Sequence and partial functional analysis of canine Bcl-2 family proteins

Abstract

Dogs present with spontaneous neoplasms biologically similar to human cancers. Apoptotic pathways are deregulated during cancer genesis and progression and are important for therapy. We have assessed the degree of conservation of a set of canine Bcl-2 family members with the human and murine orthologs. To this end, seven complete canine open reading frames were cloned in this family, four of which are novel for the dog, their sequences were analyzed, and their functional interactions were studied in yeasts. We found a high degree of overall and domain sequence homology between canine and human proteins. It was slightly higher than between murine and human proteins. Functional interactions between canine pro-apoptotic Bax and Bak and anti-apoptotic Bcl-x_L, Bcl-w, and Mcl-1 were recapitulated in yeasts. Our data provide support for the notion that systems based on canine-derived proteins might faithfully reproduce Bcl-2 family member interactions known from other species and establish the yeast as a useful tool for functional studies.

Key words: Bcl-2 family; canine; cloning; comparative sequence analysis; functional yeast study

1. Introduction

Rodent models, including genetically modified mice and xenografts in immunocompromised mice, are indispensable tools for cancer research (Rosol et al., 2003; Kelland, 2004; Sharpless and Depinho, 2006). They have evolved in recent years to a vast array of sophisticated models that are continuously broadening and deepening our knowledge of cancer biology (Cheon and Orsulic, 2011; Ruggeri et al., 2014). A number of limitations in the use of mice, however, have become apparent in recent years. These limitations include the restricted genetic diversity inherent to mouse laboratory strains as well as substantial differences between humans and mice in certain molecular regulatory pathways, both factors with potential impact on murine tumorigenesis models (Jacks, 1996; Balmain, 2002; Hahn and Weinberg, 2002; Young and Longmore, 2004; Cheon and Orsulic, 2011). Xenografts in immunocompromised mice are widely used for preclinical drug development (Kelland, 2004) although these animals provide a completely different microenvironment for tumor growth than the original host. In addition to a limited suitability for modeling metastasis, xenograft models offer a "reductionist" view since the cells used represent only a small selection of all possible combinations of genetic and epigenetic alterations leading to a certain morphological tumor subtype (Sharpless and Depinho, 2006).

Spontaneous canine neoplasms offer the opportunity to complement data from rodents. Cancer is the most common cause of death in dogs (Vail and MacEwen, 2000). Canines, similarly to humans, constitute an outbred population with a much broader genetic background than rodent laboratory strains. In addition, canines are genetically more closely related to humans than rodents as shown from whole genome comparisons of the three species (Lindblad-Toh et al., 2005), which is also reflected by a greater similarity at the metabolic level. Canines develop a range of spontaneous neoplasms often with histological characteristics and biologic behaviour

similar to human tumors, including non-Hodgkin's lymphoma, soft-tissue sarcomas and osteosarcoma (Vail and MacEwen, 2000; Rosol et al., 2003; Khanna et al., 2004). Dogs share a common environment with people. Because of their increasing significance as companion animals, canine tumor patients are increasingly subjected to therapy and hence provide a yet underexploited resource for modeling both pathogenetic and therapy-related aspects of human cancer.

Deregulated apoptosis is a hallmark of cancer (Hanahan and Weinberg, 2000) and plays roles in tumorigenesis and the development of resistance to therapy (Johnstone et al., 2002). The B-cell lymphoma-2 (Bcl-2) family is a large group of proteins which exert a key regulatory function in intrinsic apoptosis by controlling the integrity of the outer mitochondrial membrane (Cory and Adams, 2002). The family encompasses three major subgroups with different functions and displaying different subsets of conserved sequence motifs known as Bcl-2 homology (BH) domains which are the hallmark of this protein family (Kelekar and Thompson, 1998; Daniel et al., 2003). Most anti-apoptotic members (which comprise Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1) contain BH domains 1 to 4. The multidomain pro-apoptotic members (Bax, Bak and Bok) are required for the execution of apoptosis through the mitochondrial pathway (Wei et al., 2001) and contain BH domains 1 to 3 (Chan and Yu, 2004). The third subgroup is composed of several pro-apoptotic molecules (including Bad, Noxa and many others) called BH3-only proteins since they display only the BH3 domain. They either inhibit the anti-apoptotic members or activate the multidomain pro-apoptotic members. Following an appropriate apoptotic stimulus, the characteristic balance between anti-apoptotic and multi-domain pro-apoptotic members of normal cells is perturbed. As a result, the complete pro-apoptotic molecules oligomerize and form channels in the outer mitochondrial membrane which allow the release of apoptogenic factors (e.g. cytochrome c) from the intermembranous mitochondrial space into the cytosol (Westphal et al., 2011).

Subsequently, specific proteolytic enzymes, the caspases, are activated and elicit the demise of the cell. The BH domains are the basis for the interactions between the members: a hydrophobic groove formed by specific residues of the BH1, BH2 and BH3 domains of the anti-apoptotic members interacts with the amphipathic helix of the BH3 domain of molecules of other subgroups (Cory and Adams, 2002). Comparatively little information is available, however, on this protein family and other molecules involved in apoptosis in canines. A small number of Bcl-2 family members have been cloned and partially characterized in the dog, including the complete coding sequences for Bcl-2, Bcl-x_L, Mcl-1 and Bax (Sano et al., 2003; Sano et al., 2004). Differences at the molecular level within the intrinsic apoptotic pathway between humans and dogs have been reported in rare instances. For example, a consensus Akt phosphorylation site in human caspase-9 reported to mediate suppression of apoptosis is lacking in the dog (Cardone et al., 1998; Rodriguez et al., 2000). However, awareness of such variations is important because on one side they possibly impinge on the function of the pathway under physiological and neoplastic conditions, on the other side they may interfere with targeted therapies.

The budding yeast *Saccharomyces (S.) cerevisiae* has been extensively used to functionally characterize human and murine Bcl-2 family members. Toxicity of Bax expression for yeasts and its abrogation by coexpression of Bcl-2, Bcl-x_L and Mcl-1 first became evident in yeast two-hybrid studies of mammalian Bcl-2 family members (Sato et al., 1994). Subsequent investigations have shown that the mechanisms leading to yeast cell death caused by Bax partly rely on ancestral programmed cell death (PCD) machinery components (Ligr et al., 1998; Khoury and Greenwood, 2008). Several agents can induce yeast PCD, including hydrogen peroxide, UV radiation, the absence of nutrients, hyperosmotic stress, acetic acid and aging (Farrugia and Balzan, 2012). Yeast PCD share some phenotypical and biochemical similarities with mammalian apoptosis, including the release of mitochondrial cytochrome c

into the cytosol (Ludovico et al., 2002). This particular phenomenon - a hallmark of mammalian Bax action – has been demonstrated following Bax expression in yeast (Manon et al., 1997). To date, several studies of mammalian Bcl-2 family proteins in *S. cerevisiae* have focused on genetic analyses of Bax, Bcl-x_L and Bcl-2, while this model has rarely been used, if at all, to characterize further members such as Bak, Bcl-w, and Mcl-1 for instance (Bodrug et al., 1995; Tao et al., 1997; Beaumont et al., 2013).

In order to contribute to ongoing efforts in developing the canine model as a tool for cancer research, we herein perform an analysis of the sequences, some of which are novel for the dog, and study the function in the yeast, of selected canine Bcl-2 family proteins.

2. Materials and methods

2.1. Cell lines and cultures

The Madin-Darby canine kidney (MDCK) cell line originates from an adult female Cocker Spaniel dog and it was purchased from the American Type Culture Collection (ATCC) (www.ATCC.org/). The cells were grown in Iscove's Mod. Dulbecco's Medium (Sigma, St. Louis, USA) supplemented with 10% fetal calf serum inactivated at 60°C, 2.5% HEPES buffer (Sigma, St. Louis, USA), 1% L-Glutamine (Sigma, St. Louis, USA) and 1% Penicillin-Streptomycin solution (Sigma, St. Louis, USA). The canine normal keratinocyte line used originates from the skin of a healthy Beagle dog (Kolly et al., 2005). The cells were grown in Dulbeccos' Modified Eagles Medium (Sigma, St. Louis, USA) supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin and 15% fetal calf serum. All cell culture reagents were obtained from Gibco BRL life Sciences (Basel, Switzerland). Both cell lines were grown at 37 °C and a 5% CO_2 atmosphere.

2.2. RNA extraction, RT-PCR and cloning

Total RNA was isolated from MDCK cells and/or from normal canine keratinocytes with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Then 40 U Protector RNAse Inhibitor (Roche, Mannheim, Germany) was added to each RNA extract. cDNA was synthesized from 1µg of total RNA using the 1st strand cDNA Synthesis Kit for RT-PCR (AMV) and an oligo-dT-primer according to the manufacturer's instructions (Roche, Mannheim, Germany).

Primers including the putative Start and Stop codons as deduced from publicly available sequence information and taking the human sequence as a reference (Tables 1 and 2) were designed either manually or by using the Primer3 website (http://frodo.wi.mit.edu/) (Rozen and

Skaletsky, 2000). Reverse transcription PCR reactions were accomplished with 1µl of cDNA samples and Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). Basic cycling parameters were as follows: initial denaturation at 94 °C for 1.5 min; 35 cycles of denaturation at 94 °C for 50 sec, annealing for 50 sec at 50-70 °C (a gradient was chosen on dependance of the primer melting temperature) and extension at 72 °C for a time adjusted to the expected size of the product (1 min per 1000 bp); final extension for 10 min at 72 °C. Each PCR reaction was optimized by adjusting the annealing temperature and the final magnesium concentration to yield a band with the size corresponding to the expected principal transcript for each molecule. After agarose gel separation, the amplificates obtained were recovered with MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) and sent to a company (Microsynth AG, CH-9436 Balgach) for direct sequencing (Cycle Sequencing / Capillary Electrophoresis) with the primers used for amplification. If the amplification product corresponded to the expected sequence, the PCR was repeated using primers containing restriction enzyme sites suitable for cloning into the GST-tagged pGEX-4T2 vector (Invitrogen, Carlsbad, USA). Amplicons were cloned and transformed into DH5 alpha competent cells. Miniprep plasmid DNA was sequenced (Microsynth AG, CH-9436 Balgach) using the pGEX 5'- and 3'-sequencing primers indicated by the manufacturer. Final cDNA sequences were derived from the consensus of at least three individual clones and were deposited in GenBank (Table 2). The correct identity of the clones was further confirmed at the protein level with immunoblot analyses with appropriate antibodies using lysates of transformed BL-21 Star *E. coli* harvested after induction with beta-D-thiogalactopyranoside as previously described by Keller et al. (2007).

