions. For the RACE-PCR, the Gene Racer kit was used according to the manufacturer's protocol. Gene-specific primer was used in RACE-PCR that contained a known sequence of the gene to be cloned, and a primer supplied in the Gene Racer Kit, which binds to the 5 'and 3' ends of the cDNA. The RACE PCR was performed according to the manufacturer's protocol at the highest possible temperature to obtain maximum specificity.

5.3. Methods for bacteria

5.3.1. Cultivation of E. coli

E. coli cells were cultured at 37°C on LB agar plates with antibiotics or in 170 to 220 rpm shaken LB medium with antibiotics at 37°C.

5.3.2. Preparation of electro-competent bacteria

Electro-competent bacteria produced by incubation of 10ml LB/tetracycline medium with XL1 Blue MRF '(5 mg/ml of tetracycline) (Glycerol stock bacteria or LB-tetracycline plate with bacteria) and shaken overnight at 37°C. Next morning 500 ml of LB medium were incubated with 10 ml of the pre culture and grown at 37°C to reach OD (optical density) 600 = 0.6. Afterward, bacteria cells are harvested with 30 min centrifuge at 4000 rpm, in 4°C. The bacterial pellet was re-suspended in 10ml ice-cold sterile ddH2O, transferred into a 50ml falcon and filled with ice-cold ddH2O

to 50ml. This step repeated four times. In the fourth step, the bacterial pellet was re-suspended in a small amount ddH2O and transferred to an Eppendorf tube. This was followed by centrifugation for 20 min at 10,000 rpm and 2°C. Finally, the bacterial pellet re-suspended in the same volume of 10% glycerol, aliquoted to 50µl and frozen on dry ice or liquid nitrogen and stored at -80°C.

5.3.3. Electro-transformation of bacteria

For electroporation, an aliquot electrocompetent cells thawed on ice. 10-100 ng DNA or 5µl of a ligation approach reached to 100µl with ddH2O added and incubated for 1 min on ice. Electroporation was carried out at 200 Ω, 25 uF and 2.5 kV in 0.2 cm cuvettes. After transformation, 1ml of pre-warmed (37°C) SOC medium was added and the cells shaken for 1 hr at 37°C horizontally. Thereafter, the bacterial cells were plated on LB plates with antibiotics and incubated overnight at 37°C.

5.3.4. Transformation of bacteria by means of heat shock

For transformation by heat shock, an aliquot chemically competent cells was thawed on ice, DNA was added (10-100 ng or 5µl of a ligation mixture) and incubated for 30 min on ice. The heat shock was performed at 42 °C for 45 seconds, followed by 2 minute incubation on ice. After transformation, 1 ml of pre-warmed (37°C) SOC medium was added and the cells shaken for 1 hr at 37°C horizontally. Thereafter, the bacterial cells were plated on LB plates with antibiotics and incubated overnight at 37°C.

5.3.5. Preparation of bacteria plasmid DNA (Mini preparation)

Each a bacterial colony dissolved in 4 ml of LB medium inoculated with the appropriate antibiotic and shaken overnight at 37°C and 180 rpm. After centrifugation of the bacterial cells, the pellet was dissolved in 100µl of ice-cold solution-I (50 mM glucose, 25 mM Tris / HCl pH 8, 10 mM EDTA pH 8). 200µl fresh solution-II (0.2 M NaOH, 1% SDS) added and mixed by inverting five times. 150µl ice-cold Solution-III (60 ml 5M KAc, 11.5 ml of glacial acetic acid, 28.5 ml of ddH2O) added and mixed briefly and incubated for 5 min on ice. It was followed by a centrifugation at 12000g for 10 min. The supernatant containing plasmid DNA was precipitated with 2 volumes of 100% ethanol for 2 min at room temperature and then mixed, centrifuged again and the DNA pellet washed with 500µl 70% ethanol and then dried. The DNA re-suspended in 35 µl ddH2O.

5.3.6. Preparation of bacteria plasmid DNA (Maxi preparation)

Maxi-preparations of plasmid DNA from bacteria that contained the plasmid was carried out with Maxi preparation kit from Macherey-Nagel according to the provided protocol and DNA concentration determined afterward.

