Alternative splicing of medaka bcl6aa and its repression by Prdm1a and Prdm1b

Xiaomei Ke
Central China Normal University
Runshuai Zhang
Central China Normal University
Qiting Yao
Central China Normal University
Shi Duan
Central China Normal University
Wentao Hong
Central China Normal University
Mengxi Cao
Jianghan University
Qingchun Zhou
Central China Normal University
Xueping Zhong
Central China Normal University
Haobin Zhao (zhaohb@mail.ccnu.edu.cn)
Central China Normal University School of Life Sciences https://orcid.org/0000-0003-4672-7654

Research Article

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Abstract

Bcl6 and Prdm1 (Blimp1) are a pair of transcriptional factors that repressing each other in the mammals. Prdm1 represses the expression of *bcl6* by binding a cis-element of *bcl6* gene in mammals. The homologs of Bcl6 and Prdm1 have been identified in teleost fish. However, whether these two factors regulate each other by the same way in fish as that in the mammals is not clear. In this study, the regulation of *bcl6aa* by Prdm1 was investigated in medaka. The mRNA of *bcl6aa* has three variants (*bcl6aaX1-X3*) at the 5'-end by alternative splicing with different promoters detected by RT-PCR. The three variants can be detected in adult tissues and developing embryos of medaka. The predicted proteins of Bcl6aaX1-X3 may have modification such as acetylation, C-mannosylation, phosphorylation, and sumoylation in the N-terminuses with different half-lives and relative translation efficiencies. *Prdm1a* and *prdm1b* are expressed in the tissues and embryos where and when *bcl6aa* is expressed. The expression of *prdm1a* was high while the expression of *bcl6aa* was low, and vice versa, detected in the spleen after stimulation with LPS or polyI:C. In vitro reporter assay indicated that *bcl6aa* could be directly repressed by both Prdm1a and Prdm1b in a dosage-dependent manner. After mutation of the key base, G, of all predicted binding sites in the core promoter region of *bcl6aa*, the repression by Prdm1a and/or Prdm1b disappeared. The consensus binding site of Prdm1 in *bcl6aa* gene is GAAAA(T/G). These results indicate that both Prdm1a and Prdm1b directly repress the expression of *bcl6aa* by binding the consensus binding site where the 5'-G is critical in medaka fish.

Introduction

Bcl6 (B-Cell Lymphoma 6) is a member of the POK (POZ and Krüppel) /ZBTB (zing finger and BTB) protein family (Lee and Maeda 2012). Prdm1 (positive regulatory domain I-binding factor or PR domain-containing protein 1) also called Blimp1 (B lymphocyte-induced maturation protein 1) is belonging to PRDM family (John and Garrett-Sinha 2009). Bcl6 and Prdm1 are two transcription factors functioning in diverse tissues such as the immune system and bone, etc. in the mammals. The effect of Bcl6 is opposite to that of Prdm1. For example, Bcl6 inhibits but Prdm1 promotes osteoclastogenesis in mice (Miyauchi et al. 2010). In the immune system, Prdm1 is highly expressed in the Th2 (T helper 2) cells and is required for normal Th2 humoral responses in vivo by repression of Bcl6 and Tbx21 (T-box transcription factor 21) which are necessary for Th1 cells (Cimmino et al. 2008). Bcl6 promotes differentiation of CD4 (cluster of differentiation 4) + T follicular helper (Tfh) cells and B cells in mice. Contrarily, Prdm1 inhibits Tfh differentiation and B cell maturation (Johnston et al. 2009). STAT3 (Signal transducer and activator of transcription 3) can upregulate PRDM1 coordinately with down-regulation of BCL6 to control human plasma cell differentiation (Diehl et al. 2008). Prdm1 and Bcl6 repress one another in CD4 T cells. Bcl6 directly inhibits *prdm1* expression or binds to Bach2 (BTB domain and CNC homolog 2) to repress *prdm1* and represses plasmocytic differentiation (Ochiai et al. 2008; Tunyaplin et al. 2004). Conversely, Prdm1 directly represses *bcl6* by binding to the *bcl6* gene in both CD4 T cells and B cells (Cimmino et al. 2008).

Hobit (Homolog of Blimp1 in T cells) or ZNF (Zinc finger protein) 683 is the homolog of Prdm1 in mammals. Hobit was initially identified in natural killer T (NKT) cells of mouse (van Gisbergen et al.

2012). Hobit functions in repression of IFN (Interferon)-γ expression and induces granzyme B expression in mice (van Gisbergen et al. 2012). Human HOBIT was identified in NK cells and effector-type CD8 + T cells (Vieira Braga et al. 2015). Hobit recognizes similar regulatory sequences of the target genes of Prdm1 in mouse lymphocyte (Mackay et al. 2016). Hobit cooperates with Prdm1 in differentiation and maintenance of CD4 + or CD8 + tissue-resident memory T (Trm) cells (Behr et al. 2019; Kragten et al. 2018; Mackay et al. 2016; Zundler et al. 2019).

In teleost fish, the homologs of Prdm1 are Prdm1a, Prdm1b, and Prdm1c. Prdm1a and Prdm1b are closely related to mammalian Prdm1 and Hobit respectively. Prdm1c is evolved from duplication of Prdm1a in fish (Perdiguero et al. 2020). Prdm1a has been reported in fugu (Ohtani and Miyadai 2011; Ohtani et al. 2006a), zebrafish (Ingham and Kim 2005; Page et al. 2013; Roy and Ng. 2004; Wilm and Solnica-Krezel 2005), rainbow trout (Diaz-Rosales et al. 2009; Perdiguero et al. 2020; Zwollo 2011), medaka (Zhao et al. 2014), Nile tilapia (Wu et al. 2019), and Japanese flounder (Liu et al. 2016). Prdm1a plays important roles in embryonic development such as fin, muscle, and cloaca, etc. of zebrafish (Ingham and Kim 2005; Mercader et al. 2006; Pyati et al. 2006; Roy and Ng 2004; Wilm and Solnica-Krezel 2005). Prdm1a was detected in IgM + CD8α- cells in fugu kidney (Odaka et al. 2011). Prdm1a is expressed in the plasma CD45 + B cells with expression of IgM in zebrafish (Page et al. 2013). Previously, we reported the expression of *prdm1a* (ENSORLG00000015684, JX402912) and *prdm1c* (ENSORLG00000012948, JX402913, NP_001265739) in medaka (Zhao et al. 2014). *Prdm1a* could be upregulated in the liver of medaka and zebrafish by lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (polyI:C), and grass carp reovirus (GCRV) (Zhao et al. 2014). Prdm1 was also detected in the IgM + B cells of the head kidney of tilapia and was stimulated with LPS in vitro (Wu et al. 2019). Rainbow trout *prdm1a* could be upregulated by IL-2 (Diaz-Rosales et al. 2009). Rainbow trout *prdm1a*, *prdm1b*, and *prdm1c* transcripts were identified in the B and T cells and were upregulated in the head kidney and spleen after infection of Viral hemorrhagic septicemia virus (VHSV) (Perdiguero et al. 2020).