2.3. Computational analysis

Putative canine protein sequences were derived from the cDNA sequences using a translation tool accessed via the ExPASy homepage (http://www.expasy.org/). Canine nucleotide coding sequences (cds) and deduced protein sequences were compared with human and murine counterparts (Table 2) using the EMBOSS sequence analysis tool provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/align/). The nucleotide and the protein sequences were aligned with the needle method and the Blosum62 matrix algorithm, respectively. The exon/intron structure was derived by aligning the cds with the respective genome sequence using Splign, a mRNA-to-genomic alignment program available at NCBI (http://www.ncbi.nlm.nih.gov/) and confirmed using BLAT (Blast like alignment tool) on the MGC genome browser (http://mgc.ucsc.edu/) (Kent, 2002). The genomic sequence releases were CanFam3.1 [GCF_000002285.2] for canines, Mus musculus GRCm38.p1 [GCF_000001635.21] for murines, and [GCF_000001405.22 GCF_000306695.1 GCF_000002135.2 GCF_000002125.1] for humans. For domain comparisons we used human domain signatures indicated in UniProtKB/Swiss-Prot and additional relevant features as suggested from the literature.

2.4. Subcloning and expression in S. cerevisiae

The cDNAs containing the cds for the canine multidomain pro-apoptotic proteins Bax and Bak and the anti-apoptotic proteins Bcl-x_L, Bcl-w, and Mcl-1 were subcloned by sticky-end ligation in a yeast expression vector (epitope tagging vector pESC, Invitrogen) containing the *URA3* selection marker. This vector contains two multiple cloning sites which are located downstream of the galactose inducible divergent promoters GAL1 and GAL10, respectively. Each cDNA was introduced individually in the vector. In addition, vectors were constructed with Bax or Bak paired with each one of the cDNAs for the anti-apoptotic proteins. Proper introduction of

the cDNAs was verified by sequencing (Microsynth AG, CH-9436 Balgach) miniprep plasmid DNA using the pESC 5'- and 3'-sequencing primers indicated by the manufacturer. The yeast used throughout this study was strain BY4743 *Δhis3/leu2/met15/ura3* purchased from Euroscarf (D-60438 Frankfurt; Acc. No. Y20000). It was routinely cultured in YPAD (1% yeast extract, 2% Bacto peptone, 2% glucose, 40 mg Adenine sulfate) medium as described (Guscetti et al., 2005). The yeasts were transformed by electroporation in presence of 1 M sorbitol (Gene Pulser with Pulse Controller, Bio-Rad) according to a previously described protocol (Becker and Lundblad, 1994) with the empty plasmid and all plasmids described above. Selection was done on minimal 2% glucose medium without the selection marker URA. To monitor the effect of expression of the Bcl-2 family proteins, transformed yeasts were grown under aerobic conditions (shaken at 220 rpm, 30 °C) in 3 ml of liquid minimal medium containing 2% galactose (inducing) instead of glucose (repressing) and the optical density at 600 nm (OD_{600}) was measured every 6 hours until saturation. Each experiment was carried out in duplicate. In addition, a clonogenic assay was carried out by plating aliquots of 500 cells on minimal glucose medium, each in triplicate, after growth in inducing liquid medium for 24 and 48 hours as previously described (Guscetti et al., 2005). All clonogenicity experiments were carried out at least in three independent instances. Statistical significance of clonogenicity data for yeasts expressing either Bax or Bak alone vs. all other proteins either individually or in combination was calculated by the student´s t test. Significance was set at a value of $p = 0.05$.

2.5. Western Blots

To verify expression of the respective protein after induction, representative culture samples collected at 24h or 48h after start of induction were analysed by western blot as previously described with minor modifications (Dettwiler et al., 2013). Briefly, protein extracts were prepared by mechanical lysis of the yeast cells (mixed to equal volumes of a lysis buffer

containing 100 mM Tris-HCl at pH 6.8, 10% (v/v) glycerol, 3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 1% (w/v) bromophenol blue using glass beads and a Vortex mixer at maximum speed for 2 min. The extracts were run on 12% SDS-polyacrylamide gels, transferred to PVDF membranes (Immobilon-P Transfer Membrane, Millipore Corporation, Billerica, MA) by electroblotting. Following primary antibodies, all previously shown to cross-react with the recombinant canine protein expressed in bacteria, were used: anti-human Bax Ab-1 mouse monoclonal antibody, clone 2D2 (cat. no. MS-711-P0, Lab Vision Corporation, Thermo Fisher Scientific, Fremont, CA 94539, USA), applied at 0.5 ug/ml; anti-human Bak rabbit polyclonal antibody (cat. no. 06-536, Merck Millipore, Billerica, MA 01821, USA), applied in a 1:400 dilution; anti-mouse Bcl-x_L Ab-2 mouse monoclonal antibody, clone 7D9 (cat. no. MS-1334-PO, Lab Vision Corporation, Thermo Fisher Scientific, Fremont, CA 94539, USA), applied at 0.5 ug/ml; anti-human Bcl-w (16-29) rabbit polyclonal antibody (cat. no. 197209, Merck Chemicals Ltd., San Diego, CA 92121, USA), applied in a 1:400 dilution; and anti-human Mcl-1 rabbit polyclonal antibody (cat. no. HPA008455-100UL, Atlas Antibodies AB, SE-106 91 Stockholm, Sweden), applied in a 1:400 dilution. After incubation with the appropriate primary antibodies, horseradish-peroxidase labelled secondary antibodies were applied and binding was visualised using a chemiluminescent substrate. The PageRulerTM Plus Prestained Protein Ladder (Fermentas Life Sciences, Lot 00070238; Thermo Fisher Scientific, Fremont, CA 94539, USA) was used to assess protein sizes.

3. Results

3.1. Nucleotide sequence comparison of canine Bcl-2 family members with the human and murine orthologues

We have identified and cloned the coding sequence (cds) of seven canine Bcl-2 family members. Three sequences (Bcl-x, Bax and Mcl-1) have previously been reported by others (Sano et al., 2003; Sano et al., 2004), the remaining four (Bcl-w, Bak, Bad and Noxa) are novel for the dog and have been deposited in GenBank. All sequence accession numbers are indicated in Table 2. In this section we report basic data derived from our cDNA clones, and, for the sake of completeness, from the complete mRNA sequence for canine Bcl-2 previously deposited in GenBank by others. Alignment of the sequences with the dog genome showed 100% identity and canonical splice sites except where otherwise stated. Expected amino acid exchanges resulting from base mismatches are mentioned. In addition, a comparison with the reference sequences of the human and murine counterparts is presented (Table 3).

Bcl-2 isoform α. The canine complete mRNA sequence for Bcl-2 available from GenBank was derived from peripheral mononuclear blood cells of an adult dog. The dog genome sequence displays a gap in the region containing the Bcl-2 gene resulting in 102 bases of the cds (from base 157 through 258) that could not be aligned. Mismatches were observed at positions 146 (T instead of C as in the genome sequence, resulting in a Phe instead of a Ser), 148 (T instead of G, resulting in a Ser instead of an Ala), 327 (C instead of G), 330 (C instead of T) and 441 (T instead of C) of the cds. In addition a single base insertion was found at position 331 (G, eliciting a frame shift) of the cds. Comparison with the human Bcl-2 isoform α cds (720 bp) revealed 92% identity for the canine sequence (720 bp) which is close to the homology observed between human and murine (711 bp) sequences (89%).

Bcl-x_L (BCL2L1). The complete mRNA sequence for the canine anti-apoptotic Bcl-2 family member Bcl-x_L available in GenBank was derived from lymphocytes of an adult dog. All our own clones (source: MDCK cells) showed a single nucleotide mismatch (C instead of T) at position 666 of the cds when compared to the GenBank sequence. The dog genome reference sequence indicates a C at this position. In all three species, the Bcl-x_L cds (702 bp) is distributed over two exons. Overall, canines show a slightly higher homology with humans than with mice (97% and 94%, respectively).

Bcl-w (BCL2L2). The cds of the canine anti-apoptotic Bcl-2 family member Bcl-w generated in this study (source: MDCK cells) is, like the human and murine cds, 582 bp long and distributed over two exons. The identity of the canine Bcl-w cds with its human counterpart is 95%, while the identity between human and mouse is 94%.

Mcl-1. The complete mRNA sequence for the canine anti-apoptotic Bcl-2 family member Mcl-1 available in GenBank was derived from lymphocytes. Alignment of this sequence with our Mcl-1 cds (source: MDCK cells) showed a one base deletion, a one base insertion and four single nucleotide mismatches. Alignment of our sequence with the dog genome indicated one nucleotide mismatch at position 436 (C instead of T) in our sequence. Alignment of the GenBank cds with the dog genome revealed a single bp insertion at position 31 and a single base deletion 9 bp further on as well as five mismatches at positions 6 (T instead of C), 119 (G instead of A), 436 (C instead of T), 641 (A instead of G) and 978 (C instead of T). Two of these mismatches would lead each to a amino acid (aa) substitution (position 119: Arg instead of Lys; position 641: Gln instead of Arg), the latter involving the BH3-domain. The insertion at position 31 leads to a short frameshift resulting in the exchange of aa 11-13 (Arg-Thr-Gln instead of Gly-Leu-Asn); this region does not code for any known domain. In both humans and dogs, the Mcl-1 cds encompasses 1053 bp, in contrast to 996 bp in mice. In all three species

investigated, the cds is distributed over three exons. The canine cds shares a higher homology with the human counterpart than the murine sequence (89% and 83% identity, respectively).

Bak (BAK1). The cds of the canine multi-domain pro-apoptotic Bcl-2 family member Bak generated in this work (source: MDCK cells) consists, like its human counterpart, of 636 bp distributed over five exons. The murine cds is six bp shorter (630 bp). The human sequence is more similar to the canine than to the murine sequence (90% and 80% identity, respectively).