5.4. Standard methods for *Hydra vulgaris*

5.4.1. Culture of *Hydra vulgaris*

Hydra vulgaris was cultured at 18°C in Hydra medium with 12 hour light cycle according to the standard method (Lenhoff and Brown, 1970). Each *Hydra* strain was kept in a separate plastic dish. Polyps were fed 5 days a week with newly hatched *Artemia nauplii* from Sanders Brine Shrimp Company. Artemia larvae were hatched from cysts during 24 hours of incubation in artemia medium at 21°C. Five to six hours

after feeding the animals were washed. Once a week the hydras were transferred into new dishes. All animals were starved at least 24 hour prior to be used in experiments.

5.4.2. Acridine orange staining

Living *Hydra* polyps were incubated in 3.3 µM solution of acridine orange in hydramedium for 15 minutes in dark. Then the animals were washed with hydra medium 4 times and mounted on a glass slide in a drop of 2% urethane solution. Living animals were then viewed under fluorescence microscope with a FITC filter set. Acridine orange is a cationic acidophilic dye, which specifically stains apoptotic cells within phagocytic vacuoles with low pH value (Clerc and Barenholz, 1998).

5.4.3. Biolistic transformation of plasmid-DNA

Preparation of microcarriers:

For the preparation of gold stock solution, 30mg gold particles of 1.0 µm diameter gold weighed and mixed with1ml of 70% ethanol and then vortexed vigorously for 3 to 5 minutes. The mixture stands for 15 minutes at room temperature and followed by centrifugation at 1000g for only 5 seconds. The liquid was discarded and the pellet of gold beads washed in total of three times: 1mL sterile ddH2O, mix 1 min, 1 min to settle, centrifuge briefly. Finally the beads resuspended in 500µl sterile 50% glycerol in Hydra medium and stored at 4°C. The final gold solution concentration was 3mg/50µl.

Precipitation of plasmid DNA on microcarriers:

40µl of the gold stock solution were vortexed for 5 minutes. Subsequently, at least 10µg and a maximum of 20µg of the desired plasmid DNA was added in a maximum

of 15µl and mixed well. To associate the DNA with the gold particles 50µl of 2.5 M CaCl2 and 20µl 0.1M Spermidine was added dropwise and mix well for 10 minutes on the shaker. Subsequently centrifuge for 5 seconds at 500 g and the supernatant discarded. The DNA-gold pellet mixed in 140µl of 70% ethanol and again centrifuge at 500 g for 5 seconds, the supernatant discarded. The pellet washed with 140µl of 100% ethanol. At the end, the pellet was re-suspended in 50µl or 75µl of 100% ethanol. 25 µl of this DNA solution was dropped in the middle of a macrocarrier and dried in room temperature.

Biolistic transformation:

Transformation of about 100 starved animals was performed with the PDS-1000/He Particle Delivery System from Bio-Rad at a pressure of 850 to 900psi. The vacuum pump and the particle delivery system were switched on and the rupture disc soaked in 100% isopropanol was added to the disc and the retaining Cap macrocarrier placed in the macrocarrier launch assembly. Petri dish with about 100 hydras (they must without medium in the middle of the petri dish) placed on the target shelf. It was then applied vacuum, and the firing button pressed until the rupture disc was broken. The transformation was carried out for 2 to 3 times with the same animals. The hydras were incubated in hydra medium at 18°C in the dark. The expression of green or red fluorescent protein examined under Fluorescence binocular.

5.4.4. Treatment with Benzo[*a*]pyrene and Semi quantitative real time PCR method

Benzo[*a*]pyrene solution (1000 µg/mL in acetone) was used as a stock solution and about 100 hydra polyps were treated with 45 µg/L of benzo a pyrene solution for 48 hours. Afterward, mRNA was extracted and cDNA prepared.

Semi quantitative real time PCR performed with normal pcr in parallel with 25, 26, 27, 28, 29 and 30 cycles. 450 µl/L acetone treated cDNA used as standard and actin used as housekeeping gene for comparison.

The method involves reverse transcription using an oligo-dT or random hexamer primer. The resulting cDNA thus represents both house-keeping gene transcripts as well as specific transcripts one is quantitating. The RT reaction is then amplified in a pair of PCR series - one series is to amplify the house-keeping gene cDNA (using GAPDH-, etc., specific primers), and the other is for the specific cDNA of interest.

