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

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Abstract Bcl6 and Prdm1 (Blimp1) are a pair of transcriptional factors that repress each other in mammals. Prdm1 represses the expression of bcl6 by binding a cis-element of the bcl6 gene in mammals. The homologs of Bcl6 and Prdm1 have been identified in teleost fish. However, whether these two factors regulate each other in the same way in fish like that in 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 detected by RT-PCR. The three variants can be detected in adult tissues and developing embryos of medaka. 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 binding site of Prdm1 in the bcl6aa gene is GAAAA(T/G). These results indicate that both Prdm1a and Prdm1b directly repress the expression of bcl6aa by binding their binding sites where the 5′-G is critical in medaka fish.

Keywords Prdm1/Blimp1 · Bcl6 · Gene expression · Cis-element · Reporter assay · Medaka

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 the 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 in mammals. The effect of Bcl6 is opposite to that of Prdm1. For example, Bcl6 inhibits but Prdm1 promotes osteoclastogenesis in mice (Miyauuchi et al. 2010). In the immune system of
the mouse, 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 the natural killer T (NKT) cells of the mouse (van Gisbergen et al. 2012). Hobit functions in the 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 the 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. 2013; Roy and Ng 2004; Wilm and Solnica-Krezel 2005), 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. Prdm1a is necessary for fin, muscle, cloaca, etc., during organogenesis 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 the fugu kidney (Odaka et al. 2011). Prdm1a is expressed in the plasma CD45+B cells with the 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 (Bcl6aa) is required for optic cup formation (Lee et al. 2013) and is a key factor for cold response (Hu et al. 2015). Zebrafish bcl6aa and bcl6ab were detected in the spinal cord, heart, and retina. Zebrafish bcl6ab was upregulated after a heart injury (Hui et al. 2017). 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 the GCRV challenge in grass carp (Zhu et al. 2019). Senegalese sole bcl6 could be induced by the sulfated ulvan polysaccharide 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 a possible Bcl6 binding site in the 5'-regulation region of the 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 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), and polyI:C (Sigma-Aldrich), respectively (Zhao et al. 2014, Zhang et al. 2019b). LPS and polyI:C were dissolved in PBS at 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.

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. SYXX(E)2015–0012].

Extraction of total RNA

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 the Ultrapure RNA kit (CoWin Biosciences, Beijing, China) as the protocol provided by the manufacturer. The cDNA was synthesized according to the protocol of the Fast-Quant 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 the 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).

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 (ddH2O). The cycling program was 95 °C 3 min; 30 cycles of 95 °C 30 s, 62 °C 30 s, and 72 °C 25 s; and 72 °C 5 min. Quantitative RT-PCR (qRT-PCR) of triplicate samples was performed with a 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 °C 2 min followed by 39 cycles of 95 °C 10 s, 62 °C 30 s, and 65 °C 30 s. Relative expression of the genes in the samples was calibrated/normalized against RPS18 by using the 2−ΔΔCt method (the quantity in the samples of fish received PBS referred to 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 an internal control (Zhao et al. 2014).

Bioinformatic assay

The variants of bcl6aa were translated into protein isoforms, Bcl6aaX1-X3. 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/).

The variants of bcl6aa in other animals were checked on the Ensembl (http://www.ensembl.org/). The sequences of the protein isoforms of Bcl6aa were downloaded and aligned with medaka Bcl6aa by the software Bioedit (https://bioedit.software.informer.com/).

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 theoretically predicted promoter 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 P112–P4700 (Fig. 2). 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. 3).

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 cell line used is the human embryonic kidney (HEK)-293 T cell line. The 293 T 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 using 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 ≥ 3). The differences among the treatments were calculated by one-way analysis of variance (ANOVA).

### Results

The variants of \textit{bcl6aa} in medaka

RT-PCR results showed one band of \textit{bcl6aa-201} in adult tissue of medaka. The sequencing result was the same as the predicted one and was named \textit{bcl6aaX1} (Fig. 1A). \textit{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. 1B).

RT-PCR results of \textit{bcl6aa-202} showed three bands of 292, 377, and 462 bp in size (Fig. 1B). The sequence of 292 bp was the predicted \textit{bcl6aa-202}
and was named 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 same as the genomic sequence of bcl6aa. This sequence of 462 bp is a possible variant or contamination of genomic DNA of bcl6aa.