Bax variant α. The mRNA sequence comprising the complete cds of Bax available in GenBank was derived from a canine osteosarcoma cell line. Our own cds sequence is 100% identical to the GenBank sequence. The dog genome sequence displays a gap in the region containing the bax gene; where proper alignement was possible (position 1 through 477 of the cds) our sequence (source: MDCK cells) was 100% identical to the genome sequence. In all three species investigated, the cds is 579 bp long. In murines and humans it is distributed over six exons. The cds of both dogs and mice show a similar degree of homology with the human Bax var. alpha (93% and 90% identity, respectively). We additionally detected a splice form of the canine Bax cds, which is similar to the human Bax γ version (GenBank accession no. NM_138762). Like its human counterpart, the cds consists of 126 bp divided into two exons. It shares 91% identity with the human Bax γ cds. In silico translation predicts, like in humans, an hypothetical protein of 41 aa (identity human/canine: 83%).

Bad. The cds of the canine BH3-only Bcl-2 family member Bad generated from MDCK cells in this study consists of three exons, spanning 504 bp in length. This is three bp shorter than the human cds and 111 bp shorter than the murine cds, which spans over four exons. Since several gaps occur in the alignment between human and canine cds, we generated additional clones from a normal canine keratinocyte cell line (Kolly et al., 2005) to exclude a MDCK-related artifact. Clones from both cell types yielded the same sequence. The canine Bad cds is 86% identical to its human counterpart, whereas the identity between human and mouse is only

65%, due to the additional coding exon at the 5'-end in the murine sequence. When only overlapping parts were compared, identity between human and mouse sequences was 82%.

Noxa (PMAIP1). The cds of the canine BH3-only Bcl-2 family member Noxa, generated from a normal canine keratinocyte cell line (Kolly et al., 2005) in this study, is distributed, like its human counterpart, over two exons with a total of 165 bp. It yields 87% identity with the human sequence. The murine cds is divided into three exons and spans 312 bp. The mouse sequence differs remarkably from the human (40% identity), which ensues from the presence of an additional coding exon at the 5'-end in the murine sequence. Comparison of overlapping parts only yielded 70% identity between human and mouse sequences.

3.2. Protein sequence comparison of canine Bcl-2 family members with the human and murine orthologues

We further analyzed in silico the protein sequences of the canine Bcl-2 members as deduced from the nucleotide sequences described above. The degree of conservation between humans, canines and mice of the whole proteins as well as of the most relevant domains, motifs and residues were determined (Table 4). All sequences and respective alignments are reported in Supplemental Figure 1.

The overall identity of these proteins ranged from 76% to 100% between canines and humans, and from 34% to 99% between mice and humans. In all but one (Bcl-2 isoform alpha)cases there was a higher degree of homology between human and canine sequences than between human and murine sequences. The length of the Bcl-2 family proteins is relatively conserved in canines compared to humans, while it markedly differs for some murine proteins, in particular the BH3-only members Bad and Noxa. The anti-apoptotic family members are best conserved, with the two proteins Bcl-x_L and Bcl-w 100% identical at the amino acid (aa) level between humans and dogs. In murines, three out of 15 Bcl-2 Homology (BH) domains analyzed

in this subgroup differed from the human counterpart by one aa, while there were no differences in dogs. The transmembrane domains showed either 100% conservation in all three species (Bcl-x_L, Bcl-w, Mcl-1) or slight differences with the human counterpart in the canine version (Bcl-2). The multi-domain pro-apoptotic members Bak and Bax showed a slightly lower degree of interspecies conservation than the anti-apoptotic members. In the mouse, all BH domains of these two molecules showed at least one aa exchange, three BH domains showed two aa exchanges. In the dog only two out of these six BH domains showed one aa exchange. The transmembrane domain of Bax is 100% conserved across the three species, that of Bak shows an exchange of four aa in mice and of two aa in dogs. Interestingly, the lowest overall and domain identity in the Bcl-2 family was observed with the two BH3-only proteins Bad and Noxa. This is particularly true for the murine molecules which are markedly longer than the canine and human counterparts. Comparison of these proteins between murines and humans yielded 58% and 34% identity for Bad and Noxa, respectively (while their overlapping regions showed 73% and 64% identity, respectively).

Several human Bcl-2 family proteins present with cleavage sites for caspase-3 and other proteases. Figure 1 shows the comparative tetramer sequences preceding cleavage sites reported in the literature. Sequences 100% conserved in all three species include both caspase-3 cleavage sites of Bcl-x_L, the major caspase-3 cleavage sites of Mcl-1 and of Bad, and the calpain recognition sequence of Bax (Cheng et al., 1997; Clem et al., 1998; Fujita et al., 1998; Wood et al., 1998; Condorelli et al., 2001; Michels et al., 2004).

3.3. Partial functional characterization of canine Bcl-2 family proteins in the yeast S. cerevisiae

In this part we functionally analyzed interactions of a subset of canine Bcl-2 family members comprising the multidomain pro-apoptotic proteins Bax and Bak and the anti-apoptotic

proteins Bcl-x_L, Bcl-w and Mcl-1 in the yeast model *S. cerevisiae*. First, the proteins were expressed in inducing liquid media containing galactose, and yeast cell growth was monitored by measuring the OD_{600} at 6 h intervals. As expected, compared to yeasts containing the empty vector, cultures of yeasts expressing either Bax or Bak showed a growth delay, while the growth of yeasts expressing any of the anti-apoptotic proteins was unaffected (Fig. 2a). The growth delay caused by Bax or Bak was completely abrogated when any of the anti-apoptotic proteins was expressed concomitantly (Fig. 2b). This kind of growth delay has previously been described for human and/or murine Bax and Bak, and it has been attributed to reproductive cell death (Ligr et al., 1998; Xu et al., 1999; Guscetti et al., 2005). This notion was herein confirmed for the canine orthologs by using a clonogenic assay following 24 and 48 hours of protein expression. The data is presented in Fig. 3a and 3b. Expression of either Bak or Bax alone resulted in a reduction of the percentage of viable and reproduction-competent yeasts to 20-10% (Bak) or 3-1% (Bax) of yeasts carrying the empty vector. In contrast, Bcl-x_L and Mcl-1 showed a slight degree of toxicity which was most apparent at 48 h of expression (Fig. 3b). The effect of Bax and Bak was abrogated through concomitant expression of each of the anti-apoptotic proteins Bcl-w, Bcl-x_L, and Mcl-1. Extracts from yeasts transformed with the corresponding cDNAs showed distinct bands for each protein in Western blots indicating their expression (Fig. 4).

4. Discussion

We have carried out a comparative sequence analysis of a representative number of canine Bcl-2 family members, at both the nucleotide and protein level. A high sequence homology was found between the canine proteins and their human and murine counterparts. Notably, the canine sequences showed a higher degree of homology with the human sequences than the murine sequences, which is consistent with a faster mutation rate in mice as compared to dogs and people (Lindblad-Toh et al., 2005) and supports in principle the dog as a model organism for diseases involving deregulation of mitochondrial apoptosis. Bcl-2 family proteins have been assigned a central importance in the pathogenesis of various human cancer syndromes (Kelly and Strasser, 2011; Nys and Agostinis, 2012). Likewise, they have been implicated in the pathogenesis of diverse canine tumors including for instance hemangiosarcoma, mammary tumors, tumors of neural and hematopoetic origin (Sano et al., 2003; Kumaraguruparan et al., 2006a; Kumaraguruparan et al., 2006b; Yan et al., 2006; Murakami et al., 2008; Ide et al., 2010; Strefezzi et al., 2012; Dettwiler et al., 2013).

Proteins are assigned to the Bcl-2 family owing to the presence of at least one of four BH domains (Kelekar and Thompson, 1998; Daniel et al., 2003). These domains mediate the interplay between the Bcl-2 family members, which controls the integrity of the mitochondrial membrane. Structural studies have shown the basis for interactions between the different subgroups (Cory and Adams, 2002). The fact that several domains participate in forming a functionally important scaffold-like structure might explain the limited overall and domain interspecies variation found in anti-apoptotic members of the Bcl-2 family. In contrast, the BH3-only members Bad and Noxa showed the least degree of overall interspecies conservation. This might derive from the fact that the BH3-only subgroup members exert their apoptosis-related function through one single domain as opposed to members of the other subgroups.

Both murine Bad and Noxa significantly differ from their human and canine counterparts by displaying an additional coding exon. In addition, murine Noxa displays a second BH3-domain motif (Oda et al., 2000). In contrast, regulatory serine residues (Ser $^{75, 99, 118, 134}$ in humans) in Bad are conserved among the three species at nearly the same locations. Phosphorylation of these residues upon a pro-survival stimulus maintains Bad in an inactive state and bound to cytosolic 14-3-3Sigma proteins (Bae et al., 2001). Recent studies have revealed that the anti-apoptotic members of the Bcl-2 family each bear a unique pattern of interaction with peptides derived from BH3 domains of BH3-only molecules (Certo et al., 2006) providing the basis for the development of therapeutic compounds targeting these interactions (Liu and Wang, 2012). The strong degree of conservation of the dog proteins suggest that this species is potentially well-suited for modeling interactions of Bcl-2 family members and related therapeutic peptides.

Apoptosis is executed through cleavage of proteins at specific recognition sites through specific proteases, mainly through caspases (Cory and Adams, 2002). Several members of the Bcl-2 protein family display protease cleavage sites, and their cleavage products have been shown in general to enhance apoptotic activity (Cheng et al., 1997; Clem et al., 1998; Fujita et al., 1998; Wood et al., 1998; Condorelli et al., 2001; Michels et al., 2004) and in some cases to mediate apoptotic activity (Ofengeim et al., 2012). Sequence comparison between human, canine and murine Bcl-2 family members has revealed a number of fully conserved cleavage sites. Some of the remaining cleavage sites conserved to less than 100%, such as e.g. the motif present in canine Bcl-2, might still be functional based on the sequence, while others, such as for example the minor cleavage site of Bad, are likely not functional. The impact of sequence variants on function should be verified experimentally. Conservation of protease recognition motifs across species support their biological relevance on one side and the validity of the canine model on the other side.