The homologs of Bcl6 have been reported in several fish including fugu (Odaka et al. 2011; Ohtani et al. 2006b), zebrafish (Lee et al. 2013), medaka (Zhang et al. 2019b), grass carp (Zhu et al. 2019), and Senegalese sole (*Solea senegalensis*) (Ponce et al. 2020). Zebrafish Bcl6a is required for optic cup formation (Lee et al. 2013) and is a key factor for cold response (Hu et al. 2015). Fugu Bcl6 was identified in the immune organs or tissues (Ohtani et al. 2006b), and in the leukocyte cells expressing secretory-type IgM and Prdm1 (Odaka et al. 2011). Transcription of bcl6 could be promoted by T-cell factor (TCF) 7 in response to GCRV challenge in grass carp (Zhu et al. 2019). Senegalese sole *bcl6* could be induced by the sulfated polysaccharide ulvan from a green seaweed (*Ulva ohnoi*) (Ponce et al., 2020). Previously, we reported that two homologs of *bcl6*, *bcl6aa* and *bcl6ab*, were detected in the immune organs such as the liver, kidney, and spleen, and could be induced by polyI:C and LPS in medaka (Zhang et al. 2019b).

The reports mentioned above show the conserved function of Bcl6 and Prdm1 in the immune response in fish. Fugu Bcl6aa and Prdm1a were reported as the transcriptional repressors in vitro (Ohtani and Miyadai 2011). A mutation of possible Bcl6 binding site in the 5'-regulation region of *prdm1* gene of Japanese flounder increased the reporter activity in vitro (Li et al. 2017). The possible binding sites were

also found in rainbow trout *prdm1* genes (Perdiguero et al. 2020). However, whether Prdm1 and Bcl6 repress each other in the same way as that in mammals is not reported. Although the expression of *prdm1a*, *prdm1c*, and *bcl6aa* had been reported previously (Zhang et al. 2019b; Zhao et al. 2014), the expression of *prdm1b* and the alternative splicing variants of *bcl6aa* are not reported yet in medaka. In this paper, we report the alternative splicing variants of *bcl6aa* and the expression of *prdm1b* in medaka. Moreover, the repression of medaka *bcl6aa* by Prdm1a and Prdm1b was studied in vitro. The results showed a direct repression of *bcl6aa* by medaka Prdm1a and Prdm1b binding the conserved cis-elements.

Materials And Methods

Animals

Wild strain medaka was used as experimental fish. The fish were maintained under artificial photoperiod of 14 h light and 10 h dark, at an ambient temperature of 28.0°C. Spontaneously spawned eggs were collected and incubated at an ambient temperature of 28.0°C.

Adult fish were randomly divided into three groups and were injected intraperitoneally with 10 μl of phosphate buffer solution (PBS), LPS (Sero-type: O55:B5, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), polyI:C (Sigma-Aldrich), respectively (Zhao et al. 2014; Zhang et al. 2019). LPS and polyI:C were dissolved in PBS in a concentration of 5 μg/μl respectively. Five fish of each group were randomly sampled at 1–10 days post injection (dpi) for measurement of gene expression.

The animal protocol for this study was approved by the Animal Care and Use Committee of Hubei Province in China [No.
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Extraction of total RNA

The cDNA was synthesized according to the protocol of FastQuant RT kit (Tiangen Biotech, Beijing, China).
Adult tissues were isolated from medaka fish that were killed by decapitation after anesthesia with MS-222 (Sigma-Aldrich). Total RNA from adult tissues and embryos were extracted by Ultrapure RNA kit

(CoWin Biosciences, Beijing, China) as the protocol provided by the manufacturer. The cDNA was synthesized according to the protocol of FastQuant RT kit (Tiangen Biotech, Beijing, China).

Detection of *bcl6aa*, *prdm1a*, and *prdm1b* in adult tissues and embryos by RT-PCR

There are two transcripts *bcl6aa-201* (ENSORLT00000030671.1) and *bcl6aa-202* (ENSORLT00000019521.2) of medaka *bcl6aa* (ENSORLG00000015589) predicted in the Ensembl (http://www.ensembl.org/Oryzias_latipes/). To confirm the prediction, we detected these two transcripts by RT-PCR using the primers designed on the genomic sequence of medaka *bcl6aa* (Fig. 1A, Table 1). The PCR results were purified and were ligated into pMD18-T vector (Takara Bio, Dalian, China). Positive colonies of *E coli*. transformed with the ligated vectors were sent for sequencing. Sequencing results were assembled respectively for each colony and were aligned together. Then, the variants were identified and were blasted against the genome of medaka on web (http://www.ensembl.org).

The cycling program was 95 ℃ 2 min followed by 39 cycles of 95 ℃ 10 s, 62 ℃ 30 s and 65 ℃ 30 s. Relative expression of the genes in the samples was calibrated/normalized against RPS18 by using 2−ΔΔCt method (The quantity in the samples of fish received phosphate buffer solution (PBS) referred as 1) (Livak and Schmittgen 2001).
PCR reaction was performed in a volume of 25 μl, containing 12.5 μl of 2× Es Taq master mix (CoWin), 1 μl of primers (10 μmol/L), 0.1 μl of cDNA solution, and 10.4 μl of double distilled water (ddH_2O). The cycling program was 95 ℃ 3 min; 30 cycles of 95 ℃ 30 s, 62 ℃ 30 s, 72 ℃ 25 s; and 72 ℃ 5 min. Quantitative RT-PCR (qRT-PCR) of triplicate samples was performed with CFX96 real-time PCR detection system (BioRad Laboratories, Hercules, California, USA) in a volume of 20 μl containing template cDNA, primers and 2× SuperReal Pre Mix Plus kit (Tiangen). The cycling program was 95 ℃ 2 min followed by 39 cycles of 95 ℃ 10 s, 62 ℃ 30 s and 65 ℃ 30 s. Relative expression of the genes in the samples was calibrated/normalized against *RPS18* by using $2^{-\Delta\Delta Ct}$ method (The quantity in the samples of fish received phosphate buffer solution (PBS) referred as 1) (Livak and Schmittgen 2001). The primers used were shown in Table 1. *Beta-actin* and/or the ribosomal protein *RPS18* were used as internal control (Zhao et al. 2014).