After PCR, same volume of reaction products is electrophoresed on an agarose gel.

All reactions carried out at the same time, using reagents that are prepared as a supermix to minimize any variation. PCR for different number of cycles set up together and samples removed one after one during the middle of the extension phase during the desired PCR cycle numbers. Tubes were placed in a water bath at 65-75 °C for a few minutes to ensure that extension is complete.

5.4.5. In Situ Hybridization

Probe production by linearization:

For each plasmid two restriction sites were recognized to produce antisense and sense probe. The in vitro transcription approach was used to synthesis an antisense probe which is complementary to the mRNA and can hybridize to that. The sense probe which is not complementary to mRNA, was used as a control probe. For each restriction digest, 50µg of a specific plasmid maxi preparation were used. The digestion was carried out for each of the two approaches with restriction enzyme and only one side of the specific Inserts cut. 20µg of each specific antisense and sense

digestion of the same Plasmid were loaded on a 1% agarose gel, and they were extracted from the gel by using gel peqGOLD Extraction kit or the QIAquick Gel Extraction Kit and eluted in 20µl Elution Buffer. During the subsequent in vitro transcription was used 1µg of the antisense or sense-eluate.

Table 7. Probe production information

Gene	probe	Restriction enzyme	RNA- polymerase
HyLfg-4	Anti sense	NotI	T3
	Sense	Hind III	T7
HyBI-1	Anti sense	NotI	T3
	Sense	KpnI-HF	T7
HyLfg-1ia	Anti sense	NotI	T3
	Sense	Hind III	T7
HyLfg-1ib	Anti sense	NotI	T3
	Sense	Hind III	T7
HyWnt3a	Anti sense	NotI	T3
	Sense	Hind III	T7

In vitro transcription:

In vitro transcription reaction mixture was composed of 1µg of linearized DNA, 10x transcription buffer, DIG-RNA Labeling Mix, 20U RNase inhibitor and 40U of the corresponding RNA polymerase in the total volume of 20µl with DEPC-ddH2O. After three hours Incubation at 37°C additional 20U-specific RNA polymerase added and again incubated for two hours at 37°C. Subsequently, the starting DNA was digested by adding 2µl Dnase-I (RNase-free) by incubation at 37°C for 45 minutes. The amplified RNA probes were purified using either the RNeasy Mini Kit, or by precipitation. Purification of the RNA probes was used in the purification on RNeasy spin columns according to the manufacturers proceed and eluted in 30µl RNase-free water and finally 20U RNase inhibitor added to protect probe. To precipitate

synthesized RNA samples, 2.5 µl 4M LiCl solution, 2µl 0.5M EDTA solution and 75µl 100% ice-cold ethanol-DEPC were added. The mixture was then stored at -20°C overnight. This was followed by centrifugation at 14 000g, 4°C for 30 minutes. The RNA pellet was washed twice with 50µl ice-cold 70% ethanol-DEPC and finally after drying, eluted in 20µl DEPC-ddH2O and mixed with 20U RNase inhibitor.

Dot Blot: To test the successful in vitro transcription and for determining quantity of RNA probe to be used a dot blot approach was performed. For this purpose from the purified probes, dilutions of 1:100, 1:500, 1:1000, 1:1500, 1:2000, 1:2500 and 1:3000 prepared. 1µl of each dilution step was dropped and dried a PALL Biodyne B transfer membrane. After cross linking of the RNA probes to the membrane surface by UV Stratalinker 1800 the membrane was washed with MAB for1 minute and then Blocked for 30 minutes at in MABB at room temperature Then Incubated with anti-DIG antibody solution for 30 minutes at room temperature. Finally, the membrane was washed for 1 minute with NTMT and then dyed with NBT-BCIP staining solution for 10 minutes. The staining reaction was stopped with 1 x TE buffer, and the membrane dried for documentation and stored at room temperature. The applicable amount of probe determined from the comparison with the control probe.