Comparison of the sequences generated in this study with the canine genome sequence as well as with mRNA sequences previously submitted to Genbank by others revealed a generally high degree of concordance. Except for the gap in the genome sequence in the region coding for Bax and for a single base mismatch in the cds of Mcl-1 all sequences generated in this study (4119 bases) were 100% identical with the dog genome sequence. Another gap in the genome sequence hindered proper alignment of the Bcl-2 cds available from Genbank. Nevertheless, the dog genome third release coverage has been estimated at 99.8% of the euchromatic portion of the genome, and the rate of sequencing errors within genes has been drastically reduced compared to the previous release (Lindblad-Toh et al., 2005; Hoeppner et al., 2014). Mismatches either represent sequencing errors, single nucleotide polymorphisms, somatic cell variation, or RNA editing (Furey et al., 2004). All of our novel cds were also predicted by the automated prediction program GNOMON at NCBI with the exception of Noxa. In addition, the automated program predicted an additional sequence at the 5'-end of canine Bad that was not supported by own 5'-RACE data (data not shown). Comparisons of previous GenBank entries with the dog genome revealed conspicuous mismatches for Bcl-2 and Mcl-1. An analysis of approximately 30,000 non-redundant human mRNA sequences suggested that mRNA collections may contain a substantial number of errors (Furey et al., 2004).

We have used the well-characterized eukaryotic yeast *S. cerevisiae* for the expression and functional analysis of the canine Bcl-2 proteins cloned herein. This unicellular organism is easy to manipulate genetically and it has previously been extensively used for functional studies of human Bcl-2 family proteins since considered to be devoid of homologs of the Bcl-2 family (Fleury et al., 2002; Priault et al., 2003; Guscetti et al., 2005; Khoury and Greenwood, 2008). This notion has recently been questioned since the description of a yeast protein variously referred to as Bxi1p (Bax inhibitor-1) or Ybh3p (Yeast BH3-only protein) (Büttner et al., 2011; Cebulski et al., 2011). However, while its classification and function are

still debated, it did not visibly interfere with the experiments in this study. Expression of canine Bcl-2 family members in yeast has not been described before. Here, we successfully performed a functional analysis of five canine Bcl-2 family members in this model organism, namely the pro-apoptotic members Bak and Bax and the anti-apoptotic members Bcl-w, Bcl-xL and Mcl-1. As previously described for the human and/or murine orthologs (Ink et al., 1997; Tao et al., 1997; Ligr et al., 1998), expression of canine Bak and Bax showed a lethal effect in yeast. Moreover, this effect was abrogated on co-expression of the anti-apoptotic members Bcl-xL, Mcl-1 and Bcl-w as previously demonstrated for large part of their human orthologs in yeast (Tao et al., 1997; Beaumont et al., 2013). To our knowledge, abrogation of the effect of Bak by Bcl-w has not been previously reported in this model. We therefore consider the yeast system as suitable for the functional assessment of canine Bcl-2 family members and their interactions. Future use of the model could encompass e.g. the addition of BH3-only proteins (Guscetti et al., 2005) or the comparative exploration of the effects of specific compounds on interactions between Bcl-2 family members of canine versus human origin (Silva et al., 2011) thus further assessing the potential value of the canine model. Several murine models, in particular genetically engineered mouse models, have proven invaluable for mechanistic and drug discovery studies (Cheon and Orsulic, 2011; Ruggeri et al., 2014). As a specific example, the Eμ-myc lymphoma model has been successfully used to model the efficacy of the Bcl-2 antagonist ABT-737 against an aggressive lymphoma subtype (Mason et al., 2008), and its derivative ABT-199 is currently tested in phase II and III studies (Besbes et al., 2015). Provided the canine and human molecules behave similarly, spontaneous dog tumors could provide an additional frame for testing such compounds in a clinical setting.

5. Conclusions

In summary, we have performed a comparative as well as a functional analysis of a representative set of canine Bcl-2 family members. Our analysis revealed a high degree of sequence similarity in the canine Bcl-2 family proteins compared to their human counterparts, especially within domains. Functional interactions of selected canine multidomain pro-apoptotic with anti-apoptotic members were recapitulated in a yeast model. This in vitro model might provide a valuable tool for instance for testing therapies specifically targeting this protein family and, subsequently, to further establishing spontaneous dog neoplasms as models for human cancer.

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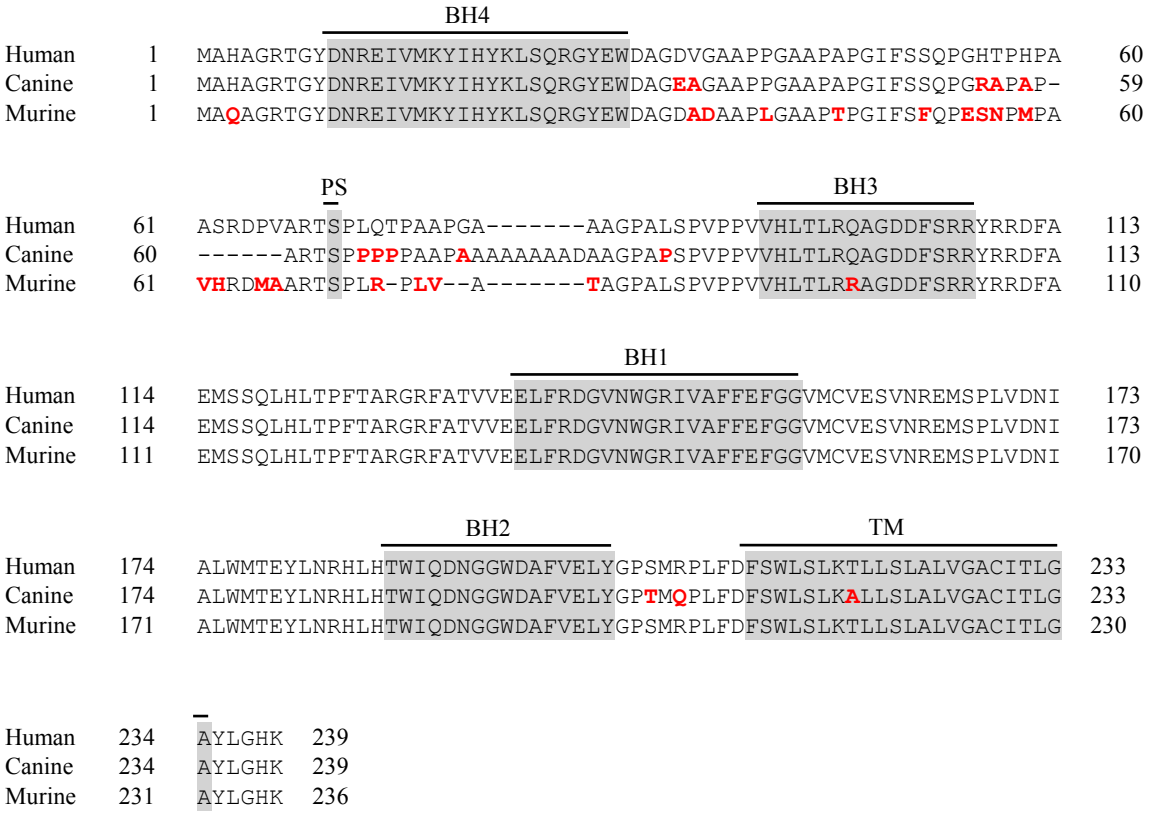
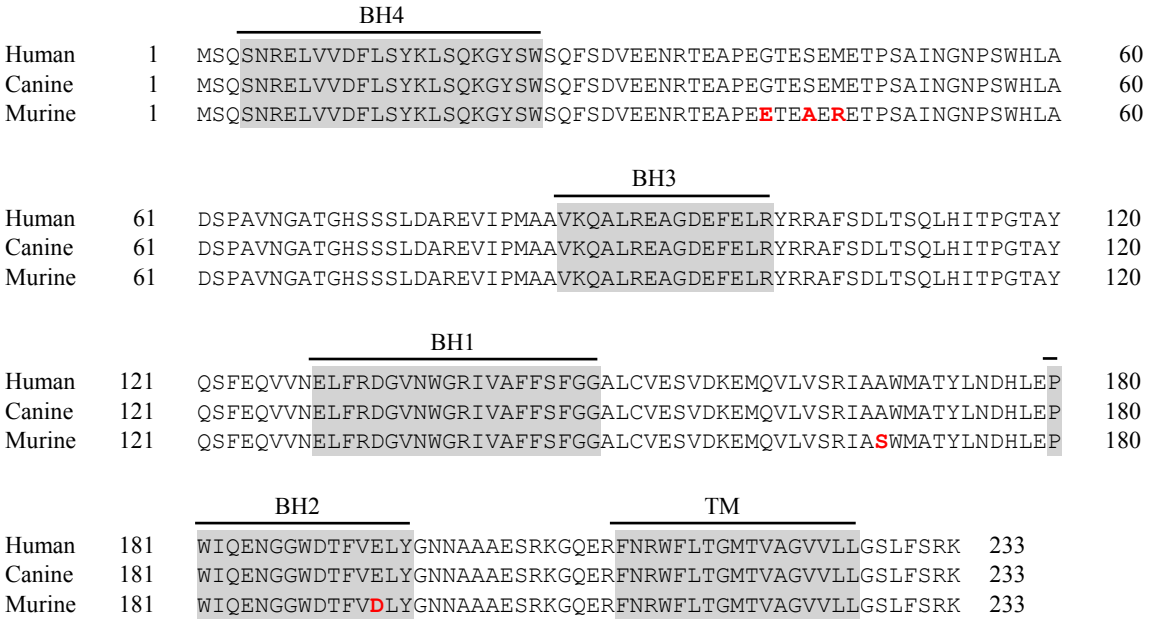
Supplemental Figure S1

Alignments of Bcl-2 proteins of human, canine, and murine origin and principal domains and motifs

Alignment for Bcl-2 isophorm alpha



Alignment for Bcl-x_L (BCL2L1)