Bioinformatic assay

The variants of *bcl6aa* were translated into protein isoforms, Bcl6aaX1-X3. The isoforms were analyzed by SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) to find the signal peptide. The possible sites of acetylation, C-mannosylation, phosphorylation, and sumoylation of the isoforms were predicted by the Servers of NetAcet 1.0 (http://www.cbs.dtu.dk/services/NetAcet/), NetCGlyc 1.0 (http://www.cbs.dtu.dk/services/NetCGlyc/), NetPhorest (http://www.netphorest.info/), and SUMOplot analysis program (https://www.abcepta.com/sumoplot). The N-terminus, protein half-life, and relative translation efficiency of Bcl6aaX1-X3 were predicted by TermiNator (https://bioweb.i2bc.paris-saclay.fr/terminator3/).

Reporter assay

A sequence of 4700 bp in length at the upstream of the start codon in the exon IV of medaka *bcl6aa* gene was isolated by PCR with a pair of primers, bcl6aa-p4700-F and bcl6aa-p4700-R (Table 1). This sequence in 4700 bp (named promoter 2) was truncated into 112 to 3814 bp in length by PCR with the forward primers (not shown) and bcl6aa-p4700-R. The sequences of 112 to 4700 bp in size were named as P112 ~ P4700 (Fig. 1). These sequences were subcloned into the vector pGL3-Basic (Promega, Madison, WI, USA) between the cloning sites *Kpn* I and *Nhe* I at the upstream of the luciferase reporter gene to make a series of reporter plasmids. A sequence of 312 bp in length at the upstream of the start codon of the exon I from − 613 to -302 (named promoter 1) was synthesized and was subcloned into pGL3-Basic as P312 (Fig. 2).

The expression vectors harboring *prdm1a* and *prdm1b* were constructed by insertion of the open reading frames (ORFs) of *prdm1a* and/or *prdm1b* isolated by RT-PCR with the primers (Table 1) into the vector pCS2 + between *Hind* III and *EcoR* I separately.

The potential binding sites of the transcription factors in the *bcl6aa* promoters were predicted by scanning in the JASPAR database (http://jaspar.genereg.net/). The 5'-G of the possible binding sites was mutated into T by synthesis.

Reporter assay was performed as the guide of the kit, Dual-Luciferase Reporter Assay System (Promega). The 293T cells incubated in high-glucose Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum were transfected with Lipofectamine 3000 (Thermo Fisher Scientific) with 5 ng pRL-TK, 100 ng pGL3-Basic, and 100 ng pCS2+-Prdm1a or pCS2+-Prdm1b vectors. Firefly luciferase values were normalized to Renilla luciferase control values when harvested 48 h post transfection. The assay was performed in three independent transfections. The plasmids were prepared by OMEGA Endo-free Plasmid Kit (Omega Bio-tek, Norcross, GA, USA).

Statistical analysis

Statistical analysis was performed with SPSS Statistics (IBM, Armonk, NY, USA). The values of gene expression were calculated and described as mean ± standard errors with at least three independent experiments ($n \geq 3$). The differences among the treatments were calculated by one-way analysis of variance (ANOVA).

Results

The variants of *bcl6aa* in medaka

RT-PCR results showed one band of *bcl6aa-201* in adult tissue of medaka. Sequencing result was the same as the predicted one and was named as *bcl6aaX1*. *Bcl6aaX1* was highly expressed in the brain, eye, and gill, was moderately expressed in the liver, intestine, and ovary, and was expressed faintly in the heart, kidney, spleen, testes, and muscle of adult fish (Fig. 1).

RT-PCR results of *bcl6aa-202* showed three bands of 292, 377, and 462 bp in size (Fig. 1). The sequence of 292 bp was the predicted *bcl6aa-202* and was named as *bcl6aaX2*. The sequence of 377 bp was a new splicing variant, which was the predicted *bcl6aa-202* enclosing the intron between the exon III and IV and was named as bcl6aaX3. The sequence of 462 bp was found as the genomic sequence of *bcl6aa*.

The expression of *bcl6aaX2* and *bcl6aaX3* was detected in all checked tissues (Fig. 1). High expression of both *bcl6aaX2* and *bcl6aaX3* was detected in the liver, spleen, intestine, and kidney. Moderate expression of *bcl6aaX2* was detected in the eye, gill, muscle, testes, and ovary. Low expression of *bcl6aaX2* was detected in the brain and heart. Moderate expression of *bcl6aaX3* was detected in the brain, eye, gill, heart, muscle, testes, and ovary.

The expression of *bcl6aaX1*, *X2*, and *X3* was detected in medaka embryos. *Bcl6aaX1* and *bcl6aaX3* were detected from the 4-cell stage throughout embryonic development. However, *bcl6aaX2* was detected later, from 4 days post fertilization (dpf) onwards. These results indicate that *bcl6aaX1* and *X3* can be deposited into the zygote maternally but *bcl6aaX2* is zygotically expressed. The expression of *bcl6aaX1*, *X2*, and *X3* reached high level from 6 dpf and was maintained until hatching.

The differences of the *bcl6aa* variants were analyzed by bioinformatics. The translated isoforms, Bcl6aaX1-X3 are different in the N-terminals. Bcl6aaX1, X2, and X3 are 743, 720, and 698 amino acids (AA) in length respectively. Bcl6aaX1 or X2 is 45 or 22 AA longer than Bcl6aaX3 in the N-terminus.

