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Functional characterization of a BCL10 isoform in the rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

The complexes formed by BCL10, MALT1 and members of the family of CARMA proteins have recently been the focus of much attention because they represent a key mechanism for regulating activation of the transcription factor NF-κB. Here, we report the functional characterization of a novel isoform of BCL10 in the trout *Oncorhynchus mykiss*, which we named tBCL10. tBCL10 dimerizes, binds to components of the CBM complex and forms cytoplasmic filaments. Functionally, tBCL10 activates NF-κB transcription factor and is inhibited by the deubiquitinating enzyme A20. Finally, depletion experiments indicate that tBCL10 can functionally replace the human protein. This work demonstrates the evolutionary conservation of the mechanism of NF-κB activation through the CBM complex, and indicates that the rainbow trout *O. mykiss* can serve as a model organism to study this pathway.

1. Introduction

The NF-κB family of transcription factors is a group of evolutionarily conserved proteins that are important regulators of the immune system function, controlling the expression of numerous proteins involved in innate and adaptive immunity [1,2]. NF-κB also transcribes genes that exert a positive effect on cell survival and proliferation, and disregulation of the mechanisms controlling its activation often results in immunoproliferative, inflammatory and autoimmune phenotypes [1,2].

The human Caspase recruiting domain (CARD)-containing protein BCL10 is a 233 amino acids protein originally identified as a target of translocation in a subset of mucosa-associated lymphoid tissue (MALT) lymphoma cells [3–5]. As a consequence of a translocation, BCL10 is overexpressed, and that results in a constitutive NF-|B activation which is eventually responsible for the neoplastic transformation [3–5]. Gene targeting of the BCL10 locus in murine strains results in immunodeficiency, having $BCL10^{-/-}$ mice severe defects in humoral and cellular immune responses and antigen-induced proliferation, due to impaired NF-|B activation following stimulation in both T and B cells [6]. Thus, BCL10 is indispensable for NF-|B activation following antigen receptor stimulation on B and T lymphocytes [6].

The biological function of BCL10 is explicated through participation at the CBM complex, a molecular complex that includes one of three members of the family of CARMA proteins and MALT1 [7]. The three CARMA proteins, CARMA1, 2 and 3, constitute a family of proteins conserved across many species and are characterized by the presence of different functional domains shared by all members of the family [8]. Functionally, all three CARMA proteins are able to associate BCL10 through an homophilic interaction between the corresponding CARD domains, and to cooperate with it in inducing the transcriptional activity of NF-κB [8].

Compared to mammalian NF-κB, very little is known about piscine regulators of this transcription factor. Recently, extensive analysis of fish genomes have reported the presence of several CARD domain containing proteins encoded by the genome of fish such as zebrafish and the rainbow trout *Oncorhynchus mykiss* [9–12]. In particular, because of multiple whole-genome duplications occurred in salmonid species [13], for the rainbow trout genome have been annotated four different genes encoding for putative proteins that share aminoacidic similarity with human BCL10. However, it is not established whether any of these genes is actually expressed, and no functional data is available regarding any of these proteins. In this work, we report on the functional

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characterization of a rainbow trout *O. mykiss* BCL10 ortholog, herein defined tBCL10.

2. Materials and methods

2.1. Ethics

All the procedures involving animals were conducted as indicated in the Italian National Guidelines (D.L. No. 100/2006, and D.L. No. 116/1992) and in the appropriate European Directives (EEC Council Directive 86/609, 1.12.1987), adhering to the Guide for the Care and Use of Laboratory Animals (United States National Research Council, 1996). All the in vivo experimental activities were approved by the Animal Ethics Committee (CESA) of Biogem (Italy).

2.2. RNA extraction and cloning of tBCL10 full-length cDNA

Total RNA was extracted from trout peripheral blood leukocytes by using Trizol reagent, and 1 μg of total RNA was reverse-transcribed to generate a first-strand cDNA. Primers used to amplify tBCL10 were the following: forward 5′-ATGGACTCCTGG TGTATCACTGAC-3′ and reverse 5′-TCAGACTCTTAAGGTCCCGG GCTC-3′. PCR conditions were as follows: 98 °C for 30 s, 30 cycles

Table 1
Loci encoding for proteins similar to human BCL10 in the rainbow trout genome.