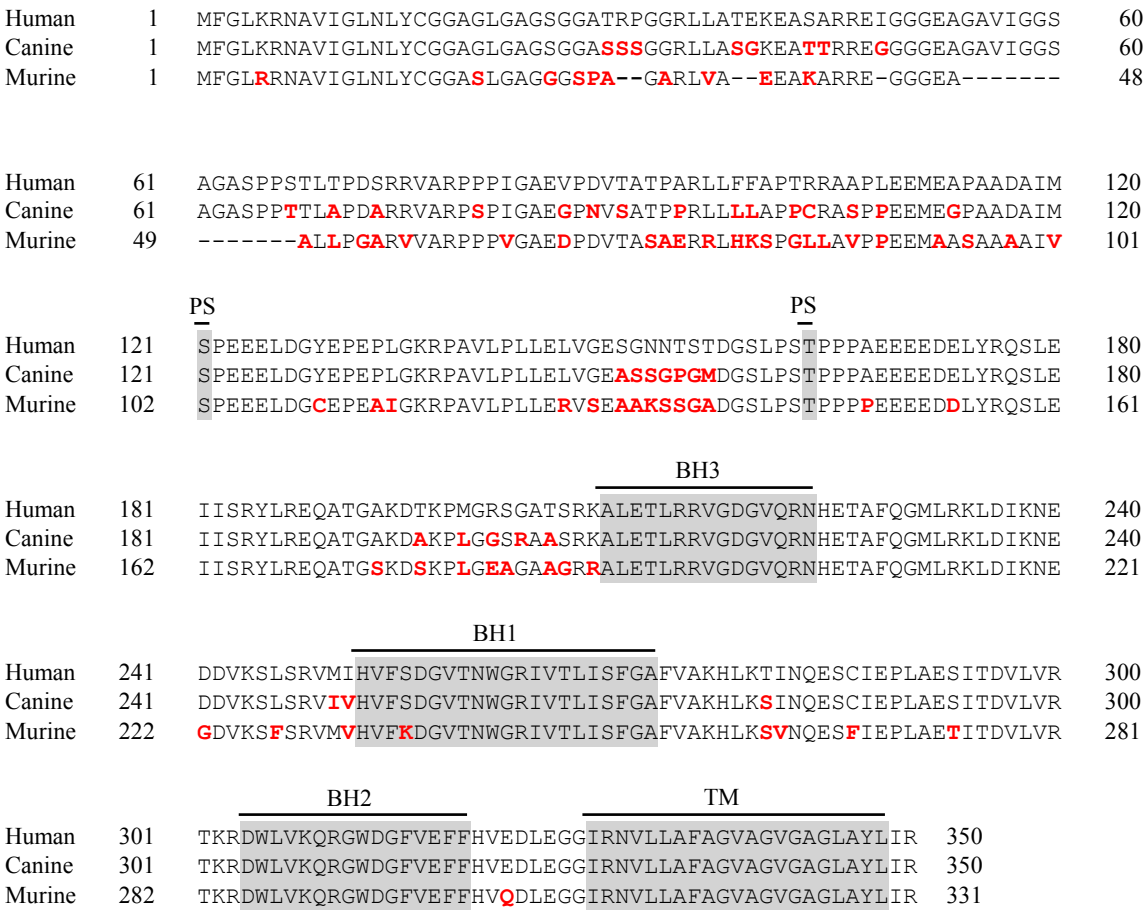
(Supplemental Figure S1 ctd.)

Alignment for Bcl-w (BCL2L2)

```
                                BH4                                 BH3
Human     1  MATPASAPDTRALVADFVGYKLRQKGYVCGAGPGEGPAADPLHQAMRAAGDEFETRFRRT  60
Canine    1  MATPASAPDTRALVADFVGYKLRQKGYVCGAGPGEGPAADPLHQAMRAAGDEFETRFRRT  60
Murine    1  MATPAS*PDTRALVADFVGYKLRQKGYVCGAGPGEGPAADPLHQAMRAAGDEFETRFRRT  60

                                                BH1
Human    61  FSDLAAQLHVTPGSAQQRFTQVSDELFQGGPNWGRLVAFFVFGAALCAESVNKEMEPLVG  120
Canine   61  FSDLAAQLHVTPGSAQQRFTQVSDELFQGGPNWGRLVAFFVFGAALCAESVNKEMEPLVG  120
Murine   61  FSDLAAQLHVTPGSAQQRFTQVSDELFQGGPNWGRLVAFFVFGAALCAESVNKEMEPLVG  120

                                    BH2
Human   121  QVQEWMVAYLETRLADWIHSSGGWAEFTALYGDGALEEARRLREGNWASVRTVLTGAVAL  180
Canine  121  QVQEWMVAYLETRLADWIHSSGGWAEFTALYGDGALEEARRLREGNWASVRTVLTGAVAL  180
Murine  121  QVQ*WMVAYLETRLADWIHSSGGWAEFTALYGDGALEEARRLREGNWASVRTVLTGAVAL  180

Human   181  GALVTVGAFFASK  193
Canine  181  GALVTVGAFFASK  193
Murine  181  GALVTVGAFFASK  193
```

(Supplemental Figure S1 ctd.)

Alignment for Mcl-1



(Supplemental Figure S1 ctd.)

Alignment for Bak

```
Human     1   MASGQGPGPPRQECGEPALPSASEEQVAQDTEEVFRSYVFYRHQQEQEAEGVAAPADPEM   60
Canine    1   MASGQGPGPPRRECGEAAPSSTSEEQVARDTEEVFRSYVFYRHRQEQEAEGAAVPADPEM   60
Murine    1   MASGQGPGPPKVGCDES--PSPSEQQVAQDTEEVFRSYVFYLHQQEQETQGAAAPANPEM   58

                                  BH3                                  
Human    61   VTLPLQPSSTMGQVGRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFE   120
Canine   61   VTLPLEPSSTMGQVGRQLAIIGDDINQRYDSEFQAMLQHLQPTAENAYEYFTKIASSLFE   120
Murine   59   DNLPLEPNSILGQVGRQLALIGDDINRRYDTEFQNLLEQLQPTAGNAYELFTKIASSLFK   118

                  BH1                                                BH2
Human   121   SGINWGRVVALLGFGYRLALHVYQHGLTGFLGQVTRFVVDFMLHHCIARWIAQRGGWVAA   180
Canine  121   SGINWGRVVALLGFGYRLALHVYQRGLTGFLGQVTRFVADFMLHHCIARWIAQRGGWVAA   180
Murine  119   SGISWGRVVALLGFGYRLALYVYQRGLTGFLGQVTCFLADIILHHYIARWIAQRGGWVAA   178

                      TM                                                
Human   181   LNLGNGPILNVLVVLGVVLLGQFVVRRFFKS   211
Canine  181   LNLGNGPILNVLIVLSVVLLGQFVVRRFFKS   211
Murine  179   LNFRRDPILTVMVIFGVVLLGQFVVHRFFRS   209
```

Alignment for Bax var. alpha

```
Human     1   MDGSGEQPRGGGPTSSEQIMKTGALLLQGFIQDRAGRMGGEAPELALDPVPQDASTKKLS   60
Canine    1   MDGSGEQPRGGGPTSSEQIMKTGALLLQGFIQDRAGRMGGETPELPLEQVPQDASTKKLS   60
Murine    1   MDGSGEQLGSGGPTSSEQIMKTGAFLLQGFIQDRAGRMAGETPELTLEQPPQDASTKKLS   60

                  BH3                                  BH1
Human    61   ECLKRIGDELDSNMELQRMIAAVDTDSPREVFFRVAADMFSDGNFNWGRVVALFYFASKL   120
Canine   61   ECLKRIGDELDSNMELQRMIAAVDTDSPREVFFRVAAEMFSDGNFNWGRVVALFYFASKL   120
Murine   61   ECLRRIGDELDSNMELQRMIADVDTDSPREVFFRVAADMFADGNFNWGRVVALFYFASKL   120

                                      BH2                  
Human   121   VLKALCTKVPELIRTIMGWTLDFLRERLLGWIQDQGGWDGLLSYFGTPTWQTVTIFVAGV   180
Canine  121   VLKALCTKVPELIRTIMGWTLDFLRERLLGWIQDQGGWDGLLSYFGTPTWQTVTIFVAGV   180
Murine  121   VLKALCTKVPELIRTIMGWTLDFLRERLLVWIQDQGGWEGLLSYFGTPTWQTVTIFVAGV   180

                  TM                
Human   181   LTASLTIWKKMG   192
Canine  181   LTASLTIWKKMG   192
Murine  181   LTASLTIWKKMG   192
```

(Supplemental Figure S1 ctd.)

Alignment for Bad

```
Human     1   ----------------------------------------MFQIPEFEPSEQEDSSSA   18
Canine    1   ----------------------------------------MFQIPEFEPSEQEDSSPA   18
Murine    1   MGTPKQPSLAPAHALGLRKSDPGIRSLGSDAGGRRWRPAAQSMFQIPEFEPSEQEDASAT   60

                                                                      PS
Human    19   ERGLGPSPAGDGPSGSGKHHRQAPGLLWDASHQQEQPTSSSHHGGAGAVEIRSRHSSYPA   78
Canine   19   NRGLGPSPTGDRPPSPGKHQQTAPGLLGEAGHQQGQPASRKHHGGAGA-ETRSRHSSFPA   77
Murine   61   DRGLGPSLTEDQP---GPY--LAPGLLGSNIHQQGRAATNSHHGGAGAMETRSRHSSYPA   115

                                  PS          BH3                 PS
Human    79   GTEDDEGMGEEP-SPFRGRSRSAPPNLWAAQRYGRELRRMSDEFVDSFKKGLPRPKSAGT   137
Canine   78   GTDEDEGMEEEELSPFRGRSSSAPPNLCAARRYGRELRRMSDEFQGSF-KGLPRPKSAGT   136
Murine   116  GTEEDEGMEEEL-SPFRGRSRSAPPNLWAAQRYGRELRRMSDEFEGSF-KGLPRPKSAGT   173
                                                        PS

Human    138  ATQMRQSSSWTRVFQSWWDRNLG--------  160
Canine   137  ATQMRQSPSWTRVIQSWWDRNLGRGGSAPSQ  167
Murine   174  ATQMRQSAGWTRIIQSWWDRNLGKGGSTPSQ  204
```

Alignment for Noxa (PMAIP1)

```
Human     1   ----------------------------------------------------MPGKKARKNAQ   11
Canine    1   ----------------------------------------------------MPGRKARKSAQ   11
Murine    1   MPGRKARRNAPVNPTRAELPPEFAAQLRKIGDKVYCTWSAPDITVVLAQMPGKSQKSRMR   60

                                    BH3              MTD
Human    12   -PSPARAPAELEVECATQLRRFGDKLNFRQKLLNLISKLFCSGT   54
Canine   12   -PGPTRAPEELEVECAIQLRKFGDKLNFRQKLLNLLSKLFRSGT   54
Murine   61   SPSPTRVPADLKDECA-QLRRIGDKVNLRQKLLNLISKLFNLVT   103
```

Legend:

Alignments are slightly modified from a CLUSTAL format alignment by MAFFT (v7.182)
Non-identical amino acids are labelled in bold red;
domains (labelled in gray): BH1, BH2, BH3, BH4 = Bcl-2 Homology Domain 1 to 4; TM = Transmembrane domain;
MTD = Mitochondrial Targeting Domain; PS = phosphorylation site (Serine or Threonine residues)

Figure legends

Fig. 1 Interspecies conservation of protease (caspase and calpain) recognition motifs in indicated Bcl-2 family members.