A signal peptide of 22 AA of Bcl6aaX1 was predicted by SignalP. Bcl6aaX1 and X2 may have more modification such as C-mannosylation, phosphorylation, and sumoylation in the N-terminals than that in X3 (Fig. 1). These modifications may change the activities, half-lives of the isoforms (Table 2). Bcl6aaX3 has the longest half-life. The translation efficiency of Bcl6aaX2 or Bcl6aaX3 is 5 times of Bcl6aaX1's (Table 2). Comparing the structures, the N-terminal of Bcl6aaX1 has two more α-helixes and two more loops, the N-terminal of Bcl6aaX2 has one more α-helix, one more β-sheet, and one more loop compared with Bcl6aaX1. These structures may not change the main structure of Bcl6aa which is like that of mammalian Bcl6 by homology modeling (data not shown).

Expression of *prdm1a*, *prdm1b* and *bcl6aa*

At first, the expression of *prdm1a* or *prdm1b* (ENSORLG00000010025, XM_004074327) was checked (Fig. 1). In adult, *prdm1a* was detected at high level in the gill, kidney, and ovary, at moderate level in the intestine, spleen, and liver, and at low level in the brain, eye, heat, muscle, and testes. *Prdm1b* was detected at high level in the eye, gill, liver, and intestine, at moderate level in the kidney, heart, spleen, and brain, and at low level in the muscle, ovary and testes. In the embryos, both *prdm1a* and *prdm1b* were detected from the 4-cell stage until hatching. These results showed the existence of *prdm1a* and *prdm1b* with *bcl6aa* in the same tissues and the same embryonic stages.

The expression of *prdm1a* and *bcl6aa* in the spleen was checked after immune stimulation with LPS or polyI:C (Fig. 1). QRT-PCR results indicate that *prdm1a* or *bcl6aa* expression fluctuated after immune

stimulation. However, the opposite expression of *prdm1a* and *bcl6aa* was observed. While the expression of *prdm1a* was high, the level of *bcl6aa* was low; vice versa.

Repression of *bcl6aa* promoter by Prdm1

Firstly, a sequence of 4700 bp at the upstream of the start codon of *bcl6aaX3* (Promoter 2) was subcloned into pGLBasic vector as a reporter, and a series of reporters were made same way (Fig. 2A). Reporter assay showed the promoter 2 was active (Fig. 2B). The promoter activity changed with the sequences in length. A short sequence of 112 bp (P112) or the sequence of 4700 bp (P4700) could drive luciferase expression at high level. A very low expression of the reporter was driven by a sequence of 1067 bp (P1067) due to some cis- or trans-elements existed. When co-transferring expression vector of *prdm1a* or *prdm1b* with the reporters into the cells, the expression of luciferase was repressed in almost all reporters except of the P532 (Fig. 2C).

The P4700 and P112 were used for further experiments. The *prdm1a* or *prdm1b* vector was co-transferred with P4700 or P112 reporter in different amount (Fig. 2D, E). An amount of *prdm1a* or *prdm1b* vector in 150 ng could repress both P4700 and P112 reporter, and an amount of *prdm1a* or *prdm1b* vector in 300 ng could repress further.

The core binding sites of Prdm1 in *bcl6aa* promoters

To know the core binding site of Prdm1 in the sequence of *bcl6aa* gene, P112 was selected. By searching on Jaspar, two binding sites were identified as S2 and S3 containing a core sequence of GAAAA at forward and reverse strand (Fig. 3A). This core sequence had been proved as binding motif of Prdm1 in *bcl6* (Cimmino et al. 2008) and *NLRP12* (Lord et al. 2009) of mice. Single or double mutation of these two sites had not any significant change of the repression of Prdm1a or Prdm1b on the reporter (Fig. 3B). Then, we checked the sequence and found a similar site, S1, composed of GAAAT in the reverse strand. The reporter with single mutant of S1 still was repressed by Prdm1a or Prdm1b. However, the reporter with triple mutant of S1, S2, and S3 was not repressed anymore by both Prdm1a and Prdm1b (Fig. 3B).

We wondered the repression of Prdm1 on *bcl6aaX1*. A sequence of 312 bp at upstream of the start codon (P312) was selected for analysis (Fig. 3C). This fragment of *bcl6aa* gene could drive expression of the reporter. The reporter driven by P312 could be significantly repressed by both Prdm1a and Prdm1b. Triple mutation of S4 (GAAAT), S5 (GAAAA), and S6 (GAAAG), or single mutation of S7 (GAAAA) had not any effect on the repression of Prdm1. Mutation of the 5'-G of all four binding sites resulted in no repression by both Prdm1a and Prdm1b (Fig. 3D). The consensus of the binding motif is GAAAA(G/T) (Fig. 3E).

Discussion

In this study, three variants of *bcl6aa* were identified in medaka. The expression pattern of *bcl6aa* was in opposite with that of *prdm1a* in medaka spleen after immune stimulation. Medaka *bcl6aa* existed in the

tissues or embryos which expressing *prdm1a* or *prdm1b*. Moreover, the direct repression of *bcl6aa* by both Prdm1a and Prdm1b was confirmed.

Alternative splicing of *bcl6aa*

Alternative splicing of a gene is common in the organism (Black 2003). By alternative splicing, one gene can produce multiple mRNA which can be translated into different protein isoforms. Gene expression can be regulated at transcriptional level by using different promoters or alternative splicing and at translational processes and post-translation by different modifications. An isoform of human BCL6, BCL6S lacking the first two ZFs still represses the target genes of BCL6 (Shen et al. 2008). The alternative splicing of *bcl6b* was also identified in medaka (Zhang et al. 2019a). The three variants of medaka *bcl6aa* is produced by using different promoters and alternative splicing at the 5-prime. *Bcl6aaX1* is produced by using the promoter 1. *Bcl6aaX2* and *bcl6aaX3* are the products of the promoter 2 by alternative splicing.

The expression pattern of *bcl6aaX1-X3* is different in adult tissues and the embryos of medaka. *Bcl6aaX1* was mainly detected in the brain, eye, and gill. *Bcl6aaX2* and *bcl6aaX3* were mainly detected in the kidney, liver, spleen, and intestine. The expression of *bcl6aaX2* was lower than that of *bcl6aaX3*. In the embryos, *bcl6aaX1* and *bcl6aaX3* are maternal factors which can be detected at the early stages such as 4-cell stage of the embryos, but *bcl6aaX2* is zygotically expressed later. These expression differences suggested a regulation of *bcl6aa* at the transcriptional level in different tissues and embryos. However, the main structure of Bcl6aa is not changed. The different isoforms may still repress their target genes as reported in human BCL6 and BCL6s (Shen et al. 2008).