Name	Accession number	Length	Predicted MW	Similarity to human BCL10
BCL10a isoform 1	CAF31504	203	22,567	40%
BCL10a isoform 3	CDQ87110	199	22,126	38%
BCL10b isoform 1	CDQ56929	270	29,611	46%
BCL10b isoform 2	CDQ91425	262	28,612	44%

BCL10-like proteins encoded by the genome of *Oncorhynchus mykiss* and their similarity to the human protein.

Table 2
Loci encoding for proteins similar to human BCL10 in the rainbow trout genome.

Species	Protein	Length	Identities	Positives
Oncorhynchus mykiss	BCL10a isoform 2 (tBCL10)	207	83/207 (40%)	169/207 (79%)
Homo sapiens	BCL10	233		
Oncorhynchus mykiss	tBCL10 CARD (6–116)	111	59/111 (53%)	97/111 (79%)
Homo sapiens	BCL10 CARD (8–115)	108		

Amino acidic similarity between hBCL10 and tBCL10 in the entire protein and in the CARD domains.

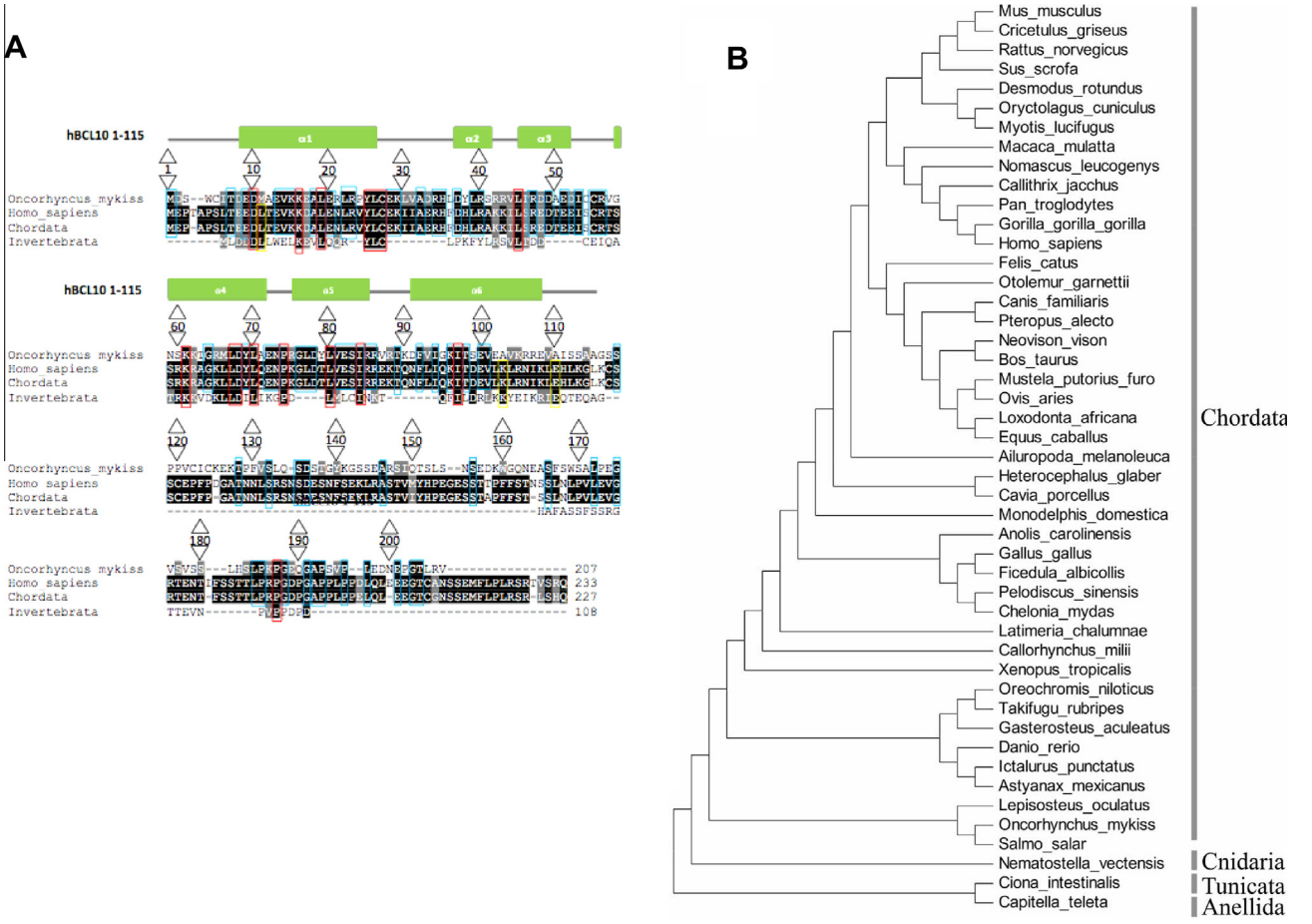

Fig. 1. *Alignment and phylogenetic tree of tBCL10.* (A) Alignment of tBCL10 sequence with the human BCL10 sequence and the consensus sequences generated by aligning the BCL10 sequences of Chordata and the CARD domains of three Invertebrata proteins. At the top of the alignment the six alpha helix regions of the CARD are shown. Amino acid numbering refers to the tBCL10 sequence. The alignment was using ClustalW and the printout from multiple-aligned sequences was done with BOXSHADE. The black background designates identical amino acids, the gray background conservative substitutions. Colored rectangles indicate amino acids conserved among the sequences examined. The sequences used for generation of