In situ hybridization reaction: All incubations were carried out in 1 ml of the appropriate solution at room temperature and 80 rpm. Starved hydras were relaxed in 2% urethane for 2-3 minutes and then fixed in 4% PFA / Hydra medium. After a 10-minute incubation at 4°C, the solution was replaced with fresh 4% PFA / Hydra medium and incubated in 4°C overnight. Fixed animals were washed three times for 5 minutes each in 1x PBS. Afterward, animals first dehydrated through an ascending series of methanol and again by a descending series rehydrated. For this purpose,

the animals were 5 minutes each with 25% MeOH / PBT, 50% MeOH / PBT, 75% MeOH / PBT, twice 100% MeOH / PBT, 75% MeOH / PBT, 50% MeOH / PBT and finally re-treated with 25% MeOH / PBT. Subsequently, the samples were washed three times for 5 minutes with PBT. To permeabilize the hydras were treated for 10 minutes with proteinase K working solution. The reaction was followed with a subsequent brief and a 5-minute treatment Glycine solution. Then again twice for 5 minutes was washed with PBT, then incubated twice for 5 minutes each with 0.1 M Triethanolamine and followed with 0.25% acetic anhydride in TEA for 5 minutes and another 5 minutes with 0.5% acetic anhydride in TEA and washing twice for 5 minutes in PBT. The second fixing of the hydra was for 20 minutes in 4% PFA / Hydra medium at room temperature without shaking. To remove the PFAs five times washed with PBT for 5 minutes each. Incubation in in 750µl 50% Hybmix / PBT carried out at 55°C in a humid chamber for 10 minutes then incubated for 10 minutes in 750µl 100% hybmix and finally least 2 hours in fresh 100% hybmix. 750µl of the desired probe solution (0.005 - 0.2 ng / ul in hybmix) were first boiled for 2 minutes at 95°C and incubated at 55°C for 10 minutes before adding to hydras then incubated up to two days at 55°C in amoist chamber. The probes were removed by washing with 100% fresh 750µl hybmix, 75% HybMix/2x SSC 50% and 25% HybMix/2x HybMix/2x SSC SSC away in a humid chamber at 62 °C. This was followed by twice incubation in 0.1% in CHAPS/2x SSC at 62°C then hydra washed with MAB twice for 10min before incubation in blocking solution for 1 hr at room temperature and at least 1 hr at 4°C. Finally, the animals were placed in anti-DIG-antibody solution and incubated overnight. The hydras were washed 6x 30min with MAB, then 2 x 5min with NTMT. It was followed by dyeing at 37°C in NBT BCIP staining solution, then washing in 100% EtOH for 20 minutes. Finally the animals washed 2x 5min with PBS

and they are embedded in a drop of PBS / glycerol on a microscope slide.

5.5. Methods for human cells

5.5.1. Cultivation of HEK 293T cells

For all experiments, HEK 293T cells (Human Embryonic Kidney) were used. These were incubated at 37°C, 5% CO2 and saturated humidity. As medium DMEM (Dulbecco's minimal essential medium) was used, which was treated with the antibiotic Pen / Strep and 10% FCS (fetal calf serum). All procedures were performed under a clean bench.

5.5.2. Splitting of HEK 293Tcells

HEK 293T cells are semi adherent cells. When the cells are 70 - 80% confluent, the medium is replaced. To detach the cells, the culture medium is removed and then the cell layer was washed once with 1X PBS. In order to solve the cells completely from the bottom of the bottle, they were rinsed thoroughly with DMEM. The cells were then either given on 6-well plates with cover slips or distributed in small bottles.

5.5.3. Transfection of HEK 293T cells with Lipofectamine

At the time of transfection, the cells were 50 - 60% confluent. Before the transfection, the medium changed with starving medium (0.5% FCS in DMEM). 8µg DNA from a maxi preparation added to 250 µl starving medium, vortexed very well and incubated for 5 minute in room temperature. In parallel 5 µl of lipofectamine 2000 added to 250 µl of starving medium and incubated as pervious. Then those two solutions mix together and incubated in room temperature for 20 minute. The mixture was then added dropwise to the cells and gently mixed. After 5 hours of incubation the medium

was changed again with 10% FCS in DMEM. The cells were used after 24-48h for the experiments.