The expression of bcl6aaX2 and bcl6aaX3 was detected in all checked tissues (Fig. 1B). 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 (Fig. 1C). 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 a 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 (Fig. 1D). 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.

Bcl6aaX1 and X2 may have more modification such as C-mannosylation, phosphorylation, and sumoylation in the N-terminals than that in X3 (Fig. 1D). 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).
prdm1b, the expression of luciferase was repressed in almost all reporters except the P532 (Fig. 2C). The activity of P532 was low with or without Prdm1b. This might be a random error or a repression cis-element existed.

The P4700 and P112 were used for further experiments (Fig. 2D, E). Both Prdm1a and Prdm1b repress the two promoter constructs’ activity in a dose-dependent manner.

The core binding sites of Prdm1 in bcl6aa promoters

To know the core binding site of Prdm1 in the sequence of the 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 the 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 a single mutant of S1 still was repressed by Prdm1a or Prdm1b. However, the reporter with a triple mutant of S1, S2, and S3 was not repressed anymore by both Prdm1a and Prdm1b (Fig. 3B).

We wondered about the repression of Prdm1 on bcl6aaX1. A sequence of 312-bp upstream of the start codon (P312) was selected for analysis (Fig. 3C). This fragment of the bcl6aa gene could drive the expression of the luciferase obviously. 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 opposite to that of prdm1a in the medaka spleen after immune stimulation. Medaka bcl6aa existed in the tissues or embryos which expressed 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 the 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 are produced by alternative splicing. The variants or isoforms of bcl6aa also exist in other fish such as fugu.

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 the 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 in different stages of the 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). 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).

**Fig. 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 the *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 the 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, shows 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
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 |
| Bcl6aaX2 | A(2) | 65 | 5 |
| Bcl6aaX3 | Ac-A(2) | 220 | 5 |

Repression of bcl6aa by Prdm1

Prdm1a was detected in all checked adult tissues and embryos. This result is like the previous report (Zhao et al. 2014). Prdm1b as a close 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 in 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 evidence that these two genes were expressed in the 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).

The 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 predicted promoters of bcl6aa.

The binding sites of Prdm1 are different in their target genes in 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), Pax5 (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, GAAAG(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 mammals (Mackay et al. 2016). Like Hobit in mammals, medaka Prdm1b recognizes the binding motives of Prdm1a in the bcl6aa gene.

In 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 and prdm1a are expressed in the same tissue or the same stage of the embryos. Bcl6aa can repress prdm1 expression in medaka too (data not shown). It seems contrary to their mutual repression that both bcl6aa and prdm1a are expressed in the same tissue. In fact, it is not a paradox. Mutual repression of Prdm1 and Bcl6 is not a zero and one but a high and low relationship in the cells. Prdm1 and Bcl6 may present in different cells in the same tissue or in the different stages of the cells. Mutual repression of Prdm1 and Bcl6 orchestrates the development of the cells. For example, Prdm1 and Bcl6 are expressed in both B and T cells. In B cells, Bcl6 is essential for GC establishment and/or maintenance (Ye et al. 1997). Prdm1 is the master regulator of plasma cell differentiation, repressed by Bcl6 and repressing Bcl6 (Boi et al. 2015). In T cells, expression of Bcl6 in CD4+ T cells is necessary and enough for in vivo Tfh differentiation and T cell help to B cells in mice (Johnston et al. 2009). Prdm1 inhibits Tfh differentiation and help, thereby...
preventing B cell germinal center (Johnston et al. 2009). Prdm1 is critical for most terminal effector cell differentiation in both CD4+ and CD8+ T cells (Boi et al. 2015). Bcl6 inhibits but Prdm1 promotes osteoclastogenesis in the bone of mice (Miyauchi et al. 2010).

As their homologs in mammals, Prdm1 and Bcl6 may function together to ensure proper development and maturation of NK cells, T cells, and B cells in fish as in mammals.

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.

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

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

Code availability Not applicable.

Declarations

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

Consent for publication All authors agree with submitting the paper for publication in the journal, Fish Physiology and Biochemistry.

Conflict of interest The authors declare no competing interests.

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