the consensus are available in Supplementary Material. (B) Phylogenetic tree analysis of BCL10 proteins. The phylogenetic tree was constructed based on the full-length amino acid sequences using the neighbor-joining method within the Mega program. The sequences used for alignment and generation of the consensus are available in Supplementary Material. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(98 °C/5 s; 63 °C/22 s; 72 °C/30 s), and then 72 °C for 5 min. The RT-PCR product of the expected size was gel purified, cloned into HA- and FLAG-tagged expression vectors using standard methodologies and confirmed by sequencing. The sequence was deposited in GenBank with the accession number GenBank: KP055818.

2.3. Sequence analysis and phylogenic analysis of tBCL10

The tBCL10 protein sequences were analyzed by using the BLAST algorithm at the NCBI web site (http://www.ncbi.nlm.nih.gov/blast), and the multiple sequence alignment was created with ClustalW program (http://www.ebi.ac.uk/clustalw/). Phylogenetic

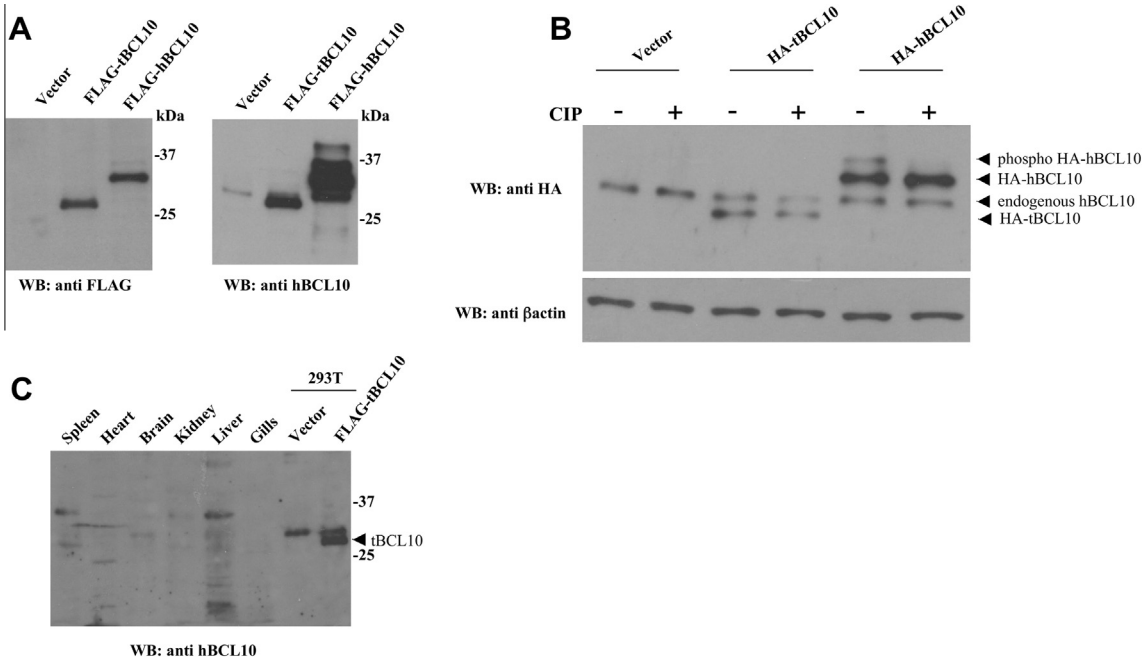

Fig. 2. *tBCL10 expression.* (A–B) Immunoblot analysis of lysates from HEK293 cells transfected with the indicated expression vectors. Were indicated, prior to SDS–PAGE separation cell lysates were treated with 10 units of calf intestinal phosphatase (CIP) for 30 min at 37 °C. (C) Immunoblot analysis of proteic extracts from rainbow trout organs probed with anti-hBCL10. Lysates from HEK293T cells transfected with tBCL10 were used as a positive control (arrow).