Medaka Bcl6aaX1 possesses a signal peptide at its N-terminal. The signal peptide is an important component for protein secretion (Dev and Ray 1990). It was wondered that whether Bcl6aaX1 might be secreted. The mouse mammary tumor virus Rem protein may give an example. The signal peptide of the mouse mammary tumor virus Rem protein is accumulated in nucleoli after release from the endoplasmic reticulum membrane (Dultz et al. 2008). This means the signal peptide is a part for protein translocation and is not only for secretion. As a homolog of BCL6, Bcl6aa must be translocated into the cell nucleus. All isoforms of Bcl6aa possess the nuclear location signal (NLS) as expected (data not shown).

Comparing with Bcl6aaX3, Bcl6aaX1 and X2 have more modification at their N-terminal, such as phosphorylation, C-mannosylation, and sumoylation. C-mannosylation is a modification for efficient protein secretion, supporting folding, and enhancing stability of thrombospondin repeats (Shcherbakova et al. 2019). The sumoylation sites are the possible sites for attachment of SUMO-1 (small ubiquitin-related modifier), a member of the ubiquitin and ubiquitin-like superfamily. SUMO modification changes the fate of the protein and regulates protein localization and activity (Gill 2004). Phosphorylation is a common modification in the proteins. Phosphorylation can regulate protein folding, protein-protein interaction, localization, stability, and activity (Liu et al. 2020; Narayanan and Jacobson 2009; Nishi et al. 2011). The half-lives of the isoforms might be due to these modifications. These modifications might be

used for regulation of the three isoforms of Bcl6aa to change their location, protein interaction, and activities, etc. in medaka cells. The actual modifications and the consequences need be studied further.

Repression of *bcl6aa* by Prdm1

Prdm1a was detected in all checked adult tissues and embryos. This result is like previous report (Zhao et al. 2014). *Prdm1b* as a closely relative of *Hobit* (Perdiguero et al. 2020) was also detected in all tissues and all stages of the embryos. The expression of *Prdm1* homologs in the immune organs of medaka is like the finding in rainbow trout (Perdiguero et al. 2020). *Bcl6aa* was also detected in the immune tissues and embryos of medaka as reported previously (Zhang et al. 2019b). *Prdm1a* and *bcl6aa* could be induced by immune stimulants as previous reports (Zhang et al. 2019b; Zhao et al. 2014). These results suggest a possible relation of Prdm1 and Bcl6aa in medaka. The expression of *prdm1a* and *bcl6aa* in the spleen gave an evidence that these two genes was expressed in a opposite way after immune stimulation with LPS or polyI:C. This is in accordance with that Prdm1 and Bcl6 repress each other in the mammals (Cimmino et al. 2008; Diehl et al. 2008; Miyauchi et al. 2010; Ochiai et al. 2008; Tunyaplin et al. 2004).

Reporter assay showed a direct repression of *bcl6aa* by Prdm1a and Prdm1b in a dosage dependent manner. Prdm1a and Prdm1b significantly down-regulated the expression of the reporters of *bcl6aa* in vitro. There are many binding sites of Prdm1 in the sequence of *bcl6aa*. All variants of *bcl6aa* can be repressed by Prdm1 because that Prdm1 can repress the activities of both promoter 1 and promoter 2 of *bcl6aa*.

The binding sites of Prdm1 are different in their target genes in the mammals. Prdm1 can bind a motif, GAAAG of the genes, *c-myc* (Kuo and Calame 2004; Shaffer et al. 2002), *IFN-β* (Kuo and Calame 2004), *Pax-5* (Kuo and Calame 2004), *Spi-B* (Kuo and Calame 2004; Shaffer et al. 2002), *Id3* (Kuo and Calame 2004; Shaffer et al. 2002), *ifng* (Cimmino et al. 2008), and *tbx21* (Cimmino et al. 2008), a motif, GAAAT or GAAAG of *CIITA* (Chen et al. 2007; Kuo and Calame 2004; Shaffer et al. 2002), and a motif, GAAAA of *NLRP12* (Lord et al. 2009) and *bcl6* (Cimmino et al. 2008). In this study, medaka Prdm1a was found binding a motif, GAAAA(G/T) of medaka *bcl6aa* gene. When The 5'-G of all binding sites was mutated, the repression effect of Prdm1a or Prdm1b disappeared. This result suggests that medaka Prdm1a or Prdm1b can bind the motif GAAAA of medaka *bcl6aa* as that in mice *bcl6* (Cimmino et al. 2008), the motif GAAAG, and the motif GAAAT found in other genes of the mammals. In the motif GAAAA(G/T), the 5'-G is critical for Prdm1 binding because its mutation diminishes the repression effect of Prdm1.

Prdm1b is closely relative to Hobit (Perdiguero et al. 2020). Hobit recognizes the similar regulatory sequences of the target genes of Prdm1 in the mammals (Mackay et al. 2016). Like Hobit in the mammals, medaka Prdm1b recognizes the binding motives of Prdm1a in *bcl6aa* gene.

In the mammals, Hobit functions in NK cells (van Gisbergen et al. 2012) and T cells (Vieira Braga et al. 2015). Hobit cooperates with Prdm1 to maintain Trm cells (Behr et al. 2019; Kragten et al. 2018; Mackay et al. 2016; Zundler et al. 2019). In fish, Prdm1a, Prdm1b, and Prdm1c, may cooperate in the immune system as their homologs Prdm1 and Hobit in the mammals (Perdiguero et al. 2020).

Bcl6aa can repress *prdm1* expression in medaka too (data not shown). As their homologs in mammals, Prdm1 and Bcl6 may function together to ensure proper development of NK cells, T cells, and B cells, they may also work together to keep homeostasis such as bone (Miyauchi et al. 2010) in fish.

In conclusion, multiple variants of *bcl6aa* exist in medaka, *bcl6aa* is the direct target of Prdm1 by binding its cis-element GAAAA(G/T), the 5'-G is critical for Prdm1 binding.