5.5.4. Immunofluorescence staining of cell culture

The medium of transfected HEK 293T cells was aspirated and the cells were washed once with 1x PBS. Then fixed with 4% PFA / PBS for 15 min and then washed twice with 1x PBS. Subsequently, incubation was carried out with permeabilisation solution for 10 min. After two further washes with 1 × PBS, the cells were incubated for 1 hour in blocking solution at room temperature. Afterward, incubation with the primary antibody (diluted in blocking solution) carried out for 1hr. After three times/10-minute washes with 1x PBS, it was incubated with the secondary antibody for 1 hr at room temperature as well, but in the dark. The cells were then washed again, and the nuclei were stained with DAPI/TO-PRO-3 and the coverslips were mounted in Vectashield.

5.5.5. Induction of apoptosis by camptothecin

To study anti-apoptotic effect of Lifeguard proteins in Hydra, camptothecin induced apoptosis was carried out in HEK cells. Camptothecin is a topoisomerase-I inhibitor. Camptothecin leads to apoptosis by activation of the mitochondrial apoptosis pathway (Stefanis et al., 1999). This method has been described in the literature (Wang et al., 2006; El-Assaad et al., 1998).

In the assay, HEK 293T cells transfected with the respective plasmid and after 24 hr (when the protein has been expressed) treated with 10 µM camptothecin for 24 hr. The transfected cells were counted on a fluorescence microscope. Changes in nuclear morphology like condensation and fragmentation of chromatin or the

shrinkage of the nucleus counted as a marker for apoptosis.

5.6. Microscopy

Confocal Laser Scanning Microscopy

Leica SP5-2 confocal laser-scanning microscope was used for Light optical serial sections. Leica SP5-2 equipped with an oil immersion Plan-Apochromat 100/1.4 NA objective lens. eGFP, FITC, Alexa488 were visiulized with an argon laser at Excitation wavelength of 488 nm and emission filter at 520 - 540 nm for eGFP, FITC and Alexa488. The helium-neon laser with excitation wavelength of 633 nm and emission filter of 660–760 nm was used for TO-PRO-3.

Emission filters at 660 - 760nm is used for Cy3, RFP and and BODIPY-TR using a Kryton laser excited at a wavelength of 561nm and emission filter at 570-580nm were visualized. 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. To obtain an improved signal-to-noise ratio, 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.

Confocal microscopy on living animals

In a live-scan, the animals were initially relaxed with 2% urethan for 2-3min and placed on a slide with a Wax-foot coverslip. In this way, Hydra wont damaged by the pressure of cover slip and also animals cannot move.

For additional staining with Golgi marker, 1µl of 500µM BODIPY-TR in Hydra medium solution injected into the hydra gastro cavity. Thereafter, polyps incubated in 5µM

BODIPY-TR ceramids solution for 30min. immediately afterward the optical sections were made.

5.7. Creating a phylogenetic tree

An alignment between full-length protein sequences of all listed organisems (in appendix) was prepared with Clustal W in *Geneious* (Biomatters). Subsequently, a phylogenetic tree was created base on the Neighbor Joining (NJ) method with 10,000 bootstraps.