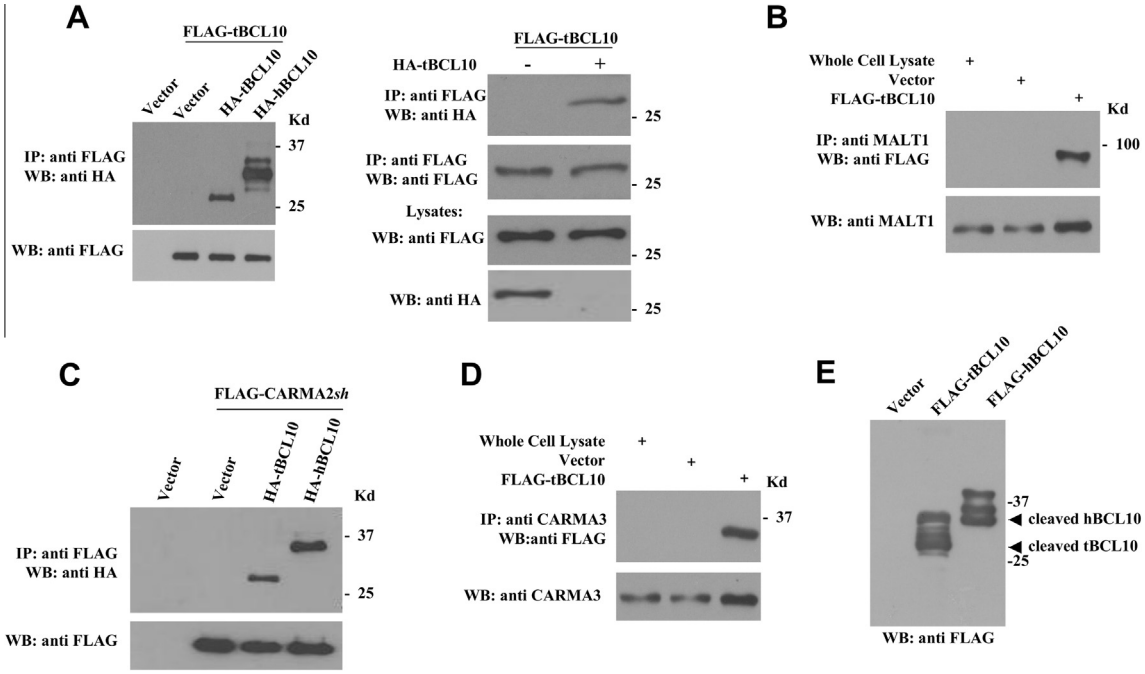

Fig. 3. *tBCL10 dimerizes and binds to CBM proteins.* (A) HEK293 cells were transiently cotransfected with FLAG-tagged or HA-tagged versions of tBCL10 and hBCL10. 24 h later, cell lysates were immunoprecipitated with anti-FLAG mAb. Immunocomplexes were separated by SDS–PAGE and transferred onto membranes subsequently probed with anti-HA antisera. The right panel shows controls for immunoprecipitation specificity. (B) Lysates from HEK293 cells transfected with tBCL10 were analyzed for coprecipitating MALT1; (C) CARMA2*sh* and (D) CARMA3. (E) Over-exposure of immunoblot experiments described in (A) shows proteolytic processing of tBCL10.

analysis was conducted with MEGA version, using distance methods and the neighbor-joining algorithm [14].

2.4. Immunoblot analysis and coprecipitation

Cell lysates were made in lysis buffer (150 mM NaCl, 20 mM Hepes, pH 7.4, 1% Triton X-100, 10% glycerol, and a mixture of protease inhibitors). Proteins were separated by SDS–PAGE, transferred onto nitrocellulose membrane, and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ). Blots were developed using the ECL system (Amersham Biosciences). For co-immunoprecipitation experiments, cells were lysed in lysis buffer and immunocomplexes were bound to protein A/G, resolved by SDS–PAGE, and analyzed by immunoblot assay. Sources of antisera and monoclonal antibodies were the following: anti-FLAG, anti-β-Actin, Sigma; anti-HA, anti-MALT1, anti-CARMA3 and anti-BCL10 (H-197 SC5611, generated against an epitope corresponding to amino acids 1–197 of human BCL10), Santa Cruz Biotechnology. The calf-intestinal alkaline phosphatase was purchased from Roche.