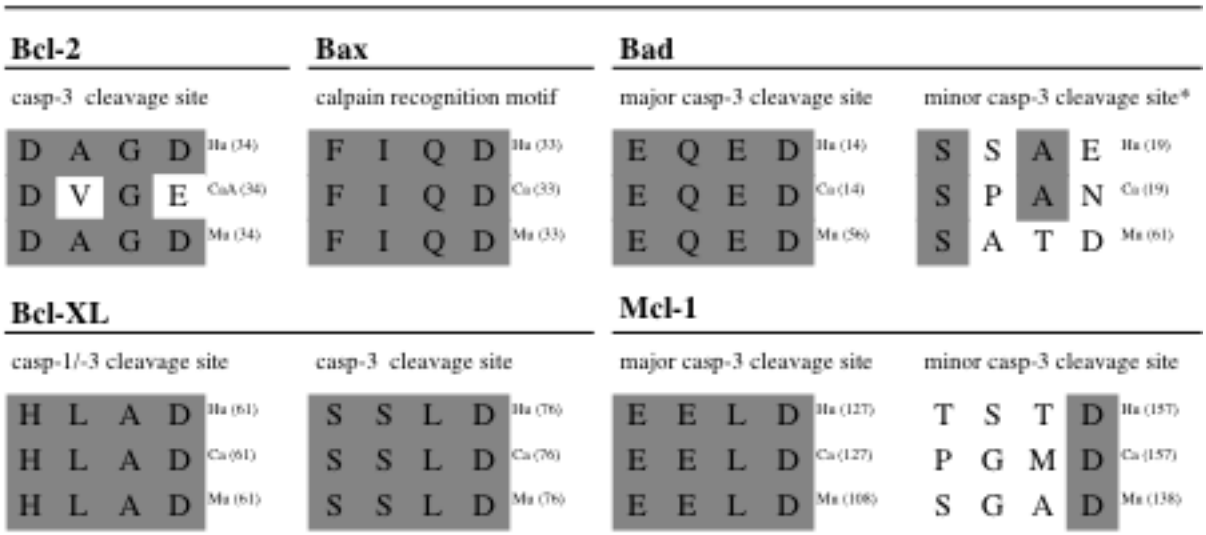
Fig. 2 Growth curves of yeast expressing Bcl-2 family proteins grown in minimal inducing medium. A: expression of proapoptotic proteins Bak and Bax impose marked growth retardation. B: co-expression of anti-apoptotic Bcl-2 family proteins abrogates the growth retardation effect of Bak and Bax. The curves are deduced from OD_{600} measurements at 6h intervals and are representative of two independent experiments.

Fig. 3 Clonogenic assay of yeast cultures induced to express different Bcl-2 family proteins by growth in liquid galactose media for 24h (a) and 48h (b), followed by plating on non-inducing, glucose containing, solid media, and growth for 72h at 30 °C. The columns indicate the mean value and standard error of the mean (SEM) of at least three independent experiments. Expression of Bak and Bax results in massively reduced clonogenicity. This effect is more pronounced after protein expression of 48h (b) compared to 24h (a) and is abrogated by co-expression of anti-apoptotic proteins. Values for Bax and Bak are significantly different from all other values (** $p<0.0001$, *$P=0.019$, student's t test).

Fig. 4 Western blots of yeasts transformed with plasmids containing the indicated canine Bcl-2 family cDNAs, cultured in liquid inducing medium for 24 h and labelled with the indicated antibodies. Relevant marker sizes are indicated. (a): lane 1, vector only; lane 2, Bcl-w; lane 3, Bcl-w+Bak; lane 4, Bcl-w+Bax; lane 5, Bcl-x+Bak; lane 6, Bcl-x+Bax, lane 7,

Mcl-1+Bak; lane 8, Mcl-1+Bax. (b): lane 1, vector only; lane 2, Bcl-w. (c): lane 1, vector only; lane 2, Bcl-x. (d): lane 1, vector only; lane 2, Mcl-1.

Figure 1



Bold: substrate; plain: specific tetramer recognition site; Hu: human; Ca: canine; Mu: murine, number in parentheses refer to position of the fourth amino acid, which directly precedes the caspase cleavage site; residues in grey are identical to human sequence; *minor caspase-3 recognition site described in murine Bad.

Figure 2a

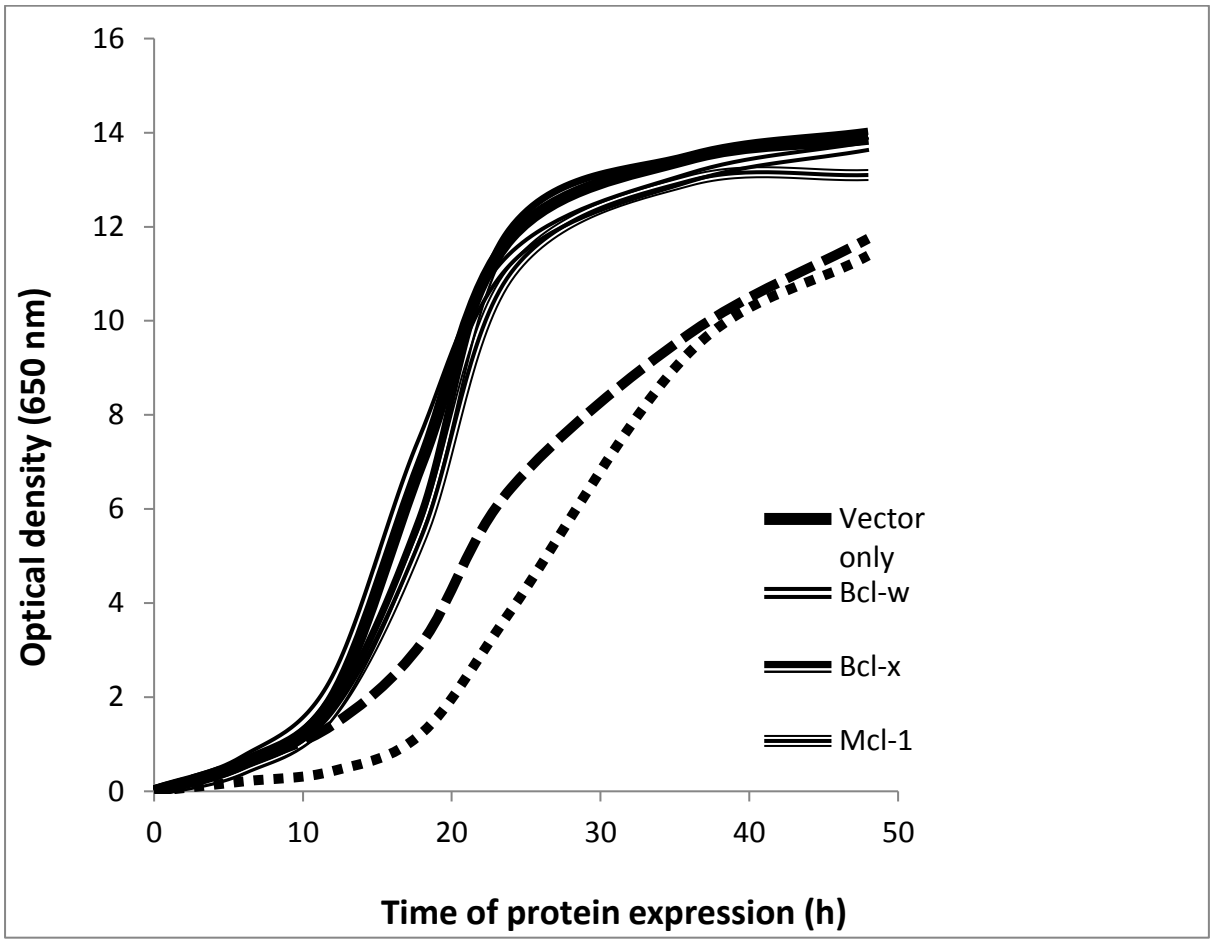

16
14
12
10
8
6
4
2
0
Optical density (650 nm)
0
10
20
30
40
50
Time of protein expression (h)
Vector only
Bcl-w
Bcl-x
Mcl-1