6. Appendix

Protein sequence	Accession number
BI-1 *Homo sapiens*	NM-003217.2
BI-1 *Mus musculus*	NP-080945.1
BI-1 *Xenopus laevis*	NM-001087329.1
BI-1 *Salmo salar*	NM-001141682.1
BI-1 *Hydra magnipapillata*	Hma1.130444
BI-1 Arabidopsis thaliana	AAM65074.1
BI-1 Rice	Q9MBD8.1
BI-1 *Drosophila melanogaster*	NP-648205.1
Lfg-4 *Homo sapiens*	NM-016056.2
Lfg-4 *Mus musculus*	NM-026617.3
Lfg-4 *Gallus gallus*	XM-001235092.1
Lfg-4 *Xenopus laevis*	NM-001095813.1
Lfg-4 Sea anemone	XM-001640172.1
Lfg-4 *Hydra Magnipapillata*	XM-002162893.1
Lfg-4 *C. elegans*	B0563.4
Lfg-4a Thale cress	AT4G15470.1
Lfg-4b Thale cress	AT4G02690.1
Lfg-4c Thale cress	AT3G63310.1
Lfg-4d Thale cress	AT1G03070.1
Lfg-4 Zea mays	NP-001149877.1
Lfg-4 Rice	NP-001055493.1
Lfg-4 Tomato	XP-004229465.1
Lfg-1i Mosquito	XP-001865933.1
Lfg-1i Sea anemone	XP-001629637.1
Lfg-1i Drosophila	XP-002050155.1
Lfg-1i Silkworm	ABD36125.1
Lfg-1ia *Hydra Magnipapillata*	Hma2.214458
Lfg-1ib *Hydra Magnipapillata*	Hma2.205245
Lfg-1 *Homo sapiens*	NP-000828.1
Lfg-1 *Mus musculus*	EDL29538.1
Lfg-1 *Salmo salar*	NP-957502.1
Lfg-1 *Xenopus laevis*	NP-001072357.1
Lfg-1ib *C. elegans*	F40F9.1b
Lfg-1ia *C. elegans*	F40F9.1a.1
Lfg-1ic *C. elegans*	F40F9.2
Lfg-2 *Homo sapiens*	NP-036438.2
Lfg-2 *Salmo salar*	NP-957502.1
Lfg-2 *Mus musculus*	NP-082500.2
Ghitm *C. elegans*	K11H12.8
Lfg-5 *Bos taurus*	NP-001070536.1
Lfg-5 *Odobenus rosmarus*	XP-004394155.1
Lfg-5 *Ovis aries*	XP-004007761.1
Lfg-5 *Mus musculus*	AAI15682.1
Lfg-5 *Homo sapiens*	EAL24151.1
Lfg-3 *Mus musculus*	BAC43762.1
Lfg-3 *Homo sapiens*	NP-071435.2
Lfg-3 *Xenopus laevis*	NP-001088462.1

Hydra Bax inhibitor-1

>Hma1.130444

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AWTENKPKNQLQRLGYLMGFSFCVGLSLGPLIGHVIKINPTIVATALFSTSLIFLCFSLSALWAEQRSYLYLGGT
LLSALSLMCLLSFINIFFKSEMIYQFHLYGGLLLFCAFILYDTQLIVEKRRMGDTDFIWHSVDLFLDFVNIFRRL
LIILGNKEEKKKNKRSE*

Hydra Lifeguard-4

>XM_002162893.1

ATGGTTGATATAGAAGGAAGTTTTTCTAAAAACGAACGAGATGATTTTTACTCTGTTACAGTTGCTCAGTCTTCA
TTGCAAGTTCGACTTGGTTTCATTAGAAAGGTGTATGGAATCTTAAGTACTCAACTTTTTATAACAACTTTGGTT
GGTGCTCTGTTTATGTATAATGATAACATTAAGCAGTTTGTGCAACAGAGTCCTAATTTGCTACTGTTTGGTTTG
ATTGCTTCTATTGGCTTAATAATTGCTCTTGGAATAAAAAGAAAAGATTCCCCAACTAATTTTTATCTGCTCGCT
GCTTTTACCTTAATTGAAGCATACACTGTTGGAACCATTGTGACGTTTTATGATCAGTTTATTGTGTTGGAGGCT
TTTGGCTTGACCATGGCTGTTGTGGTTGCACTGACAATATATACATTTCAATCGAAAAAAGATTTTAGCGCATGG
GGTGCTGGGTAA

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GAGLFAMLWIIVLAGFLQIFIRNEMFELILAVAGAILFAGFIVFDTHMMIHKLSPEEYILAAINLYLDIINLFLE
ILKILNAAKRN*

Hydra Lifeguard-1ia

>Hma2.214458

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TLCESYLIGVVSAHYNVNEVLLAMGIVAVVSLAITIFAFQTKYDFTMMGGFLLVLVIVLLCFGIFTIFFHSKIVR
LVYACLGALIFGLYLVYDTQLMMGGEKKYSISPEDYIFAALNLYLDIVMLFIYILEIVGIAGGNK*