2.5. Cell culture and transfection

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

The expression vectors used in transfection experiments for this study have been previously described [15–18].

DNA plasmids were transfected by standard calcium-phosphate method. Short hairpin RNAs targeting hBCL10 were the following: shBCL10 #3 5′-CCTTAAGATCACGTACTGTTTCTCGAGAAACAGTACG TGATCTTAAGG-3′ and shBCL10 #5 5′-GTTGAATCTATTCGGCGAGAA CTCGAGTTCTCGCCGAATAGATTCAAC-3′. Retroviral infections were carried out as previously described [19].

2.6. Rainbow trout tissues immunoblot analysis

6–9 months trouts were euthanized and dissected. Proteic extracts from selected organs were prepared using Nonidet P-40 lysis buffer (1% (v/v) Nonidet P-40, 150 mm NaCl, 50 mm Hepes, pH 7.4, 5 mm EDTA, 10% (v/v) glycerol, and complete protease inhibitor mixture (Roche). After homogenization and centrifugation (13,000 × *g*, 15 min, 4 °C), protein concentration of supernatant was determined by BCA protein assay (Pierce). A 15 μg sample of whole cell extract was separated on SDS–polyacrylamide gel and transferred to membranes. Filters were blocked for 2 h in 3% nonfat dry milk in phosphate-buffered saline (PBS) with 0.3% Tween 20. Western blot analysis was performed using a rabbit anti-hBCL10 antisera, followed by horseradish-peroxidase-conjugated mouse anti-rabbit antibody (Amersham Biosciences). Signal was developed using an enhanced chemiluminescence method (Amersham Biosciences) according to the manufacturer's instructions.

2.7. Luciferase assay

To assess for NF-|B activation, HEK293 were transfected with plasmidic DNAs together with pNF-κB-luc (Clontech) in 6-well plates. After transfection and treatments, luciferase activity was determined with Luciferase Assay System (Promega). A plasmids expressing β-galactosidase was added to the transfection mixture in order to normalize for the efficiency of transfection.

2.8. Immunofluorescence

1×10^4 HEK293 were grown and transfected in chamber slides. Sixteen hours after transfection, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then permeabilized in PBS/0.1% Triton X-100. Cells were incubated for 30 min in 5% FCS–PBS with anti-FLAG antibody (Sigma–Aldrich) followed by several washes with 5% FCS–PBS, and then incubating for 30 min with secondary antibody in 5% FCS–PBS. All steps were done at room temperature.

3. Results and discussion

Because of the multiple genome duplication events occurred in salmonids [13], the recent sequencing of the genome of the rainbow trout *O. mykiss* has revealed the presence of multiple putative isoforms of BCL10 in the genome of this specie [12]. For instance, in the rainbow trout genome have been annotated four loci encoding for proteins similar to human BCL10 (hBCL10) (Table 1). However, when we tried to amplify the cDNAs encoding for these BCL10 isoforms by RT-PCR from total *O. mykiss* mRNA, only one of them was successfully amplified. Sequence analysis showed that the amplified cDNA encodes for a further isoform of BCL10, probably generated by an alternative splicing of the same transcript encoding for BCL10a isoform 1. That sequence was therefore named BCL10a isoform 2 (GenBank: KP055818), and hereafter abbreviated as tBCL10.

The tBCL10 cDNA encodes for a protein of 207 amino acids, with a predicted molecular mass of 23 kDa (Table 2 and Fig. 1A). The overall amino acidic identity of tBCL10 to hBCL10 is 40% (Table 2). The major amino acidic differences between the two