Declarations

Acknowledgements

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Data Availability

The data will be provided upon direct request to the authors.

Code availability

Not applicable.

Authors' Contributions

Qingchun Zhou, Xueping Zhong, and Haobin Zhao: conceptualization and designing the experiment; Xiaomei Ke, Runshuai Zhang, Qiting Yao, and Shi Duan: experimental set up and execution; Wentao Hong and Mengxi Cao: data analysis; Xiaomei Ke, Runshuai Zhang, and Haobin Zhao: manuscript writing.

Ethics approval

This study was carried out in strict accordance with recommendations in the Regulation for the Management of Laboratory Animals of the Ministry of Science and Technology of China. The animal protocol for this study was approved by the Animal Care and Use Committee of Hubei Province in China [No. SYXK(E)2015-0012]. None of the fish suffered starvation, trauma or electrical shock and all the fish were completely anesthetized before tissue sampling.

Consent to participate

All names in author list have been involved in various stages of experimentation or writing.

Consent for publication

All authors agree with submit the paper for publication in the journal of Fish Physiology and Biochemistry.

Conflict of interest

The authors declare no conflict of interest.

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Tables

Table 1 The primers used in this study

Gene	Primer	Sequence (5'- 3')	Application
bcl6aa	bcl6a-201F	TTTCTTCGCCAGACGTCCCAA	RT-PCR
	bcl6a-202-F	ATGGCGCCTCCTTCTGGTGAC	
	bcl6a-R	GGTCCAGACTGATGGCATTAAGGTT	
	bcl6aa-QF	ACCGTCCACACAGGAGAGAAG	qPCR
	bcl6aa-QR	ATCAACACATGGGCACGAAG	
	bcl6aa-p4700-F	ATTTCTCTATCGAAAGGTACCTCCAGCTGTAGCACCATGTC	Isolation of the promoter
	bcl6aa-p4700-R	CTAGCTAGCAAGTTCTGCAAAAGTGGAAGAG	
prdm1a	Prdm1a-F1	ATGGGATCAAAGTTACATGGCGACC	RT-PCR
	Prdm1a-R1	TGTTGGTGCCACGCAGAATGGAAAG	
	prdm1a-QF	AGAAGCCACATGAATGTCAGGTT	qPCR
	prdm1a-QF	AGTGGAGACGCAGGTGAGTT	
	prdm1a-OF	ATTAAGCTTGATCCCACGGCTCGTCCTTATG	Expression vector
	prdm1a-OR	GGAATTCGCCATAGTTCTGGTGTCACTCC	
prdm1b	Prdm1b-F2	TCCTCTTACCCCCTCTACCCCCAGT	RT-PCR
	Prdm1b-R2	AGGTGACTGCTTGGTTTGGCGAGAG	
	prdm1b-OF	ATTAAGCTTATGTGTGGCTCCTCTCAAG	Expression vector
	prdm1b-OR	GGAATTCTTAAGTCTCTGAAACACAGCC	
β-actin	β-actin-F	CACACCTTCTACAATGAGCTG	RT-PCR
	β-actin-R	CCAGATCTGCTGGAAGGTGG	
RPS18	S18-QF	GTGTGGTGACCATCATGCAGAA	RT-PCR/qPCR
	S18-QR	TGGCAAGGACCTGGCTGTATT	

Note: The underlined letters represent the restriction sites.

Table 2 The predicted N-terminus, half-life, and relative translation efficiency of Bcl6aa by TermiNator

Protein	Predicted N-terminus of the mature protein	Predicted Half-life (h)	Translation efficiency
Bcl6aaX1	P(2)	?	1
Bcl6aaX1-SP	M(1)	5-31	1
Bcl6aaX2	A(2)	65	5
Bcl6aaX3	Ac-A(2)	220	5

Note: Bcl6aaX1-SP, Bcl6aaX1 without the signal peptide.