Figure 2b

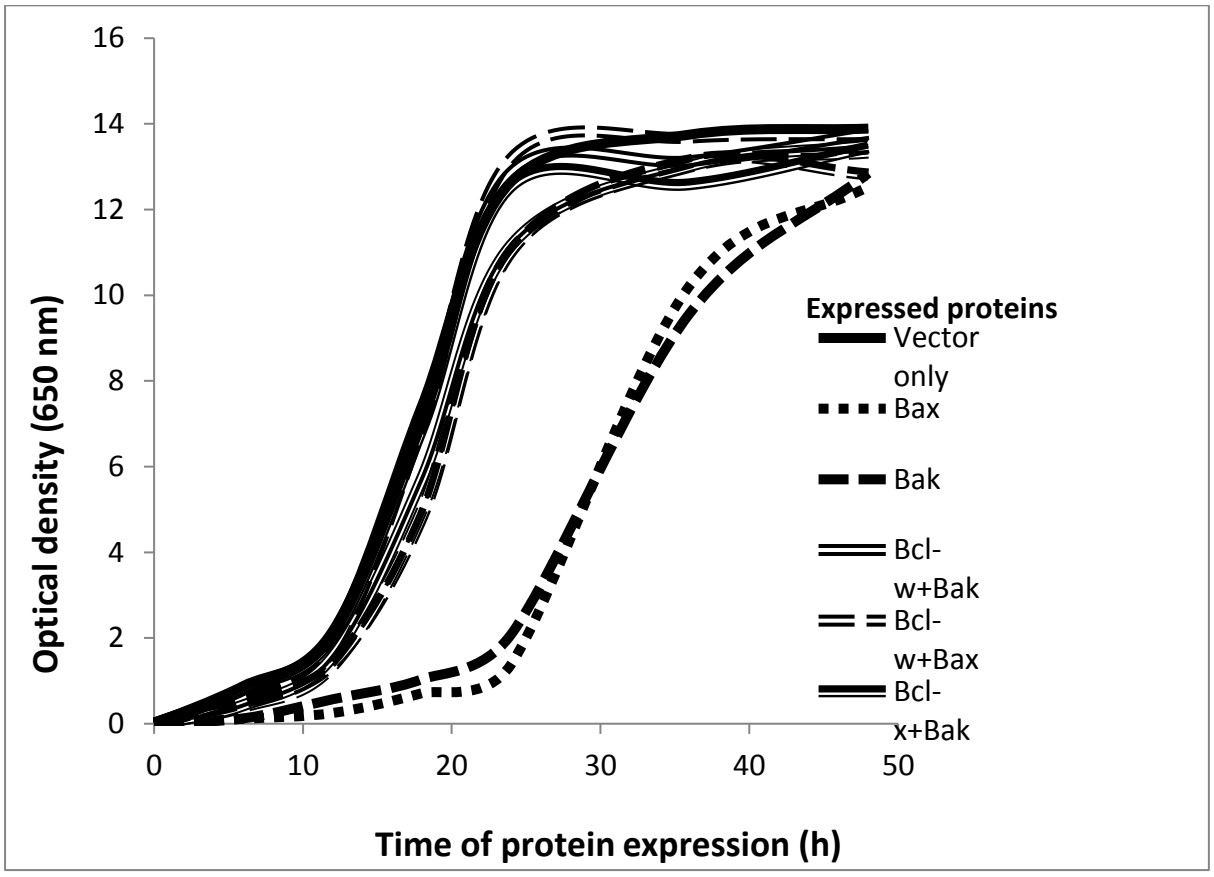

16
14
12
10
8
6
4
2
0
Optical density (650 nm)
0
10
20
30
40
50
Time of protein expression (h)
Expressed proteins
Vector only
Bax
Bak
Bcl-w+Bak
Bcl-w+Bax
Bcl-x+Bak

Figure 3a

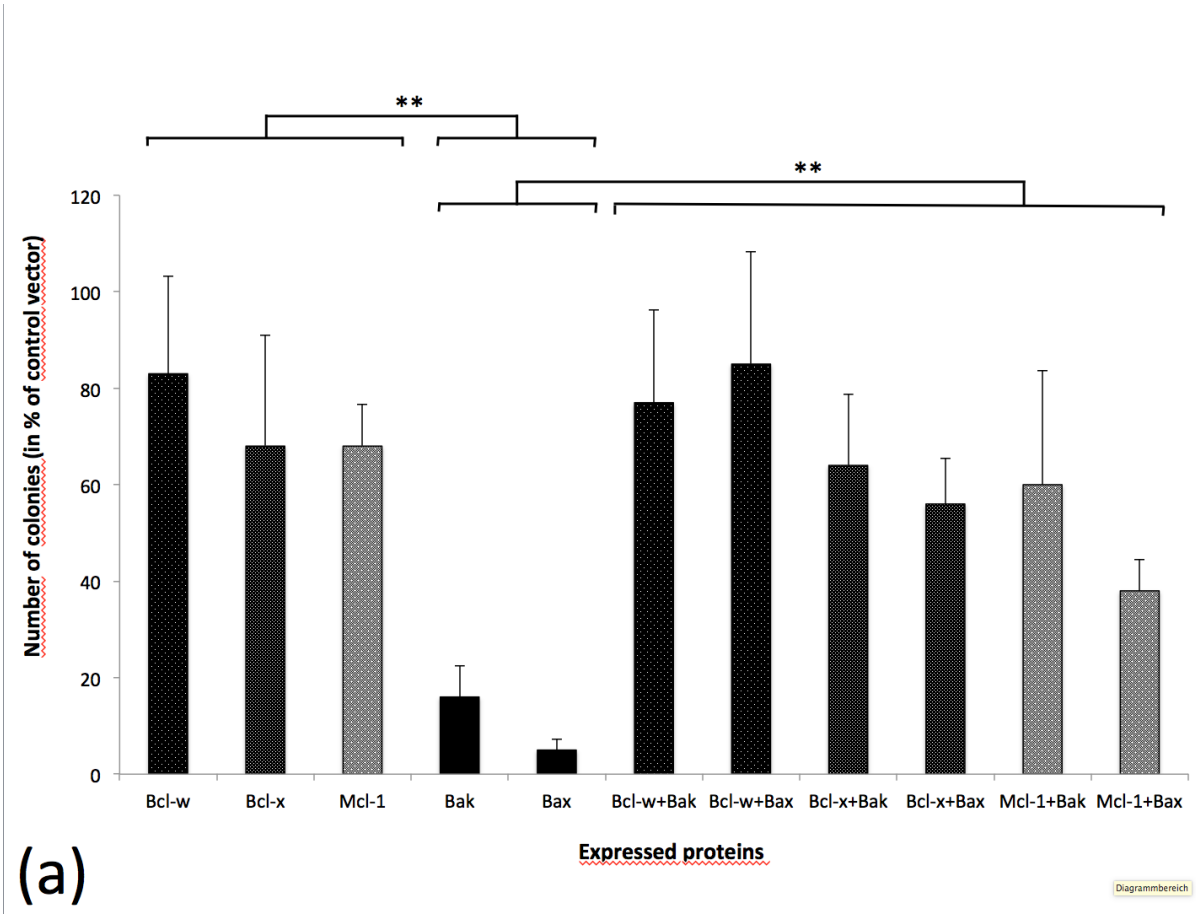
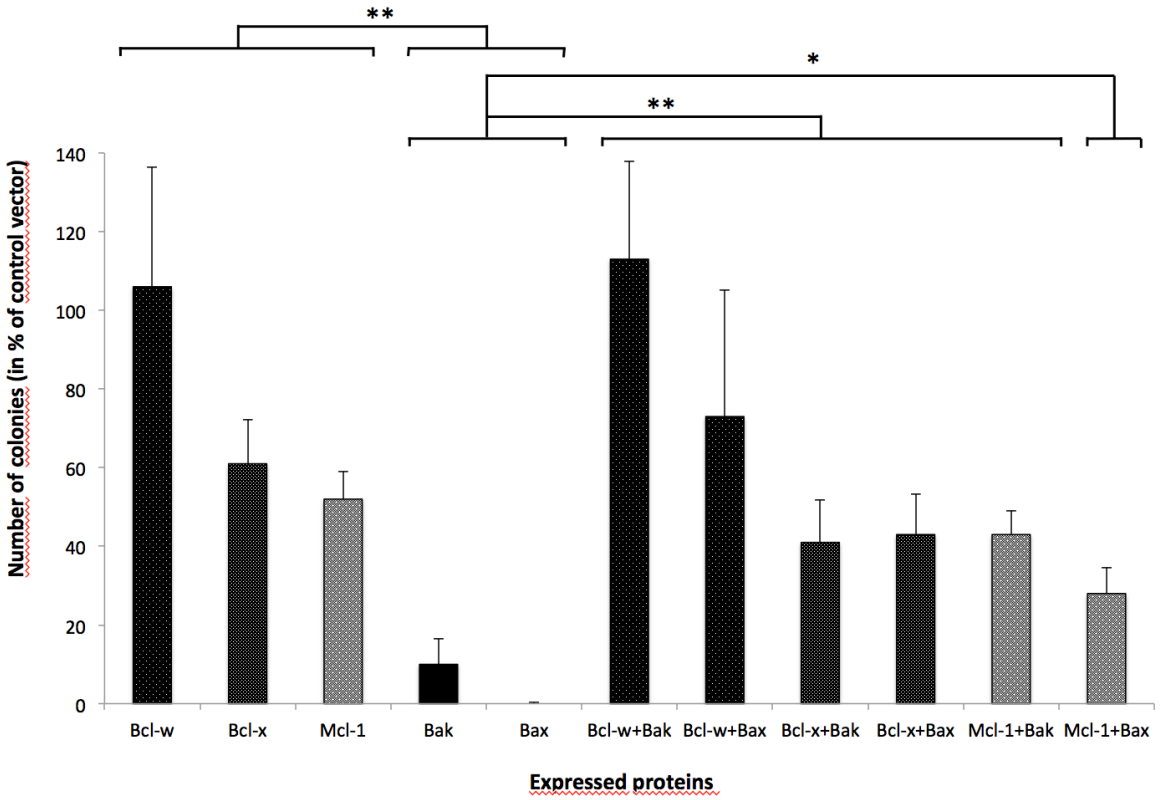

**
**
120
100
80
60
40
20
0
Number of colonies (in % of control vector)
Bcl-w
Bcl-x
Mcl-1
Bak
Bax
Bcl-w+Bak
Bcl-w+Bax
Bcl-x+Bak
Bcl-x+Bax
Mcl-1+Bak
Mcl-1+Bax
Expressed proteins
(a)
Diagrammbereich

Figure 3b


**
*
**
Number of colonies (in % of control vector)
140
120
100
80
60
40
20
0
Bcl-w
Bcl-x
Mcl-1
Bak
Bax
Bcl-w+Bak
Bcl-w+Bax
Bcl-x+Bak
Bcl-x+Bax
Mcl-1+Bak
Mcl-1+Bax
Expressed proteins