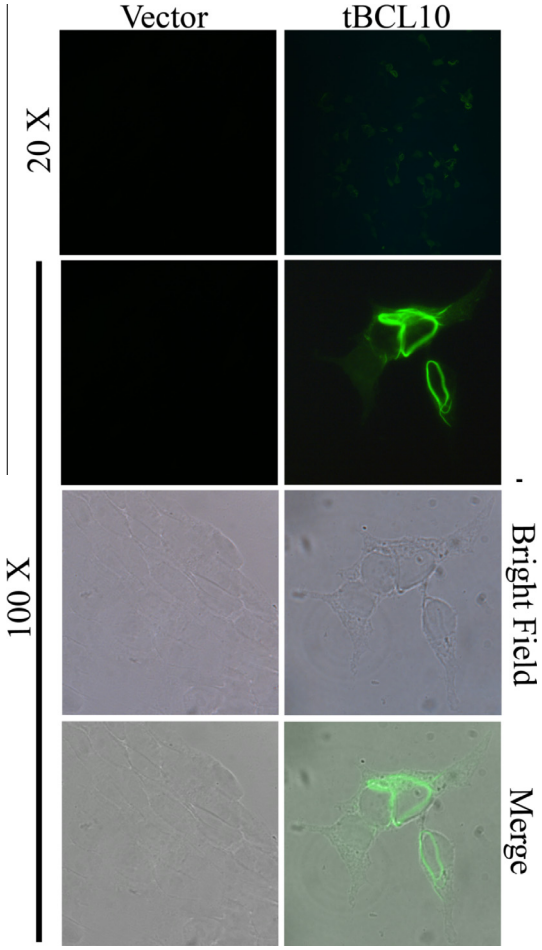

Fig. 4. *Subcellular localization of tBCL10.* HEK-293 cells were transfected with mammalian FLAG-tagged vector, empty (vector) or expressing tBCL10. 16 h after transfection, cells were stained with anti-FLAG mAb, followed by FITC-conjugated anti-mouse IgG.

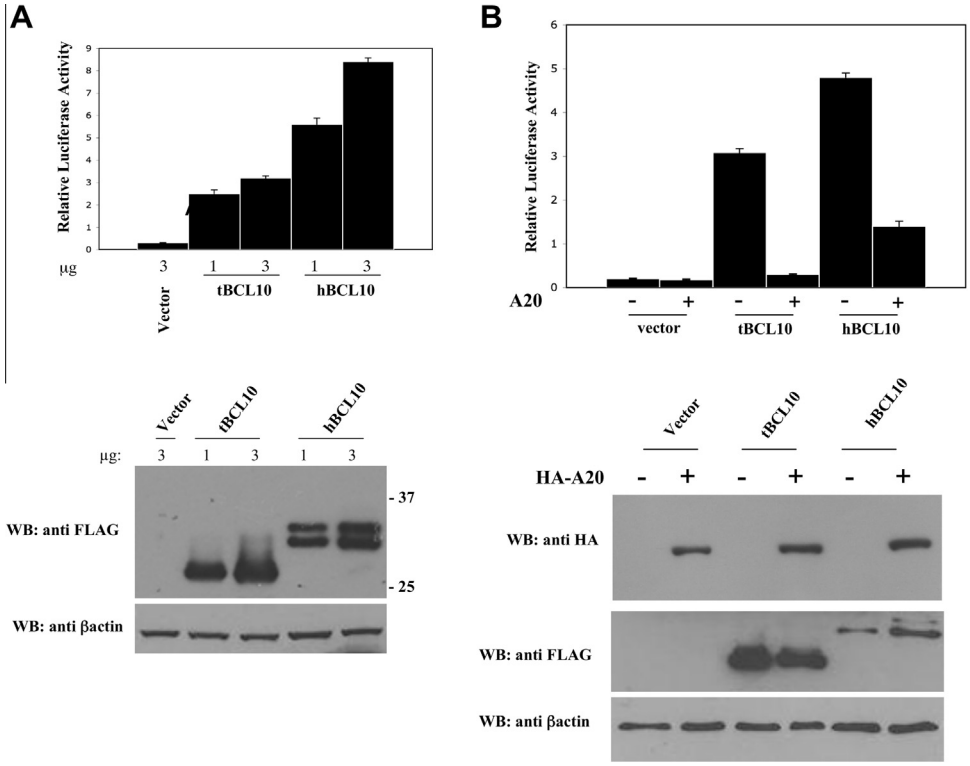

Fig. 5. (A–B) *tBCL10 activates NF-|B* HEK293 cells were transiently cotransfected with expression vectors encoding for the indicated polypeptides, together with pNF-|B-luc and pRSV-βgal reporter vectors. The total amount of transfected plasmidic DNA was maintained constant by adding empty vector. 16 h after transfection, cell lysates were prepared and luciferase activity was measured. A fraction of the cell lysates were analyzed by immunoblot to monitor protein expression, shown in the lower panels. Data shown represents relative luciferase activity normalized on β-galactosidase activity and is representative of six independent experiments done in triplicate.