Figures

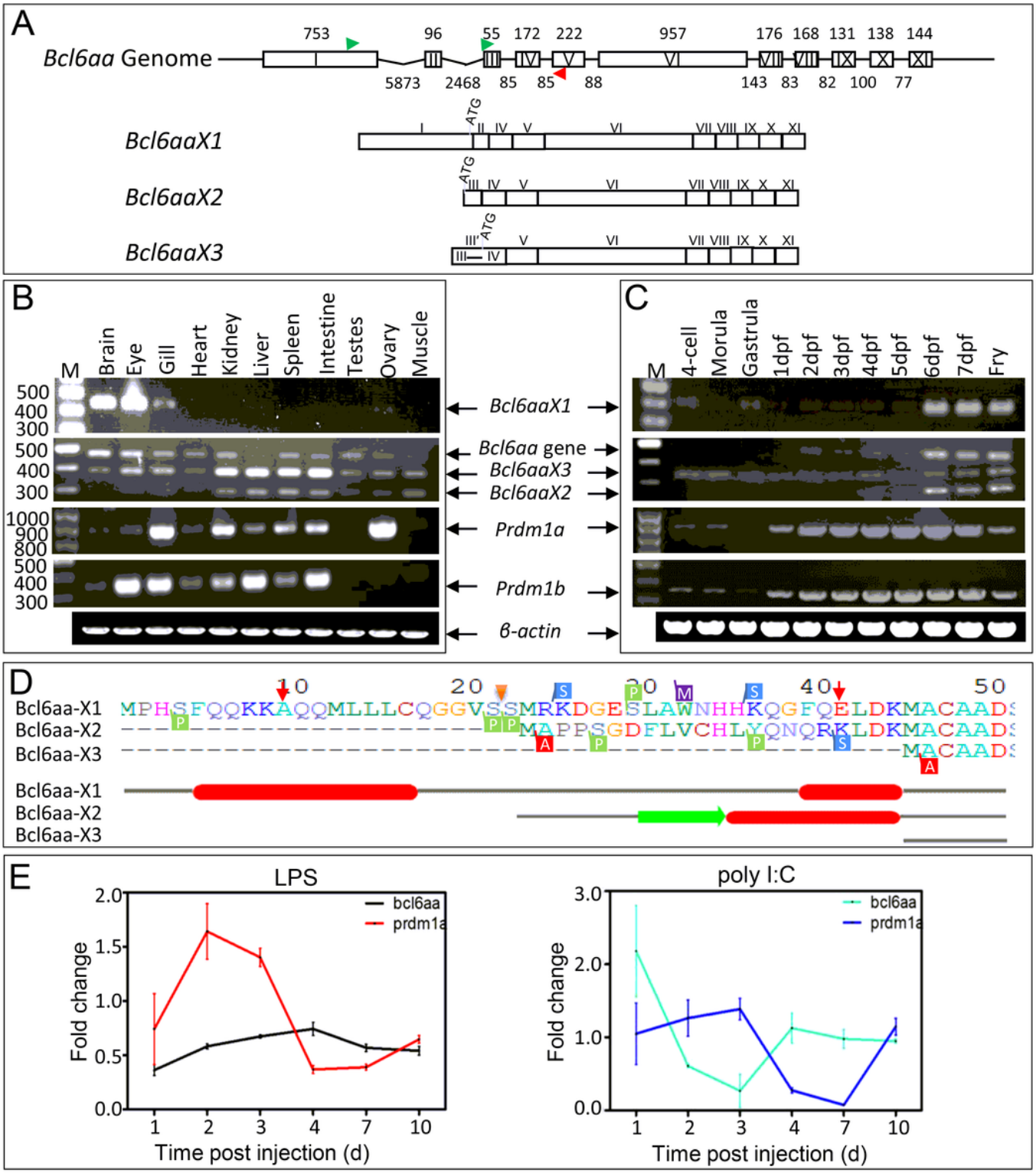

Figure 1

Schematic structure of bcl6aa gene and the expression of bcl6aa, prdm1a, and prdm1b in adult tissues and embryos of medaka. (A) Schematic structures of medaka bcl6aa gene and its transcriptional variants. The blocks with Rome numbers show the exons. The lines represent the introns. The Arabic numbers indicate the lengths of the exons or introns. Three variants, namely bcl6aaX1, bcl6aaX2, and bcl6aaX3, are the transcripts of the bcl6aa gene with different exons at the upstream. The positions of

the code ATG show the initial translation sites in the three variants. The III' is composed of the exon 3, 5, and their intron. The arrows show the positions of the primers used for RT-PCR. (B-C) The expressions of the variants of bcl6aa, prdm1a, and prdm1b detected by RT-PCR in the adult tissues (B) and the embryos (C) of medaka. M, DNA marker; the sizes in base pairs (bp) are shown beside the gel documents. Beta-actin was used as an internal control. The bands of bcl6aa gene were due to contamination of genomic DNA in the templates. (D) The alignment of Bcl6aaX1-X3 indicate the differences in the N-terminals. The arrows show the splicing sites. The arrow head shows the hydrolytic site of the signal peptide in Bcl6aaX1. The letters A, M, P, and S in the blocks show the sites with predicted acetylation, C-mannosylation, phosphorylation, and sumoylation in the N-terminals of Bcl6aaX1-X3. The structures of Bcl6aaX1-X3 predicted by Jpred4 are shown under the alignment. The red cylinders represent the α-helixes, the green thick arrow represents the β-sheet, and the lines represent the coils. (E) Relative fold change of bcl6aa and prdm1a in the spleen after intraperitoneally injection of LPS or polyI:C.