(b)

Figure 4

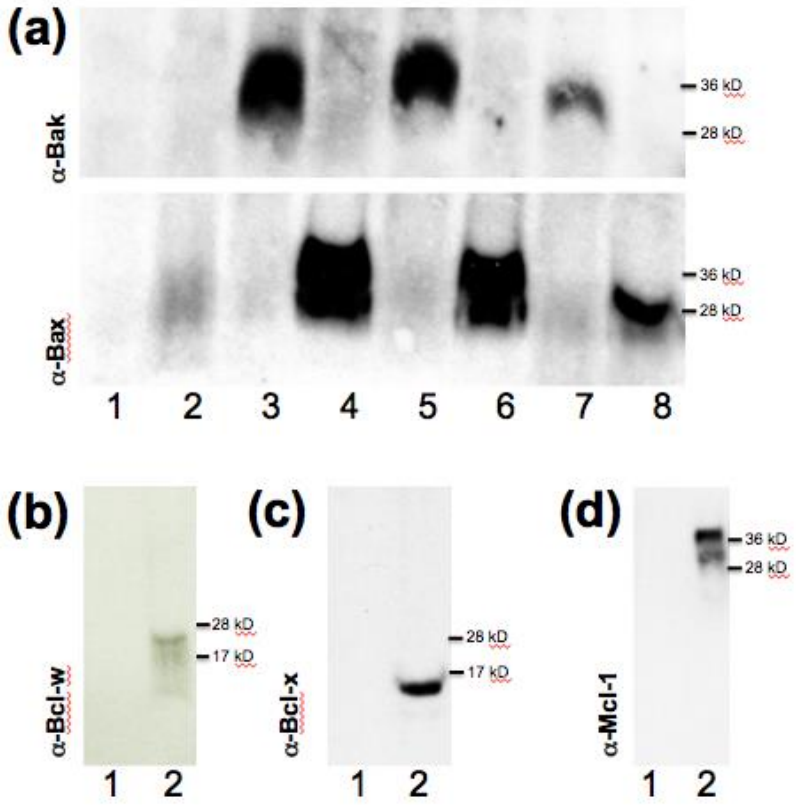

(a)
α-Bak
36 kD
28 kD
α-Bax
36 kD
28 kD
1 2 3 4 5 6 7 8
(b)
α-Bcl-w
28 kD
17 kD
1 2
(c)
α-Bcl-x
28 kD
17 kD
1 2
(d)
α-Mcl-1
36 kD
28 kD
1 2

Table 1. Primers for amplification of canine Bcl-2 family cDNAs

Molecule	Forward primer	Reverse primer
Bcl-x_L (BCL2L1)	5`-CCATCCCTATTATAAAA**ATG**TCTCA-3`	5`-GGGTAAGTGGGTGGTCAGTG-3` *
Bcl-w (BCL2L2)	5`-GG**ATG**GCGACCCCAGCC-3`	5`-GC**TCA**CTTGCTCGCAAAAAAG-3`
Mcl-1	5`-AGGAGCTTGCG**ATG**TTCGG-3`	5`-CACTTAAAAGG**CTA**TCTTATTAG-3`
Bak	5`-GAGAA**ATG**GCATCCGGGC-3`	5`-CCTCTGGGAG**TCA**TGATTTG-3`
Bax var. alpha	5`-TG**ATG**GACGGGTCCGGGG-3`	5`-GGCC**TCA**GCCCATCTTTTTC-3`
Bad	5`-AGAGC**ATG**TTCCAGATCCC-3`	5`-GGACGCGGGACGGG**TCA**C-3`
Noxa (PMAIP1)	5`-AG**ATG**CCCGGCCGGAAG-3`	5`-A**TCA**AGGTTCCTGAGCGGAAG-3`

Start- and stop-codons are indicated in bold

* Stop-codon is upstream of primer sequence

Table 2. Accession numbers of Bcl-2 family members used in this study

Molecule	Nucleotide and protein sequence accession number for indicated species		
	Human[1]	Canine[2]	Murine[1]
Bcl-2 isoform alpha	NM_000633.2, NP_000624.2	AB154172.1, BAD05044.1	NM_009741.4, NP_033871.2
Bcl-x_L(BCL2L1)	NM_138578.1, NP_612815.1	AB073983.1, BAB71819.1	NM_009743.5, NP_033873.3
Bcl-w (BCL2L2)	NM_004050.4, NP_004041.1	DQ116955.1[3], AAZ22484.1[3]	NM_007537.1, NP_031563.1
Mcl-1	NM_021960.4, NP_068779.1	AB093582.2, BAC21258.1	NM_008562.3, NP_032588.1
Bak	NM_001188.3, NP_001179.1	DQ002813.1[3], AAY19401.1[3]	NM_007523.2, NP_031549.2
Bax variant alpha	NM_138761.3, NP_620116.1	AB080230.1, BAC53619.1	NM_007527.3, NP_031553.1
Bad	NM_004322.3, NP_004313.1	DQ127247.1[3], AAZ32936.1[3]	NM_007522.3, NP_031548.1
Noxa (PMAIP1)	NM_021127.2, NP_066950.1	DQ449072.1[3], ABE02691.1[3]	NM_021451.2, NP_067426.1

[1]Reference Sequences available at NCBI

[2]GenBank accession numbers

[3]Own submission

Table 3. Interspecies comparison of Bcl-2 family members coding sequences

Molecule	No. of coding exons			Coding sequence identity	
	Hu	Ca	Mu	Hu / Ca	Hu / Mu
Bcl-2 isoform alpha	2	?	2	92%	89%
Bcl-x_L (BCL2L1)	2	2	2	97%	94%
Bcl-w (BCL2L2)	2	2	2	95%	94%
Mcl-1	3	3	3	89%	83%
Bak	5	5	5	90%	80%
Bax variant alpha	6	?	6	93%	90%
Bad	3	3	4	86%	65%
Noxa	2	2	3	87%	40%

Hu: human; Ca: canine; Mu: murine

?: alignment to genome sequence incomplete

Table 4.

Table 4. Interspecies comparison of Bcl-2 family protein sequences

Molecule	Protein length[1]			Whole protein homology[2]		Domain[3]	Domain homology[2] Hu/Ca		Domain homology[2] Hu/Mu	
	Hu	Ca	Mu	Hu / Ca (Identities; similarities)	Hu / Mu (Identities; similarities)		Identities	Similarities[4]	Identities	Similarities[4]
Bcl-2 isoform alpha	239	239	236	219/246 (89%); 222/246 (90%)	217/239 (91%); 219/239 (92%)	BH 1	20/20 (100%)	-	20/20 /100%)	-
						BH 2	16/16 (100%)	-	16/16 (100%)	-
						BH 3	15/15 (100%)	-	14/15 (93%)	15/15 (100%)
						BH 4	21/21 (100%)	-	21/21 (100%)	-
						TM	21/22 (95%)	-	22/22 (100%)	-
						PS	1/1 (100%)	-	1/1 (100%)	-
Bcl-XL (BCL2L1)	233	233	233	233/233 (100%)	228/233 (98%); 231/233 (99%)	BH 1	20/20 (100%)	-	20/20 (100%)	-
						BH 2	16/16 (100%)	-	15/16 (94%)	16/16 (100%)
						BH 3	15/15 (100%)	-	15/15 (100%)	-
						BH 4	21/21 (100%)	-	21/21 (100%)	-
						TM	17/17 (100%)	-	17/17 (100%)	-
Bcl-w (BCL2L2)	193	193	193	193/193 (100%)	191/193 (99%); 192/193 (99%)	BH 1	20/20 (100%)	-	20/20 (100%)	-
						BH 2	16/16 (100%)	-	16/16 (100%)	-
						BH 3	15/15 (100%)	-	15/15 (100%)	-
						BH 4	21/21 (100%)	-	21/21 (100%)	-
						TM	21/21 (100%)		21/21 (100%)	-
Mcl-1	350	350	331	312/350 (89%); 326/350 (93%)	267/350 (76%); 290/350 (83%)	BH 1	21/21 (100%)	-	20/21 (95%)	-
						BH 2	16/16 (100%)	-	16/16 (100%)	-
						BH 3	15/15 (100%)	-	15/15 (100%)	-
						TM	21/21 (100%)	-	21/21 (100%)	-
						PS	2/2 (100%)	-	2/2 (100%)	-
Bak	211	211	209	194/211 (92%); 201/211 (95%)	162/211 (77%); 182/211 (86%)	BH 1	20/20 (100%)	-	18/20 (90%)	20/20 (100%)
						BH 2	16/16 (100%)	-	14/16 (87%)	14/16 (87%)
						BH 3	14/15 (93%)	15/15 (100%)	14/15 (93%)	15/15 (100%)
						TM	16/18 (89%)	17/18 (94%)	14/18 (78%)	16/18 (89%)
Bax var. alpha	192	192	192	187/192 (97%); 189/192 (98%)	177/192 (92%); 181/192 (94%)	BH 1	20/21 (95%)	21/21 (100%)	20/21 (95%)	21/21 (100%)
						BH 2	16/16 (100%)	-	14/16 (87%)	15/16 (94%)
						BH 3	15/15 (100%)	-	14/15 (93%)	15/15 (100%)
						TM	21/21 (100%)	-	21/21 (100%)	-
Bad	160	167	204	128/169 (76%); 134/169 (79%)	122/210 (58%); 133/210 (63%)	BH 3	13/15 (87%)	-	13/15 (87%)	-
						PS	4/4 (100%)	-	4/4 (100%)	-
Noxa (PMAIP1)	54	54	103	45/54 (83%); 49/54 (91%)	35/104 (34%); 40/104 (38%)	BH 3	13/15 (87%)	14/15 (93%)	11/15 (73%)	12/15 (80%)
						MTD	9/10 (90%)	10/10 (100%)	10/10 (100%)	-

[1] The number of amino acids is indicated; Hu: human; Ca: canine; Mu: murine

[2] Identities refer to number/percentage of identical amino acids; Similarities further include amino acids with similar properties

[3] BH: Bcl-2 homology domain; TM: Transmembrane domain; PS: phosphorylation site (Serine or Threonine residues); MTD: Mitochondrial targeting domain

[4] -: Same as identities