proteins are located at the carboxy-terminal of the polypeptides, whereas the amino-terminal CARD domains of tBCL10 (amino acids 6–116) and hBCL10 (amino acids 8–115) share 59% identity (Table 2). Sequence analysis shows that several residues that have been demonstrated to be necessary for the biological activity of hBCL10, namely R36, D39, L41, R42, E53 and G78 [4,15,20–23], are conserved in tBCL10. On the other hand, the residue S141, which is implicated in attenuation of hBCL10 signaling [24], is not conserved in tBCL10 (Fig. 1A). A phylogenetic tree was constructed by the neighbor-joining method using MEGA [14], and it shows that the sequences of rainbow trout BCL10a and BCL10b isoforms clusterize within the fish BCL10 sequences (Fig. 1B).

When analyzed in immunoblot assay, tBCL10 expressed in mammalian cells migrates as a 28 kDa protein (Fig. 2A). Interestingly, a rabbit antisera raised against hBCL10 also recognizes tBCL10 (Fig. 2A, right panel). In these expression experiments, we noticed that while hBCL10 migrates as a doublet on SDS–PAGE due to phosphorylation of the protein [25,26], tBCL10 occurs as a single band, suggesting that tBCL10 is not target of phosphorylation events. Indeed, experiments in which lysates were treated with phosphatase prior to immunoblot analysis confirm this possibility (Fig. 2B). Finally, an immunoblot assay carried out on proteic lysates extracted from various rainbow trout organs and tissues indicates that a band corresponding to the molecular weight of tBCL10 is expressed in spleen and, less intensely, in kidney (Fig. 2C). Additional bands with higher molecular weight are detectable, possibly corresponding to other BCL10 isoforms encoded by the genome of *O. mykiss*.

In mammals, BCL10 plays a crucial role in the signal transduction pathway that leads to activation of the transcription factor NF-κB [6,7]. hBCL10-mediated activation of NF-κB requires oligomerization of hBCL10, assembly of the CBM complex and triggering of unconventional ubiquitination events [7,27], which eventually result in the recruitment of the IKK complex [28]. Indeed, transfection experiments indicate that tBCL10 is able to dimerize both with itself and with hBCL10 (Fig. 3A). Furthermore, tBCL10 associates with human MALT1 (Fig. 3B), with human CARMA2*sh* [16] (Fig. 3C) and human CARMA3 (Fig. 3D). Finally, as activation of the CBM complex includes induction of the proteolytic activity of MALT1 which processes hBCL10 after R228 [29], we monitored the possible processing of tBCL10 when expressed in HEK293 cells. As shown in Fig. 3E, overexposure of the immunoblot of tBCL10 expressed in the HEK293 clearly shows the occurrence of a proteolytic processing of tBCL10.

Fluorescence microscopy experiments and structural studies have shown that the NF-κB-activity produced by hBCL10 is regulated through formation of cytosolic filamentous structures [21,23]. We therefore verified whether also tBCL10 is able to form such structures. As shown in Fig. 4, assembly of filamentous structures is readily visible following expression of tBCL10 in mammalian cells.

Next, we tested whether tBCL10 is able to activate NF-κB in mammalian cells using a luciferase-based reporter assay. The results of these experiments, shown in Fig. 5A, indicate that tBCL10 is effective in activating NF-κB, even though less efficiently than hBCL10. As for hBCL10 [30,31], tBCL10-induced NF-κB activation requires ubiquitination(s) events, since NF-κB activation is completely abrogated following coexpression of A20 de-ubiquitinase (Fig. 5B).

To exclude the possibility that NF-κB activation mediated by tBCL10 was due to its interaction and subsequent oligomerization of hBCL10, we abolished expression of hBCL10 in the human cell line HEK293 through retrovirus-mediated expression of short hairpin RNAs (shRNA) targeting hBCL10. As shown in Fig. 6A,