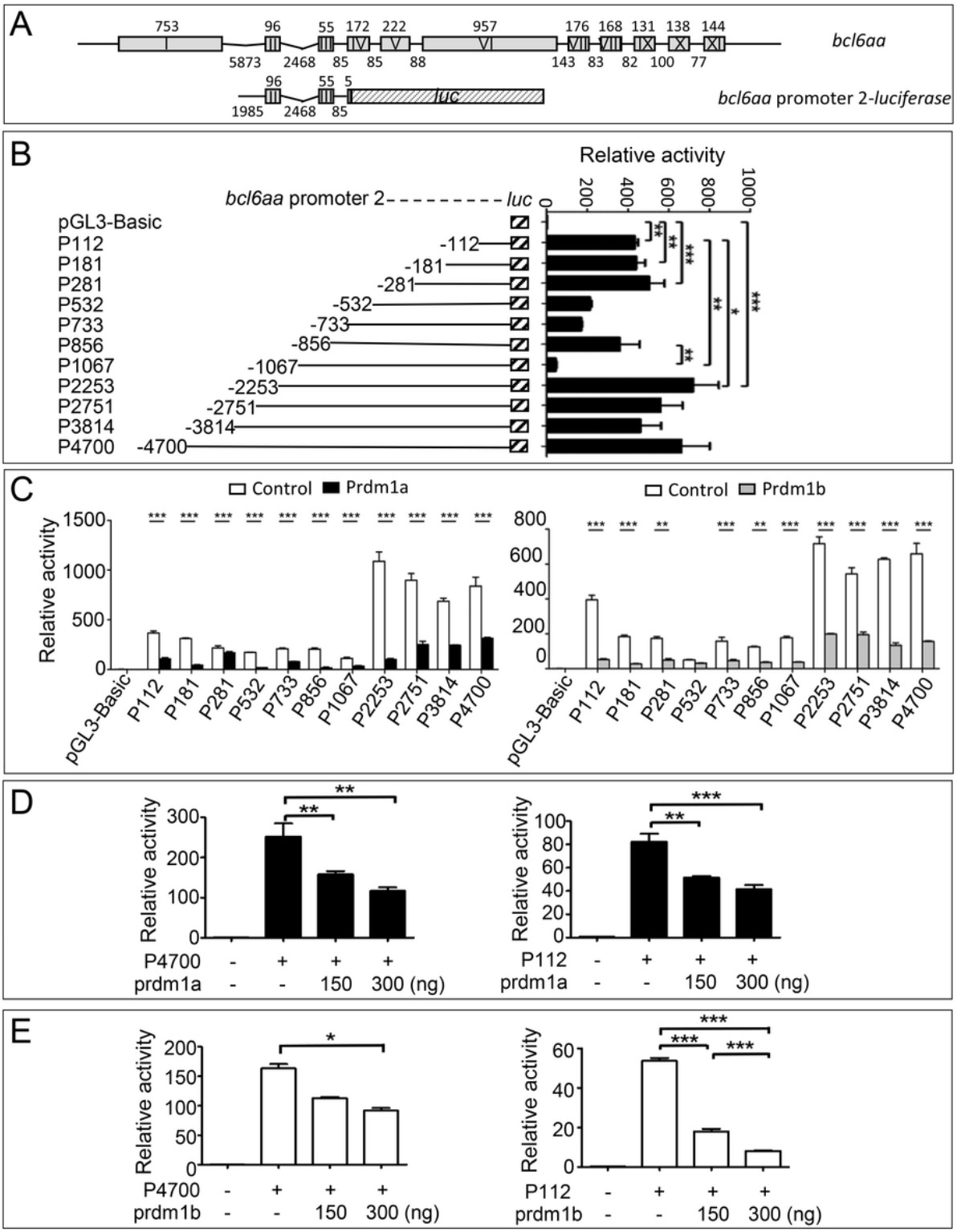

Figure 2

Luciferase reporter assay of bcl6aa promoter. (A) A schematic structure of bcl6aa gene and the reporter. The promoter 2 of bcl6aa was fused to the luciferase (luc). (B) Relative activities of the reporters with the promoters in different sizes (P114 to P4727). The control pGL3Basic was set as 1. (C) The activities of the promoters of bcl6aa in different sizes were repressed by Prdm1a or Prdm1b. (D) The repression of the activities of bcl6aa promoter by Prdm1a in different concentrations. (E) The repression of the activities of

bcl6aa promoter by Prdm1b in different concentrations. The asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

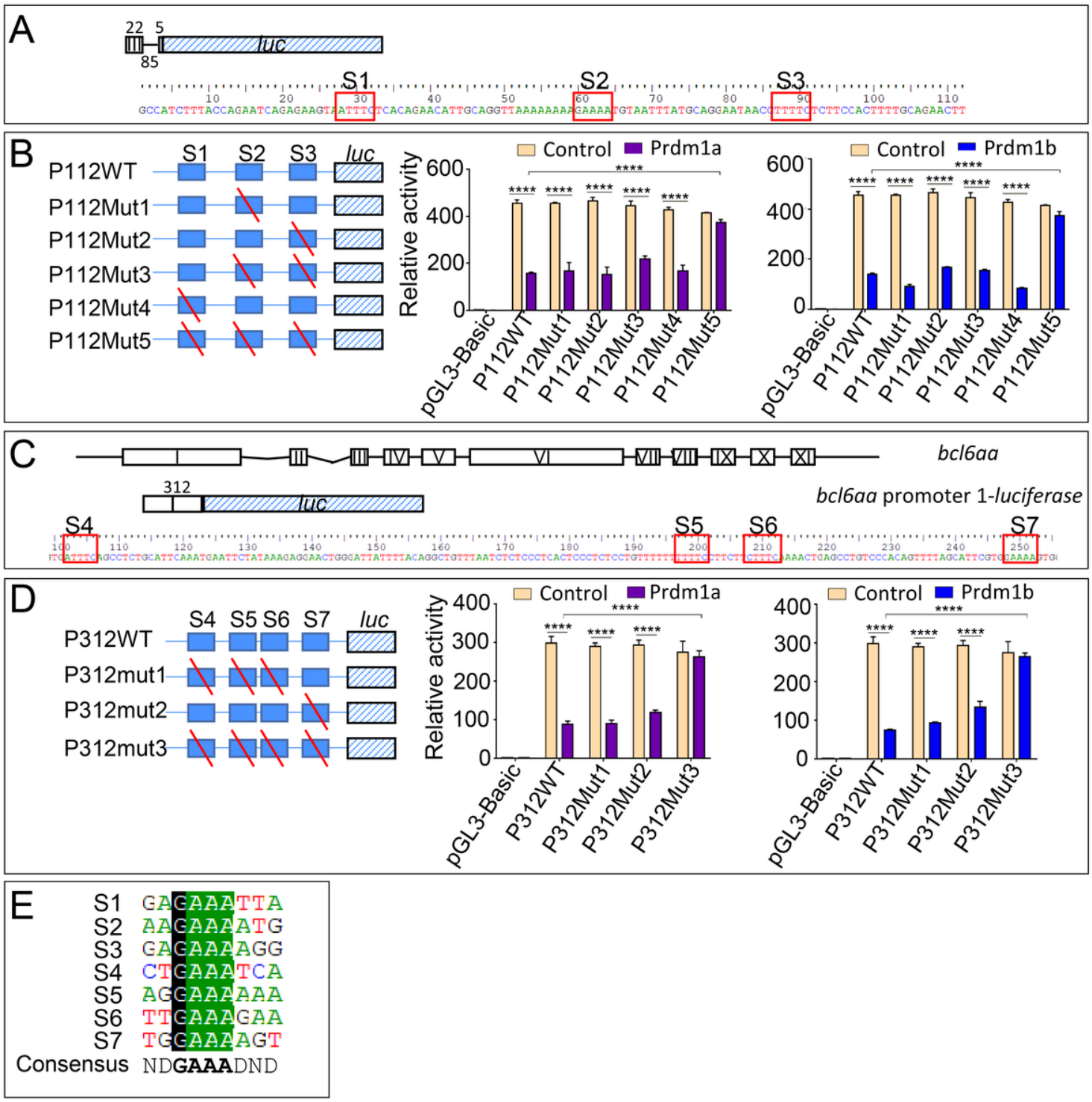

Figure 3

Luciferase assay of bcl6aa promoters. (A) and (C) The schematic structure of the reporters. The promoter P112 is 112 bp in length composed of partial exon III, exon IV and the intron (A). The promoter P312 is 312 bp in length composed of partial exon I as shown in the schematic structure of bcl6aa gene (C). The sequences are shown with the possible binding sites labeled as S1-S7 in block. luc, luciferase. (B) and (D)

The schematic structures of the reporters and the relative activities with pGL3basic set as 1. The blue blocks represent the binding sites, and the oblique lines represent mutation of the sites. The reporter activities were measured without or with co-transfer of the expression vectors of Prdm1a (Purple) and/or Prdm1b (Blue). WT, wild type; Mut, mutation. The asterisks indicate the significant differences between the control and the addition of Prdm1 (**** $p < 0.0001$). (E) Alignment of the binding sites, S1-S7, show the conservative binding motif of Prdm1. The consensus of the core binding motif is GAAAA(T/G). N, A/T/G/C; D, A/T/G.