Hydra Lifeguard-1ib

>Hma2.205245

CCCACGCGTCCGCACATACTGAAACTTTTTAGTCCCTGTGTAATAAGAATGTCAAATCAGTATAGTTATAGCCAG
CATGTTAACACTGATTTGGAAGCCAATAAACCGCCACCATACACGTCTTATAATGAATATGCAACTCAACAAAAC
CCAATGGCACCGCCACCACAATATGGTTGGATCAATCCTGATCAAGGAGCTGAATCAAATACTACTATTTTAGGA
ATATCATCATTCAGTGAAAAGTCAATCAGACAAGCTTTCATTCGTAAAGTGTATGCAATTCTTTTTTGCCAATTG
CTTGTGTCAGTTGGAATTGTTTGTCTCTTTTTACTTGTGAAGCCAATAAATACCTATGTAAAATCAAATGTTATA
ATGTTTTGGGCAGCATGGATCTTAACAATAGTTTTGATGATTGCACTCATTTGTTGCGAAAGTGTGAGGAGAACT
TTTCCCATGAACTTCATAATGTTATCATTATTTACATTATGTGAAAGTTATCTTATTGGTGTTGTTTCTGCTCAT
TATGAAGTCAATGAAGTGTTGCTAGCTATGGGTATTGTTGCAGTTCTTTCCTTGGCAATCACTATTTTTGCTTTT
CAAACAAAGTATGACTTCACAATGATGGGTGGATTTTTACTTGTGCTGTAATTGTGTTATTGTGCTTTGGAATTT
TCGCAATATTTTTTCACAGCAAGATTGTTCGATTAGTCTATGCTTGTCTTGGAGCCCTTATCTTTGGACTGTTCT
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MSNQYSYSQHVNTDLEANKPPPYTSYNEYATQQNPMAPPPQYGWINPDQGAESNTTILGISSFSEKSIRQAFIRK
VYAILFCQLLVSVGIVCLFLLVKPINTYVKSNVIMFWAAWILTIVLMIALICCESVRRTFPMNFIMLSLFTLCES
YLIGVVSAHYEVNEVLLAMGIVAVLSLAITIFAFQTKYDFTMMGGFLLVLVIVLLCFGIFAIFFHSKIVRLVYAC
LGALIFGLFLVFDTQMMLGGKKKYSISPEEYIFAALNLYIDIITLFLYILQIIGLAKN*

7. Abbriviation

Abbriviation	Description
Amp	Ampicillin
ATP	Adenosintriphosphat
Bad	Bcl-2-associated agonist of cell death
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell-lymphoma 2
Bid	BH3 interacting domain death agonist
BI-1	Bax inhibitor-1
Bik	Bcl-2-interacting killer
Bim	Bcl-2-interacting mediator of cell death
BNip	Bcl-2 nineteen kilo dalton interacting protein
BLAST	Basic local alignment and search tool
bp	Base paire
BP	Benzo (a) pyrene
BSA	Bovine serum albumin
°C	Degree celsius
cDNA	Complementary DNA
C. elegans	*Caenorhabditis elegans*
Cyt c	Cytochrome c
ddH_2O	MilliQ ddH_2O
DAPI	4',6-Diamidino-2-phenylindol
D. melanogaster	*Drosophila melanogaster*
DNA	Desoxyribonucleic acid
E. coli	*Escherichia coli*
EST	*Expressed sequence tag*
EtOH	Ethanol
ER	Endoplasmic reticulum
gr	Gram
GFP	Green floresence protein
hr	Hour
H. vulgaris	*Hydra vulgaris*
H. sapiens	*Homo sapiens*
kb	Kilo base
kDa	Kilo dalton
LB	Luria bertani
Lfg	Lifeguard protein
mA	Milliampere
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MYa	Million years ago
mRNA	*messenger* RNA

µg	Microgram / 10^{-6} Gram
µl	Microliter / 10^{-6} Liter
µm	Micrometer
µM	Micromolar
NBT	Nitro blue tetrazolium
nt	Nucleotide
ORF	Open reading frame
PBS	Phospate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyd
pH	Potential of hydrogen
RFP	Red fluorescence protein
ROS	Reactive oxygen species
rpm	Rounds per minute
RT	Room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
sec	Second
SDS	Sodium dodecyl sulfate
TAE	Tris-Acetat-EDTA
TMBIM	Transmembrane bax inhibitor motif containing

8. References

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