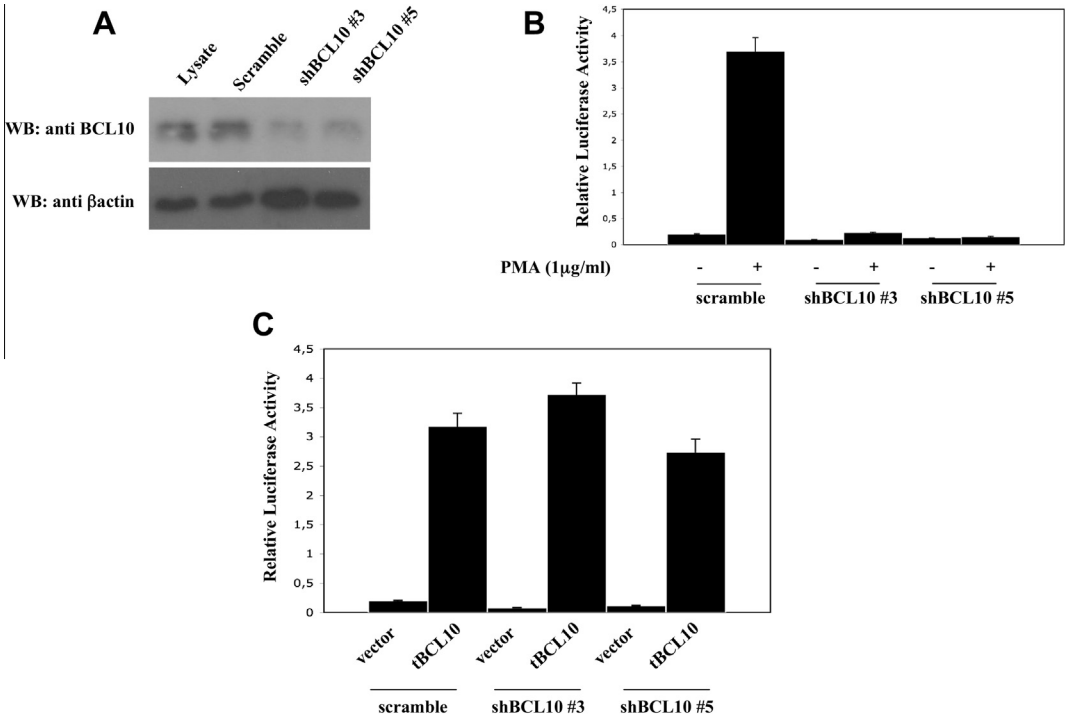

Fig. 6. *tBCL10 replaces hBCL10* (A) Cell lysates from HEK293 cells infected with retroviruses encoding for shRNAs targeting hBCL10 were monitored for hBCL10 expression by immunoblot assay. (B) NF-κB-driven luciferase activity in HEK-293 cells silenced for hBCL10 and stimulated with PMA. (C) NF-κB-driven luciferase activity in HEK-293 cells silenced for hBCL10 and transfected with tBCL10. Data shown represent relative luciferase activity normalized on β-galactosidase activity and is representative of six independent experiments done in triplicate.

introduction of hBCL10sh#3 and hBCL10sh#5 in HEK293 cells results in a great reduction of BCL10 expression. Depletion of hBCL10 in these cells abrogates their ability to activate NF-κB following exposure to phorbol-12-myristate-13-acetate (PMA) (Fig. 6B). However, introduction of tBCL10 in these hBCL10-depleted cells fully recovers their ability to activate NF-κB (Fig. 6C). Thus, tBCL10 can functionally replace hBCL10.

In the present work here presented we show that the mechanism of NF-κB activation through BCL10 and the CBM complex in conserved the rainbow trout *O. mykiss*. Given the importance of this transcription factor in both normal cell biology and autoimmune, immunoproliferative and tumoral disorders, the possibility of using additional model organism such as the rainbow trout certainly represents a field to explore further, also considering the economic value of this organism. However, an additional source of complication in the study of signal transduction pathways in the rainbow trout results from various genomic duplication events that have occurred in the evolutionary history of salmonids [12,13]. As a result, in the trout genome many mammalian genes are present in multiple copies, and that also happens for the genes encoding for the proteins of the CBM complex. On the other hand, the absence of selective pressure often leads to the functional loss of supernumerary gene copies, mostly through pseudogenization [12]. Thus, although the rainbow trout genome contains four possible homologs of hBCL10, only one of them was successfully isolated from mRNA. Obviously, this negative result does not imply that other copies of rainbow trout BCL10 can be functional. Finally, it would be certainly interesting to investigate how this phenomenon of genomic duplication has affected genes encoding for ancillary proteins that modulate the activity of the CBM complex in mammals, such as USP9X [32], CKIP-1 [33], Net1 [34], p62 [35,36], DEPDC7 [37] and various protein kinases and phosphatases [38–42], and if these proteins retain a similar function in rainbow trout.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.01.007.

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